295 CLOCK regulation of mammary epithelial cell growth.
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The circadian timing system (CTS) influences virtually all physiological processes, including lactation. However, the manner that the multitude of molecular clocks that make up the CTS both centrally and peripherally affect lactation performance is currently not understood. Preliminary studies from our lab led us to hypothesize clocks in the mammary play an important role in regulation of gland development. The objective of this study was to determine if decreasing abundance of CLOCK, a core component of the circadian clock mechanism, affects growth of mammary epithelial cells. For this study a mouse mammary epithelial cell line, HC11, was transfected with shRNA that targeted *Clock* or a negative control scramble sequence. Cells transfected with sh*Clock* expressed 70% less *Clock* mRNA than wild-type (WT) HC11 cultures, which resulted in significantly depressed levels of CLOCK protein ($P < 0.05$). Scramble had no effect on mRNA or CLOCK protein levels. Six-day growth curve analysis revealed HC11 lines carrying sh*Clock* had 4-fold higher growth rates ($P < 0.05$) and reached at least 2-fold higher cell density than scramble transfected or WT HC11 cultures. To understand how CLOCK regulates growth, sh*Clock* transfected and WT HC11 cells were plated in growth medium, serum starved for 24 h, returned to growth medium and then collected every 4 h over a 48-h period. Cell cycle analysis of propidium iodide stained cells showed sh*Clock* significantly increased response to serum starvation (90.1 ± 1.1% of sh*Clock* versus 71.3 ± 3.6% of WT-HC11 in G1 phase), and affected progression through cell cycle. Two-way ANOVA showed time and cell line had significant effects ($P < 0.0001$) on relative expression levels of the cell cycle regulators, *Ccnd1* (regulates transition from G1 to S phase), *Wee1* (regulates transition from G1 to S and G2 to M phase) and *Tp63* (a tumor suppressor gene). Moreover, basal mean expression of *Ccnd1* was 43% greater ($P = 0.002$) in sh*Clock* versus WT cell lines, and *Tp63* expression was depressed by ~3-fold in sh*Clock* cultures ($P < 0.0001$). These data support circadian clocks play a role in regulation of epithelial cell growth in the mammary gland.

Key Words: circadian, clock, mammary

296 CLOCK regulates mammary differentiation and output.
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Circadian clocks synchronize internal physiology to the environment, thus understanding role in regulation of lactation may lead to development of noninvasive approaches to improve production efficiency of dairy animals. Circadian clocks are present in all mammalian cells. The *BMAL1* and *CLOCK* genes are core components of clocks, functioning together as a transcription factor. In the mammary gland, abundance of BMAL1-CLOCK increases during the transition from pregnancy to lactation and upon lactogen-induced differentiation of mammary cells in culture. We hypothesize that in the mammary BMAL1-CLOCK regulates differentiation and milk synthesis. Our objective was to elucidate the effect of decreasing CLOCK abundance on expression of markers of differentiation (e-cadherin- CDH1) and metabolic output (fatty acid synthase- FASN; β-casein- CSN2) in a mouse mammary epithelial cell line, HC11. To decrease CLOCK abundance, HC11 cells were transfected with shRNA specific for *Clock* or a scramble sequence (negative control), and clonal lines were established. Cells transfected with sh*Clock* had a 70% reduction in *Clock* mRNA, which resulted in significantly reduced ($P < 0.05$) CLOCK protein abundance relative to wild-type cultures. Abundance of CLOCK in scramble-line was not different from wild-type. RNA and protein were collected from undifferentiated cultures, and cultures treated 96 h with dexamethasone, insulin and prolactin (differentiated). Gene expression was analyzed using RT-qPCR. Two-way ANOVA showed cell line (wild-type, scramble, sh*Clock*) and state of differentiation had a significant effect ($P < 0.05$) on relative expression of *Fasn, Csn2* and *Cdh1*. Post-hoc analysis revealed *Fasn* was significantly reduced by 2-fold ($P < 0.05$) in sh*Clock* treatments relative to wild-type cultures. *Cdh1* was also reduced more than 3-fold in sh*Clock* lines relative to wild-type cultures ($P < 0.05$). Moreover, Western blot analysis showed abundance of CDH1 protein was significantly reduced in cultures transfected with sh*Clock*. sh*Clock* sequence did not have a significant effect on *Csn2* expression. In conclusion CLOCK regulates differentiation markers and *Fasn*, future studies are needed to understand how factors affect mammary clocks and the relationship to dairy performance.

