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Effects of the Luteinizing hormone on Markers of Epithelial-Mesenchymal Transition in GCs of Pre-ovulatory Follicles

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ABSTRACT

Epithelial-to-mesenchymal transition (EMT) is a complex process in which epithelial cells lose their adhesive properties, gain the ability to migrate, and undergo structural, molecular, and biochemical changes. This transition is initiated and regulated by transcriptional repressors from the Snail, ZEB, and TWIST families, which bind to E-box sequences. Granulosa cells (GCs) are a type of epithelial cell characterized by polarity. During ovarian follicle development, CDH1, an epithelial marker, is found in oocytes, while N-cadherin (CDH2), a mesenchymal marker, is present in granulosa cells. The LH surge triggers ovulation and converts the granulosa cells to luteinization to form the corpus luteum (CL). The expression of epithelial and mesenchymal markers in bovine GCs of preovulatory follicles remained unknown. In this study, the expression of mesenchymal markers in uncultured GCs from pre- and postovulatory follicles, as well as in luteal cells at different stages of the CL were examined, ranging from early to regressed phases. Additionally, the effect of luteinizing hormone (LH) on mesenchymal marker expression in cultured GCs from preovulatory follicles were investigated. The mRNA expression of CDH1 gradually declined from preovulatory follicles to regressed CL. In contrast, the mRNA levels of Snail2 and Zeb-1 increased throughout this transition. Notably, treatment with LH (5 and 50 ng/ml) significantly reduced CDH1 mRNA expression in GCs from preovulatory follicles. Furthermore, LH (5 and 50 ng/ml) upregulated Snail2 expression, while a higher concentration of LH (50 ng/ml) specifically elevated Zeb-1 mRNA expression in GCs. These results indicates that LH is actively involved in the initiation of EMT in GCs conversion to luteal cells.

Keywords: Follicle, Granulosa cell, IGF-1, Steroidogenic enzymes, PCNA

INTRODUCTION

Epithelial-to-mesenchymal transition (EMT) is a complex process in which epithelial cells lose their adhesive properties, gain migratory ability, and undergo morphological, molecular, and biochemical changes (Kalluri and Neilson, 2003). The invasion of epithelial cells often involves EMT, which occurs alongside the downregulation of E-cadherin (CDH1) mRNA expression and the acquisition of migratory characteristics (Cano et al., 2000). A key trigger for EMT is the suppression of E-cadherin (CDH1), a homophilic protein responsible for maintaining cell-cell adhesion through interactions between its extracellular domains. The initiation and regulation of EMT are controlled by zinc-finger proteins from the Snail family, including EEB1 and EEB2, which directly repress CDH1 and other epithelial markers (Nieto, 2002), as well as transcriptional repressors from the ZEB (Browne et al., 2010) and TWIST (Ansieau et al., 2010) families, which bind to E-box sequences.The importance of Snail in triggering EMT in mammals has been confirmed, Snail was shown to convert otherwise normal epithelial cells into mesenchymal cells through the direct repression of CDH1 expression (Batlle, 2000). During early gestation in cattle, dynamic changes occur in the conceptus and endometrium, including epithelial-to-mesenchymal transition (EMT) of the conceptus following its attachment to the endometrium (Fortune, 2000). The expression

of CDH1 mRNA is tightly regulated in a spatiotemporal manner during development, and its downregulation is crucial for specific morphogenetic movements involving EMT (Kazuya et al., 2016). Granulosa cells (GCs) are a type of epithelial cell characterized by polarity, cellular adhesion, and cell-cell junctions. Previous studies have shown that during ovarian follicle development, the epithelial marker CDH1 is present in oocytes, whereas the mesenchymal marker N-cadherin (CDH2) is expressed in granulosa cells (Huber et al., 1996). As GCs mature, they enlarge and differentiate into large luteal cells within the CL (Mora et al., 2012).

