miR-375 mediates CRH signaling pathway in inhibiting E2 synthesis in porcine ovary

Chulin Yu*, Meiling Li*, Yue Wang, Ying Liu, Chengzhi Yan, Jirong Pan, Jiali Liu and Sheng Cui

State Key Laboratory of Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing, People's Republic of China

Correspondence should be addressed to S Cui; Email: cuisheng@cau.edu.cn

*(C Yu and M Li contributed equally to this work)

Abstract

The corticotropin-releasing hormone (CRH) signaling system is involved in numbers of stress-related physiological and pathological responses, including its inhibiting effects on estradiol (E2) synthesis and follicular development in the ovary. In addition, there are reports that microRNAs (miRNAs) can control the function of animal reproductive system. The aim of present study was to investigate the functions of miR-375 and the relationship between miR-375 and CRH signaling molecules in the porcine ovary. First, our common PCR results show that miR-375 and the CRH receptor 1 (CRHR1) are expressed in porcine ovary, whereas CRH receptor 2 (CRHR2) is not detected. We further have located the cell types of miR-375 and CRHR1 by *in situ* hybridization (ISH), and the results show that miR-375 is located only in the granulosa cells, whereas CRHR1 is positive in all of granulosa cells and oocytes, inferring that miR-375 and CRHR1 are co-localized in granulosa cells. Second, we show that overexpression of miR-375 in cultured granulosa cells suppresses the E2 production, whereas miR-375 knockdown demonstrates the opposite result. Besides, our *in vitro* results demonstrate that miR-375 mediates the signaling pathway of CRH inhibiting E2 synthesis. Finally, our data show that the action of miR-375 is accomplished by directly binding to the 3'UTR of specificity protein1 (SP1) mRNA to decrease the SP1 protein level. Thus, we conclude that miR-375 is a key factor in regulating E2 synthesis by mediating the CRH signaling pathway.

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Introduction

CRH is a 41 amino acid neuropeptide associated with stress response, and it is secreted mainly by the hypothalamus. The main function of CRH is to stimulate the release of adrenocorticotrophic hormone (ACTH) in the pituitary, which in turn enhances the synthesis and secretion of cortisol in the adrenal gland. There are reports that CRH also performs functions in nervous system (Chen *et al.* 2005a, 2014), immune system (Karalis *et al.* 1991, Crofford *et al.* 1992), metabolic system (Carlin *et al.* 2006, Huising *et al.* 2010, Stengel & Tache 2014) and reproduction system (Polkowska & Przekop 1997, Erden *et al.* 1998). It is documented that CRH and its receptors have been detected in testis (Yoon *et al.* 1988), the ovary (Mastorakos *et al.* 1993) and the uterus (Makrigiannakis *et al.* 1995, Zoumakis *et al.* 1996), which suggests that CRH could directly affect the gonadal functions, including the development of germ cells and steroidogenesis in both male and female gonads (Ulissee *et al.* 1989, Ghizzoni *et al.* 1997, Kiapekou *et al.* 2011, Dinopoulou *et al.* 2013). However, the mechanism of CRH affecting steroidogenesis remains unclear.

It is known that CRH functions by activation of its two known receptors, including CRHR1 and CRHR2, which are typical G-protein-coupled receptors (GPCR) containing seven transmembrane domains. The binding of CRH to the CRHRs stimulates adenylyl cyclase, increases intracellular cAMP and calcium levels and activates protein kinase-A (Spiess *et al.* 1998), which subsequently activates MAP kinase in most of the CRH-targeting cell types. In addition, it has been reported that miRNAs, such as miR-34 (Haramati *et al.* 2011), miR449a (Nemoto *et al.* 2013) and miR-375 (Zhang *et al.* 2013), may modulate cell responsiveness to CRH, and the related mechanisms are under extensive study.

miRNAs are non-coding RNAs containing 20–22 nucleotides, which reduce mRNA stability and block mRNA translation through fully or partially complementary sequence with mRNA 3'UTR (Landgraf *et al.* 2007). Previous studies have shown that miRNAs serve important roles in regulating ovary functions, such as, follicular development (Sen *et al.* 2014), cell proliferation and apoptosis (Sirotkin *et al.* 2010), and steroid hormone synthesis (Sirotkin *et al.* 2009). One such miRNA, miR-21, blocks periovulatory granulosa

cell apoptosis by decreasing cleaved caspase3 (Carletti *et al.* 2010). miR-378 negatively regulates E2 synthesis by targeting aromatase in granulosa cells (Xu *et al.* 2011), and miR-375 is expressed in developmental stages of follicles, suggesting an important role in the follicular growth or ovulation mechanism (Kang *et al.* 2013). However, the functions of miR-375 in the ovary are still unknown. In addition, our previous studies have shown that miR-375 is involved in the CRH signaling pathway, depressing proopiomelanocortin (POMC) mRNA and protein levels in the mouse pituitary (Zhang *et al.* 2013). With this in mind, we hypothesize that there are some interactions or crosstalk between miR-375 and CRH signaling molecules in the ovary, which are crucial for our understanding the role of miR-375 affecting animal reproduction.

