FSH induces the development of circadian clockwork in rat granulosa cells via a gap junction protein Cx43-dependent pathway

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Circadian clocks are cell-autonomous molecular oscillators that provide organisms with an internal mechanism to maintain temporal order in a rhythmic environment. Circadian clocks drive many diurnal rhythms of physiology and behavior, such as sleep/wake cycles, feeding, body temperature, blood pressure, release of endocrine hormones, and metabolism. In mammals, the central pacemaker controlling circadian rhythms resides in the suprachiasmatic nuclei (SCN) of the hypothalamus (62). Circadian clocks also exist in many peripheral tissues (3, 31, 68). The master clock in the SCN orchestrates peripheral oscillators via humoral and neuronal pathways. Numerous oscillators in peripheral tissues contain self-sustained operative circadian molecular machineries similar to that of the SCN. These peripheral oscillators play an integral and unique role in each of their respective tissues, orchestrating the diurnal expression of many clock-controlled genes involved in diverse physiological functions.

Over the past decade, accumulating evidence has demonstrated that functional circadian clockwork exists in mammalian ovaries (8, 11, 12, 16, 25, 31, 42, 52, 72). Originally, Fahrenkrug et al. (16) showed that rat ovaries displayed a circadian expression of the clock genes Period 1 (Per1) and Per2. Another study proved that luteinizing hormone (LH) potently induces the rhythmic expression of Bmal1 (Arnt1) and Per2 in immature rat ovaries (31). Subsequently, a report from our laboratory showed that granulosa cells and luteal cells prepared from Per2-dLuc transgenic rats displayed different Per2 oscillations after dexamethasone (DXM) synchronization (24). Immature granulosa cells sustained only one cycle of Per2 oscillation, with a quick damping rate. In contrast, luteal cells showed several robust Per2 oscillations. We concluded that circadian clockwork in ovarian cells is affected during cellular differentiation. Another study illustrated that both follicle-stimulating hormone (FSH) and LH exerted diverse specific functions on the clockwork of granulosa cells in vitro as proved by Per2 oscillations (25). Consistent with above two reports (24, 25), a study demonstrated that endocrine signals, FSH, and LH are able to transmit phase information to the ovaries and cultured ovarian cells in vivo and in vitro (72). It is interesting that FSH seems to induce the development of a circadian clock in granulosa cells, as identified by two further studies from our group (11, 12). Granulosa cells cultured with FSH for 2 or 3 days acquired more stable Per2 oscillations and higher oscillatory intensity. However, the mechanism underlining the effect of FSH on the development of the circadian clockwork in granulosa cells remains poorly understood.

Gap junctions, which are unique cell-to-cell channels between neighboring cells, exist in almost all mammalian tissues (33). An intact gap junction channel is composed of two symmetrical structures (connexins) between adjacent cells, consisting of six transmembrane protein subunits termed connexins (Cx) (7). Cx proteins, which are named on the basis of their molecular size, belong to a large Cx family that includes Cx26, Cx30.3, Cx33, Cx43, Cx50, etc. Gap junction channels formed by Cxs are important for cellular communication, which allows the direct exchange between cell ions and small metabolites up to ~1 kDa in size (e.g., calcium ions, cAMP, and inositol 1,4,5-triphosphate) (19). As an important cellular communication pathway, gap junctions play pivotal roles in a wide range of physiological functions such as cell metabolism.
cell differentiation, growth and proliferation, and hormone secretion (36, 40).

Gap junctions have also been implicated in the regulation and coordination of cellular metabolism and function during the growth and differentiation of ovarian follicles (17, 21). In mammals, follicular development, luteinization, and luteolysis are accompanied by the proliferation, differentiation, and apoptosis of granulosa and luteal cells. Cx43, the most abundant gap junction protein in ovarian follicles, plays a very important role in follicular development. For example, cultured ovaries from Cx43 knock-out mice displayed very poor follicular development (29). A subsequent study by the same group identified that Cx43 is the only Cx that makes a significant contribution to intercellular coupling in mouse granulosa cells, despite the expression of other Cxs (18).

