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Original Research Article

OESTROGENS AND PROLACTIN REGULATE MAMMARY GLAND EPITHELIAL CELL GROWTH BY MODULATION OF THE WNT SIGNAL PATHWAY

Jinjin Tong[1,3], Hua Zhang[2], Yuhong Wu[1], Yingxue Wang[1], Qingzhang Li[3], Yun Liu[1]*

[1]Department of Veterinary Surgery, College of Veterinary Medicine, Northeast Agricultural University, Harbin, [2]Animal Science and Technology College, Beijing University of Agriculture, Beijing, [3]Key Laboratory of Dairy Science, Ministry of Education, Northeast Agricultural University, Harbin 150030, P. R., China

*Corresponding Author, E-mail: abliuyun@yeah.net

Summary: Oestrogens and prolactin can regulate mammary gland development and epithelial cell growth as well as lactation. Meanwhile, the Wnt-signalling pathway and subsequent upregulation of ß-catenin driven by the downstream target of cyclinD1 also play a role in development of the mammary gland. This study aimed to assess the possible involvement of oestrogens and prolactin in the regulation of cell growth in the mammary gland. Bovine mammary gland epithelial cells (MECs) were treated with ß-estradiol (E2) and prolactin (5 μg mL^{-1}) for 48 h and then measured for cell viability. mRNA and the protein expression level of genes (E-cadherin, CyclinD1 and ß-catenin) related to the Wnt pathway were measured by qRT-PCR and Western blot, respectively, while sub-cellular localizations of the proteins in MECs were further monitored by immunofluorescence. The expressions of E-cadherin and CyclinD1 were highest at 36 h ($P<0.05$) whereas ß-catenin was lowest at 36 h after treatment with E2 and prolactin. The protein level of the E-cadherin and cyclinD1, which are the targets of the Wnt signal pathway, were unregulated. ß-catenin protein level decreased in both hormone groups. In conclusion, prolactin and E2 could efficiently affect the cell growth of MECs and increase the expression of E-cadherin and cyclinD1 at both the mRNA and protein levels. Immunofluorescence suggested that prolactin and E2 impact the nuclear expression of ß-catenin protein. The current study indicated that the proliferative efficacy of prolactin and E2 on MECs was modulated through the Wnt-signalling pathway.

Key words: mammary gland development; hormone; Wnt pathway

Introduction

The mammary gland is an organ in female mammals for milk production and contains a dynamic tissue structure derived from the epidermis that achieves full maturity in the adult (1). Embryonic-branching morphogenesis and lactation mirror the changes within the endocrine environment. Resolving the hormone interactions that modulate mammary gland growth and morphogenesis is essential for understanding the physiological basis of a successful lactation and development (2). Oestrogens and prolactin function to regulate mammary gland development, cell growth, and lactation (1), while the Wnt-signalling pathway also plays a role in the development of the mammary gland (3). For example, E-cadherin is a calcium-dependent cell-cell adhesion glycoprotein and is composed of five extracellular cadherin repeats, a transmembrane region, and a highly conserved cytoplasmic tail (2). E-cadherin is a key molecule in the cell-cell adhesion and in maintaining the morphogenesis of

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a variety of organs, including mammary epithelial cells (4). Furthermore, β-catenin is a dual function protein, regulating the coordination of cell-cell adhesion and gene transcription (5). β-catenin directly interacts with the cytoplasmic domain of E-cadherin to form an adhesion molecule complex (6) and also acts as the key mediator in the canonical Wnt-signalling pathway (7). Increased expression of β-catenin protein promoted the transcription of genes important in regulating the cell cycle (8). β-catenin is stable in the cytoplasm, and during activation, it will translocate into the nucleus (9,10) and, in turn, interact with the T-cell factor (TCF)/Lef transcription of target genes, including CD1, c-Myc, and metalloproteases that promote cell proliferation, differentiation, and tissue development (11,3).

