MicroRNA-376a Regulates 78-Kilodalton Glucose-Regulated Protein Expression in Rat Granulosa Cells

ラット顆粒膜細胞において miR-376a は GRP78 の発現を調節する

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Introduction

In females, luteinizing hormone-human chorionic gonadotropin receptor (LHR) plays a central role in maintaining reproductive functions, including steroidogenesis and ovulation (1, 2). FSH is a key factor that induces LHR expression in the granulosa cells of pre-ovulatory follicles whereby an LH surge triggers ovulation followed by corpus luteum formation (3-5). To date, numerous studies have investigated many aspects of the transcriptional and post-transcriptional regulation of LHR expression (6). Several studies have demonstrated that the cell surface expression of LHR is downregulated in granulosa cells after ovulation is induced by an LH surge or by a pharmacological dose of hCG. This loss of cell surface LHR protein is preceded by decreased LHR mRNA levels (7-9). Thereafter, both LHR protein and mRNA are dramatically recovered to sustain the functions of the corpus luteum. In contrast to extensive studies elucidating the molecular mechanisms of LHR downregulation, the mechanism of LHR upregulation following LHR downregulation has not been elucidated. Recently, our laboratory determined that the 78-kilodalton glucose-regulated protein (GRP78), an endoplasmic reticulum (ER)-associated protein that assists in proper protein folding to execute primary protein maturation in the ER (10, 11), is involved in the recovery of LHR after its down-regulation (12). In that report, cAMP mediated the primary hCG-elicited signal to induce GRP78 expression in granulosa cells for supporting corpus

luteum formation. Although GRP78 is known be an important molecule for the upregulation of LHR, the precise mechanism underlying the regulation of GRP78 expression in the ovary has not been fully elucidated.

MicroRNAs (miRNAs) are non-coding RNAs (approximately 22 nucleotides) that regulate gene expression by binding to the 3′ -untranslated regions of target mRNAs to induce the degradation of target mRNAs or to induce translational repression (13). Within the past decade, miRNAs have been recognized as important regulators in many biological and cellular processes, such as cell proliferation, differentiation, apoptosis, and tumorigenesis (14-16). Although the functions of miRNAs have not been elucidated fully, recent emerging evidence demonstrates that miRNAs are involved in ovarian follicular and luteal functions (17-20). We have also reported that miR-136-3p targets LHR mRNA to induce the transient downregulation of LHR mRNA in granulosa cells after ovulation (21). This result prompted us to search for miRNAs involved in the regulation of GRP78 expression.

In the following experiments, we profiled the miRNAs that were expressed in PMSG-primed rat ovaries in which an injection of hCG induced ovulation. From that data and data obtained from a bio-informative database, we focused on rno-miR-376a, which potentially binds to GRP78 mRNA, and characterized the function of rno-miR-376a in

cultured granulosa cells.

Materials and Methods

Hormones and reagents

Recombinant FSH and purified hCG were supplied by Dr. A. Parlow and the National Hormone and Peptide Program (National Institute of Diabetes and Digestive and Kidney Disease, National Institutes of Health, Torrance, CA, USA). Dulbecco modified Eagle medium (DMEM), DMEM/Ham's Nutrient mixture F-12, diethylstilbestrol (DES) and β-Estradiol-Water Soluble were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gentamicin sulfate and Fungizone were purchased from Invitrogen Corp. (Carlsbad, CA, USA).

hCG for the *in vivo* study was provided by Schering-Plough Corp. (Osaka, Japan), and PMSG was provided by Asuka Pharmaceutical Co., Ltd. (Tokyo, Japan).

Animals

Female 21-day-old Wistar rats (Japan SLC, Inc., Hamamatsu, Japan) were maintained according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the policies of the Gunma University Animal Care and Use Committee. The

animals were housed in a temperature- and light-controlled room (12 h light, 12 h dark cycle; lights on at 0600 h) with food and water provided *ad libitum*.

For the *in vivo* study, 21-day-old female rats received an injection of PMSG (30 IU, subcutaneous [sc]), followed by an injection of hCG (20 IU, sc) 48 h later. We then sacrificed the rats to remove the ovaries at each time point after the injection of hCG. The ovaries were stored immediately in RNAlater Tissue Collection solution (Applied Biosystems, Foster City, CA, USA).

For the *in vitro* study, 21-day-old female rats received DES injections (2 mg, sc) for 4 d, and the ovaries were removed for use in primary granulosa cell culture, as described below.

Primary rat granulosa cell culture

Granulosa cells were obtained from DES-primed rats. Both ovaries were removed from the rats, and the granulosa cells were released by puncturing the follicles with a 26-gauge needle. The granulosa cells were washed and collected by brief centrifugation, and cell viability was determined by trypan blue exclusion. The granulosa cells were then cultured in DMEM/Ham's nutrient mixture F-12 supplemented with 20 mg/L gentamicin sulfate, 500 μg/L Fungizone, and 1 g/L bovine serum albumin (BSA) on collagen-coated plates or dishes in a humidified atmosphere containing 5% CO2 and 95% air at 37°C.

miRNA microarray

Rat ovaries were removed from PMSG/hCG-treated rats to analyze miRNA expression on a microarray. The miRNA microarray expression profiling was performed by TaKaRa Bio Inc. using the Agilent Technologies Rat miRNA Microarray 8 × 15K (Santa Clara, CA, USA), which contains 350 rat miRNAs listed in the Sanger miRBase v.10.1. Total RNA, including miRNAs, was isolated from the PMSG/hCG-treated rat ovaries using the FastPure RNA Kit (TaKaRa Bio Inc., Otsu, Japan) according to the manufacturer's protocol. A quality check of the total RNA was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies). The labeling and hybridization of the total RNA samples were performed using an Agilent miRNA Microarray System (Agilent Technologies). The microarray results were extracted using Agilent Feature Extraction software, and the data were analyzed by TaKaRa Bio Inc.

miRNA target prediction

Target miRNAs that bound to rat GRP78 mRNA were predicted using MicroCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/; hosted by the European Bioinformatics Institute) based on the miRNA microarray results.

miRNA expression profiling of rat ovaries and rat granulosa cells using TaqMan miRNA assays

