MicroRNA-224 Is Involved in Transforming Growth Factor-β-Mediated Mouse Granulosa Cell Proliferation and Granulosa Cell Function by Targeting Smad4

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Many members of the TGF-β superfamily are indicated to play important roles in ovarian follicular development, such as affecting granulosa cell function and oocyte maturation. Abnormalities associated with TGF-β1 signaling transduction could result in female infertility. MicroRNAs (miRNAs), as small noncoding RNAs, were recently found to regulate gene expression at posttranscriptional levels. However, little is known about the role of miRNAs in TGF-β-mediated granulosa cell proliferation and granulosa cell function. In this study, the miRNA expression profiling was identified from TGF-β1-treated mouse preantral granulosa cells (GCs), and three miRNAs were found to be significantly up-regulated and 13 miRNAs were down-regulated. Among up-regulated miRNAs, miR-224 was the second most significantly elevated miRNA. This up-regulation was attenuated by treatment of GCs with SB431542 (an inhibitor of TGFβ superfamily type I receptors, thus blocking phosphorylation of the downstream effectors Smad2/3), indicating that miR-224 expression was regulated by TGF-β1/Smads pathway. The ectopic expression of miR-224 can enhance TGF-β1-induced GC proliferation through targeting Smad4. Inhibition of endogenous miR-224 partially suppressed GC proliferation induced by TGF-β1. In addition, both miR-224 and TGF-β1 can promote estradiol release from GC, at least in part, through increasing *CYP19A1* mRNA levels. This is the first demonstration that miRNAs can control reproductive functions resulting in promoting TGF-β1-induced GC proliferation and ovarian estrogen release. Such miRNA-mediated effects could be potentially used for regulation of reproductive processes or for treatment of reproductive disorders. **(*Molecular Endocrinology* 24: 540–551, 2010)**

Follicles are the functional units of the ovary, and each follicle consists of an oocyte surrounded by one or more layers of somatic granulosa cells (GCs). During follicular development, oocytes increase in size and progress to maturation, which is accompanied by proliferation and differentiation of their surrounding granulosa and thecal cells as well as the somatic cells acquiring the ability to secrete or respond to sex hormones. This complex process is regulated by bidirectional communication between oocyte and GCs, and granulosa and theca cells. Many extra- and intraovarian factors have been implicated in follicle regulation (1, 2), *e.g.* members of the TGF-β superfamily.

There is increasing evidence that many members of the TGF-β superfamily have roles in early follicle development (3), GC proliferation and differentiation (4, 5), and theca cell function (2). TGF-β binds to a heteromeric complex of the membrane receptors that have an intracytoplasmic serine/threonine kinase domain, type I and II TGF-β receptors (6). After ligand binding to type II receptors, the type I receptors are activated (6). Activated type I receptors phosphorylate the receptor-specific Smad2 and Smad3 (R-Smads). Phosphorylated R-Smads form heteromeric complexes with common partner Smad4 (Co-Smad) and translocate into the nucleus. The

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Abbreviations: CCK-8, Cell counting kit 8; E_2, estradiol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC, granulose cell; LNA, locked nucleic acid; miRNA, micro-RNA; snRNA, small nuclear RNA; UTR, untranslated region.

complexes bind to a Smad-binding element (SBE) and recruit some transcription factors, coactivators, or corepressors, thus leading to transcription initiation or repression (7, 8). The perturbation of components of TGF-β signaling pathway could lead to reproductive abnormalities. For example, conditional deletion of *Smad4* gene in preantral GCs leads to severe defects in subsequent follicle development and differentiation (9); mice lacking Smad3 show increased rates of cell death and abnormal cell differentiation (10); an inhibitor of Smad2/3 activation, SB431542, could block oocyte-stimulated proliferation of mural and cumulus cells (11). Thus, new insight into the regulation of the TGF-β signaling pathway will help to elucidate the mechanisms of TGF-β-mediated follicular development. Recent reports have demonstrated the association between TGF-β signaling pathway and micro-RNAs (miRNAs) (12–14).

miRNAs are a class of small noncoding RNAs ranging in size from 20 to 25 nt (15–17). The biogenesis of miRNAs initiates with transcription by RNA polymerase II and proceeds with the processing of the primary transcript (pri-miRNA) into a hairpin intermediate (pre-miRNA) by the nuclear ribonuclease III Drosha and its cofactor DGCR8/Pasha (16). The pre-miRNA is then exported to the cytoplasm by exportin-5, where it is further processed into a mature approximately 22-nucleotide miRNA by a second ribonuclease III, Dicer (18). The mature miRNA guides miRNA-ribonucleoprotein complexes to the 3′-untranslated region (UTR) of target mRNAs and influences the translation or stability of the transcripts. miRNAs are instrumental in controlling many biological processes, such as cell proliferation, differentiation, and apoptosis (18–20). Information of the roles of miRNAs in controlling gonadal functions is limited to characterization of the expression profiles of miRNAs in normal ovarian cells (21) and in ovarian carcinoma cells (22, 23). Ro *et al.* (21) profiled the mouse ovarian miRNAs and concluded that these miRNAs may play critical roles in controlling the expression of genes essential for ovarian folliculogenesis and endocrine function. These studies suggest that miRNA may play an important role in follicular development. However, involvement of miRNAs in the control of ovarian cell proliferation, apoptosis, and hormone secretion has not been well documented. In addition, the roles of miRNAs during TGF-β-mediated GC proliferation and function remains uncharacterized.

In this study, the miRNA signature of mouse preantral GCs stimulated by TGF-β1 was profiled, and one of the differentially expressed miRNAs was functionally characterized in GCs. The miRNA target was also identified.

Results

miR-224 expression is regulated by TGF-β/Smads pathway

In this study, changes in miRNA expressions of mouse (m)GCs treated or untreated by TGF-β1, were identified by using miRNA microarray assays. mGCs were collected from the follicles with 120–150 μm in diameter (Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org), as described in *Materials and Methods*. mGCs from follicles at this diameter show active cell proliferation and differentiation potential, such as mGCs could grow normally *in vitro* in our culture system (Supplemental Fig. 2). In addition, transfection efficiency of plasmid DNA/miRNA into mGCs can reach 40–60% of total cells (Supplemental Fig. 3). mGCs were treated with TGF-β1 for 6 h to obtain an miRNA signature. Microarray results revealed 16 significant differentially regulated miRNAs in mGCs, of which three were up-regulated and 13 were down-regulated during TGF-β1 treatment (for details, see Supplemental Table 2). Cluster analysis, based on these differentially expressed miRNAs, generated a tree showing a clear distinction between TGF-β1-treated and untreated mGCs (Fig. 1A). miR-712, miR-224, and miR-764-3p were all found to be significantly up-regulated, whereas miR-224 was the second most significantly elevated miRNA. To confirm the microarray data, stem-loop primer-based real-time PCR was performed to quantify mature 21-nt miR-224 molecules (Fig. 1B). Real-time PCR confirmed microarray analysis results: expression of miR-224 was up-regulated in mGCs after TGF-β1 treatment (Fig. 1C). We also examined the effects of Activin A (another member of TGF-β superfamily) on the expression of miR-224, and the results showed that Activin A could also up-regulate miR-224 expression in mGCs (Supplemental Fig. 4).

