HHS Public Access

Author manuscript

Vitam Horm. Author manuscript; available in PMC 2019 February 19.

Published in final edited form as:

Vitam Horm. 2018 ; 107: 67–87. doi:10.1016/bs.vh.2018.01.010.

Regulation of Luteinizing Hormone Receptor mRNA Expression in the Ovary: The Role of miR-122

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Abstract

The expression of luteinizing hormone receptor (LHR) in the mammalian ovary is regulated in response to changes in the secretion of follicle-stimulating hormone and luteinizing hormone by the anterior pituitary, at least in part, through posttranscriptional mechanisms. The steady-state levels of LHR mRNA are maintained by controlling its rate of degradation by an RNA-binding protein designated as LHR mRNA-binding protein (LRBP). LRBP forms a complex with LHR mRNA and targets it for degradation in the p bodies. miR-122, an 18 nucleotide noncoding RNA, regulates the expression of LRBP. Thus, the levels of miR-122 determine the cellular levels of LHR mRNA expression. This phenomenon has been examined during the induction of LHR mRNA expression that occurs during follicle maturation in response to rising levels of FSH. In this situation, miR-122 and LRBP levels decrease as LHR mRNA expression undergoes downregulation in response to preovulatory LH surge. miR-122 expression as well as LRBP levels show robust increases. The mechanism of induction of LRBP by miR-122 has also been discussed.

Keywords

LH receptor; RNA-binding protein; Posttranscriptional regulation; microRNA; Ovary; Noncoding RNA; G protein-coupled receptors; Mevalonate kinase; Reproductive function; Hormone receptor regulation

1 Introduction

The adult mammalian ovary undergoes cyclical changes throughout the reproductive period in response to constantly changing secretory patterns of the anterior pituitary hormones, LH and FSH, which are in turn regulated by ovarian steroids through feedback mechanisms. In the human, early events of follicular maturation consist principally of mitosis of granulosa cells and transformation of the surrounding stroma to layers of theca cells stimulated by gradually rising levels of FSH and low levels of LH. In rodents and humans, luteinizing hormone (LH) regulates androgen biosynthesis by the theca–interstitial cells during follicular development, and these androgens are converted to estrogens by the granulosa cells (Bjersing, 1968). The follicular growth during the second half of the follicular phase is accompanied by rising levels of estradiol and increased expression of the LH receptor (LHR). Although a large number of primordial follicles begin to develop in each cycle, all

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become atretic except the one that is destined to ovulate. The granulosa cells in the ovulatory follicles reach optimum size by mitotic activity supported by the increasing levels of estrogen produced in response to FSH and, indirectly, LH. The oocytes undergo meiotic division in the preovulatory follicles, and in most mammals, ovulation occurs when the oocyte has reached the metaphase of the second meiotic division. After ovulation, the cell types associated with corpus luteum secrete progesterone and estrogens to support the implantation of the blastocyst. In the absence of blastocyst implantation, corpus luteum regresses. The ovarian cycle repeats itself until menopause, which occurs after depletion of ovum results in the cessation of the cyclical changes in ovarian structure and function. The main driver of the ovarian cycle is the sequential and combined actions of FSH and LH. While the FSH action is confined to the granulosa cells within the ovarian follicles, LH acts on multiple targets including the theca–interstitial cells, FSH-stimulated granulosa cells, and the corpus luteum after ovulation. While FSH in concert with other paracrine factors is known to regulate the development of the primary follicles to preantral and antral stages, the LHR makes its appearance in significant amounts as a result of FSH stimulation.

The action of LH is mediated by LHR, which belongs to the glycoprotein subfamily of the large G protein-coupled receptor family with an unusually large extracellular domain, seven-membrane spanning helices, and an intracellular domain with two conserved palmitoylation sites (Kawate, Kletter, Wilson, Netzloff, & Menon, 1995; Kawate & Menon, 1994; Munshi, Clouser, Peegel, & Menon, 2005; Zhu, Wang, & Ascoli, 1995). Its expression increases during follicle growth and shows a dramatic decline in response to a bolus of LH that occurs prior to ovulation (Menon, Clouser, & Nair, 2005; Menon, Franzo-Romain, Damanpour, & Menon, 2011; Zeleznik, Midgley, & Reichert, 1974; Zeleznik, Schuler, & Reichert, 1981). During this period of transition, a refractory period of LH responsiveness occurs that is characterized by a transient loss of LHR (Hoffman, Peegel, Sprock, Zhang, & Menon, 1991; LaPolt, Oikawa, Jia, Dargan, & Hsueh, 1990; Lu, Peegel, Mosier, & Menon, 1993; Peegel, Randolph, Midgley, & Menon, 1994; Segaloff, Wang, & Richards, 1990) and consequent loss of the LH responsiveness (Azhar & Menon, 1979; Ghosh, Peegel, Dunham, Sands, & Menon, 1988; Hunzicker-Dunn, Jungmann, Derda, & Birnbaumer, 1979; Sen, Azhar, & Menon, 1979). The receptor levels then rise again, reaching maximum levels by mid-portion of the luteal phase to support progesterone production. The LHR expression then falls with the regression of the corpus luteum if pregnancy does not ensue (Azhar & Menon, 1979; Hoffman et al., 1991; LaPolt et al., 1990; Lu et al., 1993; Peegel et al., 1994). The changes in LHR expression are exquisitely regulated to ensure successful reproduction.

We have examined the mechanisms responsible for the rapid changes in the levels of LHR expression using both rodent and human models and found that a posttranscriptional mechanism, at least in part, accounts for these changes. Furthermore, we have discovered that a noncoding RNA, miR-122, plays a role in this process.

miR-122, a 22-nucleotide microRNA, was first discovered by cloning and sequencing of miRNAs from tissues in mice as a highly abundant liver-specific miRNA (Lagos-Quintana et al., 2002). It was subsequently found to be present in other tissues such as breast cancer tissues, ovaries, and granulosa cells (Fong et al., 2015; Li, Fang, Liu, & Yang, 2015; Salilew-Wondim et al., 2014; Sirotkin, Ovcharenko, Grossmann, Laukova, & Mlyncek,

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2009). miR-122 is essential for the maintenance of liver homeostasis by regulating the expression of a large number of target mRNAs involved in diverse hepatic functions and also by suppressing nonhepatic genes (reviewed in Thakral & Ghoshal, 2015). Pharmacological inhibition and genetic knockout of miR-122 have been shown to induce dysregulation of systemic and hepatic lipid metabolism, iron homeostasis, and differentiation of hepatocytes. miR-122 regulates diverse functions such as cholesterol, glucose and iron homeostasis, lipid metabolism, and infection of hepatitis C virus. Furthermore, miR-122 inhibition leads to a reduction of serum cholesterol by downregulating genes involved in cholesterol biosynthesis, including the regulatory enzyme HMG-CoA reductase, thereby protecting animals from diet-induced hypercholesterolemia. The biogenesis of miR-122 follows the canonical pathway (Filipowicz & Grosshans, 2011; Jopling, 2012). miR-122 gene is transcribed by RNA polymerase II (pol II) from a long noncoding RNA located on chromosome 18 in human and mouse, generating ~ 4.5 kb pri-miR-122, which is then processed to a 66 nt premiR-122 in the nucleus and subsequently to mature miR-122 in the cytoplasm. Our studies have shown that miR-122 regulates LHR mRNA expression posttranscriptionally by regulating the levels of LHR mRNA-binding protein designated as LRBP. Since miR-122 regulates LHR expression by altering LHR mRNA stability, a brief overview of our understanding of the posttranscriptional mechanism of LHR regulation is presented before discussing its regulation by miR-122.