Total RNA, including miRNAs, was isolated from rat ovaries or rat granulosa cells using the mirVana miRNA Isolation Kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's protocol. The concentration of the total RNA was measured using a Nanodrop-1000 spectrophotometer (Thermo scientific, Wilmington, DE, USA). The total RNA was then reverse transcribed using specific RT primers from a TaqMan MicroRNA Assay Kit (rno-miR-144: 197375, rno-miR-376a: 001069, rno-miR-451: 001141, and 4.5S RNA(H): 001716 as an internal control; Ambion Inc.). Single-stranded cDNA was synthesized from 10 ng of total RNA in a 15-μL reaction volume using the MicroRNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's protocol. The reactions were incubated for 30 min at 16°C, 30 min at 30°C, and 5 min at 85°C in a thermal cycler. Real-time RT-PCR was performed using sequence-specific primers from the TaqMan MicroRNA Assay Kit according to the manufacturer's instructions. The reactions were performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems) in a 20-μL reaction volume at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. All of the reactions were tested in triplicate. We determined the threshold cycle (Ct) values for each reaction, and their means were used to determine the relative

miRNA expression levels using the ΔΔCt method. Additionally, we calculated the percentage of rat GRP78 mRNA or miRNA expression using the ΔΔCt values for each reaction as follows:

% miRNA expression = $100 \times 2^{-\Delta\Delta Ct}$.

These values were used to calculate the, and the relative expression of GRP78 or each miRNA was normalized to the expression of hCG 0 h in the control, which was set at 1.

Expression of GRP78 mRNA expression in rat ovaries or rat granulosa cells using TaqMan Gene Expression Assays

Total RNA was isolated from rat ovaries or rat granulosa cells, and the concentrations were measured as described above. The isolated RNAs (2 µg of each sample) were treated with DNaseI (Invitrogen) to eliminate residual genomic DNA. These RNAs were reverse transcribed using random primers, 10 mM deoxynucleoside triphosphate mix, and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. The reactions were incubated for 5 min at 25°C, 60 min at 50°C, and 15 min at 70°C in a thermal cycler. To remove complementary RNA, RNase H was added to the cDNAs, and the reactions were incubated for 20 min at 37°C. Real-time RT-PCR was performed using TaqMan Gene Expression Assays (GRP78-Hspa5: Rn00565250_m1, Eukaryotic 18S rRNA:

Hs99999901_s1 as an internal control; Applied Biosystems) according to the manufacturer's instructions. Rat GRP78 mRNA expression levels were measured in a similar manner as in the miRNA assays.

miRNA precursor (Pre-miR-376a) and miRNA inhibitor (Anti-miR-376a) transfection

Primary rat granulosa cells (8.0–10 × 10^4 cells obtained from DES-primed rats) were cultured in 24-well tissue culture plates with serum-free medium. Twenty-four hours after seeding, the cells were incubated with FSH (30 ng/mL) and estradiol (10 nM) for 48 h. The cells were then transfected with Pre-miR-376a or Anti-miR-376a (50 nM each) purchased from Ambion (product IDs: PM10504 and AM10504, respectively) using siPORT NeoFX Transfection Agent according to the manufacturer's protocol. Twelve hours after transfection, hCG (30 ng/mL) was added to the culture medium to induce the downregulation or upregulation of GRP78 mRNA.

To evaluate the effects of Pre-miR-376a and Anti-miR-376a on GRP78 mRNA expression, we performed real-time RT-PCR using TaqMan gene expression assays 12 h after hCG treatment and compared the results with the mock-transfected cells. All of the reactions were tested in triplicate. Rat GRP78 mRNA expression was measured in a similar manner as in the miRNA assays.

Western blot analysis

Primary rat granulosa cells (150×10^4 cells obtained from DES-primed rats) were cultured in 3.5-cm tissue culture dishes with serum-free medium. Twenty-four hours after seeding, the cells were incubated with FSH (30 ng/mL) and estradiol (10 nM) for 48 h. The cells were then transfected with Pre-miR-376a or Anti-miR-376a (50 nM each) purchased from Ambion (product IDs: PM10504, AM10504, respectively) using Lipofectamine 2000 Transfection Reagent according to the manufacturer's protocol. Twelve hours after transfection, hCG (30 ng/mL) was added to the culture medium.

The cells were lysed in radio-immunoprecipitation assay buffer (150 nM NaCl, 50 mM Tris, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4) containing proteinase inhibitors (PMSF, pepstatin A, and leupeptin). The protein lysates were resolved on sodium dodecyl sulfate gels and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking, the expression of GRP78 was determined using a rabbit anti-GRP78 antibody (1:1000; AnaSpec, Inc.) and a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (1:40000; Bio-Rad Laboratories, Inc.). The proteins bands were visualized using enhanced chemiluminescence (Immobilon Western; Millipore). Luminescence detection was quantified by scanning the

films with a CCD camera and analyzing the digitized data using the NIH ImageJ software.

Reporter vectors and DNA constructs

To identify the rno-miR-376a-binding site at the 3′ -end of GRP78 mRNA, we generated a direct-match miRNA target site and cloned the insert into the multiple cloning site in the luciferase reporter vector from the pMIR-REPORT miRNA Expression Reporter Vector System (Applied Biosystems). The sense and antisense strands of the oligonucleotides were annealed by adding 2 μg of each oligonucleotide to 46 μL of annealing solution (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4 and 2 mM magnesium acetate) and incubating at 90°C for 5 min and then at 37°C for 1 h. The annealed oligonucleotides were digested with *Hind*III and *Spe*I and ligated into the *Hind*III and *Spe*I restriction sites the of pMIR-REPORT vector. The sequences of the inserts were confirmed by sequence analysis using a PRISM 3100 genetic analyzer (Applied Biosystems).

The following oligonucleotides were used in this studies: (1) *rno-miR-376a-binding site sequence at the 3′ -end of GRP78 mRNA,*

5′ -aatgcactagtATGGTAGAAAAAAGTTCCTACaagcttaatgc-3′ and

5′ -gcattaagcttGTAGGAACTTTTTTCTACCATactagtgcatt-3′ and

(2) *miR-376a,* 5′ -aatgcactagtACGAGGATTTTCCTCTACGATaagcttaatgc-3′ and

5′ -gcattaagcttATCGTAGAGGAAAATCCTCGTactagtgcatt-3′ .