Multiple stages of mammary developments are regulated by the Wnt-signalling pathway. Compelling evidence indicates that the Wnt-signalling pathway was activated in a subset of epithelial cells accompanying initial branch formation of the mammary gland (12) and mammary bud development, and thus, plays a major role in embryonic development, cell proliferation, and differentiation or even oncogenesis (13). Prolactin has an important biological function in promoting survival of the cells (14). Thus, oestrogens and prolactin exert their effects in the mammary gland primarily via paracrine interactions. Oestrogens are significant mediator of the development of the mammary epithelium during alveolar proliferation, ductal morphogenesis, and functional differentiation, accompanied by progesterone peptide hormones in combination with stromal signals (15). Oestrogen is a key determinant of ductal elongation in the mammary glands of mice (16) in various hormone replacement and genetic knockout studies. The principal role of prolactin in the mammary glands of mice during pregnancy is to promote alveolar development and lactation (17).

The molecular mechanisms underlying the effects of prolactin and oestrogens on E-cadherin, β-catenin, and cyclinD1 in the Wnt-signalling pathway in the mammary gland of cows have not been fully elucidated. Therefore, in this study, we hypothesized that prolactin and oestrogens may impact the Wnt/β-catenin pathway to regulate mammary gland development and cell proliferation. We then investigated this specific transduction pathway along with E-cadherin, β-catenin, and its main downstream protein, cyclinD1. These data could provide an insight into the link between oestrogens/prolactin and the Wnt/β-catenin pathway for mammary gland development and cell proliferation.

Materials and methods

Primary cell culture and treatment

Mammary gland epithelial cells from bovine (MECs) were obtained from the Key Laboratory of Dairy Science, Northeast Agricultural University, China and cultured in a cell culture medium of mixed Dulbecco's modified Eagle's medium (DMEM) with F12 (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% foetal bovine serum (Invitrogen), 5 μg mL^{-1} Hydrocortisone (Sigma-Aldrich, Bangalore, India), 5 μg mL^{-1} insulin (Sigma–Aldrich). 100 IU mL^{-1} Penicillin and 100 IU mL^{-1} Streptomycin (Invitrogen) in a humidified incubator with 5% CO_2 at 37 °C. Prolactin and 17β-estradiol (E2) were purchased from Sigma–Aldrich. The D-Hanks' medium was composed of 8.00 g L^{-1} NaCl, 0.40 g L^{-1} KCl, 0.086 g L^{-1} Na_2HPO4, 0.060 g L^{-1} KH_2PO_4, and 0.35 g L^{-1} $NaHCO_3$. For experiments, the logarithmic growth phase of MECs was inoculated at a concentration of 3×10^4 cells cm^{-2} into 6-well culture plates and grown, and the medium was refreshed after 24 h. The cells were then stimulated by adding prolactin (5 μg mL^{-1}) according to a previous study (18) for 0, 6, 12, 24, 36 or 48 h and harvested. For E2 (5 μg mL^{-1}), it was the same concentration as prolactin. The experiments were in triplicate, and the control group was replaced with fresh medium and cultured to 48 h.

Cell viability assay

Cells were harvested by trypsinization and suspended at a final concentration of 2×10^4 cells mL^{-1} in fresh DMEM. Aliquots of 100 μL/well cell suspension were plated in 96-well tissue culture plates, and cells were treated with prolactin and E2 at 5 μg mL^{-1} of each for 0, 6, 12, 24, 36, and 48 h, respectively. Cells without prolactin and E2 were used as negative controls. At the end of each experiment, 10 μL/well cell proliferation Reagent WST-1 (Roche, Basel, Switzerland) was added to the cell culture plates and further incubated for 4 h at 37 °C in a humidified atmosphere containing

5% CO_2; the cells were then mixed thoroughly for 1 min on a shaker and the absorbance of the cells against a background control was measured using a microplate reader (Thermo Fisher Scientific, United States) at 450 nm. Each experiment was repeated three times.