The present study was thus designed to investigate the miR-375 expression, functions and relationship to the CRH signaling pathway in the ovaries of pigs, which are an important farm animal and potential animal models for the related human medical research. Our results show that miR-375 is stimulated by CRH signaling and acts as a major mediator in inhibiting E2 synthesis by targeting *SP1*. These findings provide further insight into the roles and mechanisms of CRH functions in steroid hormone synthesis and provide molecular targets for exploring methods of treatment for steroid-dependent reproductive disorders.

Materials and methods

Animals

The *in vivo* experiments of this study were carried out by using 8 adult female pigs, randomly divided into the experimental group ($n=4$) and controls ($n=4$). Intravenous injections of 0.9% NaCl were administered to the controls, and injections of 50 µg CRH (1151; TOCRIS, Minneapolis, MI, USA) were administered to the experimental group (Salak-Johnson *et al.* 2004). Two hours after injection, the pigs were killed, and the blood and ovary samples were collected. The protocols pertaining to animal experiments, as we apply to this study, were approved by Chinese Animal Care and Use Committee of China Agricultural University.

Tissue collection and cell culture

The ovaries for the *in vitro* experiments were obtained from a local slaughterhouse and transported to the laboratory within 2 h of harvest and were maintained at 37°C in a sterile physiological saline solution (0.9% NaCl, 100 IU/mL penicillin, 100 IU/mL streptomycin). The collected tissues were then rinsed 3 times with physiological saline solution. For *in situ* hybridization, the collected porcine ovaries were fixed in 4% paraformaldehyde for 24 h and then placed in 30% sucrose overnight at 4°C. The tissues were embedded in Tissue Tek O.C.T. compound (4583, SAKURA, Torrance, CA, USA), and 10 µm sections were cut. For the cell culture, the

follicular fluid was aspirated from 3 to 5 mm follicles using a 20 mL syringe and centrifuged at 500g for 5 min and granulosa cells (GCs) were then collected from the bottom of centrifuge tubes. The GCs were washed with serum-free DMEM/F12 (Invitrogen) culture medium for 3 times. The cells were then pipetted up and down several times to disperse the cells. Subsequently, cell viability was determined using Trypan blue dye (T6146, Sigma), and the cells were seeded with 1×10^5 per well in 12-well plates in 1 mL DMEM/F12 supplemented with 10% fetal bovine serum (Invitrogen). The cells were cultured in a highly humidified atmosphere of 95% air and 5% CO₂ at 37°C. The porcine GCs were cultured in 10% fetal calf serum-DMEM/F12 for 72 h before further treatment, and culture medium was refreshed each 24 h.

Plasmids construction and luciferase reporter assay

The 160bp region of the *SP1* gene 3'UTR from 1139bp to 1499bp and Liver Receptor Homolog-1 (LHR1) gene 3'UTR from 237bp to 397bp 3'UTR sequences were cloned by overlap PCR. The sequences were constructed into the psiCHECK-2 vector (Promega) between the XhoI and NotI sites, and mutant 3'UTR of *SP1* and *LHR1* were also inserted as negative controls. All plasmids were transfected into the 293T cells for 24 h, and luciferase activity was analyzed by using dual-luciferase reporter assay kit (E1910; Promega) on a Modulus II Microplate Multimode Reader (Turner Biosystems, Sunnyvale, CA, USA) in accordance with the manufacturer's recommendations.

Transient transfection

The miR-375 mimic (forward, 5'-UUUGUUCGUUCGGCU CGCGUGA-3'; reverse, 5'-ACGCGAGCCGAACGAACAAA UU-3') and miR-375 inhibitor (5'-UCACGCGAGCCGAA CGAACAAA-3') were purchased from RiboBio (Guangzhou, China). The nc mimic (nc-mi) (forward, 5'-UUCUCCGAACG UGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGG AGAATT-3') and nc inhibitor (nc-in) (5'-CAGUACUUUUGU GUAGUACAA-3') were used as negative control. The *SP1* siRNA sequence (5'-GCAGCUACCUUGACUCCUATT-3') and the nc sequence (5'-UUCUUCGAACGUGUCACGUTT-3') were synthesized from GenePharma (Shanghai, China). The expression vector for *SP1* (pEGFP-*SP1* plasmid) was bought from Addgene (Cambridge, MA, USA) (Shen *et al.* 2009).