Following the LH surge, GCs lose their epithelial characteristics and undergo EMT, a process referred to as luteinization (Rodgers et al., 2001). The LH surge plays a critical role in ovulation, leading to the transformation of granulosa cells and the CL formation. In cattle, the CL develops rapidly within 2–3 days after ovulation, involving extensive angiogenesis and vascularization from the preovulatory follicle. However, the expression of epithelial and mesenchymal markers in bovine GCs from preovulatory follicles remains unclear. This study aimed to examine the expression of both epithelial and mesenchymal markers in uncultured GCs from preovulatory and postovulatory follicles, as well as in luteal cells at different stages of CL development, from early to regressed phases. Additionally, we investigated the impact of LH on the expression of epithelial and mesenchymal markers in cultured GCs from preovulatory follicles. Based on our findings, we hypothesize that LH enhances the expression of mesenchymal markers in GCs of preovulatory follicles.

MATERIALS AND METHODS

Ovarian collection from cows

Following the procedure described by Langhout et al. (191), the ovaries were procured from a nearby abattoir and immediately transported on ice to the lab for the isolation of TCs and GCs from 6 mm and 7–8 mm follicles. The ovaries were kept in saline at 4°C until GCs were aspirated and follicles were cut to recover TCs. Follicular samples were taken from each pair of ovaries days 4 post-ovulation, coinciding with the start of the first follicular wave, in order to acquire uncultured GCs (Hendriksen et al., 2003), which plays a role in selecting healthy, developing follicles. Uncultured luteal cells were isolated from the corpus luteum (CL) at different developmental stages—early, developing, mid, and regressing—following the protocol outlined by Mihm and Evans (2008).

Granulosa cells were isolated and cultured as follows:

The GCs with follicular fluid from follicles were aseptically collected using a syringe (2.5 ml). After the samples were collected, they were put in a Petri dish supplemented with 10% foetal bovine serum (FBS), 20 µg/ml amphotericin, and heparin sodium salt (50 IU). For five minutes at 4°C, the mixture was centrifuged at 800 × g. Following haemolysis, the pellet was re-suspended in Tris-NH4Cl, and the supernatant was discarded. Following another centrifugation, the cell suspensions were reconstituted in DMEM. The solution was filtered through a 100 µm × 2, 80 µm × 2 metal meshes in order to exclude cumulus-oocyte complexes. The final cell suspensions were made in phenol red-free DMEM/F-12 media supplemented with 10% FBS and 20 µg/ml gentamicin after the washing procedure was repeated twice after further centrifugation. Cell viability was evaluated by trypan blue exclusion and found to be between 75% and 80%. Within culture flasks GCs and TCs were cultivated independently at a density of 1.0×10^5 viable cells per ml in DMEM/F-12 media enhanced with 10% FBS. For two to three days, the cultures were kept at 38.5°C in a controlled environment with 95% air and 5% CO_2. The medium was changed every 24 hours. The cells were passaged in sterile phosphate-buffered saline (PBS) once they had achieved 80–90% confluence. Before being exposed to different LH concentrations, scattered cells in four duplicate wells were pre-incubated for 24 hours in serum-free media with or without LH. Untreated cells were used as controls.

Total RNA extraction and quantitative RT-PCR

The RNA was extracted using RNAiso Plus from both fresh and cultured GCs. The ReverTra Ace qPCR RT Master Mix with gDNA remover was used to reverse transcribe 2 µg of total RNA from each sample after the extracted RNA was measured. Using a previously approved technique, the mRNA expression levels of bovine CDH1, ZEB1, SNAIL2, TWIST1, β-actin (ACTB), and H2A were measured (Beg et al., 2001). Using 2 ng

of reverse-transcribed RNA as the input, the Ultra-Fast SYBR Green qPCR Master was used for the quantification. H2A was used for cultured GCs, whereas ACTB was utilised as the internal reference for uncultured GCs and luteal cells for normalisation. In both isolated and cultured bovine GCs, 5 housekeeping genes (MRPL4, H2A, GAPDH, ACTB, and 18S) were assessed in order to choose the internal control gene for qRT-PCR analysis. H2A was shown to be the best reference for cultivated GCs, whereas ACTB was the most stable reference for uncultured GCs, according to NormFinder (19) analysis. Agilent Technologies' AriaMx Real-Time PCR System was used to quantify mRNA expression. Prior to 45 cycles of amplification, denaturation at 95°C for 10 seconds, annealing at a gene-specific temperature (Table 1) for 10 seconds, and extension at 72°C for 15 seconds, the PCR process started with an initial denaturation phase at 95°C for 30 seconds. After a minute of dissociation curve analysis at 95°C, the temperature was gradually raised (Table 1) in increments of 0.5°C every 5 seconds. Each PCR result taken from an agarose gel was serially diluted (20–20,000,000 copies) as a quantitative standard to ascertain the levels of mRNA expression.