RNA isolation and qRT-PCR

Prolactin- and E2-treated cells were subjected to RNA isolation using the Trizol reagent (Invitrogen) in accordance with the manufacturer's protocol and dissolution in RNAse-free water and quantified using a spectrophotometer and qualified using Agilent (Palo Alto, CA, USA) bioanalysis. The RNA sample (1 μg) was reversely transcribed into cDNA using the PrimeScript® RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions and then subjected to qPCR amplification using SYBR® Premix Ex Taq™ (TaKaRa) on an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The primers were designed using Premier 5.0 (see Table 1), and all qRT-PCR amplifications were performed in duplicate and repeated three times. The relative expression value of each target gene was normalized to GAPDH expression.

Protein extraction and Western blotting

MECs were treated with either prolactin or E2 at 5 μg mL^{-1} in six plates for 36 h, and untreated cells were used as a negative control. Cells were lysed in a RIPE buffer (Beyotime, Shanghai, China) (100 μL mL^{-1} to the plate plus 2 μL mL^{-1} protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined with Bradford reagent (Sigma-Aldrich), and the same amount of protein samples (20 μg) was separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the nitrocellulose membranes (Amersham, Freiburg, Germany). After blocking in 5% non-fat milk in Tris-buffered saline (TBS, 0.1 mol L^{-1}, pH 7.4) for 90 min, the membranes were incubated with E-cadherin, β-catenin, cyclinD1, or GAPDH antibody at 4 ºC overnight. Antibody against E-cadherin (#sc-1500), β-catenin (#sc-53483), cyclinD1 (#sc-718) or GAPDH (#sc-25778) and a goat anti-mouse IgG-HRP (#sc-2005) and goat anti-rabbit IgG-HRP (#sc-2004), donkey anti-goat IgG-HRP (#sc-2020) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After being washed with TBST, the membranes were further incubated with a secondary antibody (an HRP-conjugated goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP or donkey anti-goat IgG-HRP antibody diluted at an appropriate dilution in 5% non-fat milk in TBS) for 2 h at room temperature. Protein bands were then detected by incubation with enhanced ECL (Amersham Pharmacia Biotech) on X-RAY film (Kodak, NY, USA). The protein abundance of GAPDH served as an internal control. Each experiment was repeated three times.

Immunofluorescence

An immunofluorescence (IF) assay was performed to assess the sub-cellular localizations of proteins related to the Wnt pathway in MECs. Briefly, cells were grown on coverslips at a density of 3×10^4 cells cm^{-2} and treated with prolactin and E2 at 5 μg mL^{-1} for 36 h, and cells without prolactin and E2 treatment were a negative control. Thereafter, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 15 min and then washed thoroughly with Tris-buffered saline with 1‰ $Triton_X$-100 (TBSTx, pH 7.4) three times for 5 min each and incubated with 5% bovine serum albumin (BSA) for 1 h and further incubated with a

Table 1: Primers used during real-time PCR

gene	Accession no.	Forward primer (5'-3')	Reverse primer (5'-3')
E-cadherin	NM_001002763	CGTATCGGATTTGGAGGGAC	CATCATCGAGGAACAAGAGCAG
β-catenin	NM_001076141	CCAAGTGGGTGGCATAGAGG	GGCTGGTCAGATGACGAAGG
CycinD1	NM_001046273	GGACCGCTTCCTGTCGCT	GCCAGGTTCCACTTGAGTTTGT
GAPDH	AB098934	TGCTGGTGCTGAGTATGTGGT	AGTCTTCTGGGTGGCAGTGAT