Because miR-224 is a highly up-regulated miRNA in TGF-β-treated mGCs (Fig. 1, A and C) and its function in GCs remains unknown, we then ask whether miR-224 expression is regulated by the TGF-β/Smads pathway. mGCs were pretreated with SB431542, a specific inhibitor of TGF-β RI kinase and thus blocking phosphorylation of the downstream effectors Smad2/3, and then treated with TGF-β1. Real-time PCR assay revealed that SB431542 can inhibit TGF-β1-stimulated miR-224 express levels (Fig. 1D), indicating that miR-224 expression is at least partly dependent on R-Smad activation. Although TGF-β1 regulates miR-224 gene expression, as demonstrated here, little is known about how TGF-β1 affects miR-224 processing. We next examined the accumulation of primary miR-224 gene transcripts (pri-miR-

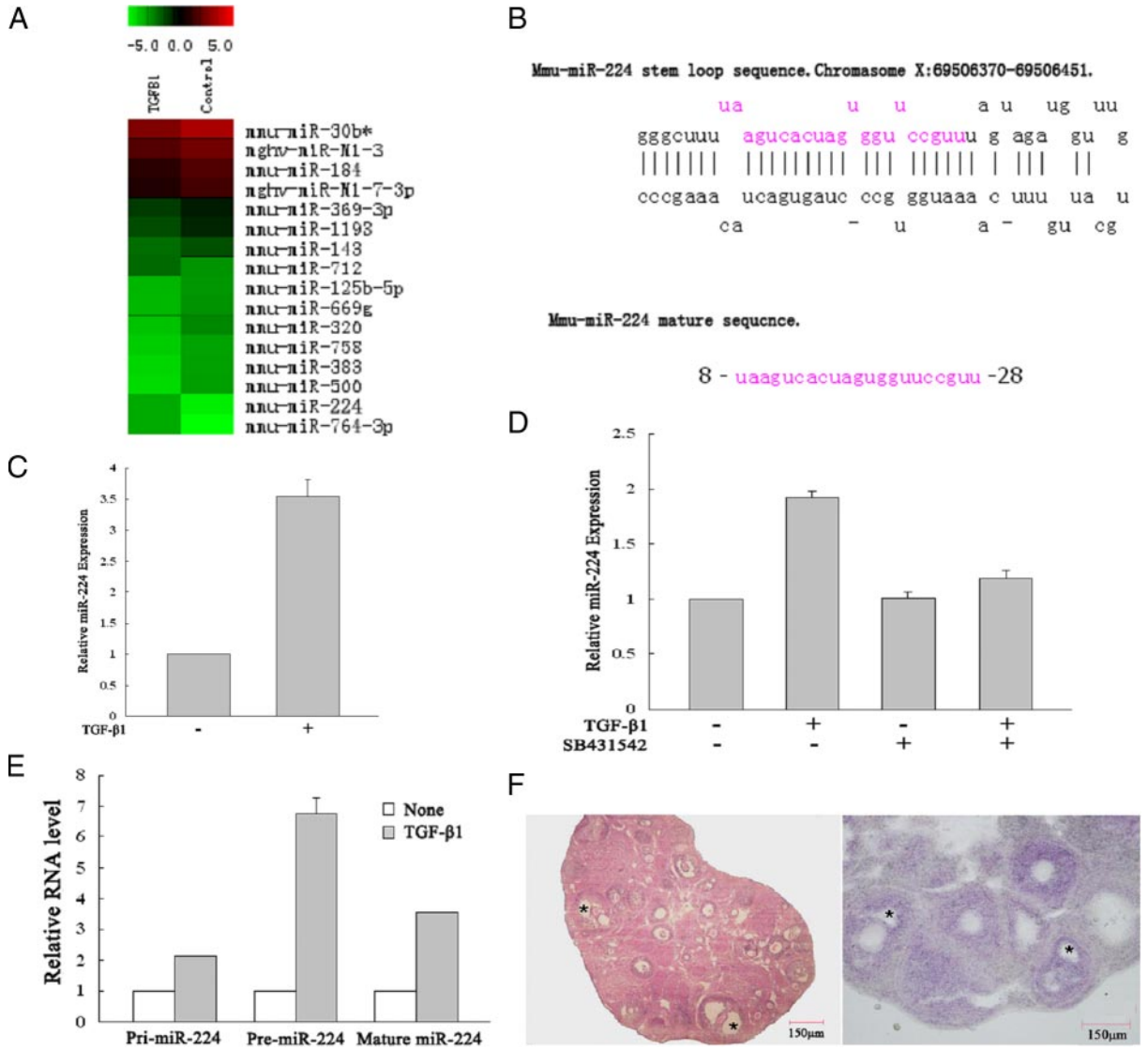

FIG. 1. miR-224 expression in TGF-β1-treated mGCs. A, Hierarchical clustering of miRNAs deregulated in mGCs treated with and without TGF-β1. mGCs were serum starved for 24 h and then treated with 5 ng/ml TGF-β1 for 6 h. Total RNA was extracted from the mGCs and used for microarray analysis. Sixteen miRNAs were identified that were differentially expressed between TGF-β1-treated and untreated mGCs. The key color bar indicates that miRNA expression levels increased from *green* to *red* compared with controls (mGCs treated with buffer alone). miR-224 was the second most significantly up-regulated miRNA. B, Stem-loop precursor sequence (*top panel*) and mature sequence of *Mus musculus* miR-224. C, Validation of TGF-β-regulated miR-224. mGCs were treated (+) with TGF-β1 or left untreated (−) for 6 h and subjected to real-time PCR analysis. The miR-224 expression levels were normalized to endogenous U6 snRNA, and data of mGCs with TGF-β1 treatment were normalized to control (without TGF-β1 treatment). miR-224 expression level was significantly up-regulated in mGCs after TGF-β1 treatment. D, Up-regulation of miR-224 is partly dependent on R-Smad. mGCs were pretreated with DMSO or 5 μM SB431542 (prepared in DMSO) for 30 min and then treated exactly as described in panel A. Extracted RNA was done with real-time PCR to quantify miR-224 levels, as described in panel C. E, Transcriptional regulation of miR-224 biosynthesis by TGF-β1. mGCs were treated with TGF-β1 for 6 h and subjected to real-time PCR analysis. The pri-miR-224, pre-miR-224, or mature miR-224 expression levels were normalized to endogenous U6 snRNA, and data of mGCs with TGF-β1 treatment were normalized to control (without TGF-β1 treatment). In C–E, data shown are representative of at least three independent experiments with triplicate samples for each treatment, expressed as mean ± SEM. +, Present; −, absent. F, miR-224 is mainly localized in GCs of ovarian follicles at various stages. miR-224 expression was performed on 10-μm frozen sections of the mouse ovary by *in situ* hybridization analysis using 5′-digoxigenin-conjugated, LNA-modified DNA probe complementary to miR-224. *Left panel*, Hematoxylin and eosin staining of normal mouse ovary; *right panel*: *in situ* hybridization signals (*purple*) of miR-224.

224), pre-miR-224, and mature miR-224 on TGF-β1 treatment by real-time PCR. The results showed that the expression levels of pri-miR-224, pre-miR-224, and mature miR-224 were all increased by 6 h TGF-β1 treatment, suggesting a transcriptional induction of miR-224 by TGF-β (Fig. 1E). Expression of miR-224 in the mouse ovary was identified by *in situ* hybridization using locked nucleic acid (LNA)-modified probes. The results show that miR-224 is expressed predominantly in GCs of follicles at different developmental stages (Fig. 1F), suggesting that miR-224 may be involved in GC proliferation and GC function during follicular development.