2 Posttranscriptional Regulation of LHR mRNA Expression

Administration of hCG, a hormone with significant chemical identity to LH, produces a rapid decline in LHR mRNA and protein in rat and human ovaries. The loss of steady-state levels of receptor expression could also be due to a temporary pause in the synthesis of new receptor protein by decreased transcription, or an increase in degradation of mRNA. Treatment of superovulated rats with 50 IU hCG led to a rapid decline of LHR mRNA expression (Hoffman et al., 1991). The Northern blot analysis of the LHR mRNA transcripts showed that the decline started at 6 h following hCG treatment and reached undetectable levels by 24 h. The recovery from downregulation was seen after 24 h and almost reached control levels by 72 h. The in situ hybridization pattern of the receptor expression in the whole ovary was in full agreement with the qPCR results and Northern blot data (Peegel et al., 1994). The process of LHR mRNA loss was very specific since other mRNAs involved in ovarian function remained unchanged (Hoffman et al., 1991). This is further supported by the finding that LHR mRNA downregulation can be achieved by chronic elevation of cyclic AMP levels by repeated administration of inhibitors of type 2 phosphodiesterase (Peegel et al., 1994). The evidence for the decrease in the steady-state levels of LHR mRNA expression occurred through posttranscriptional mechanism came from the determination of transcription rates by performing nuclear run-on assays, which showed no decrease in transcription rates during the course of downregulation when compared to the control (Lu et al., 1993). In fact, the nuclei isolated from the hCG-treated ovaries showed a robust increase in the incorporation of [^{3}H] uridine into total RNA compared to the control. These findings supported the view that the loss of LHR mRNA during ligand-induced downregulation occurred posttranscriptionally by increasing LHR mRNA degradation.

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2.1 LHR Expression During Ovarian Cycle Is Regulated Posttranscriptionally by an RNA-Binding Protein

It is now recognized that the expression of specific, highly regulated mRNAs is regulated, at least in part, at the level of mRNA degradation (Ross, 1995). In most such instances, the changes in the stability of the mRNA occur by the binding of proteins to specific sequences and/or structures of the mRNA. The specific regions that the mRNA-binding proteins interact with may be localized either on the 5′ untranslated region, the coding region, or the 3′ untranslated region of the mRNA (Ross, 1995). In general, the steady-state levels of the mRNA expression are regulated by either increasing or decreasing the degradation rate resulting from the interaction with specific RNA-binding proteins.

The possible existence of a binding protein that specifically recognizes LHR mRNA was examined by incubating ^{32}P-labeled LHR mRNA with a cytosolic fraction (100 × g supernatant) prepared from the ovary pretreated with hCG to downregulate LHR mRNA expression in the presence of ribonuclease inhibitors (Kash & Menon, 1998). After treating the reaction mixture with ribonuclease T1 to degrade the unreacted RNA probe, the ribonucleoprotein (RNP) complex was separated by electrophoresis on an 8% native acrylamide gel and subjected to autoradiography. Two RNP complexes were identified, one prominent band corresponding to 50 kDa, and a second less intense band of 45 kDa (Fig. 1). A threefold increase in the 50 kDa RNP complex was seen in samples derived from LHR downregulated ovaries, but no significant change in the intensity of the 45 kDa band was found. Because of this finding, further studies focused on the larger 50 kDa ribonucleoprotein complex, designated as LRBP (Kash & Menon, 1998). The binding of LHR mRNA to LRBP showed that the recognitionsite of the mRNA resided in the amino-terminal portion of the LHR corresponding to nucleotides 102–282. Further, truncation of this region showed that the contact site resided between nucleotides 188 and 228. RNA hydroxyl radical footprinting revealed a bipartite polypyrimidine-rich sequence (UCUCX7-UCUCCCU), corresponding to nucleotides 203–220 of LHR mRNA that specifically interacted with LRBP. Mutation of the C residues within the bipartite sequence revealed that all C residues participated in binding to LRBP (Kash & Menon, 1999). The interaction of LHR mRNA with LRBP exhibited high affinity with equilibrium dissociation constant (K_d) in the range of 4×10^{-9} M.

After establishing that the ovarian LRBP recognizes LHR mRNA with high affinity and specificity, the changes in LRBP activity in relation to tissue levels of LHR mRNA were examined. It has been established that LHR mRNA expression increases during maturation of the ovarian follicles in response to FSH. The levels then fall immediately after the preovulatory LH surge and rise again with the growth and development of the corpus luteum (Nair, Kash, Peegel, & Menon, 2002). The relationship between LHR mRNA expression and LRBP activity was examined in ovarian tissues after treating immature rats with pregnant mare serum gonadotropin to induce follicle growth and to increase LHR mRNA expression levels. At this time point, the LRBP in the cytosolic fractions prepared from the ovaries showed low levels of activity. However, when LHR expression was suppressed during ligand-induced downregulation by treatment with hCG, the LRBP activity was increased several fold~~severalfold~~ in a time-dependent manner. Thus, when the LHR mRNA expression

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level was high, the RNA-binding protein activity was at the lowest level. Conversely, the LHR mRNA-binding activity was high when the mRNA levels were low. This inverse relationship (Fig. 2) suggested that LRBP is an endogenous regulator of LHR mRNA expression.

2.2 In Vitro mRNA Degradation by the LHR mRNA-Binding Protein

The direct role of the RNA-binding protein in LHR mRNA degradation was demonstrated by employing a cell-free mRNA decay system developed by Ross (1993) under *in vitro* conditions. The assay essentially determines the ability of ribosomes to degrade exogenous RNA in the presence and absence of the RNA-binding protein. Although all mRNAs are prone to degradation, the rate of degradation varies depending on the cellular environment. The rate of decay of LHR mRNA was very rapid in ribosomes isolated from the ovaries of rats treated with hCG to downregulate LHR expression compared to the degradation of LHR mRNA by ribosomes isolated from ovaries of the saline-treated control group. The rate of decay of exogenously added ovarian RNA by ribosomes isolated from saline-treated rats was accelerated by the addition of a partially purified ovarian LHR mRNA-binding protein (Nair et al., 2002), demonstrating that the LHR mRNA-binding protein plays a role in LHR mRNA degradation.

