NR5A2 and potential regulatory miRNAs in the bovine CL during early pregnancy

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Abstract

Progesterone, which is secreted from the corpus luteum, is indispensable for the establishment and maintenance of pregnancy. The orphan nuclear receptor subfamily 5 group A member 2 (NR5A2) is a regulator of murine luteinization, but neither its regulation nor its role in the fully differentiated, mature corpus luteum (CL) have been described. Therefore, the goal of this study was to profile abundance and investigate the regulation and functions of NR5A2 in the bovine CL. Treatment of cultured luteal steroidogenic cells with a pharmacological inhibitor of NR5A2 decreased progesterone production and tended to decrease abundance of *HSD3B1* mRNA. Luteal NR5A2 mRNA increased and NR5A2 protein tended to increase between days 4 and 6 of the estrous cycle, coincident with increased steroidogenic capacity of the CL. Luteal NR5A2 mRNA decreased by 8 h after prostaglandin (PG) F_{2A} injection. During early pregnancy, luteal NR5A2 mRNA was less on days 20 and 23 compared to day 14, but protein abundance did not change. Neither 1 nor 10 ng/mL interferon tau (IFNT) altered NR5A2 abundance in cultured luteal steroidogenic cells, but 10 ng/mL PGF_{2A} decreased NR5A2. Because of discrepancies between mRNA and protein abundance of NR5A2, regulation by miRNA that changed during early pregnancy was investigated. miR-27b-3p, miR-432-5p, and miR-369-3p mimics decreased NR5A2 protein abundance and miR-369-3p also inhibited progesterone production. Overall, the results of this study show that NR5A2 may be maintained by miRNA during early pregnancy and may be an important regulator of luteal progesterone production.

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Introduction

Maintenance of luteal progesterone production is necessary for successful establishment of pregnancy, whereas a rapid decline in progesterone is the functional result of luteal regression and is required for subsequent ovulation (reviewed by Niswender *et al.* 1994). While both processes are necessary to fertility and reproduction, the molecular mechanisms governing luteal function at the crossroads of survival and death are not fully understood (reviewed by Pate 2020).

The transcription factor nuclear receptor subfamily 5 group A member 2 (NR5A2), also known as liver receptor homolog 1 (LRH-1), is an orphan nuclear receptor that can induce transcription in the absence of ligand binding. This orphan receptor is absolutely required for ovulation in the mouse (Duggavathi *et al.* 2008) and was recently identified as a potential key regulator of luteal function (Hughes *et al.* 2019). mRNA encoding NR5A2 was greater on day 17 of pregnancy than on the same day of the estrous cycle (Hughes *et al.* 2019).

In a separate transcriptome study, NR5A2 declined in response to an injection of prostaglandin (PG) F_{2A} in CL that were responsive to PGF_{2A}, but did not change in response to an injection of PGF_{2A} in day 4 CL, which have not yet acquired the capacity to regress in response to PGF_{2A} (Mondal *et al.* 2011). This is consistent with the finding of Taniguchi *et al.* (2009), who reported a decline in luteal NR5A2 in completely regressed CL, but did not investigate changes during regression. These results implicate NR5A2 as a potential regulator of luteal demise and survival.

Although NR5A2 was first identified in the liver, it plays an essential role in ovulation and luteinization. In mice with a granulosa-specific knockout of NR5A2, cumulus expansion, follicular rupture, and luteinization failed (Duggavathi *et al.* 2008). Importantly, in a conditional knockout model in which NR5A2 was depleted following the luteinizing hormone (LH) surge, CL were smaller than in the control mice, the abundance of steroidogenic transcripts was reduced, and CL produced less progesterone (Zhang *et al.* 2013, Bertolin

et al. 2014). Genes important for steroidogenesis have been identified as targets of NR5A2 in mice and human cells *in vitro* (Schoonjans *et al.* 2002, Sirianni *et al.* 2002, Peng *et al.* 2003, Kim *et al.* 2005) and luteal NR5A2 is not different in midcycle (days 8–12) as compared to late (days 15–17) CL (Taniguchi *et al.* 2009). Overall, these data indicate an important role for NR5A2 in luteal development and maintenance. However, the role of NR5A2 during early pregnancy has not been elucidated.