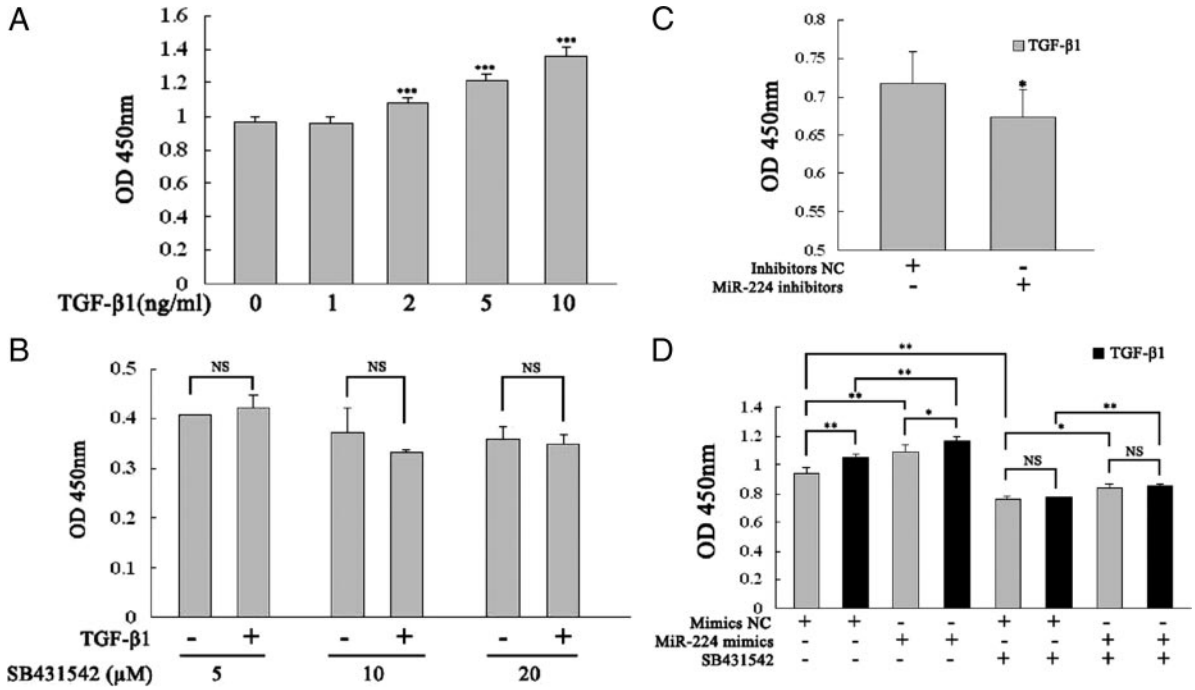

FIG. 2. miR-224 mediates TGF-β1-induced GC proliferation. A, TGF-β1 promotes GC proliferation in a dose-dependent manner. mGCs were incubated with different concentrations of TGF-β1 for 48 h after 24 h serum starvation. Cell proliferation was assayed with the CCK-8. B, SB431542 abrogated TGF-β1-mediated GC proliferation. GCs were preincubated with different concentrations of SB431542 (5, 10, and 20 μM) for 30 min, followed by incubation with 2 ng/ml TGF-β1 for 48 h. C, The knockdown of miR-224 inhibited TGF-β1-induced GC proliferation. mGCs were transfected with 100 nM each of miR-224 inhibitors and inhibitor control for 24 h and then treated with 2 ng/ml TGF-β1 for 48 h. D, The overexpression of miR-224 enhanced TGF-β1-stimulated GC proliferation, and this effect is partially dependent on TGF-β/Smad pathway. mGCs were transfected with 100 nM each of miR-224 mimics and mimics control for 24 h and then pretreated with 5 μM each of dimethylsulfoxide or SB431542 for 30 min followed by treatment with 2 ng/ml TGF-β1 for 48 h. Cell proliferation was assayed under the following conditions: mGCs treated by TGF-β1 alone, miR-224 alone, and cotreated with TGF-β1 and miR-224. Results represent the mean ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. NC, Negative control.

miR-224 is involved in TGF-β1-mediated GC proliferation and GC function

We next assessed whether miR-224 plays a role during TGF-β-induced GC proliferation. First, the effect of TGF-β1 on proliferation of primary cultures of mGCs was examined by using Cell Counting Kit-8. TGF-β1 significantly promotes GC proliferation in a dose-dependent manner, and the proliferation-promoting effects of TGF-β1 become significant at a dose of 2 ng/ml (Fig. 2A). In addition, SB431542 completely blocked TGF-β-mediated increase in GC proliferation (Fig. 2B), indicating that Smad signaling is essential for TGF-β-mediated promotion of GC proliferation. Activin A is also found to stimulate the proliferation of mGCs in a dose-dependent manner (Supplemental Fig. 5). The effect of miR-224 itself on cell proliferation was also examined by transfection of either miR-224 mimics or inhibitors into the mGCs. The level of miR-224 in cells after transfections was measured by real-time RT-PCR analysis. Transfection with miR-224 mimics increased intracellular miR-224 levels by approximately 800-fold (Supplemental Fig. 6A), whereas endogenous levels of miR-224 were decreased compared with negative inhibitor controls by more than 50% in GCs transfected with miR-224 inhibitors (Supplemental Fig. 6B). By using cell counting kit 8 (CCK-8) assay, transfection of miR-224 mimics into GCs significantly increased the proliferation potential of GCs ($P < 0.05$) (Supplemental Fig. 6C), whereas the transfection of miR-224 inhibitors reduced the level of cell proliferation (Supplemental Fig. 6D). Furthermore, miR-224 inhibitors at the higher concentration of 100 nM caused a greater inhibition of cell proliferation (Supplemental Fig. 6D). Next, mGCs were transfected with miR-224 inhibitors and inhibitor control and then treated with TGF-β1. As shown in Fig. 2C, TGF-β1-induced GC proliferation was significantly reduced by the knockdown of miR-224 ($P < 0.05$). These results indicated that miR-224 could be involved in TGF-β1-mediated GC proliferation.

To further demonstrate the effect of miR-224 on TGF-β1 function and its signaling pathway, the role of SB431542 in blocking TGF-β1/ miR-224-induced cell proliferation was examined (Fig. 2D). After 24 h of transfection with miR-224 mimics, mGCs were preincubated with or without SB431542 and then treated with 2 ng/ml TGF-β1. As shown in Fig. 2D, the ectopic expression of miR-224 alone not only increased GC proliferation but also enhanced TGF-β1-induced cell proliferation (*left side* of Fig. 2D). Although SB431542 treatment effec-

tively blocked the TGF-β1-mediated GC proliferation, the treatment could not affect GC proliferation induced by miR-224 (*right side* of Fig. 2D). miR-224 alone still promoted GC proliferation when the TGF-β/Smad pathway was blocked by SB431542, but, the proliferation potential was reduced when compared with untreated cells ($P < 0.001$). These results suggest that proliferation-promoting properties of miR-224 are partially dependent on the TGF-β/Smad pathway. To identify whether miR-224 still functions the same way in other cell types, we preformed the same experiment on KGN, a human granulosa tumor cell line (Supplemental Fig. 7). Interestingly, unlike miR-224's effects on normal primary GCs, the ectopic expression of miR-224 inhibited KGN cell proliferation. This inhibitory effect is partially dependent on TGF-β/Smad pathway, because miR-224 profoundly inhibited KGN cell proliferation when cells were treated SB431542 (Supplemental Fig. 7). However, TGF-β1's inhibition of KGN cell proliferation may be mediated through a Smad-independent signaling pathway (Supplemental Fig. 7).