Transient transfections were conducted by using Lipofectamine 2000 Reagent (Invitrogen) in accordance with the manufacturer's recommendations. All transfection experiments were conducted at least three times.

Radioimmunoassay (RIA)

The porcine GCs culture medium was replaced by serum-free DMEM/F12 for 12 h, followed by the addition of different concentrations of CRH (1151; TOCRIS) for 12 h before harvest. E2 and P4 levels in the culture medium were measured using RIA reagent purchased from the Beijing North Institute of Biological Technology (Beijing, China). The RIA was performed following the manufacturer's recommendations. The minimum

detectable concentration of E2 was 2 pg/mL and 0.2 ng/mL for P4.

Western blot

Protein samples extracted from the cultured cells were subjected to 12% SDS-PAGE and immunoblotted as described previously (Li *et al.* 2014). For SP1 analysis, the membranes were incubated with rabbit anti-SP1 antibodies (1:200; BOSTER, Wuhan, China) and mouse anti-GAPDH antibodies (1:10,000; Ambion) overnight at 4°C. The membranes were washed in Tris-buffered saline with 0.1% Tween-20 (TBST) for 30 min and TBST was changed every 10 min, and then the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse antibodies (1:5000; Zymed Laboratories, Carlsbad, CA, USA) at room temperature for 2 h. After being washed in TBST for 30 min, the membranes were exposed to nitroterazolium Blue chloride (NBT)/5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) until desired signals were achieved. We used the ImageJ2x Software to assay the relative intensity of each blot. The intensity values of each group were normalized to GAPDH (internal control) in the same group.

Common PCR and Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from the cultured cells by RNAiso Plus (9109; TaKaRa), and then 1 µg purified RNA was used for cDNA synthesis by Reverse Transcriptase M-MLV reagent (M170A; Promega). Reverse transcription was performed as its manufacturer's protocol. No-template control was included in all reverse transcription. The miRNA expression was analyzed by the method described previously (Chen *et al.* 2005b). RT-qPCR was carried out by SYBR premix Ex Taq (DRR420A; TaKaRa) in the ABI 7500 Real-time PCR system (Applied Biosystems). PCR conditions were as follows: 95°C for 10 min; 95°C for 15 s and 60°C for 1 min (40 cycles). Relative RNA quantifications were normalized to endogenous control GAPDH. The primer sequences are shown in Table 1.

CRHRs and miR-375 expression were detected by common PCR using the primer sequences shown in Table 1. And the common PCR (Bio-Rad Laboratories) was performed using the following protocol: 95°C for 5 min; 95°C for 30 s, 60°C for 30 s, 72°C for 30 s (35 cycles); 72°C for 5 min; 4°C holding. PCR products were observed on a 2% agarose gel.

In situ hybridization (ISH)

A digoxigenin-labeled locked nucleic acid (LNA) probe (38181-05; Exiqon, Woburn, MA, USA) was applied to miR-375 ISH. The CRHR1 ISH probe was 486 bp cRNA generated by DIG RNA Labeling Kit (11175025910; Roche Applied Science). The ISH was performed as described previously (Birnbacher *et al.* 1998, Javelle & Timmermans 2012). The samples were fixed for 10 min in 4% paraformaldehyde and treated with 5 µg/mL proteinase K for 5 min, followed by acetylation for 10 min. The sections were pre-hybridized at room temperature for 4 h, and then miR-375 and CRHR1 were hybridized at 57°C and 65°C

Table 1 Primer names and sequences.