MicroRNA (miRNA) are small (18–22nt) post-transcriptional regulators of mRNA abundance and translation. Otsuka *et al.* (2008) first demonstrated a role for miRNA in luteal function when they impaired miRNA processing pathways and demonstrated that mice were infertile, due in part to luteal insufficiency. Similarly, cultured bovine luteal cells with knockdown of Drosha Ribonuclease III (DROSHA), a key component of miRNA-mediated repression pathways, produced less progesterone and had higher rates of apoptosis both in the absence and presence of cytokines (Maalouf *et al.* 2016), demonstrating a role for miRNA in normal luteal function. Several studies have implicated specific miRNA in luteal progesterone production (Maalouf *et al.* 2016, Mohammed *et al.* 2017, Xu *et al.* 2018) and NR5A2 is known to be modulated by miRNA in other tissues (Tay *et al.* 2008, Li *et al.* 2015, Tian *et al.* 2017).

Among mRNA that changed in the recently published transcriptomics study comparing CL of days 17 of the estrous cycle and pregnancy (Hughes *et al.* 2019), NR5A2 emerged as a potentially important, yet minimally studied, luteal regulator. It was a predicted miRNA target in that study and, while it was in greater abundance in pregnancy, it declined in response to a luteolytic injection of PGF2A (Mondal *et al.* 2011). These findings, together with the previous data demonstrating a role for NR5A2 in murine luteinization, indicated that a study of luteal functions and regulation of NR5A2 was needed. The objectives of this study were to determine the abundance of NR5A2 during the acquisition of luteolytic capacity, luteal regression, and luteal rescue, to identify functional roles of NR5A2 in the CL, and to determine how NR5A2 is regulated in the CL, including by miRNA.

Materials and methods

Animals and tissue collection

All animal handling protocols were approved by the Institutional Animal Care and Use Committees at The Pennsylvania State University or West Virginia University. Normally cyclic cattle were observed for estrous behavior (day 0). For the luteal regression experiment, CL were removed by colpotomy on days 10–11 (midcycle, MC) at 0, 0.5, 1, 2, 4, 8, or 12 h after an intramuscular injection of a luteolytic dose (25 mg) of Lutalyse (Pfizer). Days 4 and 6 CL were collected from cows that had been synchronized for estrous with a controlled internal drug release device (CIDR; Zoetis). Six days after CIDR insertion,

cows were given a luteolytic injection of Lutalyse, and CIDRs were removed 1 day later. Two days later, cows were observed for heat, and the presence of a large, preovulatory follicle was determined by ultrasound. An ovulatory dose of GnRH (100 µg Factrel; Zoetis) was administered, and CL were collected either 4 or 6 days after the injection. Cows were slaughtered for CL collection on day 4, while CL from day 6 were collected via colpotomy. For CL of days 14, 17, 20, and 23 of pregnancy, luteal tissue samples were collected as described by Hughes *et al.* (2020). For CL of days 17 of the estrous cycle and pregnancy, luteal tissue samples were collected as described by Hughes *et al.* (2019). For all cell culture experiments, CL were collected via colpotomy on days 10–12 after observation of a natural estrous.

RNA Isolation and quantitative PCR

All reagents and supplies were ordered from ThermoFisher Scientific unless otherwise specified. RNA was isolated using the miRcury kit (Exiqon), according to the manufacturer's protocol. RNA concentration and quality were assessed with an Experion Automated Electrophoresis System (BioRad); all RQI values were greater than 9. RNA was then treated with DNase (Promega) according to the manufacturer's protocol and cDNA was synthesized using the DyNAmo cDNA Synthesis Kit (for tissue RNA samples) or AzuraQuant cDNA synthesis kit (Azura; for cultured cell RNA samples), according to the manufacturer's protocol. Quantitative PCR (qPCR) was performed, with either Bioline (for tissue RNA samples) or Azura (for culture cell RNA) SYBR green. The same SYBR green was used for a single experiment. Quantification of mRNA abundance was relative to a standard curve of serially diluted PCR product of the gene of interest. During an initial primer validation, PCR products were run on an agarose gel to visually confirm the presence of a single product of the correct size and this product was sequenced to confirm unique identity as the transcript sequence of interest. For tissue cDNA, RPL19 was used as a reference gene, while for cultured cells, GAPDH was used. In no case was the expression of the reference gene altered by treatment ($P > 0.15$). All primer sequences are shown in Table 1.