Accumulating evidence proved that the follicular stages affect the expression of Cx43 in diverse species (38, 39, 57, 67). An elevated expression of Cx43 was observed in granulosa cells with the increasing size of follicles. Considering the underlying mechanism, FSH may be involved in the increased expression of Cx43. Two studies demonstrated that FSH significantly elevates the expression of Cx43 in rat granulosa cells (32, 54), suggesting that Cx43 is an FSH-induced protein and that Cx43 plays an important role in transmitting the physiological functions of FSH. However, the manner in which the elevated expression of Cx43 stimulated by FSH regulates the physiology of ovarian follicle remains largely unknown.

Here, we hypothesized that gap junctions, which determine the cell population coupling state, are involved in the regulation of circadian clockwork. There is evidence that gap junctions provide a means to couple SCN neurons on a circadian basis (13, 63). One study found that the circadian rhythms in the release of arginine vasopressin and vasoactive intestinal peptide were disrupted in SCN slice cultures after 7 days incubation with octanol or halothane, which are both gap junction blockers (53). Another study found that Cx36 knock-out mice showed dampened circadian activity rhythms (37). These findings suggest that gap junctions play a role in the regulation of central pacemakers, SCN. However, there are few reported studies concerning the gap junction regulation of the circadian clockwork. There is evidence that gap junctions are implicated in the circadian clock mechanism (32, 54), suggesting that Cx43 is an FSH-induced protein and that Cx43 plays an important role in transmitting the physiological functions of FSH. However, the manner in which the elevated expression of Cx43 stimulated by FSH regulates the physiology of ovarian follicles remains largely unknown.

Therefore, the objective of the present study was to determine whether FSH induces the development of circadian clockwork through an elevation of Cx43 expression. We used a real-time monitoring system of gene expression to evaluate the circadian clockwork in rat granulosa cells, using transgenic rats constructed with the mouse Per2-dLuc reporter gene (24). The transcript levels of genes were examined by quantitative RT-PCR (qRT-PCR). We used immunofluorescence and Western blotting analysis to detect the Cx43 protein expression in ovarian follicles of different sizes. Our results indicate that FSH contributes to the development of circadian clockwork in rat granulosa cells, probably because of an elevated expression of Cx43. This novel finding may advance our understanding of what mechanism is involved in the development of the circadian clock in rat ovaries stimulated by FSH, and it demonstrates that gap junctions are implicated in the circadian clock in peripheral oscillators besides the central pacemaker.

**MATERIALS AND METHODS**

**Animals.** All of the experiments were performed under the control of the Guidelines for Animal Experiments by the Faculty of Medicine, Kyushu University, and in compliance with Law No. 105 and Notification No. 6 of the Government of Japan. The studies were reviewed and approved by Kyushu University. Mouse Per2 promoter region [chr1 (−): 3289505-93293019] on the Build 36 assembly by the National Center for Biotechnology Information and the Mouse Genome Sequencing Consortium, which is sufficient for circadian oscillation, was fused to a dLuc reporter gene (24). Per2-dLuc transgenic rats were generated in accordance with the method described in the patent publication no. WO/2002/081682 (Y.S., New Technology Institute, Utsunomiya, Japan). Transgenic rats were maintained under 12-h light and 12-h dark (zeitgeber time, ZT0: 0800 light on; ZT12: 2000 light off) with water and food ad libitum.

**Preparation and culture of granulosa cells.** Immature and mature granulosa cells were prepared according to a previous report (23), with minor modifications. Immature female rats (21–23 days of age) were injected subcutaneously with 1 mg of diethylstilbestrol (DES; Sigma Chemicals, St. Louis, MO) for 3 days, and immature granulosa cells from preantral follicles of oocytes were collected at ZT2 on day 4. Rats were primed with 50 IU equine chorionic gonadotropin (eCG) (Aska Pharmaceutical, Tokyo, Japan), and their ovaries were collected at ZT2 on day 4 for the preparation of mature granulosa cells from antral follicles. Oocytes were incubated in DMEM-F-12 (Invitrogen, Carlsbad, CA) containing 6 mM EGTA for 20 min and then in DMEM-F-12 containing 0.5 M sucrose for 15 min at 37°C.