Steroidogenesis is a major function of GCs in the developing follicles. The involvement of miR-224/TGF-β1 in estrogen and progesterone release by GCs was examined in this study. As shown in Fig. 3, TGF-β1 significantly promoted estradiol (E_2) release (Fig. 3A) and in-

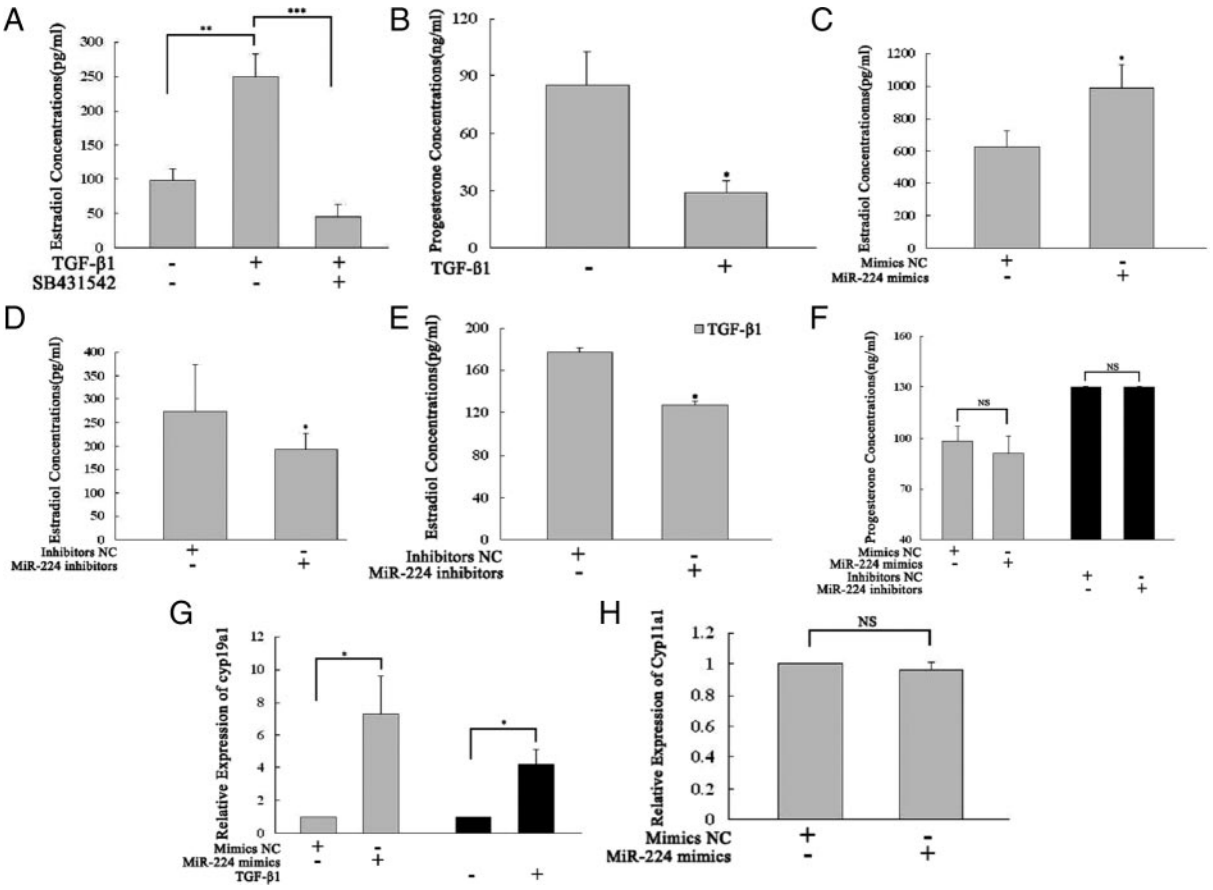

FIG. 3. Effect of miR-224/TGF-β1 on the release of steroids by cultured mouse ovarian GCs. TGF-β1 promoted E_2 release (A) and inhibited progesterone release (B) by mGCs. SB431542 decreased TGF-β1-induced E_2 release (A). GCs were preincubated with 5 μM SB431542 for 30 min, followed by incubation with 2 ng/ml TGF-β1 for 48 h. The culture medium was then collected for measurement of hormones. C and D, The ectopic expression of miR-224 stimulated E_2 release (C), and the knockdown of miR-224 inhibited E_2 release (D). GCs were transfected with 100 nM each of miR-224 mimics and mimics controls, or 150 nM each of miR-224 inhibitors and inhibitor controls. The culture medium was collected for measurement of E_2 levels at 48 h after transfection. E, The knockdown of miR-224 inhibited TGF-β1-induced E_2 synthesis. mGCs were transfected with 100 nM each of miR-224 inhibitors and inhibitor control for 24 h and then treated with 2 ng/ml TGF-β1 for 48 h. F, miR-224 had no effect on progesterone release by mGCs. mGCs were transfected with 100 nM each of miR-224 mimics and mimics controls, or 150 nM each of miR-224 inhibitors and inhibitor controls. The culture medium was collected for measurement of progesterone levels at 48 h after transfection. G, Both miR-224 and TGF-β1 could increase *CYP19A1* mRNA levels in GCs. To examine the effect of miR-224 on *CYP19A1* mRNA expression levels, mGCs were transfected with 100 nM each of miR-224 mimics and mimics controls for 48 h. To examine the effect of TGF-β1 on *CYP19A1* mRNA expression levels, mGCs was stimulated with 2 ng/ml TGF-β1 for 6 h after 24 h serum starvation. Total RNA was extracted from GCs, and the *CYP19A1* mRNA levels were measured by real-time PCR. H, The ectopic expression of miR-224 could not alter the mRNA expression levels of CYP11A1 in GCs. mGCs were transfected with 100 nM each of miR-224 mimics and mimics controls for 48 h. Total RNA was extracted from GCs, and the *CYP11A1* mRNA levels were measured by real-time PCR. Results represent the mean ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. $P > 0.05$, NS, No significant difference; NC, negative control.

hibited progesterone release (Fig. 3B) by mGCs, whereas SB431542 decreased TGF-β1-induced E_2 release (Fig. 3A), indicating that TGF-β1-induced estrogen release may be mediated in part through Smad pathway. Estrogen release was also significantly increased by the overexpression of miR-224 in mGCs (Fig. 3C). In contrast, the knockdown of miR-224 considerably inhibited E_2 release (Fig. 3D). Furthermore, TGF-β1-induced estrogen release was abrogated by the knockdown of miR-224 (Fig. 3E). miR-224 also promotes E_2 synthesis in mural GCs (Supplemental Fig. 8). However, both miR-224 mimics and inhibitors had no effect on progesterone release by mGCs (Fig. 3F). These results indicate that miR-224 plays an important role in TGF-β1-induced steroid production in the GCs. To exclude the possibility that the change in E_2 levels was due to the cell proliferation stimulated by miR-224/TGF-β1, variation in the expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of each group was examined. Cell extracts were immunoblotted for GAPDH protein levels. No significant differences in the levels of GAPDH were observed between treated and untreated cells (data not shown).

To address the mechanism of miR-224/TGF-β1's role in the release of hormones from GCs, the mRNA expression levels of CYP19A1, a key enzyme in E_2 biosynthesis, were identified between treated and untreated GCs. Real-time PCR showed a significant increase in *CYP19A1* mRNA levels in miR-224- or TGF-β1-treated GCs compared with vehicle-treated GCs (Fig. 3G). However, *CYP11A1* (a key enzyme in progesterone biosynthesis) mRNA levels in GCs were not affected after miR-224 treatment (Fig. 3H).

Smad4 is a target of miR-224 in GCs

Because miR-224 is significantly up-regulated in mGCs treated by TGF-β1 (Fig. 1, A and D) and mediates TGF-β1-stimulated GC proliferation (Fig. 2C) and GC function (Fig. 3E), potential targets that are components of TGF-β signaling pathway and known to play a role in cell proliferation or apoptosis were identified by using PicTar (24), miRBase Targets (25), and TargetScan (26). Among the candidate miR-224 targets, we found that *Smad4* gene (a critical cofactor of TGF-β signaling pathway) seems to be the most appropriate candidate because Smad4 is found to induce apoptosis in many cell types (27). The 3′-UTR of the *Smad4* gene contains two highly conserved regions that may be binding sites for miR-224 (Supplemental Fig. 9), as predicted by at least two algorithms.