Western blot analysis

Tissue samples were homogenized in urea lysis buffer (7 M urea, 2 M thiourea, 5 mM dithiothreitol, 2% w/v CHAPS) and cellular debris was pelleted out. Protein concentration was assessed by Bradford assay (BioRad Protein Assay, BioRad Laboratories) according to the manufacturer's protocol. Cultured cell samples were collected in RIPA buffer (purchased at 10× concentration from Abcam and diluted to 1× immediately before use) and cellular debris was pelleted out. Cultured cell protein concentration was assessed using a BCA assay according to the manufacturer's protocol. Proteins were separated on a Mini-PROTEAN TGX Precast Stain-Free Gel (BioRad) and transferred to a polyvinylidene fluoride (PVDF) membrane using an iBlot Dry Blotting System (Invitrogen). Western analyses were initially performed with anti-NR5A2/LRH-1 primary antibody (Abcam, product number ab62601),

Table 1 All primers used for qPCR experiments.

Gene	Forward primer 5'–3'	Reverse primer 5'–3'	Amplicon size	Accession number
<i>GAPDH</i>	AGATGGTGAAGGTCGGAGTGA	CCTTGACTGTGCCGTGAACT	180	NM_001034034.2
<i>NR5A2</i>	GTCCTGCCCAAGGCTTCAAA	AGCCCGTAATGGTACCCAGA	259	NM_001206816.1
<i>RPL19</i>	ATCGATCGCCACATGTATCA	GCGTGCTTCCTTGGTCTTAG	196	NM_001040516
<i>HSD3B1</i>	TCCACACCAGCACCATAGAA	AAGGTGCCACCATTTCAG	178	NM_174343.3
<i>CYP11A1</i>	GACCCTCTCTCATCCACA	CCTAGCCAGAAAGGTGCAAG	290	NM_176644.2
<i>SRB1</i>	CAGACATGGGCAACCTCTCT	TGGATGATCCCCTCAGGGTT	244	XM_005217738.4
<i>SOD2</i>	GGATCCCCTGCAAGGAACAA	TGGCCTTCAGATAATCGGGC	110	NM_201527.2

but subsequent Western blots were performed using the Thermo anti-NR5A2 antibody (Thermo PA5-73018), which produced cleaner blots. All blots shown in the 'Results' section were generated with the latter antibody. Both antibodies were used at 1 µg/mL, and donkey anti-rabbit IgG horseradish peroxidase secondary antibody (GE Healthcare) at a 1:10,000 dilution. Both antibodies were validated to bind specifically to human recombinant NR5A2 protein (Abcam, product number ab81844) and did not bind to the negative control tissue used (bovine skeletal muscle). All antibody validation can be seen in Supplementary Fig. 1A, B, C and D (see section on [supplementary materials](#) given at the end of this article). Sample bands were the same molecular weight as this recombinant protein band. Moreover, because BSA migrates at a similar molecular weight as NR5A2, each antibody used was preadsorbed with a 1.5 molar excess of BSA prior to use. Membranes were developed with SuperSignal West Femto Maximum Sensitivity Substrate and visualized using a ChemiDoc XRS System (BioRad). When BioRad Stain-Free gels were not used for normalization, the membrane was stripped with Restore Stripping Buffer and reprobed with mouse anti-beta-actin primary antibody and sheep antimouse IgG horseradish peroxidase secondary antibody and developed as previously described. Total protein from Stain-Free gels (BioRad) and NR5A2 abundance from bands was quantified using BioRad's Image Lab 6.0 software.

Cell culture, viability assay, and progesterone ELISA

Luteal cells were isolated and cultured as previously described (Pate 1993). For cell viability and progesterone production assays, treatments were initiated immediately after plating and cells were treated for 18–22 h. The NR5A2 inhibitor, Calbiochem #505601, was added to cells in dimethyl sulfoxide (DMSO; 0.004% v/v). For luteinizing hormone (LH)-responsiveness assays, cells were cultured for 6 h in glass culture tubes in a shaking water bath. For assessment of the regulation of NR5A2 by interferon tau (IFNT) and PGF2A, cells were cultured overnight, washed, and treatments were applied for 24 h, after which cells were collected. IFNT was generously provided by Dr Fuller Bazer, Texas A & M University. Concentrations of treatments were as shown in the 'Results' section. A progesterone ELISA was performed to assess progesterone secretion into culture media, as previously described (Petroff *et al.* 1997), with the following modifications: samples and antibodies were diluted in a buffer of 0.04 M 3-(N-morpholino)propanesulfonic acid, 0.12 M NaCl, 0.01 M EDTA, 0.05% Tween 20, 0.005%

chlorhexidine digluconate, and 0.1% gelatin. Antibodies were goat antimouse IgG antibody (2 µg/mL; EMD Millipore) and monoclonal progesterone antibody (57.8 ng/mL; East Coast Bio). Progesterone conjugate (East Coast Bio) was diluted 1:750. Functional range of the progesterone standard curve (Cayman Chemical) was 0.16 to 10 ng/mL and all samples were diluted so that they fit within the functional range of the assay. Cell number was assessed using an MTT cell viability assay, according to the manufacturer's protocol, with serially-diluted untreated cells serving as a standard curve relative to which treated wells were quantified.