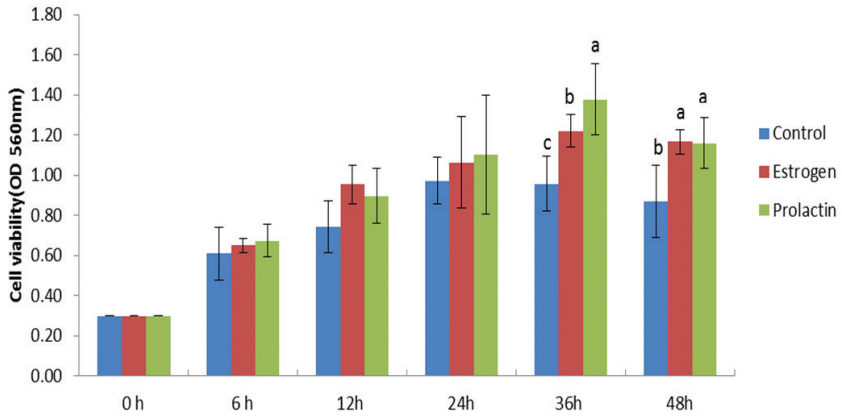
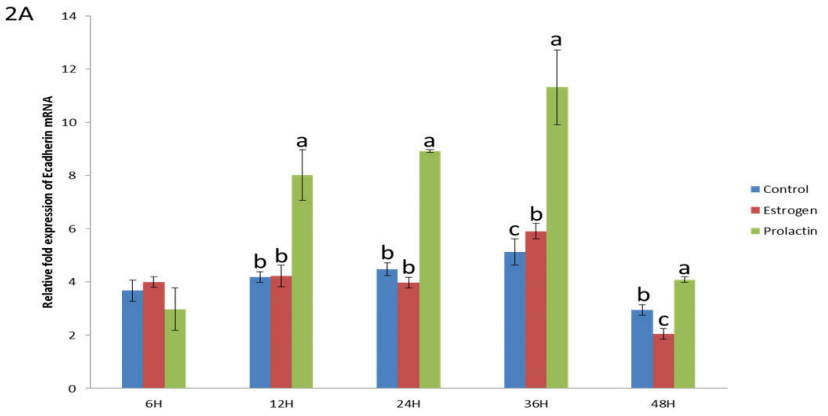
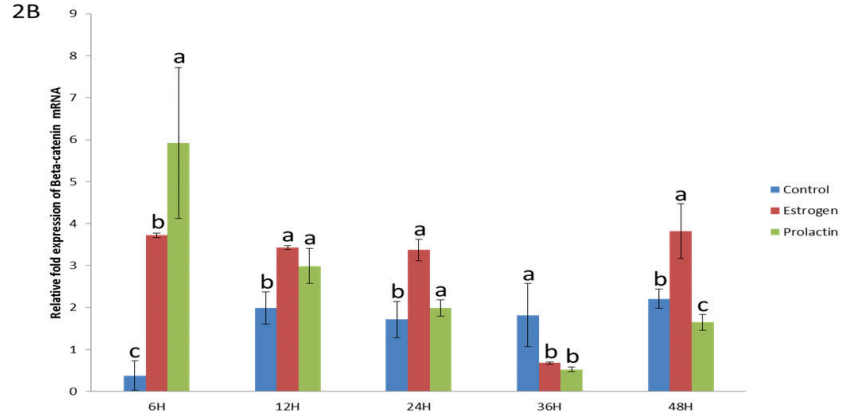


Figure 1: MECs Growth curve *in vitro* by prolactin and E2 (5 μg mL^{-1}) from 0 h to 48 h. The data were represented as mean ± SEM. Different letters indicated a significant level of P<0.05 in comparison to the control





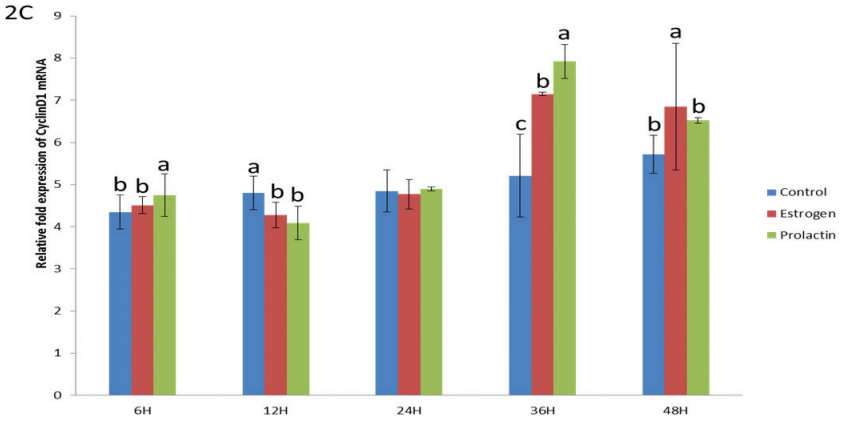


Figure 2: mRNA expression levels of E-cadherin (2A), β-catenin (2B) and cyclinD1 (2C) with treatment of prolactin or E2 for 0, 6, 12, 24, 36 and 48 h. GAPDH was used for normalization. Different letters indicate a significant level of P<0.05 in comparison to the control