Luciferase assay

We transfected HEK293 cells with 200 ng of each vector (pMIR-REPORT luciferase vectors, as described above, and the pMIR-REPORT βgal vector as a control for transfection normalization) and 50 nM Pre-miR-376a or Anti-miR-376a using Lipofectamine 2000 Transfection Reagent according to the manufacturer's protocol. To measure the luciferase activity, we harvested the cells 24 h after transfection and conducted the assay using the pMIR-REPORT miRNA Expression Reporter Vector System (Applied Biosystems).

Data analysis

The microarray data were first filtered by subtracting the control probe data. To identify miRNAs, a one-way ANOVA was performed for differentially expressed miRNAs between four groups (hCG 0 h, hCG 6 h, hCG 12 h, hCG 24 h). False discovery rates (FDR) were assessed using the Benjamini and Hochberg method. Hierarchical clustering was then performed using the complete linkage method.

Comparisons between groups were performed using one-way ANOVA followed by Dunnett's multiple comparison test. The data represent the mean ± SE from at least three

independent experiments. A value of $P < 0.05$ was considered significant.

Results

We utilized a miRNA microarray analysis to identify miRNAs that were expressed in rat ovaries. Based on the differentially expressed miRNAs, a cluster analysis was executed to generate subgroups characterized by the expression patterns in four groups (hCG 0 h, hCG 6 h, hCG 12 h, and hCG 24 h). A total of 44 miRNAs were found to differ significantly in response to hCG treatment, as measured using the Benjamini and Hochberg method, suggesting that these miRNAs potentially affected the expression of GRP78. From these 44 miRNAs, we focused on 23 miRNAs that increased 6 h after hCG treatment and then decreased by 24 h (Table 1), and 19 miRNAs that decreased 6 h after hCG treatment and then increased by 24 h (Table 2). The results of the bioinformatics database inquiry (MicroCosm, http://ebi.ac.uk/) revealed that rno-miR-144, rno-miR-376a, and rno-miR-451 can bind to the 3′-UTR of GRP78 mRNA (from bp 2439–2459) and negatively regulate GRP78 expression.

In both *in vivo* and *in vitro* experiments, we previously demonstrated that GRP78 mRNA levels peaked while LHR mRNA was downregulated by hCG and that the increase of LHR protein levels was dependent on the increment of GRP78 protein (12). Therefore, we

measured the expression of GRP78 mRNA, rno-miR-144, rno-miR-376a, and rno-miR-451 using real-time RT-PCR to examine their expression patterns in the context of LHR downregulation (Fig. 1). Consistent with previous studies, GRP78 mRNA upregulation peaked 12 h after the administration of an ovulatory dose of hCG and subsequently decreased. In a similar manner, rno-miR-144 and rno-miR-376a expression peaked 12 h after the hCG treatment, and rno-miR-451 expression peaked 24 h after the hCG treatment.

Next, we investigated the effects of rno-miR-144, rno-miR-376a, and rno-miR-451 on GRP78 mRNA expression in granulosa cells isolated from DES-treated immature rats (Fig. 2). GRP78 mRNA and rno-miR-376a expression increased significantly 12 h after the addition of hCG into the culture medium and subsequently decreased, which was consistent with *in vivo* study (Fig.1). In contrast, rno-miR-144 and rno-miR-451 expression decreased significantly after the hCG treatment.

We then examined the effects of rno-miR-376a on GRP78 mRNA levels following the transfection of granulosa cells with either Pre-miR-376a or Anti-miR-376a (Fig. 3). In this experiment, the granulosa cells were cultured in a similar manner to the cells utilized in Fig. 2 except that the cells were transfected with Pre- or Anti-miR-376a 12 h prior to hCG treatment. The results indicated that the transfection with Pre- or Anti-miR-376a did not affect GRP78 mRNA expression. Furthermore, to ascertain the expression of GRP78 protein,

western blot analysis (Fig. 4) was performed using the same animal model designed for the real-time RT-PCR analysis. The results indicated that the transfection of the granulosa cells with Pre-miR-376a significantly decreased GRP78 protein. However, the transfection of granulosa cells with Anti-miR-376a upregulated GRP78 protein expression 24 h after the hCG treatment.

To confirm the presence of an rno-miR-376a binding site on GRP78 mRNA, we constructed a reporter vector that contained a putative rno-miR-376a binding site sequence (bp 2439–2459) in the 3′ -UTR downstream of a *Renilla* luciferase coding region. To avoid the influence of endogenous rno-miR-376a, we used HEK293 cells rather than rat granulosa cells (Fig. 5). To verify the effect of the miRNA transfection, we generated a reporter vector that included the whole miR-376a sequence. The results indicated that Pre-miR-376a reduced the luciferase activity to approximately 0%, while Anti-miR-376a did not affect the luciferase activity. These data demonstrate that the translation of GRP78 was blocked by Pre-miR-376a via a binding site that was the complementary sequence to rno-miR-376a.

The luciferase activity in the cells transfected with the reporter vector containing the putative rno-miR-376a binding site was reduced by approximately 50% 24 h after the cells were transfected with Pre-miR-376a. In contrast, the transfection with Anti-miR-376a did not affect the luciferase activity. This result indicates that rno-miR-376a binds to the 3′ -end of

GRP78 mRNA from bp 2439–2459 in the 3′ -UTR.

Discussion

Within a follicle after ovulation, many different cell types are subjected to dramatic changes, including proliferation, apoptosis, and differentiation. The transition from the ovulatory follicle to corpus luteum formation is a critical phase for maintaining female reproductive functions. The LH surge triggers ovulation and concurrently provokes the downregulation of LHR, resulting in the rapid and transient induction of several genes by cAMP, which functions as a primary signal downstream of LHR. Although the precise mechanism underlying the recovery of LHR from the down-regulation has not been elucidated fully, our recent finding indicates that GRP78 is involved in the mechanism (12). Therefore, to better understand the regulation of GRP78 expression, we focused on miRNAs that are induced in the ovary after ovulation because increasing evidence suggests that miRNAs play pivotal roles in the regulation of a wide range of biological processes (20, 22, 23).