2.3 Characterization of LHR mRNA-Binding Protein

After exhausting several approaches to successfully purify LRBP by a variety of techniques, including affinity purification using covalently linked RNA-binding sequence to sepharose, conventional techniques were used to purify the LHR mRNA-binding protein from the 100 × *g* supernatant fraction of the ovarian homogenates. The ovaries were initially downregulated by treatment with the ligand in order to increase the yield of the binding protein (Kash & Menon, 1999). The supernatants were subjected to chromatography on a strong cation exchange resin (Macro-Prep High S support) and eluted with 150 mM KCl. The eluates were concentrated and subjected to SDS-PAGE to separate the proteins. The ^{32}P-LHR mRNA-binding activity associated with the protein band on the gel was identified by an overlay assay (Northwestern blot) using ^{32}P-labeled LHR mRNA fragment (203 – 220) as the probe. After extensive standardization of the assay, the corresponding protein band showed the RNA-binding activity was cut, eluted, and renatured. The eluted protein was electrophoresed again to determine the purity of the preparation. The electrophoretically homogeneous protein band was then subjected to amino-terminal analysis, as well as MS-MALDI analysis, to establish its identity. Both analyses revealed the purified protein to be mevalonate kinase (Nair & Menon, 2004). The gene encoding the rat mevalonate kinase was then cloned and overexpressed in 293 T cells (Nair & Menon, 2004). The recombinant protein exhibited a concentration-dependent increase in the binding LHR mRNA probe (Nair & Menon, 2004). The binding exhibited all the characteristics of the expected LHR mRNA-binding protein with respect to specificity for binding to the previously identified contact site (nucleotides 203–220), competition by unlabeled LHR mRNA fragment (203–220), dependence on C residues in the ligand-binding site, and immunoreactivity of the recombinant protein similar to that seen for the electrophoretically purified rat mevalonate kinase. Mevalonate kinase is a metabolic enzyme involved in cholesterol biosynthesis which catalyzes the conversion of mevalonate to phosphomevalonate, the precursor of cholesterol

and many natural products. In fact, we have shown that mevalonate kinase expression is regulated by LH in the corpus luteum both at the mRNA level and at the protein level (Wang & Menon, 2005). During LH/hCG-induced downregulation, mevalonate kinase expression was induced in the corpus luteum. The induction of expression of other sterol response element (SRE)-containing genes such as HMG-CoA reductase, farnesyl pyrophosphate synthase, and LDL receptor was also seen during this phase (Wang & Menon, 2005). These results suggest that mevalonate kinase expression plays a regulatory role in LHR mRNA expression in the corpus luteum.

Depletion of mevalonate kinase expression by 25 hydroxycholesterol resulted in the abrogation of LHR mRNA downregulation in cultured human granulosa cells (Wang, Nair, & Menon, 2007). The inhibitory effect of mevalonate kinase on LHR expression was documented independently by Ikeda et al. (2008), who showed that overexpression of mevalonate kinase can abrogate an FSH-induced increase in LHR mRNA expression in rat ovarian follicles.

The notion that mevalonate kinase, a metabolic enzyme, acts as an mRNA-binding protein is consistent with a similar role of several other metabolic enzymes that have been described (Menon et al., 2005). The iron-response element-binding protein, which plays a role in iron homeostasis, is an enzyme in the citric acid cycle (Hentze & Kuhn, 1996). Similarly, thymidylate synthase, dihydrofolate reductase, glyceraldehyde-3-phosphate dehydrogenase, glutamate dehydrogenase, and lactate dehydrogenase have all been shown to serve as RNA-binding proteins that regulate different aspects of RNA metabolism (Chu, Schmitz, Ju, & Copur, 1999; Nagy & Rigby, 1995; Pioli, Hamilton, Connolly, Brewer, & Rigby, 2002).

3 Role of miR-122 in LHR Expression

Since LRBP plays a crucial role in LHR expression during ligand-induced downregulation (Fig. 3), the regulatory factors controlling LRBP expression would be expected to have a major influence on LHR mRNA expression. While searching for possible regulatory mechanisms of LRBP expression, a study by Krutzfeldt et al. who tested the biological significance of silencing miR-122 using antagomirs in mice came to our attention (Krutzfeldt et al., 2005). They identified miR-122 as a positive regulator of many enzymes in the cholesterol biosynthetic pathway such as HMG-CoA reductase, phosphomevalonate kinase, and mevalonate kinase, among several other targets in the liver. Since miR-122 was shown to be a liver-specific regulator of mevalonate kinase in that study, we examined whether miR-122 is also expressed in the ovarian tissue and its potential role in regulating LHR mRNA expression. Since mevalonate kinase has been identified as the protein that binds LHR mRNA, we will be referring to mevalonate kinase as LRBP in the context of LHR mRNA regulation in this chapter.

3.1 LH/hCG Increases miR-122 Expression

In order to determine a possible role for miR-122 in LRBP and LHR regulation, we examined the changes in the levels of miR-122 during LHR downregulation using real-time PCR. As shown in Fig. 4A, there was a significant increase in the expression of miR-122 immediately after hCG treatment. The increase was initially evident at 30 min post-hCG and

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peaked around 1–2 h, but started to decline thereafter. The expression of miR-122 in the ovary and its upregulation in response to hCG were further confirmed by fluorescence in situ hybridization (FISH), followed by confocal microscopic analysis of frozen sections from normal and hCG-treated rat ovaries, using a 5′-FITC-labeled miR-122 probe (Menon et al., 2013). The results (Fig. 4B) showed that while there was low or no expression of miR-122 in the control ovaries, its expression showed an increase in response to hCG treatment. It is noteworthy that control ovaries showed no detectable levels of miR-122 expression in real-time PCR assays or in FISH analyses. These findings suggest that miR-122 is hormonally regulated during the ovarian cycle. The failure to detect miR-122 expression in normal ovaries as reported in some studies (Landgraf et al., 2007) could be attributed to this fact, since the levels of miR-122 expression in the ovary is undetectable when LHR expression occurs maximally.

Analysis of the temporal relationship between miR-122 expression and ligand-induced downregulation of LHR mRNA showed a close relationship between the time frame of expressions of miR-122, LRBP, and LHR mRNA (Fig. 5). miR-122 expression showed an increase at 30 min post-hCG and remained steady until 2 h. A rapid surge in LRBP mRNA levels was then observed. The levels of LRBP mRNA increased at 2 h after hCG treatment and remained high until 4 h. An increase in LRBP protein expression was then seen at 4 h and sustained until 6 h of hCG treatment. Most importantly, LHR mRNA levels started to decline at 4 h post-hCG, and a minimum decrease was observed by 12 h. Thus, an increase in the expression of miR-122 was followed by an increase in LRBP mRNA and protein expression, culminating in decreased levels of LHR mRNA regulation. This temporal relationship suggested that miR-122 might play a role in mediating an increase in LRBP levels during LHR mRNA downregulation.

The signaling pathway involved in hCG-induced expression of miR-122 showed the involvement of cAMP/PKA and ERK pathways (Menon, Gulappa, & Menon, 2015; Menon et al., 2013). For example, pretreatment with H-89, a PKA inhibitor, as well as inhibition of ERK1/2 using U0126, abrogated hCG-mediated increases in miR-122 in primary cultures of human granulosa cells. A significant increase in the expression of miR-122 was seen at 1 h following hCG treatment. This increase was abrogated when cells were pretreated with U0126 or H-89 1 h before hCG treatment. This is in agreement with our previous findings that the cAMP/PKA pathway plays a crucial role in the induction of LRBP expression and its binding to LHR mRNA during hCG-mediated LHR mRNA downregulation. These findings suggest that hCG treatment activates the cAMP/PKA/ERK1/2 signaling cascade and results in an increase in miR-122 levels, culminating in increased LRBP expression and leading to LHR mRNA downregulation.