To determine whether *Smad4* gene is a true target of miR-224, mGCs and HEK293T cells were transfected with miR-224 mimics/control, or miR-224 inhibitor/control. Immunoblotting and real-time PCR analysis revealed that Smad4 protein (Fig. 4, A and B) but not *Smad4* mRNA (Fig. 4C) expression was significantly lower in miR-224-transfected mGCs or HEK293T cells compared with the control cells, whereas the knockdown of miR-224 was able to increase Smad4 protein expression in HEK293T cells (Fig. 4B). These results confirm that *Smad4* gene is a miR-224 target. miR-224 regulates *Smad4* gene expression at the posttranscriptional level (Fig. 4C). We also examined whether miR-224 affects the protein expressions of other Smads such as Smad2/3 and Drosha in mGCs. As shown in Supplemental Fig. 10, miR-224 did not change the Smad2/3 protein and Drosha protein expression levels in mGCs. These results suggest that Smad2/3 may not be miR-224 targets, and Smad4 may not regulate the expression of Drosha involved in miR-224 biogenesis.

To further identify that Smad4 is negatively regulated by miR-224, we constructed *Renilla* luciferase reporters that contained the entire wild-type 3′-UTR of *Smad4* as well as mutant forms of two highly conserved seeding sites (Supplemental Fig. 11). HEK293T cells were transfected with the reporter plasmids along with miR-224 mimics/control, or miR-224 inhibitor/control, and were harvested 30 h later for dual luciferase assay. The results showed that the ectopic expression of miR-224 significantly reduced reporter activity (Fig. 4D), whereas miR-224 inhibitor was able to rescue the inhibition of wild-type Smad4 3′-UTR reporter activity by miR-224 (Fig. 4D). However, the luciferase reporter activity was not inhibited by miR-224 when the seeding sites were mutated (Fig. 4E).

So far, we observed that miR-224 expression is regulated by TGF-β1 and demonstrated that miR-224 is involved in TGF-β1-mediated GC proliferation and function and reduces expression of *Smad4* gene. We proceeded to evaluate the functional relevance of Smad4 in TGF-β1-stimulated mGCs. First, Smad4 protein expression in the mouse ovary was evaluated by using immunohistochemistry. The results show that Smad4 protein was mainly localized in GCs at various stages of follicular development (Fig. 4F), which was very similar to the miR-224 expression in the ovary (Fig. 1E). The colocalization of miR-224 with Smad4 protein indicates that they may interact with each other in mGCs. Next, we examined whether the expression level of Smad4 in mGCs was regulated by TGF-β1. As expected, Smad4 protein levels were reduced in GCs after 2 h, 4 h, 6 h (the time also used in identifying miRNA expression profiles from TGF-β1-treated mGCs), and 12 h treatment with TGF-β1, but rebounded after 24 h treatment (Fig. 4G), indicating that other regulatory mechanisms exist at this time point. Meanwhile, miR-224 levels were all up-regulated by TGF-β1 across time points examined (Fig. 4G). Because miR-224 down-regulates Smad4 to promote GC proliferation and GC function, it is reasoned that the overexpression of Smad4 can reverse this phenomenon. Indeed, both cell