Granulosa cells were harvested by puncturing the follicles with a 27-gauge needle. The released cells were recovered by centrifugation and washed three times with DMEM-F-12. Approximately 1 × 10^6 granulosa cells were plated on a 35-mm collagen-coated dish (Ikawa, Tokyo, Japan) with 2 ml DMEM-F-12 supplemented with 1× antibiotic-antimycotic (AA; Nacalai Tesque, Kyoto, Japan) and 5% charcoal-treated FBS (Invitrogen). Cultures were carried out in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Cells were cultured for 24 h before other treatments.

**Real-time monitoring of Per2-dLuc oscillations.** Attached granulosa cells were synchronized by 100 nM DXM for 2 h and then exposed to serum-free medium supplemented with 15 mM HEPES, 0.1 mM luciferin (Wako, Tokyo, Japan), 0.1% BSA (Sigma Chemicals), and 1× AA in the presence of 1× insulin-transferrin-selenium (Life Technologies, Grand Island, NY) for luminescence determination. In some experiments, the monitoring was performed in the presence of lindane, carbenoxolone (CBX), or dimethyl sulfoxide (DMSO). Luciferase activity was chronologically monitored at 37°C with a Kronos Dio AB-2550 luminometer (ATTO, Tokyo, Japan) interfaced to a computer for continuous data acquisition, as described (10). The data are presented as photon counts per minute. A baseline correction was calculated using a 24-h moving average, which removed the first 12 h of data. The time of the first phase was determined from the peak that appeared between 12 and 36 h of culture after DXM synchronization. The amplitude of Per2-dLuc was documented by the single Cosinor method using Timing Series Single 6.3 (Expert Soft Technologies, Richeieu, France).

**RNA extraction and qRT-PCR.** Cultured cells were harvested at indicated time points, and total RNA was isolated using an RNeasy Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer’s protocol. RNA samples were treated with RNase-free DNase (Qiagen). The cDNAs were generated by RT with oligo(dT)15 and random primers using a GoTag 2-Step RT-qPCR System (Promega, Madison, WI). The primer sets used for the qRT-PCR are listed in Table 1. The qRT-PCR was performed in a 50-μl volume containing a 20-ng cDNA sample in GoTag qPCR Master Mix and 250 nM specific primers.
Table 1. Primer sequences for the targeted genes in qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Sequence 5′-3′</th>
<th>Amplicon, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmal1 (Arntl)</td>
<td>NC_005100</td>
<td>F: CGCTGGACCAAGGGAAGTGA R: CTGTTAACGCTGGGAGGTT</td>
<td>97</td>
</tr>
<tr>
<td>Per2</td>
<td>NM_031678</td>
<td>F: AGACGTTGCGAAGCAAGGA R: CGCCTTCTAGGTTGATAGGAT</td>
<td>90</td>
</tr>
<tr>
<td>Clock</td>
<td>NM_021856</td>
<td>F: GGGCCAGAATAGCGCCGAGAT G: ACTTGGCAGACAGGCGGCC</td>
<td>136</td>
</tr>
<tr>
<td>Rev-erba (Nrl1)</td>
<td>NM_031134</td>
<td>R: AGACGTGACGACCCCGAGATC</td>
<td>102</td>
</tr>
<tr>
<td>Gr (Nr3 cl)</td>
<td>NM_012576</td>
<td>R: CATGGCCGATGTTGAGATTCT</td>
<td>76</td>
</tr>
<tr>
<td>Lhcg</td>
<td>NM_012922</td>
<td>G: GGCTGATMAACTTCTGGAAGGCTGTTACG</td>
<td>120</td>
</tr>
<tr>
<td>Cx43 (Gja1)</td>
<td>NM_012567</td>
<td>F: GCGGAGCTTGGAGGCGGCAAA R: ATCCGGGACTGGCTGACG</td>
<td>243</td>
</tr>
<tr>
<td>Gapdh</td>
<td>NM_017008</td>
<td>F: AACCCTGGCAAGTATGATGACATCA R: AGAACCTGGGCTGCTGTTGGA</td>
<td>111</td>
</tr>
</tbody>
</table>

Per, Period gene; Gr, glucocorticoid receptor; Cx, connexin; F, forward; R, reverse.