To identify miRNAs that potentially regulate GRP78 expression, microRNA microarrays were performed using ovaries from PMSG-primed rats injected with an ovulatory dose of hCG. The array data along with the bioinformatic analysis provided by MicroCosm Targets,

which indicated that several miRNAs bind to the GRP78 mRNA 3′ -UTR, led us to focus on rno-miR-144, rno-miR-376a, and rno-miR-451. We narrowed the focus to rno-miR-376a based on the results of the *in vitro* experiments (Fig. 2). Initially, miRNAs were thought to bind within the 3′ -UTR of their target mRNAs, but recent evidence has demonstrated that the 5′ -UTR (24) or coding region (25) can also be the target sites for miRNAs. As more than 60% of human protein coding genes are predicted to be under the control of miRNAs by binding within their 3′ -UTR (26, 27), GRP78 mRNA in the rat ovary is regulated post-transcriptionally by miRNAs in a same manner as the majority of human mRNAs.

Predicting miRNA targets using computational analysis, which was employed for this study, is efficient but can result in tens or hundreds of targets with high false-positive rates (28). To avoid this issue, we executed a luciferase assay to identify an rno-miR-376a-binding site in the 3′ -end of GRP78 mRNA, which confirmed that the 3′ -end of GRP78 mRNA from bp 2439 to 2459 is the binding site for rno-miR-376a. As mentioned above, miRNAs mainly control their target genes through base pairing to the 3′ -UTRs of target mRNAs, resulting in the repression of translation of target proteins or the degradation of target mRNAs. In initial reports, the extent of complementarity between miRNA and its target mRNA is thought to govern either translational mRNA cleavage or repression (29): in plants, nearly perfect complementarity results in the degradation of target miRNAs (30), whereas in

animals, partial complementarity results in a translational block (31). However, recent studies have reported that miRNAs can also induce mRNA degradation in animals and, conversely, translational repression in plants, although the topic remains controversial due to conflicting data (32, 33). Moreover, the state of the complementarity between target mRNA and miRNA has been suggested to affect mRNA degradation (34), which is applicable to rno-miR-376a because the introduction of the complete identical sequence of rno-miR-376a into a reporter vector abolished the luciferase activity, whereas the luciferase activity was reduced to 50% using the reporter vector containing the 3′ -UTR rno-miR-376a binding site of GRP 78 mRNA (Fig. 5).

The results presented in this study (Fig. 3 and Fig. 4) demonstrate that rno-miR-376a decreases GRP78 protein production by translational repression without altering GRP78 mRNA levels and that the transfection of rno-miRNA-376a into granulosa cells repressed protein expression by approximately three-fold (Fig. 4). Although the three-fold reduction of GRP78 protein significantly impaired the recovery of LHR expression in the granulosa cells after the LHR downregulation induced by ovulation (12), this miRNA-mediated protein repression is modest, which is consistent with previous reports concluding that the magnitude of repression by miRNA rarely exceeds two-fold (26, 35). Moreover, it has been accepted that a single miRNA can downregulate hundreds of mRNAs and the production of hundreds

of proteins (26, 36). Combined with the MicroCosm analysis of miRNA targets, which revealed that 64 miRNAs can bind to the 3′ -UTR of GRP78 mRNA, we speculate that multiple miRNAs may constitute a network that is involved in the regulation of GRP78 mRNA, whereas rno-miR-376a is solely identified to be evoked by the ovulatory signal. In the regulation of gene expression, many intermediate steps occur during the processing of RNA to protein. GRP78, which is regarded as a stress-inducible house-keeping gene, is transcribed constitutively. Therefore, the post-transcriptional control of GRP78 by rno-miR-376a has a significant impact on the regulation of GRP78 protein expression and not gene expression, which is evident by the lack of GRP78 transcriptional regulation.

In conclusion, the results from the current study have demonstrated that the induction of rno-miR-376a by hCG represses the translation of GRP78, which is involved in the recovery of LHR from the downregulated state after ovulation in granulosa cells. Although the precise mechanism regulating GRP78 has not been elucidated, our finding provides novel data to better understand the complicated mechanism of LHR regulation after ovulation.

Summary

The 78-kilodalton glucose-regulated protein (GRP78) is a molecular chaperone that assists in protein assembly, folding, and translocation. Recently, our laboratory reported that GRP78 regulates the expression of luteinizing hormone-human chorionic gonadotropin receptor (LHR) in the early stage of corpus luteum formation. In this study, we investigated whether microRNAs (miRNAs), which post-transcriptionally regulate mRNA, are involved in the regulation mechanism of GRP78 in the ovary. A miRNA microarray was performed to analyze the overall miRNA expression profile, and the results indicated that 44 miRNAs were expressed highly after ovulation was induced. The results from a bio-informative database analysis and *in vitro* granulosa cell culture studies led us to focus on rno-miR-376a for further analysis. In both *in vivo* and *in vitro* studies, rno-miR-376a levels increased 12 h after human chorionic gonadotropin (hCG) administration. To elucidate whether rno-miR-376a induced mRNA destabilization or translational repression of GRP78, rno-miR-376a was transfected into cultured granulosa cells, resulting in decreased GPR78 protein levels without an alteration in GRP78 mRNA levels. To confirm that rno-miR-376a binds to GRP78 mRNA, we cloned the 3′ -end of GRP78 mRNA (nucleotides 2439–2459) into a reporter vector that contained a Renilla luciferase coding region upstream of the cloning site. The luciferase assays revealed that rno-miR-376a bound to the 3′ -end of

GRP78 mRNA.

From these data, we conclude that rno-miR-376a potentially negatively regulates GRP78 protein expression through translational repression at an early stage transition from the follicular phase to luteinization.

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Figure legends

Fig. 1. Time course of rat GRP78 mRNA, rno-miR-144, rno-miR-376a, and rno-miR-451 expression in rat ovaries induced by PMSG and hCG.