PGE2 Extraction

Three volumes (900 µl) of diethyl ether were added after 300 µl of follicular fluid was extracted from preovulatory follicles. Then, using a Shaker BC-730 (2-14-9 Itabashi, Itabashi-ku, Tokyo), the mixture was shaken for 30 minutes at 120 round trips per minute. The samples were shaken, let to stand for fifteen minutes, and then frozen for an hour at -80°C. Carefully, the top diethyl ether fraction was decanted and evaporated. For PGE_2 enzyme immunoassays (EIAs), the residual material was dissolved in 300 µl of EIA assay buffer.

Enzyme immunoassay (EIA)

Enzyme immunoassays (EIAs) were used to measure the amounts of PGE_2 in follicular fluid as desribed by (Bao et al., 1997). The standard curve for A4 comprised a level range of 0.0390625 to 10 ng/ml. Follicle fluid from pre-ovulatory follicles had average intra-assay and inter-assay coefficients of variation of 6.2% and 13.04%, respectively.

Experiment.1: ESR2 mRNA expression in uncultured granulosa and luteal cells

Preovulatory and postovulatory follicles, along with CL samples spanning early to regressed stages, were harvested from healthy cows. GCs were retrieved by bisecting the follicles. Subsequently, GCs from follicles and luteal cells from CLs were separated through centrifugation. The resulting cell pellets were immediately re-suspended in RNAiso Plus (400 µl) and kept until RT-PCR analysis at -80°C.

Experiment 2: Impact of LH on CDH1, ZEB1, SNAIL2, and TWIST1 mRNA expression in cultured GCs

GCs derived from pre-ovulatory follicles were cultivated in 24-well plates at a density of 1.0×10^5 cells per millilitre in 1 millilitre of DMEM/F-12 mixture in each well. The medium was changed after a 24-hour incubation period at 38.5°C to a solution including 0.1% BSA, 5 µg/ml holotransferrin, and 2 µg/ml insulin. For a further twenty-four hours, the cells were treated to varying doses of LH (5 and 50 ng/ml). Following treatment, each group's cells were collected in 1.5 ml tubes with 400 µl of RNAiso Plus and stored until the RNA extraction at -80°C.

STATISTICAL ANALYSIS

The mean ± SEM is used to express all experimental results. The Shapiro-Wilk test and the Brown-Forsythe test in R wer used to determine the normality of data and homogeneity of the variance (Kadokawa et al., 2014). One-way ANOVA and Tukey's multiple comparison tests in R were used to evaluate the data. At $p < 0.05$, statistical significance was taken into account.

RESULTS

We conducted the retrospective study first to check the expression of LHCGR mRNA in GCs of pre and post LH surg of stage four (Day 17-20) follicles (Fig. 1). We also determined the PGE2 concentrations in the follicular fluid of the same follicles (Fig. 2).

Experiment 1. CDH1, ZEB1, and SNAIL2 mRNA expression in uncultured granulosa and luteal cells

The GCs from preovulatory and postovulatory follicles and luteal cells from early to regressed CL were isolated. The expression of CDH1 mRNA was lower in GCs of preovulatory compared to postovulatory follicles. The expression of CDH1 mRNA decreased gradually in luteal cells from the stage early to stage regressed in CL. The expression of CDH1 mRNA significantly ($p<0.05$) decreased in luteal cells from stage mid and regressed compared to preovulatory and postovulatory GCs of follicles presented in Fig.3. CDH1 mRNA expression was higher in preovulatory follicles compared to postovulatory and luteal cells from early to regressed CL. Controversy, the expression of SNAIL2 gradually increased in GCs from preovulatory and postovulatory follicles and luteal cells of the stage early to regressed CL. The luteal cells of regressed stage CL had the highest expression of SNAIL2 mRNA while the GCs from the preovulatory follicle had the lowest expression of SNAIL2 mRNA Fig. 4. The mRNA expression of ZEB1 a mesenchymal marker was decreased in the GCs of preovulatory and postovulatory follicles while mRNA of ZEB1 increased in the luteal cells from the stage early to regressed CL. The luteal cells from the stage regressed CL had the highest expression of ZEB1 mRNA Fig. 5.