primary antibody against E-cadherin and β-catenin at 4 °C overnight. On the next day, cells were washed with TBSTx thrice and then incubated with the rabbit anti-goat FITC conjugated (#sc-2777), and goat anti-mouse TRITC (#sc-2092) antibodies (Santa Cruz Biotechnology (Santa Cruz, CA, USA)) secondary antibody in a 1:200 dilution in TBSTx for 1 h. The nuclei of MECs were stained with DAPI for 15 min at room temperature. After that, the coverslips were mounted with a fluorescence-quenching mounting medium (Beyotime, Shanghai, China) and reviewed under a confocal microscope; the images of cells were captured by the Z-stacking function for serial confocal sectioning at 2 μm intervals (Leica TCS SP2, Buffalo Grove, IL, USA) and then analysed with Leica software. The experiments were performed in triplicate and repeated at least once.

Statistical analysis

Comparison of differences between treatment and control was statistically analysed by using SPSS 16.0 software (SPSS, Chicago, IL, USA). Association among the expression of E-cadherin, β-catenin, and cycinD1 before and after treatment was analysed using the ANOVA two-way measures. Cell proliferation in different time points (0, 6, 12, 24, 36 and 48 h) after prolactin and E2 treatment was evaluated using the analysis of ANOVA repeated measures. For protein intensity, the data were analysed with one-way ANOVA repeated measures with the bandscan 5.0 software and the data were summarized as mean ± SEM. A p-value ≤ 0.05 was considered to be statistically significant.

Results

Effects of prolactin and E2 on regulation of cell proliferation

MECs growth curve revealed that the largest cell population was reached at 36 h after treatment with 5 μg mL-1 prolactin and E2. In comparsion to the controls, an increase of cell population in both prolactin and E2 groups was observed. Notably, numbers of cells at 36 h were significantly higher than those of other time points in prolactin and E2 treatment (Figure 1). Therefore, the optimal treatment time of prolactin and E2 was determined to be 36 h.

Effects of Prolactin and E2 treatment on expression of E-cadherin, ß-catenin and cyclinD1 in MECs

The effects of prolactin and E2 treatment on the expression of E-cadherin, β-catenin and cyclinD1 in MECs were then examined. The relative mRNA expression of E-cadherin and cyclinD1 was slightly increased in comparison to the control group, during the period from 12 h to 48 h after the treatment (Figure 2AC). Notably, during treatment with either prolactin or E2, mRNA expression levels of β-catenin were increased in comparison to the controls between 6 and 24 h, but remarkably reduced at 36 h after the treatment (Figure 2B). However, there is no significant difference between the prolactin and E2 groups in the effect of the treatment.

The protein expression of E-cadherin, β-catenin and cyclinD1 were observed in MECs after treatment of 5 μg mL^{-1} prolactin and E2 at 36 h. Western blot and the desitometric analysis results are depicted in Figure 3. Desitometric analysis indicated a decrease in β-catenin protein during either prolactin or E2 treatment in comparison with control cells ($P<0.05$). Moreover, E-cadherin and cyclin D1 protein quantities were greater than in the control cells ($P<0.05$). However, the protein amount of E-cadherin, β-catenin and cyclinD1 did not differ between the prolactin and E2 groups ($P>0.12$).

Immunofluorescence

Using laser-scanning confocal microscopy, we explored whether prolactins and E2 could modulate the expression of Wnt pathway components. Our results showed that within 36 h of treatment of either prolactin or E2, the fluorescence of E-cadherin protein was strong (green fluorescence) in perinuclear of MECs (Figure 4B, 4C). However, red signals were the tetramethylrhodamine isothiocyanate (TRITC)-labeled β-catenin, which was expressed in the nuclear compartment. It was suggested that the expression of β-catenin be decreased receiving either prolactin or E2 treatment (Figure 4B, 4C). Therefore, these results revealed that the expression of E-cadherin in MECs treated with prolactin and E2 was upregulated, however, β-catenin expression was lower than in the control cells.