Female 21-day-old rats injected subcutaneously with PMSG (30 IU/rat), followed by hCG (20 IU/rat) 48 h later, were sacrificed at the indicated times. The ovaries were removed, and total RNA was isolated. Rat GRP78 mRNA (A), rno-miR-144 (B), rno-miR-376a (C), and rno-miR-451 (D) expression levels were measured using real-time RT-PCR as described in the Materials and Methods. The amount of rat GRP78 mRNA, rno-miR-144, rno-miR-376a, and rno-miR-451 in the hCG 0 h group was set at 1. Data were normalized to 18S rRNA (for GRP78 mRNA) and 4.5S RNA(H) (for rno-miR-144, rno-miR-376a, and rno-miR451) levels in each sample and represent the mean ± SE of three independent experiments. *, significantly different from the control value at hCG 0 h, $P < 0.05$.

Fig. 2. Rat GRP78 mRNA, rno-miR-144, rno-miR-376a, and rno-miR-451 expression in primary rat granulosa cells induced by FSH and hCG.

Primary rat granulosa cells were prepared, and the indicated reagents were added to the medium after 24 h of culture. Cells were then incubated with FSH (30 ng/mL) and estradiol (10 nM) for 48 h. Subsequently, hCG (30 ng/mL) was added to the culture medium, as

described in the Materials and Methods. Total RNA was isolated, and GRP78 mRNA (A), rno-miR-144 (B), rno-miR-376a (C), and rno-miR-451 (D) expression levels were measured using real-time RT-PCR as described in the Materials and Methods. The amounts of GRP78 mRNA, rno-miR-144, rno-miR-376a, and rno-miR-451 in the hCG 0 h group were set at 1. Data were normalized for 18S rRNA (for GRP78 mRNA) and 4.5S RNA(H) (for rno-miR-144, rno-miR-376a, and rno-miR-451) levels in each sample and represent the mean ± SE of 3 independent experiments. *, significantly different from the control value at hCG 0 h, $P < 0.05$.

Fig. 3. Effects of Pre-miR-376a and Anti-miR-376a transfection on rat GRP78 mRNA expression in primary rat granulosa cells.

Primary rat granulosa cells were prepared, and the indicated reagents were added to the medium after 24 h of culture. The cells were incubated with FSH (30 ng/mL) and estradiol (10 nM) for 48 h. Pre-miR-376a or Anti-miR-376a was transfected into the cells, and 30 ng/mL hCG was added 12 h later. The effects of Pre-miR-376a and Anti-miR-376a on the expression of GRP78 mRNA were measured using real-time RT-PCR, as described in the Materials and Methods. The expression of GRP78 mRNA at hCG 0 h in the control was set at 1. Each value represents the mean ± SE of three independent experiments.

Fig. 4. Effects of rno-miR-376a on GRP78 protein in granulosa cells.

(A) Primary rat granulosa cells were prepared, and the indicated reagents were added to the medium after 24 h of culture. The cells were incubated with FSH (30 ng/mL) and estradiol (10 nM) for 48 h. Pre-miR-376a or Anti-miR-376a was transfected into the cells, and 30 ng/mL hCG was added 12 h later. Cells were harvested 24 h after the addition of hCG, and GRP78 protein levels were quantified using western blot analysis. (B) Levels of GRP78 protein were quantified by densitometric scanning. The expression of GRP78 protein in the control (hCG 0 h) was set at 1. Values represent the mean ± SE of three independent experiments. *, significantly different from the control value, $P < 0.05$.

Fig. 5. Luciferase assays for the identification of the rno-miR-376a-binding site in the 3′ -UTR of GRP78 mRNA.

(A) Arrangement of rno-miR-376a and GRP78 mRNA and a schematic drawing of the predicted rno-miR-376a-binding site in the 3′ -UTR of GRP78 mRNA. (B) Schematic drawings of the pMIR-REPORT luciferase vectors used in our experiment. To identify the rno-miR-376a-binding site in the 3′ -UTR of GRP78 mRNA, luciferase reporter vectors were generated as described in the Materials and Methods. (C) Luciferase activity was

measured to identify the rno-miR-376a-binding site in the 3′ -UTR of GRP78 mRNA. HEK293 cells were prepared, and the cells were transfected with 200 ng of each reporter vector with 50 nM Pre-miR-376a (precursor) or Anti-miR-376a (inhibitor) as described in the Materials and Methods. For transfection normalization, the cells were also transfected with the pMIR-REPORT βgal vector. Luciferase activity was measured 24 h after transfection. The activity of the control (empty vector) was set at 1. Each value represents the mean ± SE of three independent experiments. *, significantly different from the control value, $P < 0.05$.

Table 1. List of differentially expressed (upregulated) miRNAs in rat ovaries following treatment with hCG for 6 h

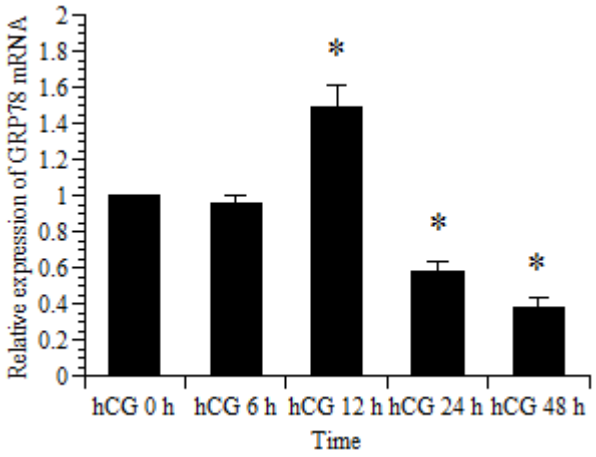
miRNA	Fold change	*q*-value	miRNA	Fold change	*q*-value
rno-miR-222	1.28	0.001	*rno-miR-410*	1.60	0.039
rno-miR-379	1.45	0.014	*rno-miR-541*	1.46	0.031
rno-miR-376b-3p	1.46	0.014	*rno-miR-132*	1.52	0.005
rno-miR-411	1.68	0.030	*rno-miR-146b*	1.39	0.020
rno-miR-136-3p	1.58	0.010	*rno-miR-125b**	1.50	0.043
rno-miR-434	1.56	0.042	*rno-miR-99a**	1.35	0.042
rno-miR-136-5p	1.43	0.020	*rno-miR-341*	1.39	0.039
rno-miR-431	1.37	0.029	*rno-miR-652*	1.25	0.081
rno-miR-376a	1.50	0.014	*rno-miR-181d*	1.09	0.358
rno-miR-154	1.30	0.038	*rno-miR-181c*	1.11	0.231
rno-miR-127	1.31	0.019	*rno-miR-503*	1.05	0.648
*rno-miR-21**	2.30	0.019			