Primer names	Sequence
U6	
RT primer	AACGCTTCACGAATTTGCGT
Sense	CTCGCTTCGGCAGCAC
Antisense	AACGCTTCACGAATTTGCGT
miR-375	
RT primer	CTCAACTGGTGTCGTGGAGTCGGCAATTCGTTGA GAGCGCACT
Sense	ACAGAGATTGTTCGTTCCGGC
Antisense	CTCAACTGGTGTCGTGGAGTC
Pri-miR-375	
Sense	CTCTCCGACCCGTATGGC
Antisense	CGTTTGTTCGTTCCGGCTCG
CRHR1	
Sense	AACTCCATCTCCGCCTCC
Antisense	GCCGTTGTTGTGGTATTGT
CRHR2	
Sense	TCTTGGACGGTTGGG
Antisense	CATTCTCGGTAGGCATT
SP1	
Sense	GACAGTGGGGCAGGTT
Antisense	GCAAGACGGGCAATAC
GAPDH	
Sense	GGTGAAGGTCTGGAGTGAACG
Antisense	CCTTGACTGTGCCGTGGAAT
CYP19A1	
Sense	GTGACCGTCTGTGCCG
Antisense	CCCCAGGAAGAGGTTGTTA
CYP11A1	
Sense	GAACACGGAGGTAATGG
Antisense	AGGCGAAGCGAAACA
HSD3B	
Sense	CGCCTGCGAAGAAGA
Antisense	AGAGCCCAGCCGTTA
HSD17B	
Sense	AGAGTCTGGCGGTTGTG
Antisense	GCGGGAGAAGAGGTCA

overnight. The signal was detected by an alkaline phosphatase-conjugated anti-DIG antibody (11093274910; Roche). The sections were incubated in NBT/BCIP at 4°C for several hours until the desired signal was achieved and then photographed under a microscope (IX71, Olympus).

MicroRNA target prediction

The two computational prediction algorithms used were miRanda (www.microrna.org) and pictar (pictar.mdc-berlin.de), and human SP1 and LHR1 were predicted to be the targets of miR-375. Notably, the predicted miR-375 target sites in both SP1 and LHR1 were conserved by homology comparison. Subsequently, we used the RNAhybrid to predict the minimum free energy of hybridization between miR-375 and its putative target genes.

Statistical analysis

Statistical analysis in this study was conducted using GraphPad Prism 6.0. Two group comparisons were performed by Student's *t*-test. More than two groups were compared using a one-way analysis of variance (ANOVA) followed by Student's *t*-test. All experiments were independently conducted for at least three times. All values were expressed as means $\pm$ S.E.M. *P* < 0.05 was considered significant.

Results

Expressions of miR-375 and CRHRs in the porcine ovary

To investigate the functional relationship between miR-375 expression and the CRH signaling pathway in the porcine ovary, we initially examined the expressions of miR-375, CRH receptors, including *CRHR1* and *CRHR2*, in the porcine ovary by common PCR. The results showed that miR-375 and *CRHR1* were expressed in the porcine ovary, whereas *CRHR2* was not detected (Fig. 1A, C). Further, we located the *CRHR1* and miR-375 by ISH, and the results showed that miR-375 was located only in the granulosa cells (Fig. 1B), whereas *CRHR1* was positive in all the granulosa cells and oocytes (Fig. 1D). These results indicate that miR-375 and *CRHR1* are co-expressed in porcine granulosa cells and suggest that miR-375 is potentially involved in the regulating effects of CRH on porcine granulosa cells.

Both miR-375 and CRH negatively regulate E2 synthesis

To determine the effects of miR-375 on porcine granulosa cells, miR-375 was over-expressed and knocked down by transfecting the miR-375 mimic and inhibitor respectively. The results showed that the miR-375 mimic increased miR-375 level by many thousands of times, and the miR-375 inhibitor decreased the miR-375 level by ~66% compared with the control, but had no effect on miR-125b and

miR-143 expression (Fig. 2A, B). As the production of E2 and P4 are the important functions of the granulosa cells, we assayed the effects of the miR-375 mimic and inhibitor on E2 and P4 levels. The RIA results revealed that the miR-375 mimic decreased E2 level in the culture medium by ~47% (Fig. 2C), whereas the miR-375 inhibitor increased E2 level ~1.4 times. However, it was out of our expectation that neither the miR-375 mimic nor inhibitor would have significant effects on P4 level (Fig. 2D). These results suggest that miR-375 specifically inhibits E2 synthesis in porcine granulosa cells. Further, we assayed the expressions of the related enzymes that are required for E2 synthesis, including 17 beta-hydroxysteroid dehydrogenase (*HSD17B*) and *CYP19A1*, 3 beta-hydroxysteroid dehydrogenase (*HSD3B*), cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*) by RT-qPCR. The results showed that miR-375 overexpression inhibited the *CYP19A1* mRNA level by ~37%, but it increased ~1.9 times when miR-375 was knocked down. However, neither the miR-375 mimic nor the miR-375 inhibitor had significant effects on *HSD17B*, *HSD3B* or *CYP11A1* expression (Fig. 2E, F). These results indicate that miR-375 negatively regulates E2 synthesis by affecting *CYP19A1* expression in porcine granulosa cells.