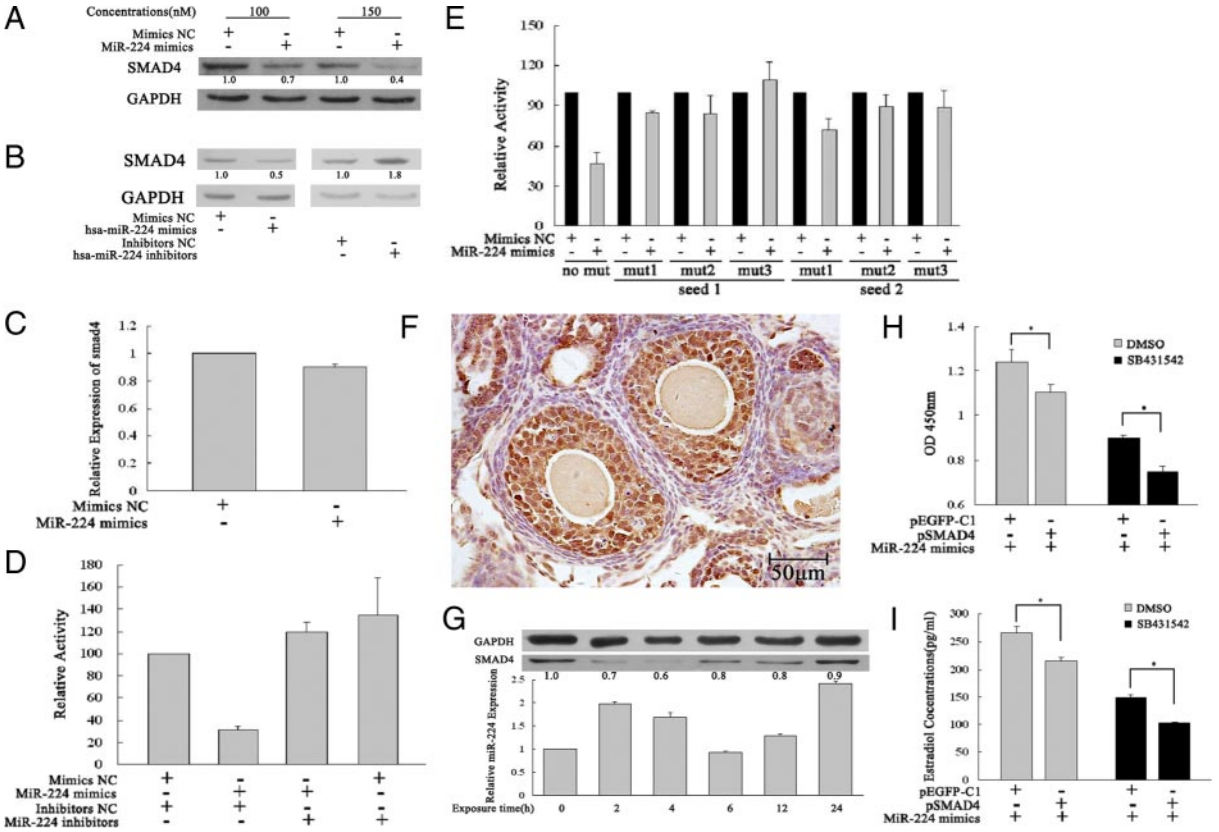

FIG. 4. Smad4 is a target of miR-224. miR-224 reduces Smad4 protein in mGCs (A) and HEK293T cells (B), but not *Smad4* mRNA expression (C). The knockdown of miR-224 increases Smad4 protein expression in HEK293T cells (B). mGCs were transfected with different concentrations of miR-224 mimics and mimics control. HEK293T cells were transfected with 100 nM each of miR-224 mimics and mimics control, or 150 nM each of miR-224 inhibitor and inhibitor control. The expression levels of Smad4 protein were determined by Western blotting analysis. The relative expression levels of Smad4 protein are shown at the *bottom of the bands* as normalized by GAPDH level. Real-time PCR analysis was performed to determine *Smad4* mRNA levels of cells transfected with 100 nM each of miR-224 mimics and mimics control. D and E, miR-224 inhibits *Smad4* gene 3′-UTR luciferase activity (D), whereas the knockdown of miR-224 (D) or the mutant forms for the seeding sites (E) no longer inhibited luciferase reporter activity. Cells were cotransfected with wild-type *Smad4* 3′-UTR (D) or mutant *Smad4* 3′-UTR reporter constructs (E) and miR-224 mimics/control, or miR-224 inhibitor/control. At 30 h after transfection, the cells were harvested and assayed for firefly and *Renilla* luciferase activities. The *Renilla*/firefly ratio was calculated and normalized against the control. F, Immunohistochemical localization of Smad4 in the mouse ovary. Smad4 proteins (*stained brown*) were found to be expressed mainly in the GCs at various stages of follicular development. G, TGF-β1 down-regulated Smad4 protein expression in mGCs. Cells were treated with 2 ng/ml TGF-β1 for the indicated time periods, and the expression levels of Smad4 and miR-224 were then examined by Western blotting and real-time PCR analysis, respectively. The relative expression levels of Smad4 protein are shown at the *bottom of the bands* as normalized by GAPDH level. Smad4 protein levels were reduced in GCs after 2 h, 4 h, 6 h, and 12 h treatment with TGF-β1 but rebounded after 24 h treatment (*top panel*). Meanwhile, miR-224 levels were all up-regulated by TGF-β1 across time points examined (*bottom panel*). H and I, The ectopic expression of Smad4 decreased GC proliferation and estrogen production induced by miR-224. mGCs were cotransfected with pEGFP-Smad4 or control vector and miR-224 mimics for 24 h, and then treated with dimethylsulfoxide or SB431542 for 48 h. Cell proliferation was assayed using a CCK-8 kit. The culture medium was collected for measurement of hormones. Data shown are representative of at least three independent experiments. In panels A–E and G–I, the data are expressed as the means mean ± SEM. *, $P < 0.001$. DMSO, Dimethylsulfoxide; mut, mutant; NC, negative control.

proliferation and estrogen production were attenuated when the expression vector pEGFP-Smad4 (Supplemental Fig. 12) was introduced into mGCs that were simultaneously treated with miR-224 mimics alone for 48 h or miR-224 mimics and SB431542 (Fig. 4H for cell proliferation; Fig. 4I for E_2 production). These results further suggest that Smad4 is a target of miR-224 and mediates the roles of miR-224 to influence GC proliferation and function.

Discussion

TGF-β superfamily members have been implicated in regulating GC proliferation (5) and terminal differentiation (4) that are critical for normal ovarian follicular development, ovulation, and luteinization. Several recent reports have shown that miRNAs are involved in TGF-β signaling pathway (12–14). In this study, miRNA expression profiles of TGF-β1-stimulated mGCs were identified for the first time, of which 16 miRNAs were found to be significantly differentially regulated. Furthermore, miR-224, the second most significantly up-regulated miRNA, was shown to play an important role in TGF-β1-induced GC proliferation and E_2 release by targeting Smad4. In addition, the overexpression of Smad4 in mGCs inhibited miR-224-induced GC proliferation and function. The findings of this study provide the first evidence that miR-

224 participates in regulation of mouse preantral GC proliferation and hormone secretion through targeting Smad4 (a critical factor of TGF-β signaling). Thus a direct link between miRNAs and TGF-β pathways during ovarian follicle development is established for the first time.

miRNA profiling has been established in mGCs treated with TGF-β in this study. In a previous study, miRNA expression profiles in normal murine mammary gland epithelial cells treated with TGF-β were identified, of which nine miRNAs were up-regulated and 19 were down-regulated by TGF-β (28). Among the 28 deregulated miRNAs, only one miRNA (*e.g.* miR-30b) showed a change in expression consistent with our results (data not shown in the cluster). This discrepancy may reflect the cell type-specific difference or may be due to the duration of TGF-β treatment. miRNAs have been shown to play important roles in several biological events. For example, miR-125b, a significantly down-regulated miRNA in our study, is involved in regulation of cell proliferation and differentiation in human breast cancer cell lines (20, 29, 30); miR-21 has been reported to play a role in driving fibroblasts into the cell cycle (31). miR-21 expression was also down-regulated in our study, although it was not statistically significant. miR-224 is found to be overexpressed in various cancers, such as thyroid (32), hepatocellular (33, 34), and prostate cancer (35), acute myeloid leukemia (36), as well as pancreatic ductal adenocarcinomas (19), indicating that miR-224 could function as a proliferation-promoting factor in cancers. Two targets of miR-224, apoptosis inhibitor 5 (*API-5*) (33) and *CD40* (19) have been identified. However, the functions of miR-224 in ovarian GCs have not been characterized. In the current study, miR-224 expression was also significantly up-regulated in mGCs treated by TGF-β1 *in vitro*, and this expression is partly dependent on TGF-β/Smad pathway. miR-224 is expressed predominantly in GCs of follicles at different developmental stages, suggesting that miR-224 may be involved in GC proliferation and GC function during follicular development. Indeed, our study provides the first evidence that miR-224 mediates TGF-β-induced GC proliferation and function.

TGF-β is known to induce apoptosis of many human cancer cell lines (37, 38). In this study, TGF-β1 was shown to inhibit KGN cell proliferation, but promote primary GC proliferation, suggesting that TGF-β1 plays different roles in different cell types. To investigate the roles of miR-224 in TGF-β1-induced GC proliferation, miR-224 was functionally characterized in mGCs. Our results show that overexpression of miR-224 significantly increases the proliferation potential of GCs, and the proliferation-promoting properties of miR-224 are partially dependent on the TGF-β/Smad pathway. In addition, we also observed that the knockdown of miR-224 inhibited TGF-β1-stimulated GC proliferation. These results indicated that miR-224 is involved in TGF-β/Smad pathway-mediated GC proliferation. Similar to these findings, a recent study has shown that the ectopic expression of miR-224 also promotes HCT116 cell proliferation (33). Further study is awaited to determine the mechanism of the action of miR-224 on GCs and its link to the TGF-β/Smad pathway.

GCs are the main source of steroidogenesis, such as E_2 and progesterone production in the ovary. E_2 is a vital steroid hormone that regulates numerous physiological processes in female reproduction (39). For example, E_2 plays an obligatory role in normal GC proliferation (40); healthy developing follicles are characterized by increased E_2 secretion (41, 42). Thus, the regulation of steroidogenesis is critical to the development of a dominant follicle and a matured ovum (43). TGF-β superfamily members are suggested to play an important role in steroid production, either alone or in combination with gonadotropins (44, 45). Some miRNAs were recently reported to influence steroid hormone release by human primary ovarian GCs (46). They found that 51 miRNAs suppressed E_2 release, whereas none of the miRNAs tested in their experiments stimulated it (46). Furthermore, they identified that 36 miRNAs inhibited progesterone release in GCs, and 10 promoted progesterone release (46). In this study, we found that both TGF-β1 and miR-224 promoted E_2 release by mGCs, whereas the knockdown of miR-224 abrogated TGF-β1-induced E_2 release. TGF-β1 inhibited progesterone release by mGCs, but miR-224 had no effect on progesterone release. We subsequently explored the mechanisms of miR-224/TGF-β1 effects by quantifying the levels of transcripts for key genes involved in steroid hormone synthesis or release, such as CYP19A1 (a key enzyme in E_2 biosynthesis) and CYP11A1 (a key enzyme in progesterone biosynthesis). The results showed that miR-224/ TGF-β1 increased *CYP19A1* mRNA levels but could not alter *CYP11A1* mRNA levels. This is the first demonstration that miR-224 can enhance E_2 release by ovarian GCs, thus being a potent regulator of ovarian functions.