RESULTS

Functional molecular machinery of a circadian clock exists in rat mature granulosa cells after DXM synchronization. The circadian rhythm of in vitro Per2 promoter activity was first investigated using mature granulosa cells. After synchronization by DXM, the Per2 circadian oscillations were clearly observed in mature granulosa cells, albeit with a rapid and steady decrease of amplitude (Fig. 1A). To further analyze the clock system in mature granulosa cells, we used a qRT-PCR approach to examine the circadian gene expression patterns using RNA samples collected at the indicated time points.
according to the Per2 oscillation between 12 and 36 h. The results are presented in Fig. 1A. The majority of circadian oscillator genes as well as a clock-controlled gene (Bmal1, Per2, Rev-erba, Star) showed robust rhythmic expression (P < 0.01). In contrast, Clock and Lhcgr transcription did not follow a significant circadian pattern. In addition, the Per2 transcript profile was consistent with Per2 oscillation. Circadian rhythms of the Per2 and Bmal1 transcripts were obviously antiphase in mature granulosa cells. The Rev-erba mRNA levels were also in phase opposition with those of Bmal1.

Development of a clock system in granulosa cells during the period of cell maturation upon FSH stimulation. To investigate the development of a clock system during the maturation of granulosa cells, we examined the FSH control of the clock system by using immature and mature granulosa cells prepared from preantral and antral follicles, respectively. Immature and mature granulosa cells were divided into four groups with or without the presence of FSH as indicated by the experiment protocol. All of the cells displayed several oscillations after synchronization by DXM (Fig. 2A).

However, it is of interest that the mature granulosa cells cultured with FSH for 2 days showed better sustainable oscillations than the other three groups, concerning the number of oscillation and amplitude. Notably, the FSH treatment had a significant effect on the peak time and amplitude of Per2 oscillations in the granulosa cells. The Per2 oscillations of mature granulosa cells cultured with 5% FBS for 1 day and then FSH for 2 days displayed significantly larger first peak amplitude relative to the Per2 oscillations of the immature granulosa cells cultured with 5% FBS for 2 days (Fig. 2B). The Per2 and Rev-erba mRNA levels confirmed this result (Fig. 2C). In addition, the FSH treatment induced Per2 oscillation phase advance shifts in the peak time of the first phase (Fig. 2B).

To exclude the possibility that the elevation of Per2 oscillation and Per2 transcription was because of increasing DXM synchronization ability, we measured the transcript levels of glucocorticoid receptor (Gr: Nr3c1) in immature granulosa cells...
(FBS, 2 days) and mature granulosa cells (FBS, 1 day; FSH, 2 days) by qRT-PCR, and we observed that there was no significant change in the Gr mRNA level in the two groups (Fig. 2D).

Alterations of gene expression upon FSH stimulation. To further analyze the development of a clock system in granulosa cells upon FSH treatment, we collected RNA samples of immature granulosa cells cultured with 5% FBS and mature granulosa cells cultured with FSH at the indicated times for qRT-PCR. The transcript levels of Lhcg, Cx43, and the core clock genes were measured, and the results are shown in Fig. 3. The FSH treatment of the mature granulosa cells significantly increased the expressions of Per2, Rev-era, Bmal1, and Cx43 relative to the immature granulosa cells cultured with 5% FBS for 2 days. Notably, there was a huge increase in Lhcg expression (15-fold). Conversely, the expression of Clock mRNA was not changed.

Dynamic changing distribution of Cx43 protein in the ovaries from hormone-treated rats with different follicular developmental stages. We also examined the Cx43 protein expression in ovaries from hormone-treated rats with different follicular developmental stages using immunofluorescence and Western blot analysis. As shown in Fig. 4A, the control ovaries had mostly primordial and primary follicles. The DES-treated group had their ovaries filled with preantral follicles. In the ovaries of the eCG group, the follicles were at the early antral to late antral stage.