It is thought that CRH has negative effects on ovary steroidogenesis (Calogero *et al.* 1996, Dinopoulou *et al.* 2013). To confirm the CRH functions in porcine granulosa cells, the cultured cells were treated with CRH for 12 h. The results showed that 100 nM CRH decreased E2 level by 42% in the culture medium. Further, we administrated 50 µg CRH to the adult pigs for 2 h (Salak-Johnson *et al.* 2004), and were then assayed to know the effects of CRH on E2 synthesis. The results showed that the CRH decreased global E2 concentration by 25% *in vivo* (Fig. 2G, J). We also examined the related enzyme expression. The RT-qPCR results showed that *CYP19A1* mRNA were decreased after CRH treatment both *in vitro* and *in vivo*, whereas the CRH had no obvious effect on the expressions of *HSD17B*, *HSD3B* and *CYP11A1* (Fig. 2H, K). Besides, our Western blot results demonstrated that the protein level of *CYP19A1* was decreased after CRH treatment both *in vitro* and *in vivo* (Fig. 2I, L). These data demonstrate that both miR-375 and CRH play negative roles in E2 synthesis in the porcine ovary.

CRH upregulates miR-375 expression via PKA-p38 MAPK signaling pathway

To examine whether CRH signaling affects miR-375 expression in porcine granulosa cells, cultured cells were treated with 0, 0.1, 1, 10 and 100 nM CRH for 12 h (Ghizzoni *et al.* 1997). The results showed that 100 nM CRH increased Pri-miR-375 and miR-375 expression in cultured cells by ~3 times (Fig. 3A, B). Further, to confirm the physiological significance of CRH and

Figure 1 Expressions of miR-375 and CRHRs in the porcine ovary. (A) miR-375 expression was examined by common PCR (U6 small nuclear RNA amplified as internal reference) in the porcine ovary and granulosa cells (GCs), and adrenal is positive control. (B) miR-375 expression was located by ISH in the porcine ovary. (C) *CRHR1* and *CRHR2* were detected in the porcine ovary and granulosa cells (GCs) by common PCR. (D) *CRHR1* was localized in the porcine ovary by ISH. Scale bar is 50 µm.

Figure 2 Effect of miR-375 on steroid hormone synthesis in cultured porcine granulosa cells. (A and B) Quantification of intracellular miR-375, the miR-125b, and the miR-143 levels were assayed by RT-qPCR after the cultured granulosa cells transfected with the miR-375 mimic (miR-375-mi) (A) and miR-375 inhibitor (miR-375-in) (B) for 24 h. Con was non-transfection control. The versus cells were transfected with nc mimic (nc-mi) and nc inhibitor (nc-in) as negative control. (C and D), E2 (C) and P4 levels (D) in the culture medium were assayed by RIA after transfected with the miR-375 mimic or inhibitor for 24 h. (E and F) Quantification of intracellular *HSD17B*, *CYP19A1*, *HSD3B* and *CYP11A1* mRNA expressions were analyzed by RT-qPCR after the cultured cells transfected with the miR-375 mimic (E) or inhibitor (F) for 24 h. (G) E2 levels in the culture medium were measured by RIA after the cultured granulosa cells exposed to 0, 0.1, 1, 10 and 100 nM CRH for 12 h. (H) Quantification of intracellular *HSD17B*, *CYP19A1*, *HSD3B* and *CYP11A1* mRNA levels were analyzed by RT-qPCR after the cultured granulosa cells were exposed to 100 nM CRH for 12 h. (I) The CYP19A1 protein levels were examined by Western blot after the cultured granulosa cells were exposed to 100 nM CRH for 12 h. (J, K and L) After the pigs injection with CRH for 2 h, E2 levels in serum were assayed by RIA (J), *HSD17B*, *CYP19A1*, *HSD3B* and *CYP11A1* mRNA were assayed by RT-qPCR (K), and CYP19A1 protein levels were analyzed by Western blot (L). Data are as shown as means $\pm$ S.E.M. ($n=4$). Significance differences are indicated by $*P<0.05$, and NS means not significant (t test).

miR-375, the *in vivo* results showed that the CRH increased the ovary Pri-miR-375 and miR-375 levels by ~ 3 times (Fig. 3C, D), consistent with the *in vitro* results.