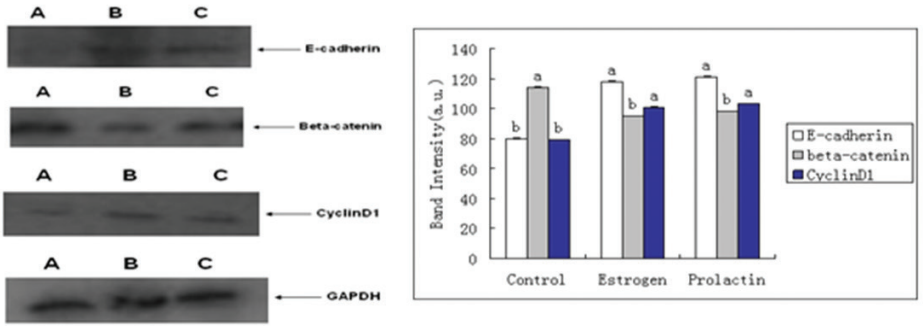


Figure 3: Western blot of E-cadherin, β-catenin and cyclinD1 in response to prolactin or E2 treatment (36 h). A: Control group; B: E2 treatment for 36 h; C: prolactin treatment for 36 h. GAPDH was used for normalization. Values are represented as mean ± SEM. Different letters indicate a significant level of $P<0.05$ in comparison to the control

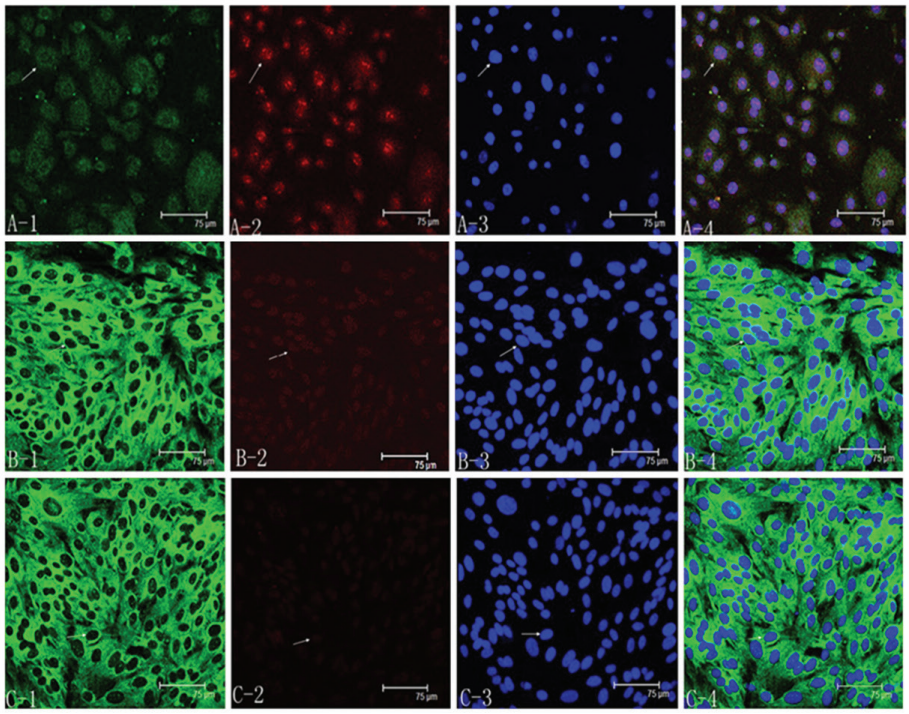


Figure 4: Immunofluorescence analysis revealed sub-cellular localizations of the proteins involved in Wnt-signalling in MECs with the treatment of prolactin and E2 for 36 h respectively. (A) Control group without prolactin and E2 treatment; (B) E2 treatment group; (C) prolactin treatment group (A-C original magnification 200×). Green, red, and blue fluorescent signals represent E-cadherin, β-catenin and DAPI-dyed nuclei, respectively