Key Words: mammary, circadian, differentiation

297 Expression of putative stem cell marker, hepatocyte nuclear factor 4 alpha, in mammary gland of water buffalo.
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Buffaloes account for more than 56% of total milk production in India. Cyclic remodeling of mammary glands of human, mice, cow, sheep and goat is determined by mammary stem cells. It is logical to assume that buffalo mammary gland will have mammary stem/progenitor cells. Thus far, no report exists on identification of buffalo mammary stem cells. Hepatocyte nuclear factor 4 α (HNF4A) is a candidate marker for hepatic progenitor cells and has recently been suggested as a marker of bovine mammary stem/progenitor cells. We hypothesized that (1) HNF4A identifies putative buffalo mammary stem/progenitor cells and (2) the number of mammary stem/progenitor cells increases during mastitis. Thirteen buffalo mammary samples were collected from a local slaughterhouse. Hematoxylin and eosin staining were performed on 5-μm-thick sections. Based on histomorphology of mammary glands, physiological stages were estimated to be nonlactating (n = 4 animals) and mastitis (n = 9 animals). In total, ~22,000 cells were counted (10 microscopic fields/animal; n = 13 animals), of which 40% cells were mammary epithelial cells (MEC) and 60% cells were the stromal cells. The percentage of MEC in nonlactating animals was higher compared with that in mastitic animals (47.3 vs. 37.3%), which was likely due to loss of MEC caused by infection. HNF4A staining was observed in

nuclei of MEC of ducts, alveoli and stromal cells. Basal location and low frequency of basally located HNF4A positive cells (ranges from 0.4 to 4.5%) was consistent with characteristics of mammary stem cells. HNF4A-positive MEC (basal and luminal; light and dark stained) tended to be higher during nonlactating stage than mastitis (8.73 ± 1.71 vs. 4.29 ± 1.19%; $P = 0.07$). MEC proliferation (assessed by immunohistochemical expression of Ki67) was higher in mastitic glands in comparison to nonlactating glands (15.3 ± 5.7% vs. 0.53 ± 0.1%; $P = 0.03$). This is the first report outlining the expression of HNF4A as a putative mammary stem/progenitor cells of buffalo mammary gland; and evaluation of MEC proliferation in naturally infected mastitic buffaloes.

Key Words: buffalo, mammary stem/progenitor cell, HNF4A

298 Optimization of transfection and real-time monitoring of fluorescent proteins in bovine cells: An untapped molecular biology approach for dairy sciences. J. S. Osorio* and M. Bionaz, *Oregon State University, Corvallis, OR.*

The study of nuclear receptor activation by specific dietary compounds via gene reporter technology (GRT) is essential in nutrigenomics research. The GRT requires inserting into cells an artificial plasmid containing a promoter region with the response element for the nuclear receptor of interest and DNA coding for luciferase or a fluorescent protein. The main challenge of using GRT is the low efficiency and high variability of plasmid transfection; thus limiting the sensitivity and increasing intra-essay variability. To investigate the efficiency of transfection in immortalized (MacT) and primary mammary bovine (BMEC) cells we have tested several concentrations of transfection reagents in combination with several concentrations of a plasmid for the constitutively expressed enhanced green fluorescent protein (EGFP). Cells were seeded 24 h prior transfection at 30,000 cells/well in a 96-well plate and treated with a nuclear staining (NucBlue Live). The transfection reagents Lipofectamine 2000, Lipofectamine LTX, and TransIT-X2 were used at 0.2, 0.3, 0.4, or 0.5 mL/well with 50, 100, 200, or 300 ng/well of EGFP. Using a robotic inverted fluorescent microscope for live imaging (Leica DMI6000B), 2 pictures/well were taken every hour for 30 h post-transfection. Cell number, viability, and quantification of transfection efficiency were assessed using the CellProfiler sotfware. Data were analyzed using GLIMMIX of SAS. EGFP protein was visualized as early as 4 h after transfection and plateau expression was observed >7 h post-transfection. We observed high variability in transfection efficiency ranging from 1 to 30%. Transfection efficiency was best using Lipofectamine 2000 in BMEC and Lipofectamine LTX in MacT. In general we observed that high dose of transfection reagent and plasmid provided best results; however, high dose of transfection reagent tend also to kill the cells. Overall, our data confirmed the large intra- and inter-cells variation in transfection efficiency and prompt for a more precise approach to obtain high reliable data for nutrigenomics studies in dairy cows.