Analysis of miRNA and target prediction

A dataset of miRNA predicted to target NR5A2 was obtained using Targetscan version 7.2 (Agarwal *et al.* 2015). Given the clear discrepancy between mRNA and protein abundance during early pregnancy, this time was targeted as the likeliest time for NR5A2 translation to be regulated by miRNA. Nanostring profiling was used according to the manufacturer's protocol to assess the abundance of all miRNA in the CL on days 14, 17, 20, and 23 of pregnancy (Hughes *et al.* 2020). These differentially abundant miRNA were used to screen the list of predicted NR5A2-targeting miRNA to identify a list of candidate miRNA that may regulate NR5A2 protein during early pregnancy.

Transfection and flow cytometry

Luteal cells were cultured as described previously, with the addition of 10 ng/mL LH and 10% newborn calf serum to medium, and in the absence of gentamicin. Luteal cells were transfected on day 1 of culture, with TRANS-IT TKO transfection reagent (Mirus Bio) and 500 nM of each miRNA mimic or inhibitor (Ambion) according to the manufacturer's protocol. Transfection medium was removed after 18 h and was replaced with serum-free medium including gentamicin. Transfection efficiency was $83 \pm 7.5\%$. The most effective knockdown of protein abundance was observed 48 and 72 h after the end of transfection, with little effect 24 h after the end of transfection. Therefore, protein abundance results 48 h after the end of transfection are shown. Progesterone production was assessed 72 h after the end of transfection to allow time for the knockdown of protein, if present, to have an effect on subsequent progesterone production. For flow cytometry, luteal cells were removed using trypsin, fixed and permeabilized, and stained for NR5A2 using the same antibody used for Western blotting, at 5 µg/mL. This concentration of

antibody resulted in increased fluorescence relative to the isotype and secondary antibody-only controls (Supplementary Fig. 1E). All events were plotted on a scatterplot of forward scatter vs side scatter and luteal cells were gated on the basis of size to separate them from the cellular debris generated by trypsinization, typically resulting in more than 10,000 events to analyze. Mean fluorescence intensity of this population was used as a measure of NR5A2 abundance.

Statistical analysis

All statistical analyses were performed using the mixed model of SAS 9.4 (Statistical Analysis System Institute). An ANOVA test with mRNA abundance of ribosomal protein L19 (*RPL19*) or glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a covariate in the model was used when analyzing mRNA abundance. Similarly, total protein loading, as assessed on the BioRad Stain-Free gel was used as a covariate for NR5A2 protein in the model when assessing protein abundance. mRNA and protein abundance are expressed as LS means $\pm$ S.E.M. from these analyses. In cases in which Western blots were run on multiple membranes for one experiment, or for cell culture experiments in which cells from each animal got the same treatment, animal or membrane was included in the model as a block. Dunnett's test was used to compare each time of regression to the midcycle CL to determine how NR5A2 changes during luteolysis relative to a functional CL. A Tukey test was used to compare the times of early pregnancy. In all cases data are reported as least square means $\pm$ pooled S.E.M. and differences were considered significant when $P < 0.05$, with statistical tendencies at $P < 0.1$. P values between 0.1 and 0.15 are shown, for the information of the reader, but not considered tendencies.

Results

To determine if NR5A2 regulates luteal steroidogenesis in fully differentiated luteal cells, the effects of a pharmacological NR5A2 inhibitor on cell viability, progesterone production, LH responsiveness, and abundance of mRNA related to steroidogenesis were assessed. This inhibitor binds to NR5A2 and prevents it from taking on the active conformation required to induce transcription (Benod *et al.* 2013). The inhibitor (Calbiochem #505601), used at 10 μ M, did not alter viability of cultured luteal cells (data not shown; $P=0.78$), but reduced basal progesterone in 18–22 h cultures (Fig. 1A) and reduced both basal and LH (10 ng/mL)-stimulated progesterone in 6-h incubations (Fig. 1B).