Table 2. List of differentially expressed (upregulated) miRNAs in rat ovaries following treatment with hCG for 12 h

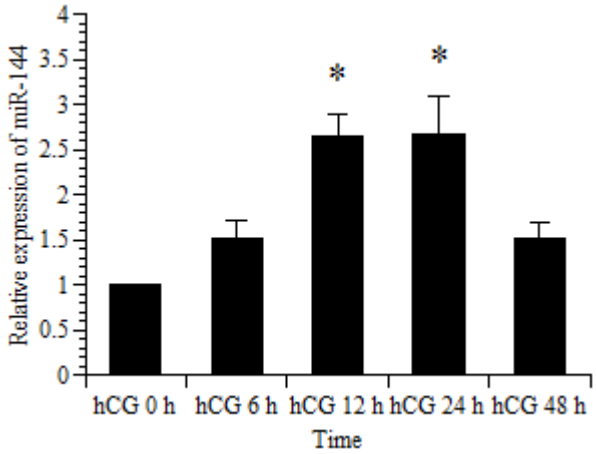
miRNA	Fold change	*q*-value	miRNA	Fold change	*q*-value
rno-miR-185	0.97	0.002	*rno-miR-33*	1.11	0.010
rno-miR-34a	1.09	0.010	*rno-miR-22*	1.05	0.010
rno-miR-144	1.22	0.030	*rno-miR-140*	1.04	0.002
rno-miR-451	1.21	0.030	*rno-miR-140**	1.00	0.001
rno-miR-7a	1.32	0.030	*rno-miR-193*	1.13	0.010
rno-miR-425	1.10	0.010	*rno-miR-877*	1.07	0.040
rno-miR-28	1.00	0.010	*rno-miR-29b*	1.10	0.008
rno-miR-29a	1.06	0.030	*rno-miR-24-2**	1.23	0.010
rno-miR-18a	1.20	0.010	*rno-miR-21*	1.18	0.010
rno-miR-27a	1.04	0.030			

Fig. 1

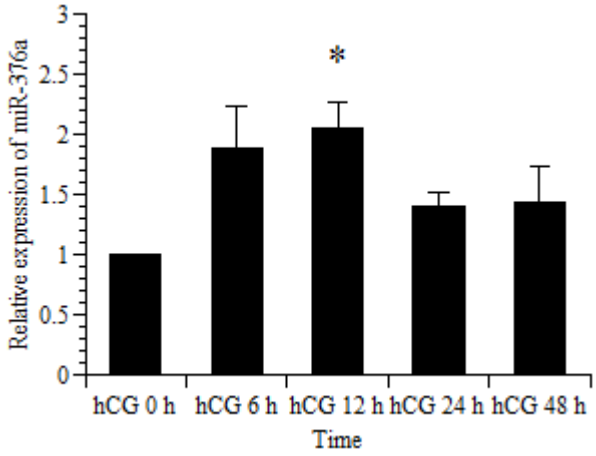
(A)


Relative expression of GRP78 mRNA
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
hCG 0 h
hCG 6 h
hCG 12 h
hCG 24 h
hCG 48 h
Time

(B)


Relative expression of miR-144
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
hCG 0 h
hCG 6 h
hCG 12 h
hCG 24 h
hCG 48 h
Time

(C)


Relative expression of miR-376a
3
2.5
2
1.5
1
0.5
0
*
hCG 0 h
hCG 6 h
hCG 12 h
hCG 24 h
hCG 48 h
Time

(D)

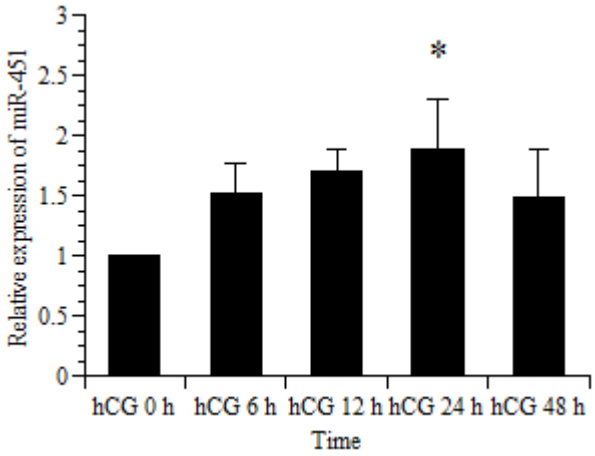
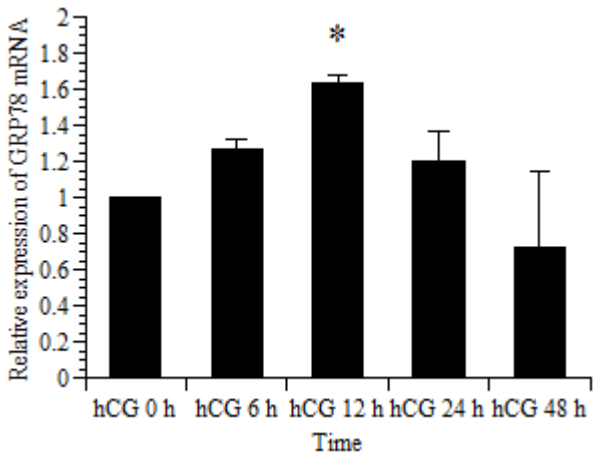
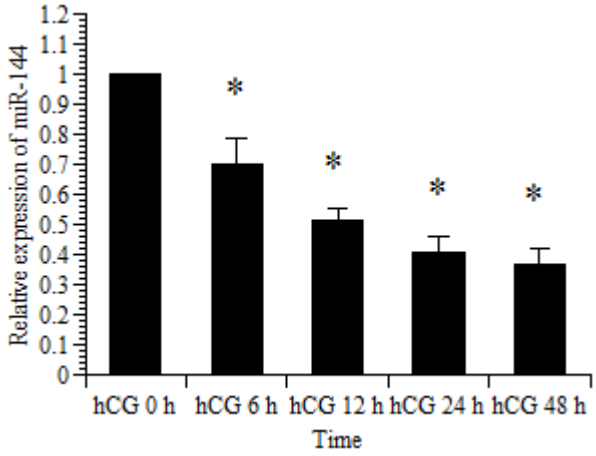