3.2 Inhibition of miR-122 Abrogated hCG-Induced LRBP Induction and LHR mRNA Downregulation

The role of miR-122 as an upstream regulator of LRBP comes from studies inhibiting miR-122 expression, followed by determining the changes in hCG-induced LRBP expression. miR-122 expression was totally blocked by pretreating superovulated rats with an LNA-conjugated antagomir of miR-122 24 h prior to treatment with hCG. Importantly,

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abolishment of miR-122 expression by treatment with the antagomir abrogated the increase in LRBP mRNA and protein levels seen in response to hCG, further supporting the finding that the increased LRBP expression required for LHR mRNA downregulation is mediated by miR-122. Since miR-122 inhibition prevented hCG-induced LRBP upregulation, it would be expected that the decrease in LRBP might lead to a decrease in the association of LRBP with LHR mRNA. RNA electrophoretic gel mobility shift assay using cytosolic (S100) fractions of ovarian cell lysates and a ^{32}P-labeled LHR mRNA probe that is known to interact with LRBP showed a marked increase in the RNP complex formation between LRBP and labeled LHR mRNA probe following hCG treatment. This RNP complex formation was significantly reduced by miR-122 antagomir treatment.

Further studies showed that the inhibition observed for LRBP–LHR mRNP complex formation in the presence of miR-122 antagomir also led to abrogation of LHR mRNA downregulation. As shown in Fig. 6, the levels of LHR mRNA in the hCG-treated samples were reduced by almost 80%. This was significantly reversed by pretreatment with an antagomir, which reduces miR-122 expression, bringing the LHR mRNA levels very close to the control levels. These results support the notion that miR-122-mediated upregulation of LRBP plays a crucial role in hCG-mediated LHR mRNA downregulation.

3.3 miR-122 Increases LRBP Expression Through the Activation of SREBP-1a/ SREBP-2

As stated earlier, mevalonate kinase (LRBP) is a member of the family of enzymes encoded by genes containing oxysterol responsive element. As shown by the elegant work of Brown and Goldstein (1997), oxysterols act by suppressing the proteolytic cleavage of the precursor of sterol response element-binding protein (SREBP) to its active form. The cleavage product, the active form of SREBP, binds to SRE, which causes transcriptional activation of genes regulated by SREBP. Conversely, when cellular concentrations of oxysterols are low, the cleavage of the precursor of SREBP is enhanced to produce an active form of SREBP, which then accelerates transcription of genes containing SRE elements in the promoter region. Therefore, it is likely that miR-122 might act at the level of SREBP processing to increase the expression of LRBP. Examination of the effect of miR-122 inhibition on SREBP levels showed that, as expected, hCG increased the activation of both SREBP-1a and SREBP-2, and that when miR-122 expression was inhibited using antagomir pretreatment, the activation of both SREBP-1a and SREBP-2 was completely blocked. This supports the notion that SREBP activation is an intermediary step in miR-122-mediated upregulation of LRBP in response to hCG treatment. This was further verified by demonstrating that hCG-mediated activation of both SREBP-1a and SREBP-2 was inhibited by pretreatment with U0126 or H-89, indicating that SREBP activation is downstream of cAMP/PKA-ERK1/2-miR-122 pathways and leads to LH/hCG-induced LHR downregulation.

The role of miR-122 in SREBP-mediated LRBP induction was further demonstrated by analyzing the binding of SREBP-1a to the promoter region of LRBP by ChIP assay, followed by real-time PCR to monitor LRBP expression. These studies showed that binding of SREBP-1a to the LRBP promoter was increased after hCG treatment. Most interestingly, the increase was almost completely abrogated by pretreatment with miR-122 antagomir prior to hCG treatment (Fig. 6). Thus, SREBP binds to the putative SRE element in the

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promoter region of the LRBP gene, which then increases the LRBP gene transcription. Inhibition of miR-122 y the antagomir treatment resulted in the inhibition of LRBP gene expression by decreasing the association of active SREBP to the LRBP promoter (Menon et al., 2015).

3.4 Downregulation of miR-122 During LHR Upregulation in Response to FSH Treatment

As stated earlier, it is now well established that during follicle growth in response to FSH, the growing follicles increase the expression of LHR. We now show that miR-122 plays a role in FSH-mediated upregulation of LHR expression by decreasing the expression of LRBP. The finding that rat granulosa cells treated with a dose of FSH (50 ng/mL) along with 17β-estradiol (E2) showed a time-dependent increase in LHR mRNA expression, and that this increase was accompanied by decreased levels of miR-122 levels, clearly shows that miR-122 levels remain suppressed when robust expression of LHR mRNA occurs. Analysis of miR-122 levels showed that FSH treatment produced a decline in miR-122 expression as predicted, prior to increasing LHR mRNA. However, this increase was not sustained throughout the FSH treatment period. After 24 h of FSH treatment, levels of miR-122 were comparable to that of the control. Thus, a decrease in miR-122 preceded the induction LHR mRNA in response to FSH treatment, suggesting that suppression of miR-122 expression triggers factors that increase LHR mRNA expression.

We have reported that an increase in miR-122 is always followed by a corresponding increase in LRBP expression, and that this increase in LRBP leads to an increase in its association with LHR mRNA, culminating in the accelerated degradation of LHR mRNA (Azhar & Menon, 1979; Menon et al., 2015; Menon, Gulappa, & Menon, 2017; Menon et al., 2013). Thus, it would be expected that a decrease in miR-122 levels would lead to a decreased association of LHR mRNA with LRBP. This in fact is the case, since analysis of the binding of LRBP with LHR mRNA by RNA electrophoretic mobility shift assay revealed a significant decrease in the association of LHR mRNA with LRBP during FSH-mediated induction of LHR mRNA. The intensity of the band representing the LRBP–LHR mRNP complex in the control was significantly higher compared to that seen after FSH treatment. This supports our hypothesis that the decrease in miR-122 is accompanied by a decrease in LRBP–LHR mRNP complex formation and inhibits LHR mRNA degradation, resulting in increased levels of LHR mRNA levels.

The suppressive effect of miR-122 on in LHR expression was further confirmed by overexpressing miR-122 *in vitro* by employing an adenoviral vector (Menon et al., 2017). Treatment of AdmiR-122-infected cells and cells infected with a GFP-tagged empty vector with FSH for another 24 h showed higher levels of LHR expression in the control group and complete suppression in the miR-122-infected group (Fig. 7). Furthermore, while FSH treatment decreased LRBP mRNA expression, overexpression of miR-122 reversed FSH-induced reduction in LRBP gene expression. Consistent with this finding, a decrease in the association of LHR mRNA with LRBP in the FSH-treated group was observed, but this decrease was abolished when miR-122 was overexpressed prior to FSH treatment. In summary, these findings suggest that overexpression of miR-122 initiated a series of events that culminated in accelerated degradation of LHR mRNA and prevented FSH-mediated

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induction of LHR mRNA. Thus, miR-122 regulates LHR levels by controlling LRBP levels (Fig. 8).