Discussion

Oestrogens and prolactin function to regulate mammary gland development and cell growth and lactation, while the Wnt-signalling pathway also plays a role in the development of the mammary gland. Overall, the Wnt/ β-catenin-signalling pathway is a known regulator of cellular functions including embryonic development and cell proliferation, differentiation, survival and adhesion (19,20). E-cadherin and β-catenin as a complex of the Wnt/β-catenin signalling pathway are specifically involved in regulating cell adhesion, and they have a close relationship with the development of the mammary gland. The current study explored the E2 and prolactin effects on MEC proliferation and the underlying molecular event *in vitro*, which could provide insightful information regarding the potential molecular actions of the mammary gland of cows. In the current study, we found that E2 and prolactin at a concentration of 5 μg/mL of each impacted MEC proliferation and the expression of E-cadherin, β-catenin, and cyclinD1. Future studies will investigate whether knockdown of the Wnt/β-catenin signalling pathway gene expression could alter the effects of oestrogens and prolactin on mammary gland development and cell growth.

Oestrogens and prolactin are required for the growth and morphogenesis of bovine mammary glands via the essential regulation of mammary epithelial cell proliferation and differentiation. Our current data has demonstrated that 5 μg/mL treatments with prolactin or E2 markedly in-

creased MEC viability and also significantly impacted the expression of E-cadherin, β-catenin, and CyclinD1, which could be crucial for exploiting oestrogens and prolactin-related mammary gland development. Specifically, postnatal mammary gland development is under the control of various hormones, including oestrogens, progesterone, growth hormone (GH), prolactin, and cell fate-determining signalling pathways (21). Our results were in accordance with previous studies showing that prolactin, by regulation of the PRL-mediated Jak-Stat signalling pathway-mediated expression of the milk proteins and oestrogens, promoted ductal extension and lobular alveolar morphogenesis (22). It has been indeed well-characterized that the bovine mammary gland-secreted oestrogens during the last week of pregnancy affects some functions of bovine neutrophils (23), which could account for our present observation that oestrogens increased cell proliferation. We found that prolactin and E2 can promote cell proliferation and survival, as well as increase cell motility, which is in accordance with Tworoger (24) who showed that that estradiol increased cell proliferation 1.5 times compared to control cells in a breast cancer cell line. Our data further revealed that the expression of cyclinD1 was affected by these two hormones, which is consistent with the observed cyclinD1 as the Wnt-signalling target gene (19). Indeed, cyclinD1 has been considered to be one of the most important factors in the regulation of cell cycle progression (25). Thus, the hormones activated β-catenin by translocation of the cytoplasmic β-catenin into the nucleus and in turn regulated gene expression, including cyclinD1, for the promotion of cell proliferation, differentiation, and organ development (26). In line with this, MEC proliferation was observed in the present study (Fig. 1). Interestingly, down-expression or destruction of E-cadherin and β-catenin is one of the changes that characterize an invasive phenotype. These genes are also considered to be invasion/tumour suppressor genes (17). Our data demonstrated that E2 and prolactin increase the expression level of E-cadherin and CyclinD1 and arrest β-catenin (Fig. 2), suggesting that prolactin and E2 enhance the Wnt-signalling activity. These results are consistent with a previous study that the proportion of the mammary epithelial cells after E2 treatment was increased and imply that Wnt signalling can substitute for oestrogens to drive total population growth (27). In addition, prolactin and E2-treated MECs showed that E-cadherin expression was markedly increased. It is possible that the proliferative effects of prolactin and E2 are mediated by the promotion of cyclinD1 expression in MECs, which further supported a previous study showing that oestrogens significantly increased expression of both c-Myc and cyclinD1 proteins and then contributed to cell cycle progression (28). Our current data showed that prolactin and E2 markedly increased the expression levels of cyclinD1 in a time-dependent manner in MECs.