To determine the signaling pathway involved in CRH upregulating miR-375 expression, we pre-incubated the cultured cells with the inhibitors of the reported key

Figure 3 MiR-375 is enhanced by CRH both *in vitro* and *in vivo*. (A and B) Quantification of intracellular Pri-miR-375 and miR-375 levels were analyzed by RT-qPCR after the cultured granulosa cells exposed to 0, 0.1, 1, 10 and 100 nM CRH for 12 h respectively. (C and D) Quantification of intracellular Pri-miR-375 and miR-375 levels were analyzed by RT-qPCR after the pigs injection with CRH for 2 h. (E) After several signal pathway inhibitors including 20 μ M of H89 (PKA), PD98059 (ERK), SB203580 (p38 MAPK) and SP600125 (JNK) (final concentration 20 μ M) and 100 nM CRH treatment for 12 h, intracellular miR-375 levels in the granulosa cells were assayed by RT-qPCR. Data are as shown as means $\pm$ S.E.M. ($n=3$). Significant differences are indicated by * $P<0.05$ and NS means not significant (t test).

downstream molecules of the CRH signaling pathways (Dermitzaki *et al.* 2002, Park *et al.* 2005, Cao *et al.* 2006), including 20 μ M of H89 (PKA), PD98059 (ERK), SB203580 (p38 MAPK) and SP600125 (JNK) for 1 h before CRH treatment. The results showed that only PKA inhibitor H89 and p38 MAPK inhibitor SB203580 blocked the stimulation of miR-375 expression by CRH (Fig. 3E). Further, we used different doses of H89, SB203580 and LY2228022 (p38 MAPK) to pre-treat the cultured cells for 1 h before 100 nM CRH treatment. The results demonstrated that the inhibitors attenuate the effect of CRH-stimulating miR-375 expression, which showed dose-dependent effects. These data indicate that CRH enhances miR-375 expression through the PKA-p38 MAPK signaling pathway in porcine granulosa cells.

MiR-375 mediates CRH negatively regulating E2 synthesis

To find out whether miR-375 was involved in CRH regulating E2 synthesis, the cultured cells were

transfected with the miR-375 mimic or inhibitor for 24 h before adding 100 nM CRH for another 12 h. The RIA results demonstrated that both the miR-375 mimic and CRH inhibited E2 levels (Fig. 4A), whereas these inhibitory effects of CRH were reversed by the miR-375 inhibitor (Fig. 4B). These data demonstrate that CRH inhibits E2 synthesis mainly through the miR-375 in porcine granulosa cells.

SP1 is a direct target of miR-375 involved in CRH signaling pathway

To determine the potential target of miR-375 affecting E2 synthesis in porcine granulosa cells, we used computational prediction algorithms, including miRanda and pictar. Among the putative target genes, *SP1* and *LHR1* play important roles in ovarian steroid hormone synthesis. Because the porcine *SP1* sequence was not available on the NCBI website, we designed the primers for the porcine *SP1* 3'UTR region using the available human and mouse *SP1* homologous sequences, and the PCR product was sequenced. The results showed that the miR-375-binding site in *SP1* 3'UTR was conserved between human and pigs (Fig. 5A). Based on porcine *SP1* and

Figure 4 Effects of the miR-375 and CRH on E2 synthesis in the porcine granulosa cells. (A) E2 levels in the culture medium were assayed by RIA after the cells were transfected with the miR-375 mimic for 24 h and added 100 nM CRH for another 12 h. (B) E2 levels in the culture medium were assayed by RIA after the cells were transfected with the miR-375 inhibitor for 24 h and added 100 nM CRH for another 12 h. Data are as shown as means $\pm$ S.E.M. ($n=3$). Significant differences are indicated by * $P<0.05$, and NS means not significant (t test).

LHR1 3'UTR sequences, the putative miR-375-binding sites in *SP1* and *LHR1* 3'UTR were predicted (Fig. 5B). Further, we used the RNAhybrid to predict the minimum free energy of hybridization between miR-375 and predicted targets (Fig. 5C). The putative targets' 3'UTR

(wild type and mutant type) were then cloned into the psiCHECK-2 vector at downstream of the luciferase gene (Fig. 5D). The dual-luciferase reporter assay showed that the luciferase activity was suppressed by ~30% in 293T cells after co-transfecting with the miR-375 mimic and

Figure 5 The direct target gene of miR-375 in the porcine granulosa cells. (A) The miR-375-binding site in *SP1* mRNA 3'UTR of pigs. (B) The predicted miR-375 target sites of 3'UTR in *SP1* and *LHR1* mRNA, the seed sequence labeled as red. (C) The minimum free energy of hybridization between miR-375 and *SP1* mRNA was predicted by the RNA hybrid. (D) Schematic representations of 3'UTR insert sites of target genes in the psiCHECK-2 vector. (E and F) The 293T cells were co-transfected with the miR-375 mimic and 3'UTR of putative targets (wild type or mutant) for 24 h, and luciferase activities were measured by using dual-luciferase reporter assay kit. (G, H, I) Quantification of intracellular of *SP1* mRNA and protein levels were respectively analyzed by RT-qPCR (G) and Western blot (H, I) after the cells transfected with the miR-375 mimic or inhibitor for 24 h. Data are as shown as means $\pm$ S.E.M. ($n=4$). Significant differences are indicated by * $P<0.05$, and NS means not significant (t test).

