Rapid Communication: Period2 gene silencing increases the synthesis of αs-casein protein in bovine mammary epithelial cells

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ABSTRACT: Period2 (PER2), a core clock gene, encodes a circadian rhythm protein which has been shown to control mammary metabolism in rodents. Whether PER2 regulates milk component synthesis such as αs-casein protein in bovine mammary cells is unknown. Thus, we used gene silencing technology to determine if PER2 silencing could affect αs-casein synthesis and cell growth in cultured primary bovine mammary epithelial cells (BMEC). The BMEC were established by enzymatic digestion of mammary tissue from mid-lactation cows. A transient-transfection technique was used to insert a small interfering RNA (siRNA) oligonucleotide specific for PER2 to inhibit transcription. Control and siRNA-transfected cells were cultured for 48 h. qRT-PCR and ELISA analysis showed that PER2 silencing enhanced the synthesis of 2 kinds of αs-casein (P < 0.05) through upregulating the mRNA level of CSN1S1 and CSN1S2 (P < 0.01). Furthermore, the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) results demonstrated that cell proliferation was not affected (P > 0.05). These data led us to hypothesize that PER2 protein may potentially play an important role in the control of milk protein synthesis and, hence, represents a target that can be used to regulate protein synthesis rate during lactation.

Key words: αs-casein, bovine mammary epithelial cell, period2 gene, small interfering RNA

INTRODUCTION

Mammary epithelial cells, the basic structural and functional unit of lactation (Qi, 2012), synthesize over 90% of proteins secreted into milk, with casein accounting for 76 to 86% of the total protein. Therefore, understanding better the regulatory mechanisms of casein synthesis is an important step to improve milk protein content. αs-casein, consisting of CSN1S1 and CSN1S2 in a 4:1 ratio, accounts for about 50% of the total amount of casein in milk. It can influence total milk volume through its role in the synthesis of total milk solids, protein and non-fat solids (Clark and Sherbon, 2000). In recent years, researchers have provided evidence that mammary function and milk composition during lactation are partly modulated by circadian clock genes, i.e., the fact that clock-mutant mice cannot adequately nourish neonates during lactation (Dolatshad et al., 2006) supported a hypothesis that the circadian system plays a role in timing the coordinated changes in metabolism and hormonal milieu needed to initiate lactation (Casey et al., 2009; Patel et al., 2011).

The Period2 gene (PER2), a core component of the circadian oscillator (Moriyama et al., 2008; Sakamoto et al., 2009), plays a key role in regulating biological circadian rhythm fluctuations in physiological processes mainly at the transcriptional level (Bae et al., 2001; Zheng et al., 2001). Suppressed PER2 expression through RNA interference (RNAi) accelerates cell proliferation and viability through inhibiting apoptosis especially in tumor cells (Oda et al., 2009). It is also thought to improve milk protein yield via its effect on the circadian rhythm (Plaut and Casey, 2012). Despite the available evidence, direct demonstration for a role of PER2 in cell proliferation and milk protein synthesis remains unknown. Thus, we used gene silencing technology to determine if PER2 silencing could affect αs-casein synthesis and cell growth in cultured primary bovine mammary epithelial cells (BMEC). The BMEC were established by enzymatic digestion of mammary tissue from mid-lactation cows. A transient-transfection technique was used to insert a small interfering RNA (siRNA) oligonucleotide specific for PER2 to inhibit transcription. Control and siRNA-transfected cells were cultured for 48 h. qRT-PCR and ELISA analysis showed that PER2 silencing enhanced the synthesis of 2 kinds of αs-casein (P < 0.05) through upregulating the mRNA level of CSN1S1 and CSN1S2 (P < 0.01). Furthermore, the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) results demonstrated that cell proliferation was not affected (P > 0.05). These data led us to hypothesize that PER2 protein may potentially play an important role in the control of milk protein synthesis and, hence, represents a target that can be used to regulate protein synthesis rate during lactation.

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synthesis in bovine mammary epithelial cells (BMEC) is still lacking. The main objective of the present study was to generate experimental data for further research on the control of PER2 in the mammary gland in the context of development and metabolism.

MATERIALS AND METHODS

The use of animals in the present study was approved by the Animal Care and Use Committee of Yangzhou University, China.