Furthermore, a previous study (29) showed that soy isoflavone genistein increased E-cadherin expression through an ERβ-mediated pathway, upregulated E-cadherin–β-catenin cell adhesion complex formation, but decreased Wnt-induced cytosolic and nuclear β-catenin accumulation, and the transcription of proliferation-associated cyclinD1 and c-Myc genes. Therefore, prolactin and E2 are effective for MEC proliferation. The Wnt/β-catenin signalling is necessary for for growth survival and differentiation of the mammary gland, and it can lead to cell proliferation. Moreover, E-cadherin can sequester β-catenin away from the nucleus, acting as a tumour suppressor (30). The cadherin-catenin complex is a group of membrane proteins to regulate cell-cell adhesion. Disruption of this cell-cell adhesion in malignant, transformed cells could contribute to enhanced cell migration and proliferation. This can be confirmed by our current data that β-catenin was affected by prolactin and oestrogens after 36 h treatment, indicating that the Wnt/β-catenin pathway was activated and the downstream target gene, cyclinD1 was increased (17). Thus, further study of the Wnt/β-catenin pathway could lead to better understanding of mammary gland development, milk production, and carcinogenesis.

In summary, our current data demonstrated that the effects of prolactin and E2 on the proliferation of mammary epithelial cells and on modulation of the key components of the Wnt signalling. E2 could increase the mammary gland epithelial cells validity and the expression of a cell-cycle key gene- cyclinD1. We also emphasized the interactions between hormone-activated pathways as the principal determinants of the mammary gland development. Our results highlighted the importance of the Wnt components E-cadherin, β-catenin, and cyclinD1, which may serve as future targets for regulating the cell development and lactation of mammary glands.

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The authors declare that they have no conflict of interest.

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ESTROGENI IN PROLAKTIN URAVNAVAJO RAST EPITELIJSKIH CELIC MLEČNE ŽLEZE S SPREMEMBO SPOROČILNE POTI WNT

J. Tong, H. Zhang, Y. Wu, Y. Wang, Q. Li, Y. Liu

Povzetek: Estrogeni in prolaktin lahko uravnavajo razvoj mlečne žleze ter razvoj in rast epitelijskih celic, pa tudi laktacijo. Poleg tega ima vlogo pri razvoju mlečne žleze znotrajcelična pot sporočanja preko dejavnikov Wnt ter povečanja izraženosti ß-katenina, povzročeno preko molekule ciklina D1. Ta študija je bila namenjena oceni morebitne udeležbe estrogenov in prolaktina pri uravnavanju rasti celic v mlečni žlezi. Epitelijske celice mlečne žleze goveda (MECS) so bile 48 ur tretirane z ß-estradiolom (E2) in prolaktinom (5 μg mL^{-1}), nato pa je bila pri njih izmerjena sposobnost njihovega preživetja. mRNK in beljakovinska raven izražanja genov (E-kadherina, ciklina D1 in ß-katenina) v povezavi s potjo Wnt sta bili izmerjeni s qRT-PCR in metodo Western blot, medtem ko je bila subcelična lokalizacija proteinov v MECS opazovana s pomočjo imunofluorescence. Izražanje E-kadherina in ciklina D1 je bilo najvišje po 36 urah ($p < 0,05$), medtem ko je bila raven izražanja ß-catenina najnižja 36 ura po dodajanju E2 in prolaktina. Raven beljakovin E-kadherina in ciklina D1, ki sta tarči znotrajceličnega signaliziranja Wnt, je bila neuravnana. Beljakovinski nivo ß-katenina se je zmanjšal v obeh hormonskih skupinah. Zaključimo lahko, da prolaktin in E2 učinkovito vplivata na rast celic MECS ter povečanje izražanja E-kadherina in ciklina D1 na obeh stopnjah - stopnji mRNK in beljakovinski stopnji. Rezultati imunofluorescence kažejo na to, da prolaktin in E2 vplivata na izražanje beljakovine ß-catenin v jedru. Sedanja študija je pokazala, da je bila proliferacijska sposobnost prolaktina in E2 na MECS uravnana preko NT-signalne poti.

Ključne besede: razvoj mlečne žleze; estrogeni; progesteron; pot Wnt