Experiment 2. Effects of LH on the expression of mRNA of SNAIL2 and CDH1 in cultured granulosa cells

Pre-ovulatory follicle GCs were exposed to varying concentrations of LH for a full day. When 5 and 50 ng/ml LH were administered to cultured GCs originating from preovulatory follicles, the findings demonstrated a substantial ($p < 0.05$) enhanced the mRNA expression of SNAIL2 (Fig. 6). Interestingly, SNAIL2, a mesenchymal marker, was markedly elevated in the GCs of pre-ovulatory follicles even at the lower dosage of LH (5 ng/ml) ($p < 0.05$) (Fig. 7). In GCs of preovulatory follicles, LH therapy also decreased the expression of CDH1 mRNA, an epithelial marker. Furthermore, in GCs of preovulatory follicles, LH (50 ng/ml) increased ZEB1 mRNA expression (Fig. 8).

Table. List of primers' Sequences

Table 3. The sequences of primers used for real-time RT-PCR.

Genes	Primer's sequence 5' to 3'	Accession No.	Size (bp)	Annealing (ºC)
H2A	F: CTCTCAAGGGCATTCTAGGC R: TGACAAAGTGGTCGTTGAGG	NM_001034034.2	120	60
CDH1	F: GATTGCAAGTTCCCGCCATC R: ACATTGTCCCGGGTGTCATC	NM_001002763.1	145	60
SNAIL2	F: CATCAGAACTCACACTGGGGA R: GAAAGGCGTCTGCACGAGTA	NM_001034538.2	235	58.5
ZEB1	F: TTTGTCTCCCAGTCAGCCAC R: GTAGGTTCACGGAATCGGCA	NM_001206590.1	119	59

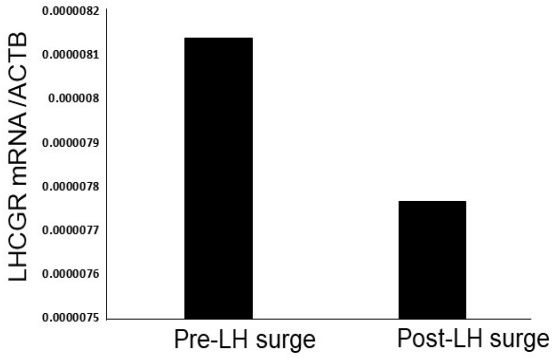

Fig. 1. Gene expression of LHCGR in GCs of pre- and post-LH surge at stage four (Days 17-20) follicles. The GCs from the pre-LH surge stage exhibited higher expression levels of LHCGR compared to the GCs from the post-LH surge stage.

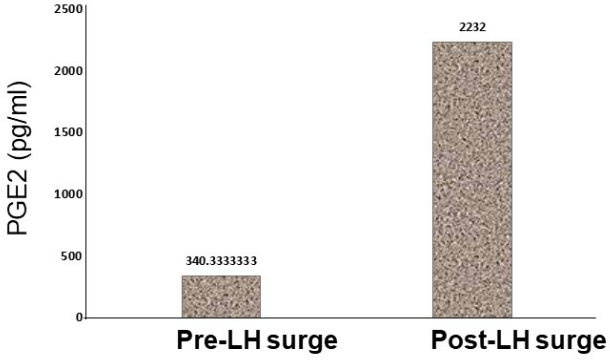

Figure 2: GCs from pre- and post-LH surge follicles at stage four (Days 17–20) produce prostaglandin E_2 (PGE_2). PGE_2 synthesis was noticeably greater in granulosa cells from the pre-LH surge stage than in those from the post-LH surge stage.