Abundance of four potential targets of NR5A2 in inhibitor-treated cells was measured by qPCR, to assess the mechanism by which NR5A2 inhibition reduces progesterone production. Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*HSD3B1*) tended to be reduced by the NR5A2 inhibitor. However, scavenger receptor class B member 1 (*SCARB1*), cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*), and superoxide dismutase 2

Figure 1 Effect of inhibition of NR5A2 on luteal steroidogenic cells *in vitro*. (A) Progesterone production in luteal steroidogenic cells treated with vehicle or NR5A2 inhibitor for 18–22 h, normalized to number of viable cells per well, at the time of medium collection ($n=5$ separate CL). (B) Progesterone production and LH-response in luteal steroidogenic cells treated with vehicle or NR5A2 inhibitor for 6 h, normalized to number of cells/tube ($n=4$ separate CL). In both A and B, vehicle was DMSO and did not alter cell number or progesterone compared to a no DMSO control. * $P < 0.05$, compared to vehicle. In B, * over individual bars indicates the main effect of inhibitor, while * on the line between bars indicates the main effect of LH.

(*SOD2*) were not altered by treatment with the NR5A2 inhibitor (data not shown).

Next, abundance of luteal NR5A2 was assessed during acquisition of luteolytic capacity, luteal regression, and luteal rescue. Luteal tissue samples from days 4 and 6 of the estrous cycle were used to encompass the acquisition of luteolytic capacity period. Luteal NR5A2 mRNA and was greater on day 6 than on day 4 of the estrous cycle. Abundance of NR5A2 protein followed a similar pattern; between days 4 and 6 luteal NR5A2 tended to increase ($P = 0.06$; Fig. 2).

To assess changes in NR5A2 during luteal regression, CL were collected 0, 0.5, 1, 2, 4, 8, 12, and 24 h after a luteolytic injection of PGF2A administered to midcycle cows. Abundance of NR5A2 mRNA decreased 8 and 12 h after PGF2A, as compared to 0 h (Fig. 3).

Discrepancies between NR5A2 mRNA and protein abundance were observed during early pregnancy. NR5A2 mRNA in the CL decreased as pregnancy progressed (Fig. 4A). Protein concentrations did not follow this pattern; NR5A2 protein abundance did not change among days of pregnancy (Fig. 4B, D and E). Comparing days 17 of the estrous cycle and pregnancy, luteal NR5A2 protein abundance did not differ ($P = 0.14$; Fig. 4C, F and G).

Given the changes observed in NR5A2 abundance during the estrous cycle, early pregnancy, and luteal regression, it was hypothesized that physiological concentrations of IFNT or PGF2A might regulate NR5A2. Neither 1 ng/mL IFNT nor 10 ng/mL IFNT altered NR5A2 protein in cultured luteal cells. However, 10 ng/mL PGF2A significantly decreased NR5A2 in cultured luteal cells, even in the presence of IFNT (Fig. 5).

Figure 2 Abundance of NR5A2 during acquisition of luteolytic capacity. (A) Abundance of NR5A2 mRNA in CL of days 4 and 6 of the estrous cycle ($n = 4$ cows per group); *Significant difference ($P < 0.05$) from day 4. (B) Abundance of NR5A2 protein in CL of days 4 and 6 of the estrous cycle (day 4 $n = 3$ cows, day 6 $n = 4$ cows). (C) A Western blot image of NR5A2 on days 4 and 6 of the estrous cycle. The farthest left day 4 sample was excluded from analysis as no NR5A2 protein was detected. (D) The stain-free membrane image (showing total protein loading) that corresponds to the blot shown.

Discrepancies between NR5A2 mRNA and protein during early pregnancy led us to hypothesize that NR5A2 protein expression may be regulated by miRNA. Thus, miRNA were profiled during early pregnancy (days 14–23) using Nanostring technology (Hughes *et al.* 2020). From this dataset, five miRNA were selected as potential NR5A2-targeting miRNA, based on TargetScan prediction (Agarwal *et al.* 2015). Among these, miR-369-3p and miR-539-5p were detected only on day 14, miR-1185-2-3p was detected only on days 14 and 17, miR-432-5p declined over time during early pregnancy, and miR27b-3p increased over time (Fig. 6). These miRNA were tested for their ability to regulate NR5A2 in cultured luteal cells in cell transfection studies.

Abundance of NR5A2 in transfected luteal cells was assessed by flow cytometry. A representative scatterplot (Fig. 7A) shows the population of luteal cells, superimposed with beads of varying sizes, which were used to determine an appropriate cutoff for luteal cells. Mean fluorescence intensity of this population of luteal cells was used to measure NR5A2 abundance in cells labeled with the NR5A2 antibody.