Primary Bovine Mammary Epithelial Cell Isolation

Three healthy Chinese Holstein cows from the Yangzhou University dairy herd at 100 ± 5 DIM were chosen for mammary biopsy and epithelial cell isolation. Mammary tissue samples were harvested by a published biopsy method (Bionaz et al., 2012) and rinsed in PBS supplemented with 100 IU/mL penicillin/streptomycin (P0389/S6501, Sigma-Aldrich Corp., Saint Louis, MO) until the washing liquid was clear. The BMEC were isolated using enzyme digestion method from our laboratory (Wang et al., 2014). Specifically, connective tissue and adipose tissue were excised with ophthalmic forceps. Parenchyma tissue was cut into about 1-mm³ pieces, placed in an ampoule and digested with 0.5% of collagenase I (17100017, Gibco, Thermo Fisher Scientific Inc., Carlsbad, CA) for 3 h at 37°C followed by filtering through a 74 μm sieve and centrifuging for 5 min at 453 × g, 15°C. The mixed cells were washed with PBS twice and inoculated at a density of 5 × 10⁵/mL in 25 cm² culture flasks with 5 mL growth medium (see Supplemental Table S1) at 37°C with 5% of CO₂. When cells covered 70% of the plate, 50 pmol siRNA and 6.6 μL soly-fecter (a mixture of lipid polymer for transfection) were homogeneously dissolved in 125 μL siRNA transfection buffer, respectively. Then, the soly-fecter dilution was added into the siRNA dilution for about 15 min to form a stable mixture and then the growth medium supplying to 2 mL mixed medium was added. Lastly, the growth medium was replaced with the same volume of mixed medium in each well before culture for 48 h at 37°C with 5% of CO₂. After verifying the siRNA transfection efficiency through comparing the mRNA abundance of three treatments with the control via qRT-PCR, the siRNA oligonucleotide inducing the lowest PER2 expression was selected for subsequent studies (sense: 5’-UUC UCC GAA CGU GUC ACG UTT-3’; antisense: 5’-ACG UCA CAC GUU CGG AGA ATT-3’).

Cell Proliferation Analysis

Cell proliferation was detected via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric kit (M6494, Invitrogen, Thermo Fisher Scientific Inc.) at 12 h, 24 h, and 48 h, respectively. The experimental and control BMEC were inoculated in 96-well plate at a density of 1 × 10⁵ and cultured at 37°C with 5% of CO₂ until 70% confluence. Then the BMEC were transfected with the siRNA oligonucleotide as mentioned above. Each treatment had 6 replicates. The silencing mixed medium was replaced with 20 μL MTT working solution (5 mg/mL) per well and incubated for 4 h each time at 12 h, 24 h, and 48 h, respectively. Then the supernatant was replaced with 200 μL of dimethyl sulfoxide (DMSO). Absorbance was read at 490 nm immediately.

Gene Expression and Protein Synthesis of αs–casein

Total RNA was isolated by the Trizol method (15596018, Ambion, Thermo Fisher Scientific Inc.), and concentration measured by NanoDrop1000. The OD260/OD280 values of RNA samples were all ≥ 1.9. Integrity of the purified total RNA was assessed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the RNA 6000 Nano Kit (Agilent Technologies). The RNA Integrity Number (RIN) values of samples used were ≥ 8.0. The RNA was reverse-transcribed into cDNA with the FastQuant cDNA kit (KR103, TIANGEN Biotech, Beijing, China). The expression levels of PER2, CSN1S1, CSN1S2, and housekeeping genes, ACTB and GAPDH, were measured using the ABI 7500 PCR sequence detector with the qRT-PCR kit (FP302, TIANGEN Biotech) and primers are reported in Supplemental Table S2. Each treatment was run in triplicate.

Total protein content in cells was extracted with the RIPA protein lysate (TIANGEN Biotech), and con-
centration detected via the BCA protein kit (P0010, Enhanced) according to manufacturer’s instructions (Beyotime Biotechnology, Shanghai, China). This consisted of preparation of protein standards and BCA working solution. After normalizing total protein concentration, CSN1S1 (XY-E80001), CSN1S2 (XY-E80004) and PER2 protein (XY-E77801) were assessed via bovine ELISA (Xinyu Biological Technology, Shanghai, China) according to manufacturer’s instructions.

**Statistical Analysis**

Differences in cell proliferation, expression of αs-casein genes and protein synthesis in BMEC between the experiment and the control group were evaluated by independent sample t test in SPSS16.0. Data are expressed as mean ± SD and significant difference was declared at P < 0.05.

**RESULTS AND DISCUSSION**

Results of the proliferation of BMEC are provided in Table 1. Cell proliferation increased by 12 h after PER2 silence but decreased 24 h later. After 48 h, proliferation remained stable although these values were not different from the control (P > 0.05). Similar results were observed in the study of Hua (2006) that although the PER2 expression in mouse SCN cells was greater than in peripheral tissues, overexpression of PER2 had no sufficient effect on the cell cycle and apoptosis in normal NIH 3T3 cells.