The immunolocalization of Cx43 in the ovaries of the control, DES, and eCG groups was shown in Fig. 4A. Small follicles from ovaries of the control and DES-treated groups were shown no or weak immunostaining signals (Fig. 4A, Ab and Ae). An intriguing finding was that large antral follicles from the eCG-treated group revealed strong dense immunolabeling of Cx43 protein that was widely distributed between granulosa cells (Fig. 4Ae). Immunofluorescence using preimmune rabbit serum instead of the antibodies showed no staining (data not shown). Similarly, Western blot analysis was consistent with immunofluorescent data (Fig. 4B). eCG substantially increased the Cx43 protein expression in immature rat ovaries compared with the control and DES treatment. Consequently, our immunofluorescence investigation and Western blotting analysis indicated that eCG greatly augments Cx43 protein expression.

Effect of gap junction blockers on the clock system of mature granulosa cells. To determine whether gap junction blockers have an effect on the modulation of circadian rhythm in mature granulosa cells, we investigated the circadian rhythm of in vitro Per2 promoter activity using mature granulosa cells upon FSH stimulation for 2 days. Mature granulosa cells were synchronized by DXM for 2 h and then they were monitored in the absence or presence of DMSO (vehicle), lindane, or CBX. There was a clear decrease of bioluminescence data of Per2 oscillations in the presence of two gap junction blockers,
lindane and CBX (Fig. 5, A and B). The first phase amplitude of Per2 oscillation was significantly downregulated in the presence of lindane or CBX, which was further confirmed by the Per2 and Rev-erbα transcript levels (Fig. 5, B and C). Interestingly, both lindane and CBX also induced phase-delay shifts of Per2 oscillations (Fig. 5B).

**DISCUSSION**

The present results indicate that FSH, as a potent regulator of follicular maturation and development, elicits the development of circadian clockwork in ovarian granulosa cells through the upregulation of Cx43, which is a vital gap junction protein.
between granulosa cells. This novel finding may advance our understanding of how FSH regulates the circadian clock system in ovaries and may provide insight into the importance of gap junctions in ovarian physiology.

There is a growing recognition that a functional circadian clock system exists in rat ovaries (11, 12, 16, 25, 72). DXM is a potent synchronization factor described in many previous studies (4, 5, 11, 12, 24, 27). Here, our result showed that several Per oscillations were generated by DXM synchronization, which indicated the presence of effective circadian clockwork in mature granulosa cells (Fig. 1A). This result was in line with two earlier reports from our laboratory (11, 12). In addition, the dynamic transcription profiles of several core clock genes across 24 h indicated that a conservative circadian clock molecular mechanism resides in mature granulosa cells (Fig. 1B). Consistent with existing evidence, our data provide a further confirmation of the presence of a functional circadian clock system in mature granulosa cells (11, 12).

To assess the circadian regulation of local physiology in specific tissues, the expression levels of two putative clock-controlled genes (Star, Lhcgr) were also measured across 24 h controlled genes (11, 12). Specifically, Star promoter contains E-box enhancers that bind to CLOCK/BMAL1 heterodimers to activate gene transcription.

In our present study, the Cosinor analysis showed that Star mRNA has a circadian rhythm transcription across 24 h (P < 0.01) in mature granulosa cells (Fig. 1B), in line with an earlier report from our group (11). Interestingly, the same report illustrated that the Star transcription profile did not show a circadian rhythm in immature granulosa cells (11). Based on previous and present information, it is conceivable that the circadian transcription of Star results from the maturation of the circadian clockwork in mature granulosa cells. It appears, therefore, that mature granulosa cells after FSH stimulation for 2 days gain functional and mature circadian clockwork.