Key Words: mammary epithelial cell, nutrigenomics, transfection

299 Palmitate and peroxisome proliferator-activated receptor (PPAR)γ synthetic agonists, but not *trans*-10,*cis*-12 CLA, activatePPAR in MacT and primary goat mammary cells. J. S. Osorio* and M. Bionaz, *Oregon State University, Corvallis, OR.*

The peroxisome proliferator-activated receptors (PPAR) can play pivotal nutrigenomics roles in ruminants owing their capacity to be activated by long-chain fatty acids (LCFA). Data on expression of putative PPARs target genes suggested that palmitate (PA) is a potent PPARs activator in cows and goats whereas *trans*-10,*cis*-12 conjugated linoleic acid (CLA) is not or is a very weak PPAR agonist. Furthermore, previous in vivo data in goats and cows suggested that 2,4-thiazolidinedione (TZD) is a weak activator of PPARγ. To test if palmitate, *trans*-10,*cis*-12-CLA, and TZD are PPAR agonists we have transfected immortalized MacT and primary mammary goat (PMG) cells with a PPRE-X3-TK-Luc plasmid. Prior transfection cells were seeded at 10^4 cells/well in a 96-well plate. We have used as transfection reagents Lipofectamine 3000 with PMG and TransIT-X2 with MacT at 0.3 μL/well with 200 ng/well of plasmid in OptiMEM deprived of fetal bovine serum (FBS). Transfected cells were treated for 24h in quadruplicates with 10% FBS or 0.1 m*M* of ethanol (control), rosiglitazone (ROSI), PA, and CLA as well as 2 doses of TZD (0.1 m*M* and 1 m*M*) in medium without FBS. All were supplemented with 10 μ*M* of 9-*cis*-retinoic acid. Cell number and transfection efficiency was obtained via CellProfiler software and luciferase was measured using luminometer and normalized by number of viable cells. Data were analyzed using GLIMMIX of SAS and significance was declared with $P < 0.05$. Transfection was higher with MacT (24 ± 3%) compared with PMG (6 ± 4%). In MacT cells, all treatments with exception of CLA activated PPAR compared with control with the highest activation observed with TZD followed by PA, ROSI, and FBS. In PMG the PPAR activation was observed with FBS, PA, and TZD, while CLA and ROSI did not affect luciferase. Overall our data confirmed that TZD is a PPARγ synthetic agonist in ruminant mammary cells. In addition, the data confirmed that PA but not CLA is a PPAR activator and that blood serum in ruminants contains potent natural PPAR agonists, likely LCFA. These findings underscore the nutrigenomics importance of PA and confirmed the PPARγ responsiveness of mammary cells in ruminants.

Key Words: nutrigenomics, PPAR, transfection

300 Protection of bovine mammary epithelial cells from hydrogen peroxide-induced oxidative cell damage by resveratrol. X. L. Jin*[1,3], Kai Wang[2], H. Y. Liu[1,3], F. L. Hu[2], F. Q. Zhao[1,4], and J. X. Liu[1,3], *[1]Institute of Dairy Science, College of Animal Sciences, Zhejiang University, Hangzhou, China, [2]Institute of Sericulture and Apiculture, College of Animal Sciences, Zhejiang University, Hangzhou, China, [3]MOE Key Laboratory of Molecular Animal Nutrition, Zhejiang University, Hangzhou, China, [4]Laboratory of Lactation and Metabolic Physiology, Department of Animal Science, University of Vermont, Burlington, VT.*