Figure 3 Abundance of NR5A2 mRNA during luteal regression. * $P < 0.05$, relative to 0 h control ($n = 4$ cows per time).

Abundance of NR5A2 in cells transfected with the scrambled mimic and scrambled inhibitor controls did not differ from that in cells treated with transfection reagent only, so each mimic or inhibitor was compared

Figure 4 Abundance of NR5A2 during pregnancy. (A) Abundance of NR5A2 mRNA ($n = 4$ cows per time). (B) Abundance of NR5A2 protein ($n = 4$ cows per time). (C) Abundance of NR5A2 protein in CL of day 17 of the estrous cycle and pregnancy ($n = 6$ cows per status). (D) A representative Western blot image showing abundance of NR5A2 during early pregnancy. (E) The representative stain-free membrane image (showing total protein loading) that corresponds to the representative blot shown for early pregnancy. (F) A representative Western blot showing abundance of NR5A2 protein in CL of day 17 of the estrous cycle and pregnancy; C day 17 cyclic; P day 17 pregnant. (G) The representative stain-free membrane image (showing total protein loading) that corresponds to the representative blot shown for CL of day 17 of the cycle and pregnancy.

Figure 5 Effect of IFNT and PGF2A on abundance of NR5A2 in midcycle luteal steroidogenic cells *in vitro*. (A) Effect of 1 and 10 ng/mL IFNT on NR5A2 protein abundance ($n = 5$ separate CL). (B) Effect of PGF2A (10 ng/mL) and IFNT (1 ng/mL) on NR5A2 protein abundance ($n = 3$ separate CL). *Main effect of PGF2A ($P < 0.05$). (C) A representative Western blot membrane image. (D) The representative stain-free membrane image (showing total protein loading) that corresponds to the representative blot shown.

to its respective scrambled control only. Three miRNA mimics, miR-27b-3p, miR-432-5p, and miR-369-3p, significantly decreased NR5A2 protein abundance, whereas the other two mimics tested had no effect (Fig. 7B). The miRNA inhibitors were generally without effect, although the miR-369-3p inhibitor decreased NR5A2 abundance (Fig. 7C).

It was hypothesized that the three miRNA that altered NR5A2 abundance may also affect luteal progesterone production. Because the miRNA-induced inhibition of NR5A2 abundance was observed at 48 and 72 hours, amount of progesterone secreted into media between 48 and 72 h following transfection was determined. The scrambled mimic tended to decrease ($P < 0.10$) and the scrambled inhibitor decreased ($P < 0.05$) progesterone relative to the cells treated only with the transfection reagent (data not shown). The miR-369-3p mimic further inhibited progesterone production, with progesterone being significantly less than the scrambled control. Although there was an apparent inhibition of progesterone in response to miR-432-5p mimic, variability in response of cells among replicates rendered this to be insignificant ($P = 0.14$; Fig. 8A). The miR-27b-3p mimic and each inhibitor had no effect on progesterone production (Fig. 8B).

Figure 6 The five miRNA predicted to target NR5A2 that change in the CL during early pregnancy ($n = 4$ cows per time). For miR-27b-3p and miR-432-5p, different letters indicate significant differences among days.

Discussion

The transcription factor NR5A2 was identified as a potential regulator of luteal fate in the recent transcriptomics study comparing changes in luteal mRNA during early pregnancy to changes during luteal regression (Hughes *et al.* 2019). These findings were the rationale for conducting this study, as they implicated NR5A2 as an important regulator of luteal survival or regression and indicated that in the future, NR5A2 could be targeted to support luteal function. In the current study, NR5A2 regulated luteal progesterone production, increased at the time of acquisition of luteolytic capacity, decreased during luteal regression, and was maintained during early pregnancy. Moreover, three miRNA that regulated NR5A2 were identified, one of which also regulated progesterone production.