Previous studies have demonstrated an essential role of PER2 in regulating mammary cell viability and proliferation. For example, the expression of PER2 in mouse mammary tissue peaked at zeitgeber time 12, and coincided with the peak of DNA synthesis and mitosis (Qi, 2012). The cell division cycle of PER2-mutant mice was shorter than wild-types (Fu et al., 2002), underscoring the importance of PER2 in cell division. Furthermore, knocking-down PER2 in human breast epithelial cells resulted in the change of the 3-dimensional morphology of cultured mammary epithelial cells (Rossetti et al., 2012) while overexpression of PER2 led to a reduction of cell differenciation causing rapid apoptosis (Kobayashi et al., 2006). Knocking-out silkworm PER2 was characterized by a reduction in metamorphosis development and acceleration in cell proliferation, indirectly indicating a role of PER2 on cell proliferation (Sandrelli et al., 2007). It is conceivable that the BMEC in the present study were not actively growing as would be the case in tumor cells where effects of PER2 manipulation have been detected (Winter et al., 2007; Yang et al., 2009). In addition, changes in physiological state owing to the culture conditions in vitro could occur over long incubation periods. It also could be possible that our sampling times missed critical stages of cell growth and division and, hence, the lack of sufficient effect of PER2 silencing as previous studies mentioned above.

Regarding αs-casein synthesis, we detected a greater mRNA expression of CSN1S1, CSN1S2, and lower PER2 mRNA expression after PER2 silencing (Table 2). In addition, PER2 silencing increased the concentration of CSN1S1 and CSN1S2 (P < 0.05) while it inhibited the synthesis of PER2 protein (P < 0.01) without affecting total protein content (Table 2).

During the transition from pregnancy to lactation in mice, analysis of core clock proteins revealed that the stoichiometric relationship between CLOCK and PER2 components remained 1:1 in liver but rose to 4:1 in mammary (Casey et al., 2014). The role of PER2 protein in mouse mammary development and lactation was underscored by a lower expression of PER2 in mammary tissue during lactation than puberty and pregnancy (Qi, 2012). Attenuated mPER2 expression appeared to be a physiological adaptation to lactation, which led us to hypothesize that the variations in mammary PER2 expression was related to the physiological changes that occurred in the dam to support lactation.

Besides the evidence of PER2 being involved in regulation of mammary cell protein synthesis, the circadian changes in the expression of PER2 also coin-

### Table 1. Cellular proliferation of bovine mammary epithelial cells

| Culture time | Control group | PER2 silence group | P-value |
|--------------|---------------|-------------------|---------|
| 12 h         | 0.24 ± 0.06   | 0.26 ± 0.04       | 0.585   |
| 24 h         | 0.34 ± 0.04   | 0.32 ± 0.10       | 0.210   |
| 48 h         | 0.42 ± 0.03   | 0.42 ± 0.03       | 0.760   |

1Cellular proliferation expressed as optical density (OD).

### Table 2. Gene expression and protein synthesis of αs-casein and PER2 in bovine mammary epithelial cells

| Item          | Control group | PER2 silence group | P-value1 |
|---------------|---------------|--------------------|----------|
| Gene symbols  |               |                    |          |
| CSN1S1        | 1.000 ± 0.044 | 1.139 ± 0.047**    | 0.001    |
| CSN1S2        | 1.003 ± 0.097 | 2.321 ± 0.132**    | 0.0001   |
| PER2          | 1.008 ± 0.143 | 0.174 ± 0.018**    | 0.0001   |
| Protein types |               |                    |          |
| Total protein | 0.210 ± 0.011 | 0.210 ± 0.038      | 0.086    |
| CSN1S1        | 0.191 ± 0.025 | 0.482 ± 0.166*     | 0.040    |
| CSN1S2        | 0.070 ± 0.008 | 0.176 ± 0.060*     | 0.040    |
| PER2 protein  | 0.363 ± 0.040 | 0.162 ± 0.031**    | 0.002    |

1Means differ at ** P < 0.01 or *P < 0.05.

2The unit of protein concentration is mg/ml.
cided with the diurnal change in milk composition (for review see Casey and Plaut, 2012). The fact that the expression pattern of PER2 is linked to casein genes on the 16th day of pregnancy and the 1st and 7th day of lactation in mice further implicated PER2 in the control of casein synthesis (Metz et al., 2006).

In summary, the present results underscore the role of PER2 in the adaptation to milk secretion and milk composition, which was characterized by enhancing αs–casein protein synthesis of BMEC and an overall increase in milk protein content. Despite these clear effects, the exact mechanisms for the PER2 effect on BMEC viability and casein mRNA warrant further investigation.

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