Figure 6 The functions of *SP1* in regulating E2 synthesis affected by CRH. (A and B) Quantification of intracellular of *SP1* mRNA (A) and protein level (B, C) in cultured cells were assayed followed by 100nM CRH treating for 0, 3, 6 and 12 h respectively. (D and E) The cultured cells were transfected with the miR-375 inhibitor for 24 h before 100 nM CRH treatment for 12 h, and then the SP1 protein levels were examined by Western blot. (F and G) The *SP1* mRNA levels were analyzed by RT-qPCR after the cells were transfected with *SP1* siRNA and the pEGFP-*SP1* plasmid for 24 h. (H) E2 levels were examined after the *SP1* siRNA transfection for 24 h and added with 100 nM CRH for another 12 h. (I) E2 levels were measured by RIA after the pEGFP-*SP1* plasmid transfection for 24 h and added 100 nM CRH for another 12 h. Data are as shown as means $\pm$ S.E.M. ($n=3$). Significant differences are indicated by $*P<0.05$, and NS means not significant (t test).

p-Luc-*SP1* 3'UTR, comparing to the cells co-transfected with the miR-375 mimic and p-Luc-*SP1* mutant 3'UTR (Fig. 5E); however, *LHR1* showed no obvious differences (Fig. 5F). In addition, the Western blot results showed that miR-375 overexpression significantly decreased SP1 protein level and its knockdown significantly increased the SP1 protein level (Fig. 5H and I). However, either miR-375 overexpression or knockdown had no significant effect on *SP1* mRNA level (Fig. 5G). These data indicate that *SP1* is a direct target of miR-375 involved in the CRH signaling pathway in porcine granulosa cells.

SP1 is involved in CRH signaling pathway in regulating E2 synthesis

To investigate whether *SP1* acts as a signaling molecule in the CRH regulating E2 synthesis signaling pathway, the cultured cells were treated with CRH for 0, 3, 6 and 12 h respectively. The RT-qPCR and Western blot results showed that both *SP1* mRNA and protein levels were downregulated after 12 h CRH treatment (Fig. 6A, B, C). Further, the cultured cells were transfected with the miR-375 inhibitor for 24 h before adding 100 nM CRH for another 12 h, and the results showed miR-375 reversing

the negative effect of CRH on SP1 protein (Fig. 6D, E). To confirm the functions of *SP1* ovarian steroidogenesis, *SP1* was respectively knocked down and over-expressed by transfecting *SP1* siRNA and expression vector. The RT-qPCR results showed that the *SP1* expression vector significantly increased *SP1* mRNA level ~1.5 times and *SP1* siRNA decreased *SP1* expression by ~60% (Fig. 6F, G). Our results further showed that both *SP1* knockdown and CRH decreased E2 synthesis in the culture medium (Fig. 6H), and the inhibitory effects of CRH on the E2 level were reversed by the overexpression of *SP1* (Fig. 6I). These results demonstrate that CRH enhanced miR-375 and decreased E2 production through miR-375 by targeting SP1.

Discussion

miR-375 expression has been detected in the ovary (Kang *et al.* 2013), but its location and function in the porcine ovary have not been studied. Detection of miR-375 in porcine ovary shows that miR-375 is localized in porcine granulosa cells, suggesting that miR-375 plays regulatory roles in steroid hormone production in granulosa cells. Our *in vitro* results further demonstrate that miR-375 negatively affects E2 production, which is involved in CRH signaling pathway. This evidence suggests that miR-375 mainly acts as an intermediary molecule in CRH regulation of E2 synthesis, but much more *in vivo* experiments are required to reveal how miR-375 affects reproductive functions.

The present study shows that miR-375 specifically decreases E2 synthesis in porcine granulosa cells but has no obvious influence on P4 levels. It is known that E2 plays key roles in female animal reproductive processes, which can be synthesized in granulosa cells. In support, our results here show that miR-375 only affects the expression of *CYP19A1*, the key enzyme of E2 synthesis. However, we still do not know the mechanisms of miR-375's affecting *CYP19A1* expression and E2 synthesis.