From the above results, it may be stated that miR-224 can control reproductive functions through enhancing GC proliferation and release of ovarian estrogen, either alone or in combination with TGF-β1. A recent report showed that in MCF-7 cells, E_2 increases cell proliferation and inhibits miR-21 expression in an estrogen receptor-dependent manner (47). Furthermore, this inhibition correlates with up-regulation of miR-21 target genes PDCD4, PTEN, and BCL-2, the key regulators of cell proliferation (47). Whether mechanisms of cooperation between miR-224/ TGF-β1-mediated cell proliferation and E_2 release exist in GCs remains to be elucidated.

The gene target of miR-224 was identified to account for the above-mentioned phenotype in this study. Because Smad4, a critical cofactor of TGF-β signaling pathway, has been shown to induce apoptosis in many cell types (27), we asked whether Smad4 mediates the effect of miR-224 in GCs. Forced expression of miR-224 and knockdown of miR-224 in mGCs decreased and increased Smad4 protein expression levels, respectively. In addition, overexpression of miR-224 reduced Smad4 3′-UTR reporter activity; however, no inhibition of reporter activity was observed when the Smad4 3′-UTR sites matching the seeding sequence of miR-224 were mutated. These results confirmed that Smad4 is a miR-224 target. miRNAs are suggested to negatively regulate their target mRNAs through base-pairing interactions, which leads to either mRNA degradation or translational inhibition (15–17). Our observations that overexpression of miR-224 did not alter *Smad4* mRNA expression levels indicate that Smad4 is regulated by miR-224 at the posttranscriptional level.

Smad4, a common mediator of TGF-β signaling, is suggested to play an important role in the regulation of ovarian follicle growth and female fertility (9). *Smad4* ovarian-specific knockout mice are subfertile with decreasing fertility over time and multiple defects in folliculogenesis, such as severe cumulus cell defects and premature luteinization of GCs (9). In this study, colocalization of Smad4 protein and miR-224 in mGCs at various stages of follicular development was verified, indicating that they may interact with each other. In addition, TGF-β1 reduced Smad4 protein expression through the up-regulation of miR-224, and Smad4 mediated the roles of miR-224 to influence GC proliferation and function. These results not only validate the functional relevance of Smad4 (a target gene of miR-224) in TGF-β1/miR-224-stimulated mGCs, but provide a new molecular mechanism on TGF-β regulation of Smad4. Recently, Wang *et al.* (33) reported that miR-224 influences both the proliferation and apoptotic potential of cells of hepatocellular carcinoma, and the regulation of the apoptotic potential is mediated via *API-5*, a known gene that regulates apoptosis. It has been suggested that a single miRNA may regulate opposing cellular processes such as cell proliferation and apoptosis through different unrelated target genes (33). In the current study, we demonstrate that miR-224 could promote cell proliferation by targeting Smad4. Whether miR-224 regulation of hepatocellular carcinoma cell proliferation potential is mediated via another target Smad4 remains to be elucidated.

In summary, we have identified the miRNA expression signature of TGF-β1-stimulated mouse ovarian GCs. Functional characterization of one of 16 deregulated miRNAs revealed that miR-224 which is up-regulated in TGF-β1-treated cells, mediated TGF-β1-induced GC proliferation and endocrine function through a target Smad4. These findings demonstrate that miRNAs play important biological roles in regulating gene expression during folliculogenesis. For example, miR-224 is likely to be involved in increasing pool of healthy follicles because it promotes granuolosa cell proliferation and E_2 release, *i.e.* it is probably a stimulator for female fertility. These effects of miR-224 on physiological processes within the ovary reveal potential application of miR-224 in the control of reproduction or in the design of nonhormonal contraceptives.

Materials and Methods

Animals

ICR mice were obtained from the Animal Center, University of Science and Technology of China (USTC), and housed in temperature (22 C)- and light cycle (14 h light, 10 h dark)-controlled quarters. Mice were provided food and water *ad libitum*. This study received ethical approval from the institutional review boards of the USTC.

Reagent, cell, and cell culture

Human TGF-β1 (R&D Systems, Minneapolis, MN) was reconstituted in acidified buffer [4 mM HCl (pH 5.7), 1 mg/ml BSA] (48) to prepare 1 mg/ml stock solutions, which was added to media to yield a final concentration. For culture control, media were treated with the acidified buffer alone in equal concentrations. Activin A was kindly donated by Dr. Yanling Wang from the Institute of Zoology, Chinese Academy of Sciences (Beijing, China).

mGCs were isolated from preantral follicles of approximately 10–12 d postpartum ovaries. Preantral follicles, 120–150 μm in diameter (a transition from preantral to early antral stage), were used in this study, because proliferation of GCs of follicles in this size range is the most active (49). Briefly, mouse was killed by cervical dislocation, and the follicles measuring 120–150 μm in diameter were isolated with no. 5 fine needles (Sigma Chemical Co., St. Louis, MO). The follicles were then treated with type IV collagenase (Sigma) and Tryple Express (Life Technologies, Inc., Gaithersburg, MD), and the isolated GCs were cultured in DMEM/F12 (1:1, Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Life Technologies) at 37 C under 5% CO_2 atmosphere. Media were changed every 2 d thereafter, and the cells were used for experiments within passage 4. Mural GCs were collected from 21 d postpartum mice primed with equine chorionic gonadotropin for 48 h. The immature GCs and mural GCs were serum starved for 24 h before TGF-β1 treatment. A steroidogenic human granulosa-like tumor cell line, KGN, was kindly donated by Dr. Yiming Mu (the General Hospital of the People's Liberation Army, Beijing, China). Mural GCs and KGN cell line were grown under the same culture conditions as described in the mGCs.

The HEK293T cell line was kindly donated by Professor Mian Wu (School of Life Sciences, USTC). The cells were grown in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Life Technologies), and cultured at 37 C under 5% CO_2 atmosphere.

miRNA microarray analysis and bioinformatics analysis

The mGCs were serum starved for 24 h and then treated with 5 ng/ml TGF-β1 for 6 h. miRNA expression profiles of mGCs treated and untreated by TGF-β1 were generated by applying the miRCURY LNA microarray platform (Exiqon, Copenhagen, Denmark). All procedures were carried out according to manufacturer's protocol. Briefly, total RNA was extracted from the cells by using a combination of Trizol (Invitrogen, Carlsbad, CA) and RNeasy mini kit (QIAGEN, Valencia, CA) according to manufacturer's instructions. Total RNA (2 μg) was dual labeled with dyes spectrally equivalent to the Cy3 and Cy5 fluorophores, using a miRCURY Array Power Labeling kit (Exiqon). Labeled miRNAs were used for hybridization on a miRCURY LNA miRNA Array containing Tm-normalized capture probes for 1700 miRNAs, perfectly matched probes for all miRNAs in all organisms as annotated in Sanger miRBase Release 11.0 (http://microrna.sanger.ac.uk). Microarrays with labeled samples were hybridized at 56 C for overnight using a heat-shrunk hybridization bag (Phalanx Hybridization Assembly, Phalanx Biotech, Taiwan, China) and washed using miRCURY Array Wash buffer kit (Exiqon).

Slides were scanned using a Genepix 4000B laser scanner (Axon Instruments, Foster City, CA), and microarray images were analyzed using Genepix Pro 6.0 software (Axon Instruments). Differentially expressed miRNAs were defined as genes expression of which in the study group (TGF-β1-treated mGCs) is consistently altered 2-fold (either greater or less) compared with the control group (TGF-β1-untreated mGCs). The 2-fold cut-off is a default for many microarray experiments because it can reflect the variability in the population of samples. Hierarchical clustering for differentially expressed miRNAs was generated by using standard correlation as a measure of similarity.