Relative expression of miR-451
3
2.5
2
1.5
1
0.5
0
*
hCG 0 h
hCG 6 h
hCG 12 h
hCG 24 h
hCG 48 h
Time

Fig. 2

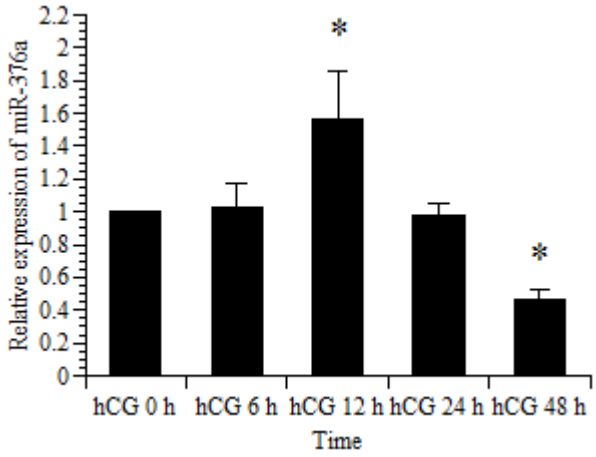
(A)


Relative expression of GRP78 mRNA
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
hCG 0 h
hCG 6 h
hCG 12 h
hCG 24 h
hCG 48 h
Time

(B)


Relative expression of miR-144
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
*
*
hCG 0 h
hCG 6 h
hCG 12 h
hCG 24 h
hCG 48 h
Time

(C)


Relative expression of miR-376a
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
hCG 0 h
hCG 6 h
hCG 12 h
hCG 24 h
hCG 48 h
Time

(D)

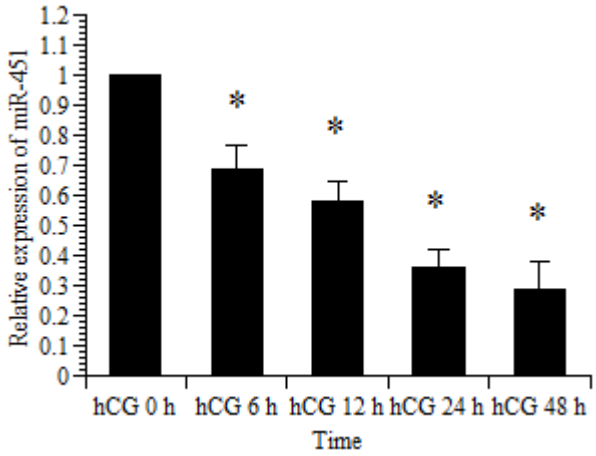

Relative expression of miR-451
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
*
*
hCG 0 h
hCG 6 h
hCG 12 h
hCG 24 h
hCG 48 h
Time

Fig. 3

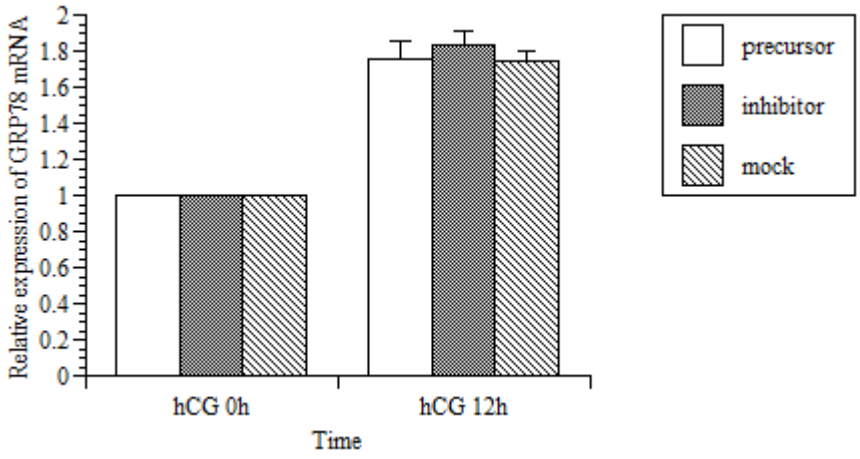
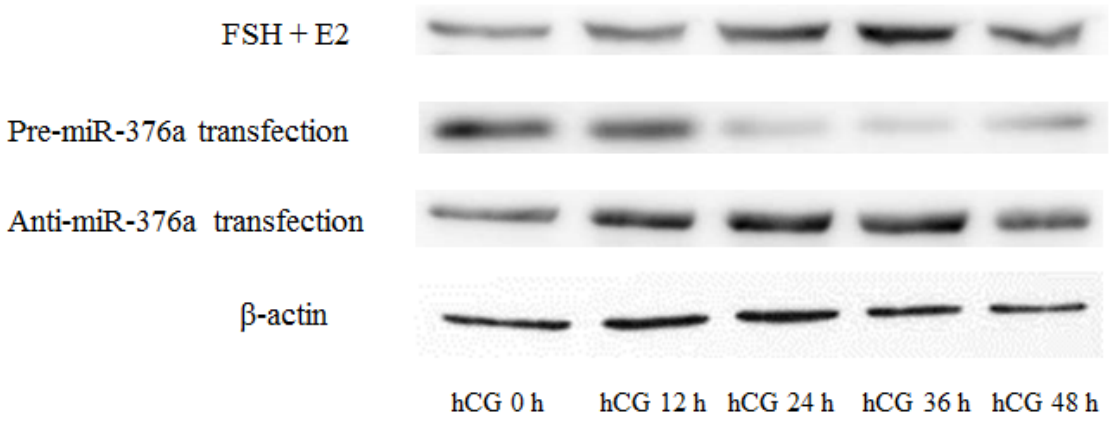