Over the past decade, luminescence-detecting systems have become a useful tool for the real-time monitoring of circadian oscillations in diverse species (26, 41, 61, 66, 69–71). Here, we used a similar technique to monitor Per2-dLuc circadian dynamics in rat granulosa cells in the presence or absence of FSH. Interestingly, our data revealed that FSH significantly increased the Per2-dLuc oscillation amplitude in a time-dependent manner (Fig. 2, A and B). Consistent with the above results, the mature granulosa cells treated for 2 days with FSH showed higher Per2 and Rev-erbα mRNA expression than the immature granulosa cells at the first phase (Fig. 2C).
Intriguingly, the FSH treatment also induced a phase-advance shift of Per2 oscillations (Fig. 2B). This result strongly suggested that FSH provides a “gate” to develop the functional cellular clock in granulosa cells, in line with two earlier reports from our group (11, 12). Here, DXM was used as a synchronization factor in granulosa cells, and therefore the expression of Gr may determine the DXM synchronization ability. Thus there is a possibility that the elevation of Per2 oscillation and Per2 mRNA in mature granulosa cells may be due to the increased transcription of Gr. This possibility was excluded by the lack of different expression of Gr in immature and mature granulosa cells before DXM synchronization (Fig. 2D). A previous report confirmed our present finding that Gr expression was unchanged by gonadotropin treatment in rat ovaries (58).

To investigate how FSH regulates the development of a circadian clock in granulosa cells, we further compared several genes’ expression in immature and mature granulosa cells before DXM synchronization. We found that the majority of the genes’ expressions were significantly elevated by FSH treatment, except for Clock (Fig. 3). The high expression of circadian clock genes (Bmal1, Per2, and Rev-erba) in the FSH-treated mature granulosa cells forecasted the presence of a functional circadian clock in mature granulosa cells. A common recognition has been reached on the point that FSH greatly augments Lhcgr transcription in rat granulosa cells. In accord with this recognition, we observed a great increase in the transcription of Lhcgr in mature granulosa cells after FSH treatment (Fig. 3). This result indicated the different maturation states of granulosa cells between immature and mature granulosa cells. In addition, LH is regarded as a strong endocrine signal to orchestrate circadian rhythms in ovaries, as shown by several elegant studies (11, 25, 72). Thus we speculate that a large increase in Lhcgr transcription by FSH stimulation in mature granulosa cells is a preparation for synchronization with LH surge.

Intriguingly, we observed increased transcription of Cx43 in mature granulosa cells treated with FSH (Fig. 3). In addition, our immunofluorescent data showed a large elevation of Cx43 protein in the large antral follicles of the eCG-treated ovaries but a weak signal or no signal in the small follicles of the control and DES-treated groups (Fig. 4). In recent decades, numerous studies have shown that FSH causes the upregulation of Cx43 function through mRNA, protein, and phosphorylation level (20, 28, 54). Given that increased expression of Cx43 is accompanied by the maturation of circadian clockwork in granulosa cells, we speculate that there is a connection between the gap junction pathway and the circadian clock in rat ovaries. Lindane and CBX are two common gap junction inhibitors that have been proved by a substantial number of studies (9, 14, 15, 22, 32, 56). Thus, we used these two gap junction blockers to evaluate their potential roles in the circadian rhythms of mature granulosa cells. Our results showed that both lindane and CBX significantly attenuated the Per2 oscillation amplitude in mature granulosa cells, which was further demonstrated by the low Per2 and Rev-erba mRNA levels (Fig. 5). In addition, lindane and CBX treatment markedly delayed the Per2 oscillation phase time in the mature granulosa cells compared with the control treatment. Interestingly, we found that the mature granulosa cells treated with lindane or CBX shared a similar Per2 oscillation-phase time delay tendency with the immature granulosa cells. Therefore, the above results may indicate that Cx43 expression is involved in the development of the circadian clockwork in granulosa cells. Our results are reasonably in line with several studies illustrating that gap junction channels are vital to maintain and synchronize circadian rhythms in diverse tissues (6, 51, 63, 73).