Dinopolou and coworkers (Dinopoulou *et al.* 2013) have reported that both *Crhr1* and *Crhr2* mRNA were detected in mouse preantral follicles, but the *Crhr1* expression was significantly higher than that of *Crhr2*. Our results show that only *CRHR1* is expressed in the porcine ovary, whereas *CRHR2* mRNA is not detected. These findings suggest that *CRHR1* may be involved in a more important role in the regulation of ovarian functions. With regard to steroidogenesis, it is reported that CRH suppresses FSH-promoted E2 production in the rat granulosa cells at a concentration of 10 nM, whereas 0.1 nM CRH significantly inhibits E2 release in the human granulosa-lutein cells (Calogero *et al.* 1996, 2002). And in our study, the results show that 100 nM CRH inhibits *CYP19A1* expression and E2 levels in the porcine ovary, which could be probably due to different animal species showing different sensibilities to CRH.

The results presented here demonstrate that *CRHR1* co-expresses with miR-375 in porcine granulosa cells. We infer that there is some crosstalk between miR-375 and the CRH signaling pathway. Further, our data show that CRH upregulates endogenous expression of miR-375 both *in vitro* and *in vivo*. It is well known that miRNAs are processed from their long RNA transcript, namely primary miRNA (Pri-miRNA) (Nothnick 2012). Our results show that CRH could upregulate Pri-miR-375 expression. These findings suggest that CRH stimulates miR-375 expression by enhancing Pri-miR-375 transcription. It has been reported that PKA-p38 is involved in the CRH signaling pathway in mast cells (Cao *et al.* 2006), keratinocytes (Park *et al.* 2005), PC12 cells (Dermitzaki *et al.* 2002) and these reports are consistent with the data presented here, although there are also reports suggesting that the binding of CRH to its receptors activates the PKA-ERK signaling pathway in pituitary (Kovalovsky *et al.* 2002). In addition, the present study demonstrates that miR-375 knockdown attenuates the negative effects of CRH on E2 synthesis and suggests that miR-375 acts as a downstream molecule of CRH affecting the E2 synthesis signaling pathway in granulosa cells. These data collectively indicate that miR-375 mediates the CRH-PKA-p38 MAPK signaling affecting E2 synthesis.

To find the target gene of miR-375, the *SP1* and *LHR1* were predicted as potential targets through use

Figure 7 A schematic of miR-375 mediates the signaling pathway of CRH inhibiting E2 synthesis. CRH activates PKA-p38 signaling pathway by binding to CRHR1, which in turn enhances miR-375 expression. Our results have shown that miR-375 plays a negative role in E2 synthesis via its direct target gene *SP1* in this pathway. Arrows mean stimulation and T bars indicate inhibition.

of bioinformatics methods. The *in vitro* experiment results show that *SP1* is a direct target of miR-375 in the porcine ovary; these findings agree with the results in cervical SiHa and CaSki cells (Wang *et al.* 2011). Our results further show that *SP1* overexpression promotes E2 synthesis and its knockdown inhibits E2 production in porcine granulosa cells. These results indicate that transcription factor SP1 performs a positive role in miR-375 regulating E2 synthesis in these cells. However, the mechanisms of SP1's promotion of E2 synthesis will require additional research. Further, we show that 100 nM CRH downregulates both *SP1* mRNA and protein levels in cultured granulosa cells, suggesting that SP1 is a downstream molecule involved in the CRH signaling pathway. Our study also shows that the miR-375 inhibitor could reverse the negative effects of CRH on SP1 protein levels and E2 synthesis *in vitro*. Taken together, these collective data suggest that *SP1* is a direct target of miR-375 in the CRH signaling pathway in porcine granulosa cells.

To conclude, the present study shows that miR-375 and *CRHR1* are expressed in porcine granulosa cells and that miR-375 plays a negative role in regulating the effect of CRH on E2 synthesis. In addition, we have shown that the signaling of CRH is transduced through *CRHR1* and PKA-p38, subsequently enhancing *miR-375* and *miR-375* expression, targeting SP1 and thus decreasing E2 synthesis in the porcine ovary (Fig. 7). As our study suggests, miR-375 serves as an important regulator in E2 synthesis by mediating the CRH signaling pathway in the porcine granulosa cells. These findings will help identify potential molecules for the treatment of reproductive disorders.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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