Relative expression of GRP78 mRNA
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
hCG 0h
hCG 12h
Time
precursor
inhibitor
mock

Fig. 4

(A)


FSH + E2
Pre-miR-376a transfection
Anti-miR-376a transfection
β-actin
hCG 0 h
hCG 12 h
hCG 24 h
hCG 36 h
hCG 48 h

(B)

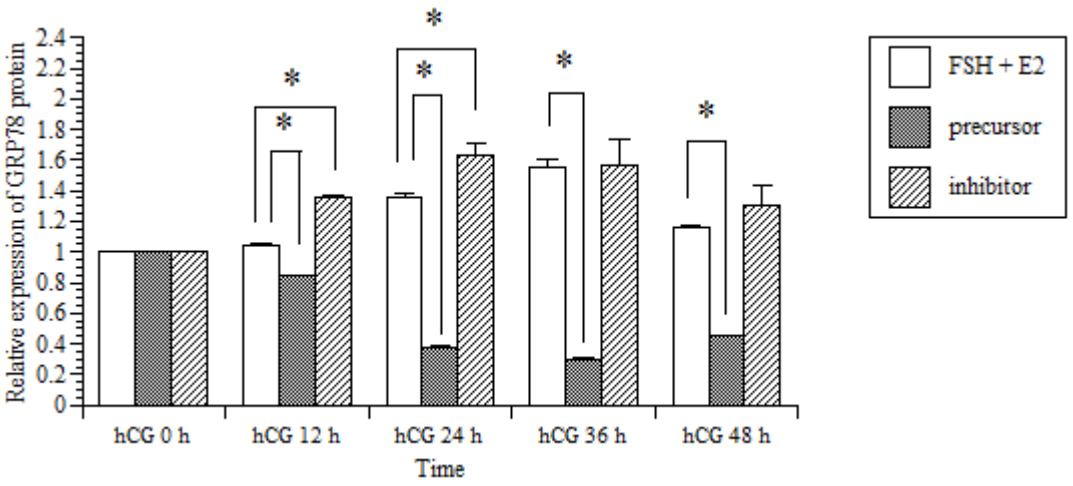
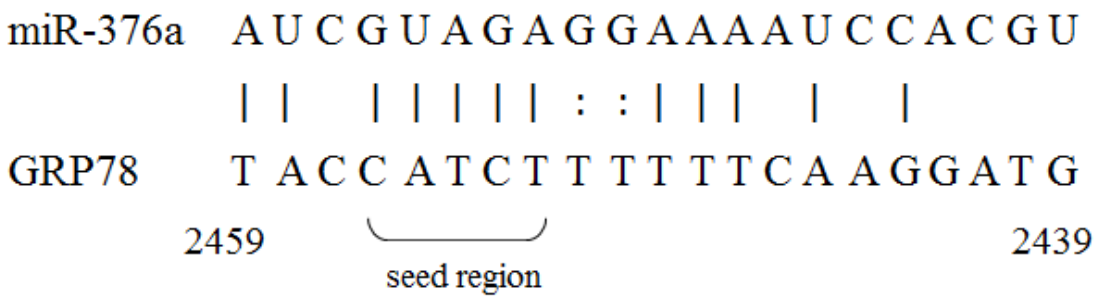
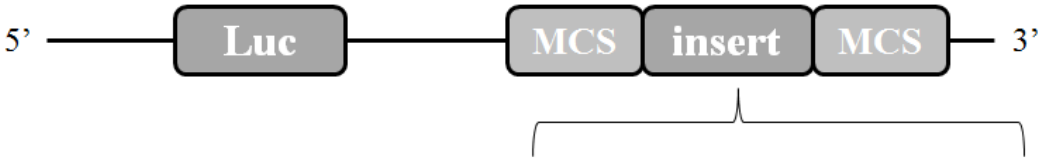

Relative expression of GRP78 protein
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
hCG 0 h
hCG 12 h
hCG 24 h
hCG 36 h
hCG 48 h
Time
FSH + E2
precursor
inhibitor

Fig. 5

(A)

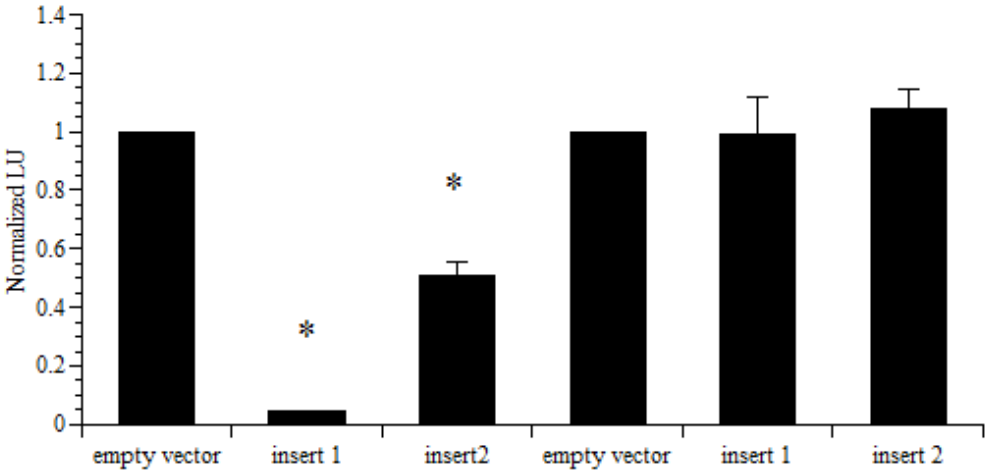


(B)



(1) Luc vector 1 (binding site sequence) : GTAGGAACTTTTTTCTACCAT
(2) Luc vector 2 (miR-376a sequence) : ATCGTAGAGGAAAATCCTCGT
(3) Luc vector 3 (empty vector) : -

(C)



precursor	+	+	+	-	-	-
inhibitor	-	-	-	+	+	+