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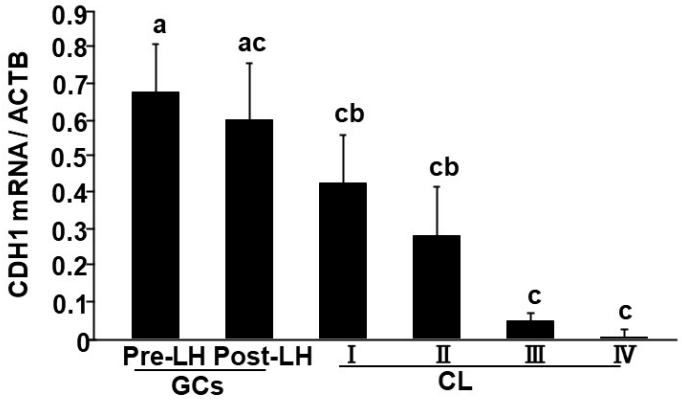

Figure 3: CDH1 mRNA gene expression in relation to ACTB in isolated granulosa cells from stage 1 to stage 4, as well as pre- and post-LH surge cells. Expression values for n = 5 are shown as means ± SEM. Significant differences ($p < 0.05$; ANOVA) are indicated by different superscripts (a-b), which are followed by Tukey's multiple comparison test.

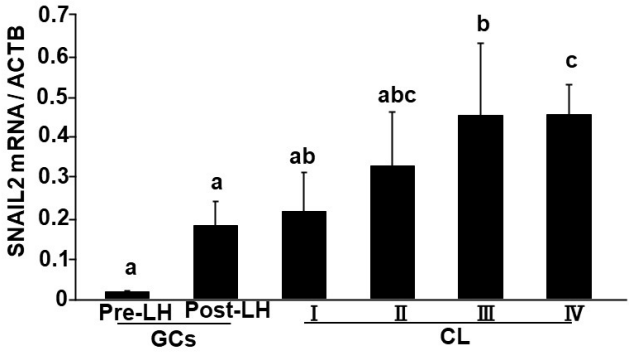

Figure 4. Isolated granulosa cells from pre-LH surge, post-LH surge, and luteal cells from stage 1 to stage 4 exhibit SNAIL2 mRNA gene expression in relation to ACTB. Expression values for n = 5 are shown as means ± SEM. Significant differences ($p < 0.05$; ANOVA) are indicated by different superscripts (a-b), which are followed by Tukey's multiple comparison test.

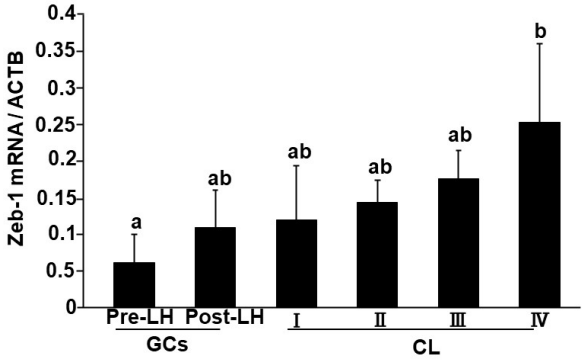

Figure 5 shows the ZEB1 mRNA gene expression in relation to ACTB in isolated granulosa cells from stage 1 to stage 4, as well as post-LH surge and pre-LH surge cells. Expression values for n = 5 are shown as means ± SEM. Significant differences ($p < 0.05$; ANOVA) are indicated by different superscripts (a-b), which are followed by Tukey's multiple comparison test.