4 Conclusion and Future Directions

miR-122 is an important molecule with therapeutic potential that has already been established in the liver, and studies are currently in progress on developing novel technologies for targeted delivery of miR-122 to liver and cancer cells. However, its function in the ovary and its role in fertility remain unexplored. On the basis of our studies, it can be speculated that miR-122 might serve as a gatekeeper for controlling LHR expression in the ovaries. Thus, approaches to alter endogenous miR-122 levels might potentially be beneficial in the treatment of infertility. We speculate that miR-122 could serve as a physiological mechanism to regulate LHR expression at the appropriate levels, thereby preventing ovarian hyperstimulation, a common complication encountered in assisted reproduction.

Acknowledgment

Supported by NIH Grant HD 06656.

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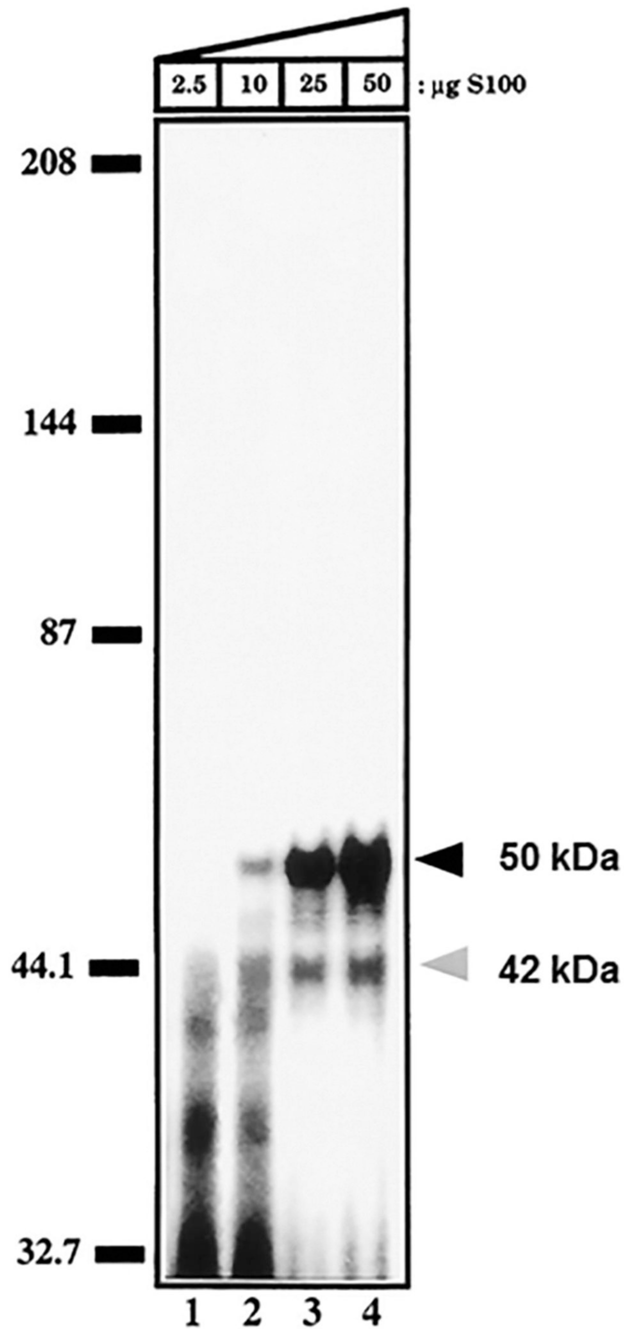

Fig. 1.

LHR mRNA binding to cytosolic fraction of the ovary during LHR downregulation. Autoradiogram of RNA gel mobility shift analysis performed by incubating with different concentrations (2.5 μg (lane 1), 5 μg (lane 2), 10 μg (lane 3), 25 μg (lane 4), or 50 μg (lane 5)) of cytosolic proteins with 32P-labeled (1×10^5 cpm) RNA. Two RNP complexes, corresponding to 50 and 45 kDa, are indicated by arrows (*Source*: Kash & Menon, 1998).

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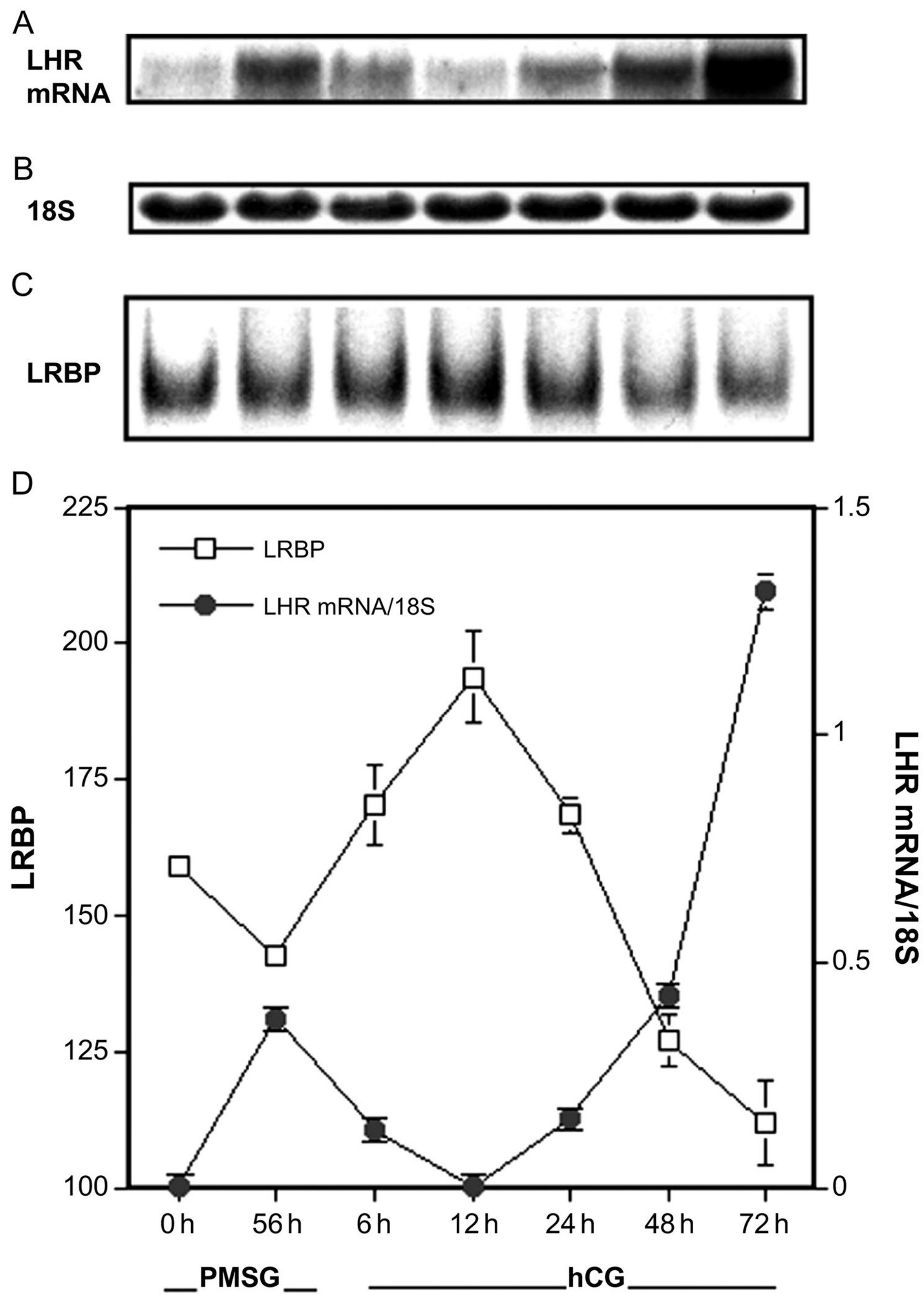

Fig. 2.