Putative mRNA targets of differentially expressed miRNAs were predicted by three algorithms: miRBase Targets (http://microrna.sanger.ac.uk/targets/v5/), TargetScan (http://www.targetscan.org/), and PicTar (http://pictar.mdc-berlin.de/). The intersections of the three algorithms were obtained from miRGen (http://www.diana.pcbi.upenn.edu/miRGen.html).

Plasmid construction

To experimentally verify whether the *Smad4* gene is an *in vivo* target of miR-224, the 3′-UTR of the *Smad4* gene was amplified from the genomic DNA of HEK293T cell line using primers as shown in Supplemental Table 1. Mutants Smad4 3′-UTR were also generated on each of the two miR-224 target recognition sites (seed sequences) by site mutations, as shown in Supplemental Fig. 8. Both the wild-type and mutated 3′-UTRs of *Smad4* gene were cloned into the psiCHECK-2 dual luciferase reporter vector using *Sgf* I and *Pme*I restriction sites. For construction of expression vectors for *Smad4* gene, mRNAs were isolated from mouse liver tissues and reversely transcribed to cDNA. The full-length cDNA was amplified by PCR using the primers indicated in Supplemental Table 1 and then cloned into the pEGFP-C1 vectors digested by *Xho*I and *Eco*RI restriction enzymes. The constructs generated were confirmed by sequencing.

Transient transfection and luciferase activity assay

Primary mGCs and KGN cells were transfected with either miRNA mimics/inhibitors or plasmids using HiPerFect Transfection Reagent (QIAGEN GmbH, Hilden, Germany) following the manufacturer's protocol. HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen). The miRNA mimics are chemically synthesized, double-stranded RNAs that mimic mature endogenous miRNAs after transfection into cells, whereas the miRNA inhibitors are chemically modified antisense RNA oligonucleotides optimized to specifically target miRNA molecules in cells. miRNA mimics and inhibitors were chemically synthesized by Shanghai GenePharma (Shanghai, China). miRNAs and/or DNA plasmids were diluted in Opti-MEM I reduced serum medium (Life Technologies). Briefly, the day before transfection, cells were plated with normal growth medium such that they were 70–80% confluent at the time of transfection. The time when transfection commenced was considered as time 0. After incubation in medium containing transfection reagent for 12 h, the media were changed into normal growth medium.

For reporter assays, HEK293T cells were transiently transfected with reporter constructs together with miRNA mimics/inhibitors and mimics/inhibitor control. Cell extracts were prepared 30 h after transfection, and the ratio of *Renilla* to firefly luciferase was measured with the Dual-Luciferase Reporter Assay System (Promega Corp.).

Cell proliferation assay and hormone analysis

Cell proliferation assays were performed by using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Cells were seeded in 96-well plates at approximately 1–1.5 $\times$ 10^4 cells per well and cultured in the growth medium. At the indicated time points, each well were added with 10 μl of the CCK-8 solution and then incubated for 2 h. The cell numbers were measured in a 96-well format plate reader (ELX 800 universal microplate Reader; BioTek, Inc., Highland Park, IL) done in triplicate by measuring the absorbance at a wavelength of 450 nm (OD_{450}).

To determine the effects of TGF-β/miRNAs on GC function, concentrations of E_2 and progesterone in culture medium were determined by using the Access Immunoassay System (Beckman Coulter, Inc., Brea, CA), an automated random-access chemiluminescence-based assay. The intra- and interassay coefficients of variation were less than 10% and 15%, respectively.

Real-time PCR assay

For real-time PCR assays, total RNA was extracted from cultured cells using Trizol (Invitrogen). cDNA was synthesized from 500 ng of purified RNA using a PrimeScript RT reagent kit (TaKaRa Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. Real-time PCR was performed in an Applied Biosystems StepOne real time PCR system using a SYBR Premix Ex Taq II Kit (Takara Bio, Inc., Shiga, Japan). Each sample was analyzed in triplicate and the experiment was repeated three times. The primers for Smad4, CYP19A1, CYP11A, mouse pri-miR-224, mouse pre-miR-224, GAPDH, and β-actin are listed in supplemental Table 1. PCR conditions were as follows: 95 C for 15 sec, followed by 40 cycles at 95 C for 5 sec, 60 C for 34 sec, and 95 C for 15 sec. Expression levels were normalized for GAPDH expression. Taqman miRNA Assays (Applied Biosystems, Foster City, CA) were used to quantify mature miR-224 expression levels. PCR conditions were as follows: 95 C for 10 min, followed by 40 cycles at 95 C for 15 sec and 60 C for 1 min. Data were analyzed by using the comparative C_T method (50), and the expression levels of miR-224 were normalized to the endogenous control U6 small nuclear RNA (snRNA).

Western blotting

Cells were rinsed with ice-cold PBS (pH 7.4) and lysed with lysis buffer radioimmune precipitation assay buffer [(50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Triton X-100; 1% sodium dodecyl sulfate; 1% sodium eoxycholate and 1 mM EDTA)], proteinase inhibitors (10 μg/ml each of aprotinin, pepstatin, and leupeptin) and 1 mM phenylmethylsulfonyl fluoride). Protein lysates were separated by SDS-PAGE, transferred to Hybond ECL Nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany), immunoblotted with antibodies, and visualized using a chemiluminescence detection system (Eastman Kodak, Rochester, NY). The following antibodies were used for immunblotting: anti-Smad4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Smad2/3 (Cell Signaling Technology, Beverly, MA), anti-Drosha (Abcam, Cambridge, MA), and anti-GAPDH (Cell Signaling Technology).

Immunohistochemistry and *in situ* hybridization

To determine the cellular localization of Smad4 protein, immunohistochemical analysis on ovarian sections from 21-d-old female mice was performed. The paraffin-embedded ovaries were serially sectioned at 6 μm thickness and placed on glass slides. Sections were then incubated overnight at 4 C with anti-Smad4 monoclonal antibody (Santa Cruz Biotechnology) at 1:300 dilution, followed by the incubation with biotinylated secondary antibody (Abcam) for 2 h at room temperature. The Smad4 antigen was visualized by using streptavidin-peroxidase and 3,3′-diaminobenzidine (Maixin Bio, Fuzhou, China). The sections were counterstained with hematoxylin. A negative control was included in which the primary antibody was omitted.

Expression of miR-224 in the follicles of adult female mice was performed by *in situ* hybridizations using 5′-end digoxigenin-labeled LNA-modified DNA oligonucleotides (LNAs) complementary to the mature miRNA supplied by Exigon A/S, as described by Lian *et al.* (51). LNAs had the following sequence: LNA-miR-224, 5′-aacggaaccactagtgactta-3′. Furthermore, LNA-scrambled, 5′-gtgtaacacgtctatacgccca-3′ serves as negative control. Briefly, ovarian cryosections were prehybridized for 6 h at 52 C with 700 μl prehybridization buffer (50% formamide, 5× saline sodium citrate, 5× Denhardt's, 200 μg/ml yeast RNA, 500 μg/ml salmon sperm DNA, 2% Roche blocking reagents, and diethylpyrocarbonate-treated water). Sections were then overlaid with 150 μl hybridization buffer (prehybridization buffer containing 1 pmol LNA probes) and incubated overnight at 52 C in a humidified chamber. After hybridization, sections were washed with B1 buffer three times. Hybridization signals were detected using anti-Digoxigenin-alkaline phosphotase fragments of antigen-binding fragments (Roche, Indianapolis, IN; 1:250) and the mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega).

Statistical analysis

Data presented in this study are the average of at least three independent experiments, each performed in triplicate, ± SEM. Statistical analysis was performed by Student's *t* test, using SAS Software. $P < 0.05$ was considered to be statistically significant.

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