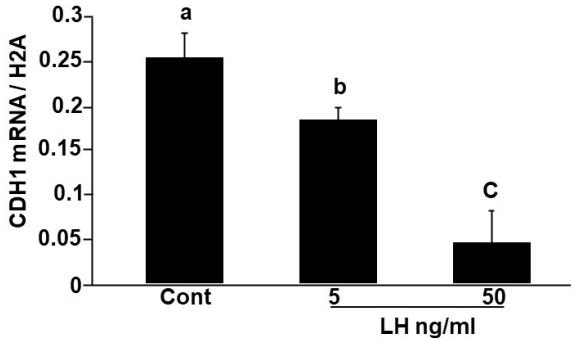

Figure 6: LH's impact on CDH1 mRNA expression in granulosa cells. For a full day, granulosa cells derived from tiny pre-LH surge follicles were exposed to different LH concentrations (5 and 10 ng/ml). Cultured granulosa cells from tiny follicles were used to evaluate the gene expression of CDH1 mRNA in relation to H2A (Cont: control; LH: luteinizing hormone). Expression values for n = 5 are shown as means ± SEM. Significant differences ($p < 0.05$; ANOVA) are shown by different superscript letters, which are followed by Tukey's multiple comparison test.

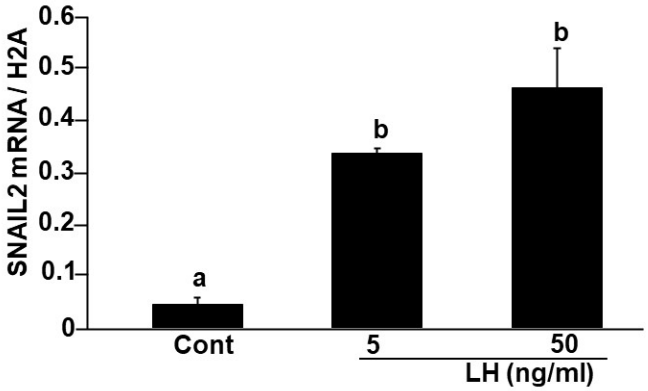

Fig. 7. LH's impact on SNAIL2 mRNA expression in granulosa cells. For a full day, granulosa cells derived from tiny pre-LH surge follicles were exposed to different LH concentrations (5 and 10 ng/ml). Cultured granulosa cells from tiny follicles were used to evaluate the gene expression of SNAIL2 mRNA in relation to H2A (Cont: control; LH: luteinizing hormone). Expression values for n = 5 are shown as means ± SEM. Significant differences ($p < 0.05$; ANOVA) are shown by different superscript letters, which are followed by Tukey's multiple comparison test.

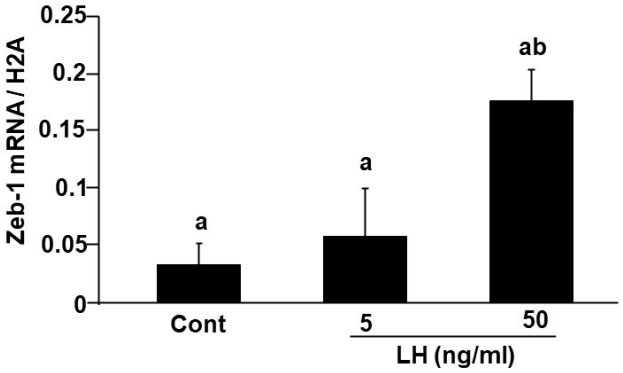

Figure 8: LH's impact on ZEB1 mRNA expression in granulosa cells. Granulosa cells derived from tiny pre-LH surge follicles were exposed to different LH concentrations (5 and 10 ng/ml). Cultured granulosa cells from tiny follicles were used to evaluate the gene expression of ZEB1 mRNA in relation to H2A (Cont: control; LH:

luteinizing hormone). Expression values for n = 3 are shown as means ± SEM. Significant differences ($p < 0.05$; ANOVA) are shown by different superscript letters, which are followed by Tukey's multiple comparison test.