LHR mRNA expression and RNA-binding activity of the cytosolic extracts from ovaries treated with PMSG and hCG. Twenty-three-day-old rats were treated with PMSG at 0 h. Ovaries were collected at 0 and 56 h later. hCG was administered at 56 h of PMSG administration, and ovaries were harvested at 6, 12, 24, 48, and 72 h. (A) Northern blots of LHR mRNA. (B) The Northern blots were normalized using 18S rRNA. (C) RNA electrophoretic mobility shift analysis performed at these time points after incubating 50 μg of cytosolic protein isolated from ovaries incubated with 32P-labeled LHR mRNA probe.

(D) 6.7-kb LHR mRNA transcript and RNA electrophoretic mobility shift analysis bands quantitated by densitometric scan and expressed in arbitrary units (*Source*: Nair et al., 2002).

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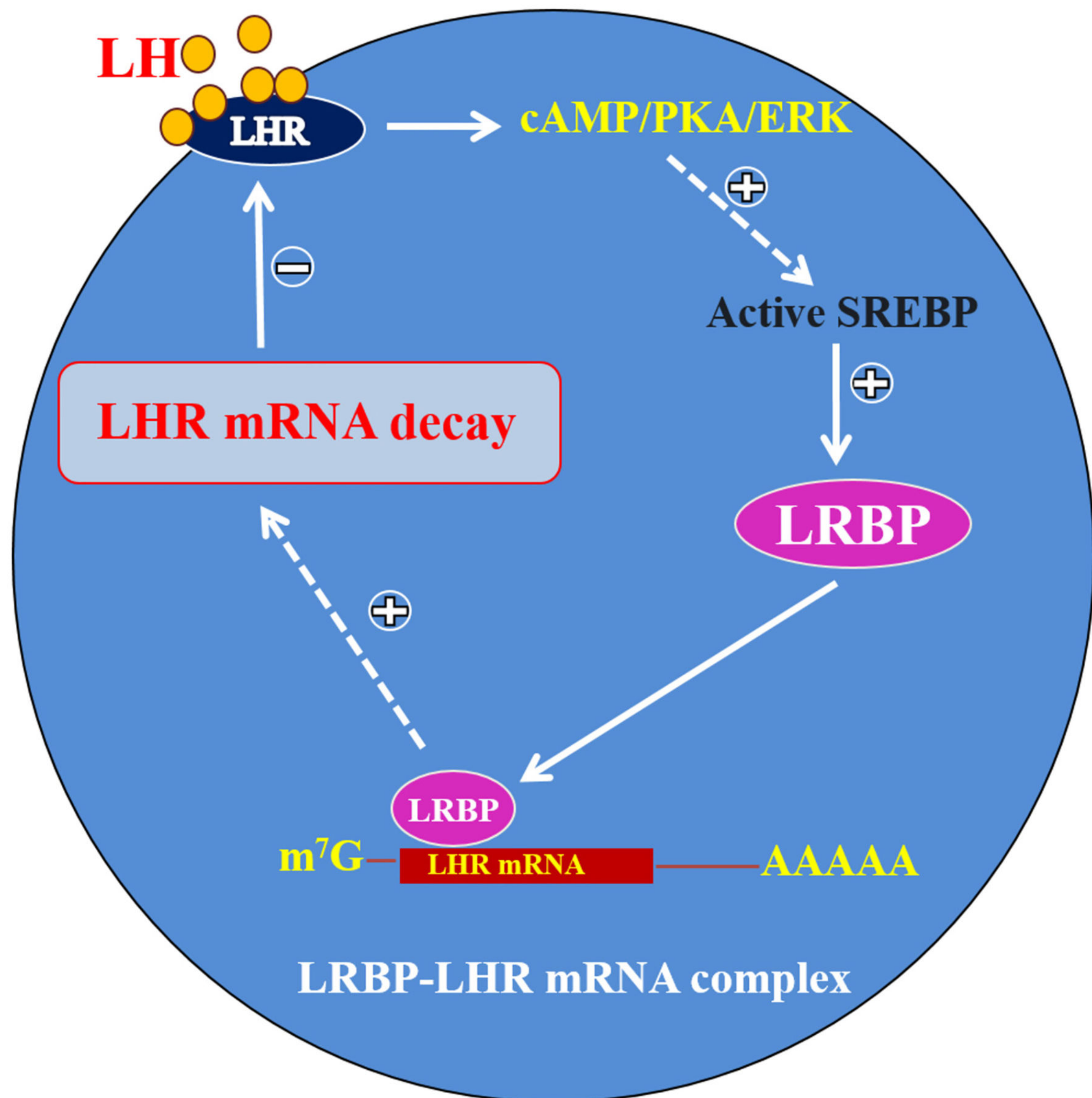

Fig. 3.

Schematic representation of LRBP-mediated regulation of LHR expression. LH/hCG binding to the LHR activates the cAMP signal cascade and increases the transcription of LRBP through the activation of the transcription factor SREBP. LRBP binds to LHR mRNA, forming an untranslated mRNP complex, which is then targeted for degradation.

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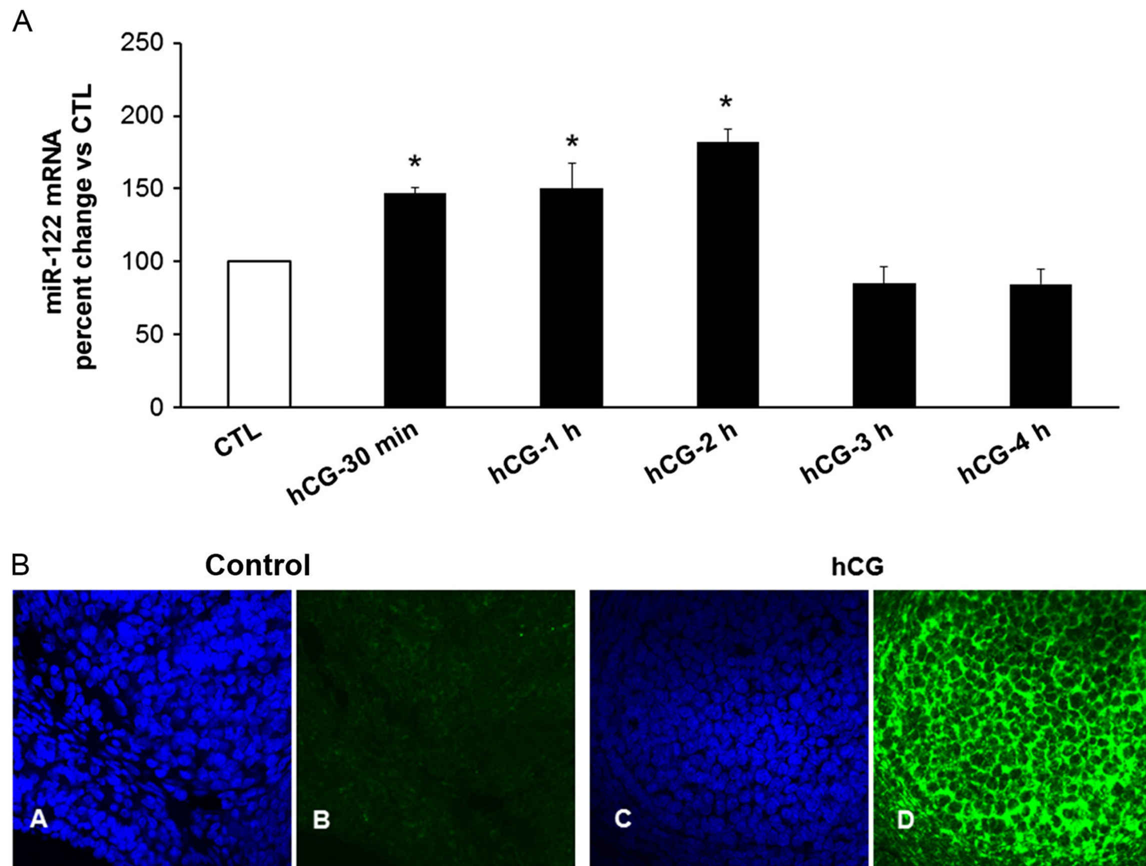