Previous studies have demonstrated an important role for NR5A2 in the regulation of steroidogenesis, via the regulation of key steroidogenic enzymes (Schoonjans *et al.* 2002, Sirianni *et al.* 2002, Peng *et al.* 2003, Kim *et al.* 2005, Duggavathi *et al.* 2008, Zhang *et al.* 2013, Bertolin *et al.* 2014, Gerrits *et al.* 2014), but none have investigated the effect of NR5A2 on progesterone production in the mature, fully differentiated CL. In the current study, an NR5A2 inhibitor reduced progesterone in the presence and absence of LH, but did not alter LH responsiveness. This effect may be mediated through transcriptional regulation of proteins involved in steroidogenesis. Taniguchi *et al.* (2009) showed a

Figure 7 Effect of miRNA mimics and inhibitors on abundance of NR5A2 in cultured luteal cells at 48 h after the end of transfection. (A) A representative scatterplot of the population of dissociated cells (red) with beads of varying sizes used to determine a size cutoff to select the population of luteal steroidogenic cells, with 15 μ M beads in dark green, 10 μ M beads in neon green, 6 μ M beads in orange, and 4 μ M beads in blue. Cells > ~12 μ M were used for further analysis. (B and C) The abundance NR5A2 in the scrambled mimic or scrambled inhibitor control is indicated by the line labeled control. *Significant difference from control ($P < 0.05$; $n = 6$ separate CL).

Figure 8 Effect of miRNA mimics and inhibitors on progesterone production in cultured luteal cells. The abundance of NR5A2 in the scrambled mimic or scrambled inhibitor control is indicated by the line labeled control. *Significant difference from control ($P < 0.05$; $n = 5$ separate CL).

correlation in the expression pattern of NR5A2 with StAR protein (*StAR*), *CYP11A1* and hydroxysteroid 17- β dehydrogenase 3 (*HSD17B3*) in bovine luteal tissue and there is extensive evidence for regulation of steroidogenic enzymes by NR5A2 in other species (Meinsohn *et al.* 2019). These proteins are highly abundant in the CL and are regulated through redundant mechanisms, making even a small reduction in progesterone production remarkable, particularly after the brief, 6-h incubation.

A few other studies have reported the presence of NR5A2 in the bovine corpus luteum and have reported declines in NR5A2 in both spontaneous (Taniguchi *et al.* 2009) and induced (Ochoa *et al.* 2018) luteolysis. In the latter study, regression was induced with intrauterine pulses of PGF2A, and both luteolysis and NR5A2 transcript abundance were rescued by infusion of PGE1 with PGF2A (Ochoa *et al.* 2018). In this study, inducing luteal regression with PGF2A and collecting samples at many times after induction of regression allowed a much higher-resolution view of changes in NR5A2 as luteal regression progressed. Moreover, NR5A2 protein abundance in cultured luteal cells was downregulated by treatment with a physiological concentration of PGF2A, indicating that PGF2A likely has a direct effect on the expression of NR5A2 in regressing CL.

In this study, changes in luteal abundance of NR5A2 during acquisition of luteolytic capacity and during early pregnancy were investigated. Although the role of NR5A2 in ovulation and luteinization has been clearly demonstrated using murine models (Duggavathi *et al.* 2008, Zhang *et al.* 2013, Bertolin *et al.* 2014), it was not clear if NR5A2 continued to increase following those early phases of luteal formation. In the current study, NR5A2 increased by about four-fold between day 4 of the estrous cycle, when CL will not regress in response to PGF2A, and day 6, when CL regress in response to PGF2A. Mondal *et al.* (2011) reported that NR5A2 declined 4 hours after an injection of PGF2A on day 11, but not on day 4. When these results are considered together, it is suggested that cellular abundance of NR5A2 may be one factor participating in the of acquisition of luteolytic capacity.

A surprising discrepancy was observed between NR5A2 mRNA and protein during early pregnancy, with NR5A2 protein remaining stable, while NR5A2 mRNA declined. Given the role of NR5A2 in luteal progesterone production, this maintenance of NR5A2 may be important in the context of luteal physiology. However, this draws into question the mechanism of action for maintenance of NR5A2 protein without maintenance of NR5A2 mRNA.

Steady-state mRNA concentrations are only a partial predictor of protein abundance; post-transcriptional and post-translational mechanisms and mechanisms of selective degradation, including miRNA-mediated events, may also play a role in determining protein abundance (Vogel & Marcotte 2012). miRNA may suppress protein synthesis, destabilize mRNA, and induce mRNA degradation, typically producing modest changes in protein expression (Baek *et al.* 2008). Translational repression of mRNA is the primary mechanism for silencing, which may or may not be followed by mRNA degradation (Wilczynska & Bushell 2015), and therefore likely accounts for the discrepancy in mRNA and protein observed in this study.