DISCUSSION

EMT is a multifaceted process involving morphological and molecular modifications that allow epithelial cells to transition into a mesenchymal phenotype. This process is primarily regulated by the Snail family of zinc-finger proteins, including ZEB1 and ZEB2 (Nieto, 2002), along with members of the ZEB (Browne et al., 2010) and TWIST (Ansieau et al, 2010) families, which function as E-box-binding transcriptional repressors. The invasion of epithelial cells is often accompanied by EMT, which occurs simultaneously with a reduction in E-cadherin (CDH1) mRNA expression and the development of migratory characteristics (Cano et al., 2000). In this study, we examined the expression levels of CDH1, Snail1, and ZEB1 mRNA in uncultured granulosa cells (GCs) from both pre-ovulatory and post-ovulatory follicles, as well as in luteal cells from corpus luteum (CL) stages 1 to 4. Our findings revealed that CDH1 mRNA expression was significantly higher in GCs of pre-ovulatory follicles, while its expression was notably lower in post-ovulatory follicles, suggesting a transition of these cells into a mesenchymal phenotype. This decline in CDH1 mRNA expression persisted after ovulation, extending from stage 1 CL to stage 4 CL (regressed CL). These observations align with previous studies indicating that, following the LH surge, GCs lose their epithelial characteristics and undergo an EMT process known as luteinization (Rodgers et al., 2001).

The Snail family of transcription factors has been previously associated with the transformation of epithelial cells into mesenchymal cells during embryonic development. It has been demonstrated that Snail facilitates this transition by directly repressing CDH1 expression (Cano et al., 2000). In the current study, we found that Snail mRNA expression was elevated in the GCs of post-ovulatory follicles compared to pre-ovulatory follicles and continued to increase in luteal cells from stage 1 to stage 4 CL. These results support prior findings that Snail mRNA plays a key role in regulating CDH1 repression in bovine ovaries. Additionally, EMT is controlled by ZEB1 and ZEB2, which directly suppress CDH1 and other epithelial markers (Nieto, 2002). Our study demonstrated that post-ovulatory follicular GCs exhibited higher ZEB1 mRNA expression, whereas pre-ovulatory follicular GCs had the lowest expression. Similarly, luteal cells at stage 4 CL displayed the highest ZEB1 expression among all CL stages. These findings indicate that mesenchymal-like cells exhibit lower CDH1 expression while showing elevated levels of mesenchymal-related marker mRNA.

In this study, we explored the impact of LH on the mRNA expression of CDH1, Snail2, ZEB1, and TWIST1 in GCs from pre-ovulatory follicles. The GCs were treated with LH (5 and 50 ng/ml) and incubated for 24 hours. A recent study on pluripotent stem cells demonstrated that an inducible hESC line effectively downregulated E-cadherin at both the transcriptional and protein levels. Their findings also revealed that silencing E-cadherin in HES3-KRABCDH1 cells led to increased expression of EMT transcription factors such as SNAI1, alterations in cell morphology toward a mesenchymal-like phenotype, and enhanced collective cell migration (Barrallo and Nieto, 2005).

The results of present study indicated that LH treatment downregulated CDH1 mRNA expression in GCs from pre-ovulatory follicles, with the higher dose of LH (50 ng/ml) exerting a stronger suppressive effect. Conversely, LH significantly upregulated Snail2 mRNA expression in these GCs, with the lower LH dose (5 ng/ml) being particularly effective in enhancing Snail2 expression. Previous studies have indicated that SNAI1's role extends beyond EMT initiation, as it also regulates morphological changes, cell adhesion, and gene expression related to tight junctions (Ohkubo and Ozawa, 2004). This implies that Snail may also contribute to these modifications and the development of an incomplete EMT state. Furthermore, the induction of ZEB1 reinforces Snail2's effects and is believed to drive the reduction of E-cadherin expression and the initiation of EMT (Aban et al., 2025). In our study, LH stimulated the expression of ZEB1 mRNA in pre-ovulatory follicular GCs, suggesting that LH plays a role in promoting the EMT process, facilitating the transformation of GCs into luteal cells.

CONCLUSION

LH markedly reduced CDH1 mRNA expression in granulosa cells (GCs) of pre-ovulatory follicles while simultaneously increasing Snail2 mRNA expression. Additionally, the higher LH concentration (50 ng/ml) significantly upregulated ZEB1 mRNA expression in these GCs. These findings indicate that LH has a crucial role in the transformation of GCs into luteal cells, thereby initiating the EMT process.

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CONFLICT OF INTEREST:

The authors confirm that there are no conflicts of interest associated with any aspect of this research.

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AUTHORS CONTRIBUTIONS:

All authors have reviewed and approved the final version of the manuscript for publication.

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