Fig. 4.

Analysis of miR-122 expression in the ovaries using real-time PCR and fluorescent in situ hybridization analysis. (A) Superovulated rats were treated with hCG on day 5. Ovaries were collected and total RNA isolated at different time intervals. The levels of miR-122 and U6 snRNA were determined by real-time PCR. (B) Fluorescent in situ hybridization analysis (FISH) of control (CTL) and hCG-treated (1 h) frozen rat ovary sections were conducted using FITC-labeled miR-122 LNA probe. Each panel shows DAPI staining (a and c; *blue fluorescence*) on the *left* and FITC (miR-122) staining (b and d; *green fluorescence*) on the *right* (Menon, Sinden, Franzo-Romain, Botta, & Menon, 2013).

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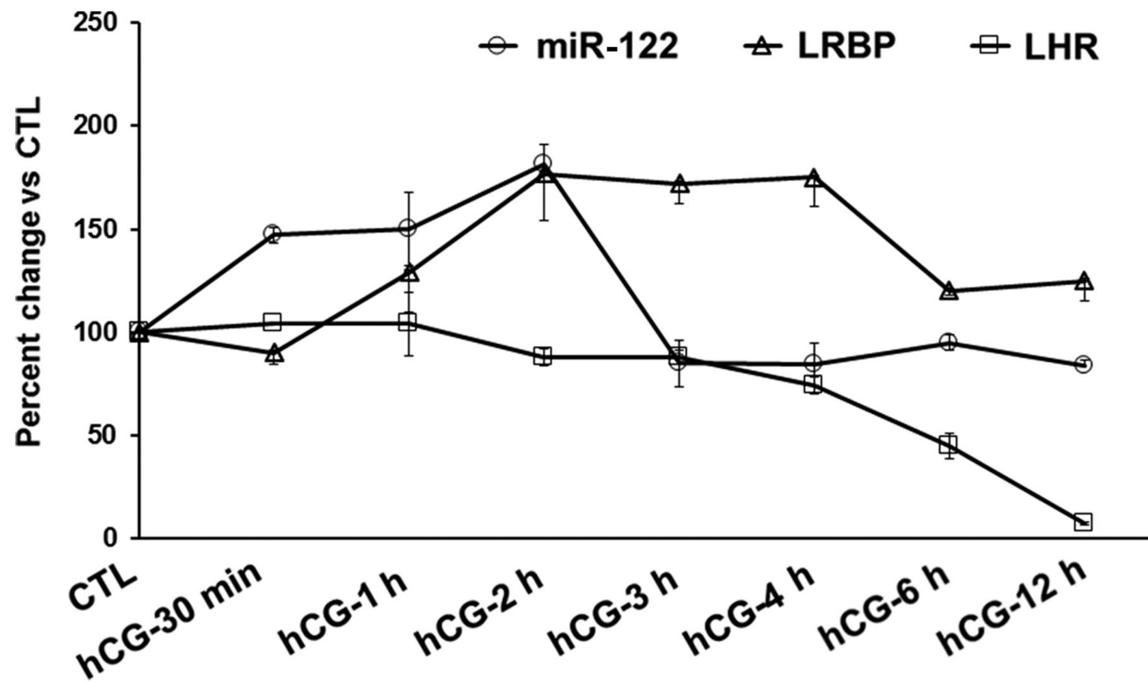

Fig. 5.

Temporal relationship of miR-122, LRBP, and LHR mRNA expressions in the ovary following hCG treatment. Superovulated rats were treated with hCG on day 5, and ovaries were collected at 0, 30 min, 1, 2, 3, 4, 6, and 12 h later. Equal amounts of RNA from the control (CTL) or hCG-treated ovaries were subjected to real-time PCR using predesigned primers and probes for miR-122, LRBP, or LHR mRNA. The *graphs* represent the ratio of LRBP or LHR mRNA levels normalized to 18S RNA and that of miR-122 normalized to U6 snRNA, and are shown as percent change vs control (Menon et al., 2013).

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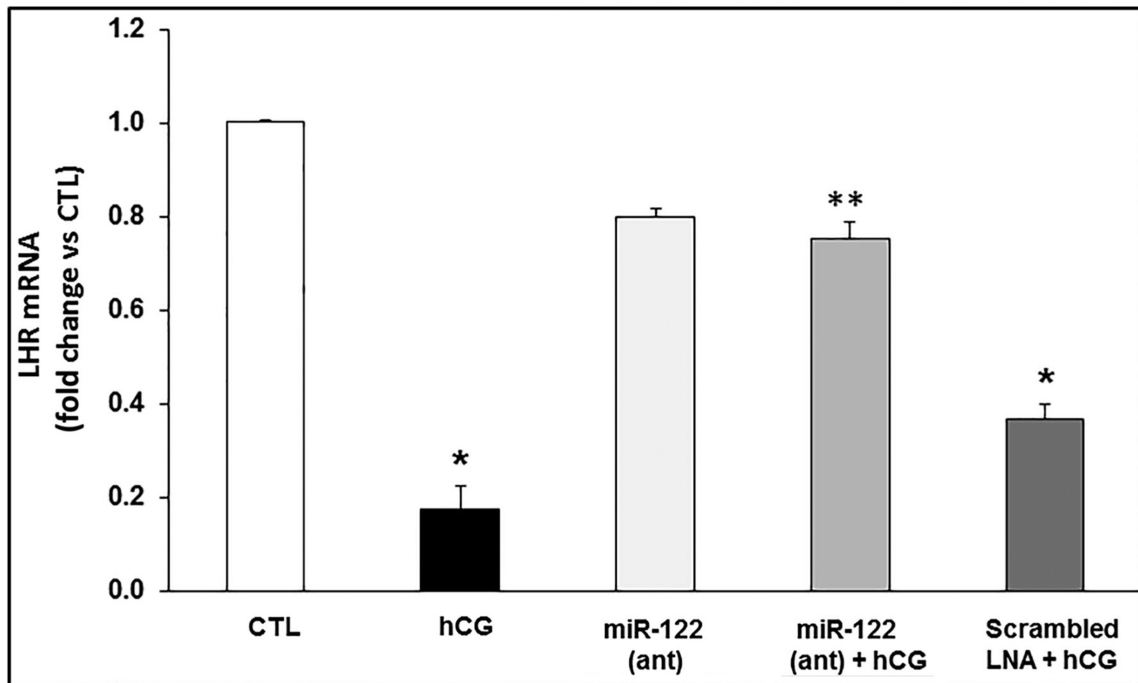

Fig. 6.