As for the specific molecular mechanism underlying how the increased expression of Cx43 contributes to the development of the circadian clockwork in rat mature granulosa cells, we think that there are two possible explanations. The first is that the increased expression of Cx43 enhances cell communication among adjacent cells, and therefore it is easy to synchronize a larger population of single cell oscillators to the same state, in turn enhancing the Per2 amplitude and Per2 mRNA level. A recent study supports this hypothesis. This splendid investigation identified that circadian rhythms in rat-1 cells and mouse primary fibroblasts are composed of single cell oscillators (65). Thus a medium change entrained most of the single cell oscillators to cluster, which contributed to an elevation of the bioluminescence rhythm amplitude. In addition, we propose that cell coupling among single cells determines the robustness of circadian rhythms. Consistent with the present study, a further investigation demonstrated that an increased amplitude of Per2 circadian rhythms upon light stimulation in mouse fibroblasts is implicated with a better synchronization state of all of the single cell oscillators (48). Interestingly, Liu and his colleagues showed that intercellular coupling among adjacent cells confers robustness against mutations in the SCN circadian clock network (35). In the present study, we found that the mature granulosa cells generated higher Per2 oscillation amplitudes than the immature granulosa cells. In addition, the mature granulosa cells treated with the gap junction blockers displayed lower Per2 amplitudes than the control group. These results may indicate that mature granulosa cells are easy to synchronize compared with immature granulosa cells. In light of the increased expression of Cx43 in the mature granulosa cells and the importance of the gap junction in determining cell coupling, we speculate that the maturation of the circadian clockwork in mature granulosa cells results from enhancing gap junction coupling upon FSH stimulation.

The second possible explanation for the specific molecular mechanism underlying how the increased expression of Cx43 contributes to the development of the circadian clockwork in mature granulosa cells is that the enhanced ability of gap junctions among granulosa cells permits more chemical signals to transmit within cells, thereby synchronizing the activity of the coupled cells and enhancing the amplitude of single cell oscillators. It is accepted that gap junction channels allow the exchange of cAMP, amino acids, nucleotides, and calcium ions within adjacent cells. Many studies concluded that cAMP and calcium ions are essential to sustain mammalian circadian rhythms through paracrine coupling, which accounts for the increased amplitude, accuracy, and robustness of circadian oscillators (2, 34, 45, 47, 59, 64). We therefore raise the possibility that the maturation of the circadian clockwork in mature granulosa cells is because of enhanced paracrine coupling via cAMP and calcium ions contributed by the high expression of Cx43.

As shown in Fig. 3, our study revealed an elevation of several core clock genes’ expression in the mature granulosa cells (more than the immature granulosa cells) before DXM synchronization, a result that firmly supports the second pos-
possible explanation. However, we cannot exclude the first possibility that the increased expression of Per2 and Rev-erbα is attributed to a better synchronization ability of population cells via the high expression of Cx43. Thus, the development of the circadian clockwork in granulosa cells is perhaps via the second possibility or a combination of both explanations. Overall, our present study supplied the first evidence that gap junction channels under FSH regulation are implicated in circadian clock genes’ expression in ovarian granulosa cells. Further studies are required to dissect the specific mechanism of the maturation of the circadian clockwork in granulosa cells promoted by the gap junction pathway.

In conclusion, our data demonstrate the existence of functional circadian clockwork in mature granulosa cells and the development of circadian clockwork in granulosa cells upon FSH stimulation through the gap junction pathway. As revealed by using the gap junction blockers, it is strongly suggested that FSH induces the development of the clock system by increasing the expression of Cx43. Our findings contribute to our understanding of the cellular and molecular mechanisms underlying the effect of FSH on the circadian clockwork in granulosa cells.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: H.C., Y.S., S.H., and M.A.H. conception and design of research; H.C., L.Z., G.C., and G.K. performed experiments; H.C., L.Z., and N.Y. analyzed data; H.C. and M.A.H. interpreted results of experiments; H.C., L.Z., and G.C. prepared figures; M.A.H. drafted manuscript; H.C. and M.A.H. edited and revised manuscript; M.A.H. approved final version of manuscript.

REFERENCES
32. Ke FC, Fang SH, Lee MT, Shyu SY, Lai SY, Chen YJ, Huang FL, Wang PS, Stocco DM, Hwang JJ. Lindane, a gap junction blocker, suppresses FSH and transforming growth factor beta 1-induced con...

Kumar NM, Gilula NB. The gap junction communication channel. Cell 84: 381–388, 1996.