In a recent study (Hughes *et al.* 2020), miRNA were profiled in the CL during early pregnancy. That dataset was combined with miRNA target prediction (Agarwal *et al.* 2015) and was used to screen all miRNA predicted to target NR5A2 and identify a list of candidate miRNA that are likely to regulate NR5A2 during early pregnancy. Among these miRNA, four declined during early pregnancy and one increased. Declining miRNA during early pregnancy would lead to decreasing inhibition of translation and thus stabilization of NR5A2 protein. Because there is evidence that miRNA can also induce translation of specific targets (Vasudevan *et al.* 2007), the miRNA that increased over time during early pregnancy was also tested for an ability to regulate NR5A2, with the hypothesis that it might increase abundance of NR5A2.

Not all predicted miRNA targets are actual targets (Steinkraus *et al.* 2016); thus it is important to confirm target predictions *in vitro* by quantifying predicted target protein abundance. This study has confirmed that three of the miRNA tested, namely miR-369-3p, mir-432-5p and miR-27b-3p, decreased NR5A2 in bovine luteal cells. Two of these, miR-369-3p and mir-432-5p also reduced progesterone production by approximately 27%. Although this degree of reduction in progesterone production may seem unremarkable, it would be highly unlikely that modulation of a single miRNA would be capable of producing profound changes in steroidogenesis. It is more likely multiple miRNA serve to fine tune steroidogenesis in concert with the primary regulators of the multiple reactions required to achieve progesterone synthesis. Regardless, the results of this study clearly show that miR-369-3p and miR-432-5p can regulate NR5A2 in bovine luteal cells, and this

is coincident with a small decrease in progesterone production. Further, these two miRNA decline during early pregnancy (Hughes *et al.* 2020), which would facilitate maintenance of NR5A2 protein, even in the face of decreased steady-state concentrations of mRNA, and support steroidogenesis.

It was surprising that NR5A2 was a predicted target of miR-27b-3p, because this miRNA increases during early pregnancy (Hughes *et al.* 2020). In fact, the miR-27b-3p mimic did decrease NR5A2 protein abundance in luteal cells, consistent with findings in breast cancer cells (Zhu *et al.* 2016). Despite the downregulation of NR5A2, there was no effect of miR-27b-3p on progesterone production. In this study, only a single target of miR-27b-3p (NR5A2) was evaluated, but there is evidence that this miRNA is also an important regulator of PI3K/AKT and MAPK/Erk signaling and cholesterol homeostasis, pathways that are relevant to luteal progesterone production (Chen *et al.* 2018). This miRNA is also a positive regulator of cellular proliferation (Yang *et al.* 2019). Therefore, it is possible that, although miR-27b-3p can target NR5A2 in luteal cells, this effect is outweighed by the multiple well-established effects of this miRNA on pathways that would facilitate luteal maintenance, and counterbalanced by the decline during early pregnancy of at least two miRNA (miR-369-3p, mir-432-5p) that would otherwise suppress both NR5A2 and progesterone production.

It was not entirely unexpected that the miRNA inhibitors had little effect in luteal cells, based on our past experience with miRNA inhibitors (Maalouf *et al.* 2016) and reports from others (Xu *et al.* 2018). Also, even single nucleotide differences in inhibitor sequences of commercially obtained miRNA inhibitors can result in off-target or lack-of-target effects (Robertson *et al.* 2010). As observed in this study, multiple miRNA can target the same mRNA or pathway, and perhaps this redundancy makes it difficult to observe the effect of inhibiting one single miRNA. Although the exact reason for lack of inhibitor effects is unknown, it is not uncommon to observe this result. Thus, for these luteal cell cultures, conclusions drawn are based on effects of miRNA mimics.

It has been demonstrated in this study that there is dynamic regulation of NR5A2 during acquisition of luteolytic capacity, luteal regression, and luteal rescue. The patterns of NR5A2 expression parallel progesterone concentrations during these transitional states of luteal function, and an inhibitor of NR5A2 produced a modest, yet significant decrease in progesterone production in luteal cells. Putative regulators of NR5A2 in bovine luteal cells, including PGF2A, miR-27b-3p, miR-432-5p, and miR-369-3p, have been identified. Overall, NR5A2 supports luteal progesterone production and thus, modulation of NR5A2 by miRNA or prostaglandins may contribute to the regulation of progesterone production during key transitions in the lifespan of the CL.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0009>.

Declaration of interest

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

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Author contribution statement

C H K H designed the experiments, performed the experiments, analyzed the data, and wrote the manuscript. A R performed the experiments and was involved with experimental design. E K I designed one of the whole animal experiments, performed that experiment, and was involved in manuscript preparation. J L P designed the experiments and was involved in manuscript preparation.

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