Knowledge in the effects of oxidative stress and antioxidants on bovine mammary epithelial cells (bMEC) is limited. The objectives of this study were to investigate the oxidative damaging effects of hydrogen peroxide (H_2O_2) and the cytoprotective effects of resveratrol, a well-known natural product rich in grape seeds, against oxidative stress in cultured bMEC (MAC-T). To establish an oxidative stress model in bMECs, 500 μ*M* H_2O_2 was added to MAC-T cells. The CCK-8 assay was applied to detect cell viability and flow cytometry method was used to detect intracellular production of reactive oxygen species. Pretreatment of MAC-T cells with resveratrol could rescue the decrease in cell viability and resulted in lower intracellular accumulation of reactive oxygen species after H_2O_2 exposure. Using qRT-PCR, we found that resveratrol helped MAC-T cells to prevent H_2O_2-induced endoplasmic reticulum stress, indicated by significantly decreased abundance of endoplasmic reticulum stress marker GRP78 and CHOP mRNA ($P < 0.01$). Resveratrol also inhibited mitochondria-related cell apoptosis by downregulating the expression of pro-apoptotic Bax gene ($P < 0.01$) and upregulating expression of anti-apoptotic Bcl-2 gene ($P < 0.01$) compared with H_2O_2 group. Moreover, resveratrol increased the

abundance of multiple antioxidant defense genes (HO-1, xCT, Txnrd1, and NQO-1) in MAC-T cells under normal/oxidative conditions. It is confirmed that Nrf2 was required for the cytoprotective effects on MAC-T cells by resveratrol, because knockdown of Nrf2 abolished resveratrol-induced cytoprotective effects against oxidative stress, accompanied by no significant differences in gene expression of Nrf2, HO-1, Txnrd, and CHOP between resveratrol+H_2O_2 and H_2O_2 groups in Nrf2 knockdown MAC-T cells. Finally, by using selective inhibitors, we confirmed that the induction of Nrf2 by resveratrol was mediated through the activation of the PI3K/Akt and ERK/MAPK pathways, but negatively regulated by p38/MAPK pathway. In conclusion, our study provided evidence that resveratrol may be potentially used as a therapeutic agent for cytoprotection of bMEC against oxidative stress.

Key Words: resveratrol, oxidative stress, mammary epithelial cell

301 Stabilization of Nrf2 by tBHQ attenuates heat shock-induced cell damage in bovine mammary epithelial cells. X.L. Jin*[1,2], H.Y. Liu[1,2], and J.X. Liu[1,2], *[1]Institute of Dairy Science, College of Animal Sciences, Zhejiang University, Hangzhou, China, [2]MOE Key Laboratory of Molecular Animal Nutrition, Zhejiang University, Hangzhou, China.*

Nrf2-ARE signaling plays a key role in cellular antioxidant-defense system, but whether Nrf2 activation has protective effects against heat shock (HS) stress remains unclear. The objective of the present study was to determine whether the Nrf2 activator, tBHQ, could attenuate the heat stress-induced responses in bovine mammary epithelial cells (bMECs). MAC-T cells were exposed to HS (42.5°C for 1 h) followed by recovery at 37°C to mimic HS stress. Compared with cells consistently cultured at normothermia (37°C), cell viability was dropped after HS treatment ($P < 0.01$), and arrived at the lowest (64.5 ± 7.68%) after 12 h recovery. Accordingly, mRNA abundance of cell-apoptosis marker genes (Bax and CHOP, $P < 0.01$) and cellular antioxidant defense genes (HO-1 and Txnrd1, $P < 0.01$) increased time-dependently in HS challenged cells and reached to the highest level after 12 h recovery, compared with normothermic cultured MAC-T cells. When the MAC-T was pretreated with tBHQ (10 μ*M*) for 2 h and performed HS following 12 h recovery, pre-incubation of tBHQ significantly prevented loss of cell viability and downregulated mRNA expression of Bax and CHOP ($P < 0.01$) than HS treated MAC-T cells in the absence of tBHQ. The presence of tBHQ also significantly blocked accumulation of reactive oxygen species induced by HS in MAC-T cells after recovering for 2 h ($P < 0.01$). More importantly, tBHQ pre-treated cells showed stronger activation of Nrf2-ARE signaling compared with the HS group, including more nuclei-accumulations of Nrf2 and higher upregulations of Nrf2-ARE driven gene expressions (including Nrf2, HO-1 and Txnrd1, $P < 0.01$). RNA silencing of Nrf2 in HS-treated MAC-T cells almost abolished the cytoprotective effects of tBHQ. These results indicated that HS could cause oxidative stress in bMECs, and stabilization of Nrf2 by tBHQ could attenuate HS-induced bMECs damage.

Key Words: heat shock, Nrf2, tBHQ