Reversal of hCG-induced LHR mRNA downregulation by miR-122 antagomir. LNA-conjugated miR-122 antagomir or scrambled LNA control was injected into the bursa of the ovaries of superovulated rats on day 4, followed by hCG on day 5. Ovaries were collected 12 h later and were processed for total RNA isolation. Total RNAs were reverse transcribed, and the resulting cDNAs were subjected to real-time PCR quantitation using specific primers and probes for LHR. The *graph* represents changes in mRNA levels normalized to 18S rRNA and shown as fold change vs control (CTL) (Menon et al., 2015).

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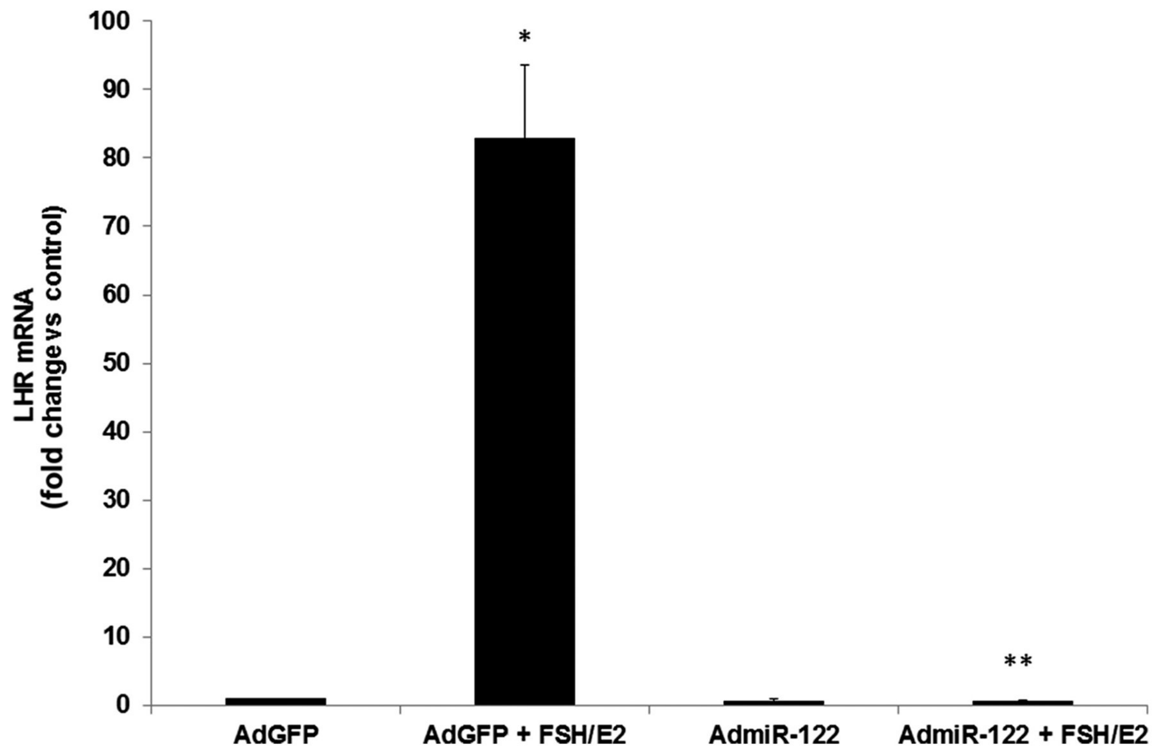

Fig. 7.

Overexpression of miR-122 inhibited LHR upregulation. Cells were pretreated with AdmiR-122 (1×10^9 pfu) or AdGFP (1×10^9 pfu) for 24 h. Media were replaced after 24 h, and cells were treated with FSH (50 ng/mL) and E2 (1 nM) for 6 h. Total RNAs were isolated and reverse transcribed, and the resulting cDNAs were subjected to real-time PCR quantitation using specific primers and probes for LHR. The *graph* represents changes in mRNA levels normalized to 18S rRNA and shown as fold change vs control (Menon et al., 2017).

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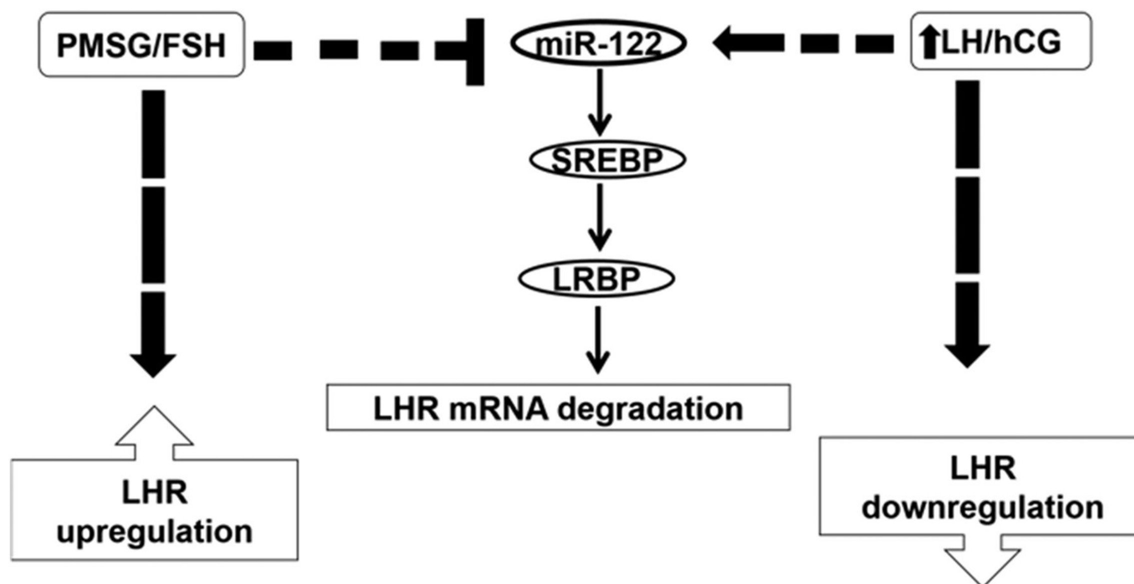

Fig. 8.
miR-122/LRBP-mediated regulation of LHR levels in the ovary. miR-122 is expressed in response to a high dose of LH or hCG, which then activates SREBP and leads to increased expression of LRBP. Increase in LRBP causes a decrease in LHR mRNA. FSH causes decreased expression of miR-122 and LRBP during follicle growth. Decrease in LRBP causes an increase in LHR mRNA by preventing its degradation.