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Effects of intramuscular administration of folic acid and vitamin B_{12} on granulosa cells gene expression in postpartum dairy cows

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ABSTRACT

The fertility of dairy cows is challenged during early lactation, and better nutritional strategies need to be developed to address this issue. Combined supplementation of folic acid and vitamin B_{12} improve energy metabolism in the dairy cow during early lactation. Therefore, the present study was undertaken to explore the effects of this supplement on gene expression in granulosa cells from the dominant follicle during the postpartum period. Multiparous Holstein cows received weekly intramuscular injection of 320 mg of folic acid and 10 mg of vitamin B_{12} (treated group) beginning 24 (standard deviation = 4) d before calving until 56 d after calving, whereas the control group received saline. The urea plasma concentration was significantly decreased during the precalving period, and the concentration of both folate and vitamin B_{12} were increased in treated animals. Milk production and dry matter intake were not significantly different between the 2 groups. Plasma concentrations of folates and vitamin B_{12} were increased in treated animals. Daily dry matter intake was not significantly different between the 2 groups before [13.5 kg; standard error (SE) = 0.5] and after (23.6 kg; SE = 0.9) calving. Average energy-corrected milk tended to be greater in vitamin-treated cows, 39.7 (SE = 1.4) and 38.1 (SE = 1.3) kg/d for treated and control cows, respectively. After calving, average plasma concentration of β-hydroxybutyrate tended to be lower in cows injected with the vitamin supplement, 0.47 (SE = 0.04) versus 0.55 (SE = 0.03) for treated and control cows, respectively. The ovarian follicle ≥12 mm in diameter was collected by ovarian pick-up after estrus synchronization. Recovered follicular fluid volumes were greater in the vitamin-treated group. A microarray platform was used to investigate the effect of treatment on gene expression of granulosa cells. Lower expression of genes involved in the cell cycle and higher expression of genes associated with granulosa cell differentiation before ovulation were observed. Selected candidate genes were analyzed by reverse transcription quantitative PCR. Although the effects of intramuscular injections of folic acid and vitamin B_{12} on lactational performance and metabolic status of animals were limited, ingenuity pathway analysis of gene expression in granulosa cells suggests a stimulation of cell differentiation in vitamin-treated cows, which may be the result of an increase in LH secretion.

Key words: dairy cow, folic acid, vitamin B_{12}, granulosa cell

INTRODUCTION

Dairy cows enter a state of negative energy balance during early postpartum period. One important challenge with regard to herd management is that females must become gravid during early lactation. A relationship between ovarian activity and energy status has been observed in high-producing dairy cows during the early postpartum period (Staples et al., 1990). This postpartum anestrus depends on several factors, such as nutrition, body reserves, milk yield, lactation status, and postpartum health (Lucy, 2001). Moreover, a period of prolonged energy deficit impairs ovarian follicle development, corpus luteum function, and the secretory activity of the reproductive tract (Wathes et al., 2003).

Folic acid and vitamin B_{12} are essential for the synthesis of DNA, RNA, and proteins, in addition to their roles in energy production. In the search for a strategy to offset the energy deficit in the dairy cow, recent studies have shown that a combined supplementation of folic acid and vitamin B_{12} improves the efficiency of energy metabolism during early lactation (Graulet et al., 2007; Preynat et al., 2009a). Propionate is the major source of glucose in cows (Amaral et al., 1990), and the enzyme methylmalonyl CoA mutase is a vitamin B_{12}-dependent enzyme that is essential for the entry of propionate into the Krebs cycle and gluconeogenesis

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pathway. Expression of the methylmalonyl-CoA mutase gene was increased by a combined treatment with folic acid and vitamin B_{12} (Preynat et al., 2010). A combined supplement of folic acid and vitamin B_{12} increased milk production, lactose, CP, and TS yields in the milk of cows during early lactation, and tended to increase whole-body glucose flux (Preynat et al., 2009a).

A limited number of studies have been carried out to investigate the effect of folic acid and vitamin B_{12} on ovarian physiology. In the mouse, ovarian follicle development was not supported in medium with a very low dose of Met, vitamin B_{12}, and folic acid (Anckaert et al., 2010). Plasma concentration of vitamin B_{12} was significantly reduced in polycystic ovarian syndrome patients with high body mass index (Kaya et al., 2009), which impairs follicular development. Among transcripts of all 12 enzymes of methionine or folate cycles, the one for betaine-homocysteine methyltransferase and methionine adenosyl transferase 1A were, respectively, absent and weak in cumulus and granulosa cells (Kwong et al., 2010), arguing against a direct effect of the supplement on Met or folate cycle in the ovarian follicle. In a large study, it was recently shown that the first breeding postpartum occurred 3.8 d earlier in multiparous cows receiving weekly an intramuscular injection of folic acid and vitamin B_{12} from 3 wk before the expected date of calving until 8 wk of lactation (Duplessis et al., 2014).

The present study was undertaken to determine the effects of a combined supplementation of folic acid and vitamin B_{12} on gene expression of granulosa cells from the dominant follicle of postpartum lactating dairy cows. To test this hypothesis, a microarray platform was used to study the effect of the vitamin treatment on granulosa cell transcriptome profiling. Results of gene expression will allow further understanding of the effect of vitamin supplementation on ovarian physiology.

MATERIALS AND METHODS

Animals and Treatments

Twenty-four multiparous Holstein cows from the herd belonging to the Center for Research in Animal Science (Deschambault, QC, Canada), kept in tiestalls, were assigned to 12 blocks of 2 cows each according to their previous milk production and planned calving dates; the two groups were designated as control (receiving saline) and treated (receiving folic acid and vitamin B_{12}). Milk production (SD) during the previous 305-d lactation period was similar for the 2 groups of cows: 10,210 (416) and 10,187 (422) kg for the control and treated groups, respectively ($P = 0.93$). At the entry in the study, 3 wk before the expected date of calving, cow BW was not different ($P = 0.80$) between treatment groups, 715 ± 24 and 724 ± 22 kg (±SD) for the control and treated groups, respectively. Body score condition was not significantly different ($P = 0.33$) between control (3.35 ± 0.34) and treated group (3.56 ± 0.44). Half the cows were treated weekly with a single intramuscular injection of 320 mg of folic acid (pteroylmonoglutamic acid, ICN Biochemicals Inc., Cleveland, OH) and 10 mg of vitamin B_{12} (cyanocobalamin, 5,000 μg/mL, Vetoquinol, Lavaltrie, QC, Canada), whereas control animals received 5 mL of saline (0.9% NaCl), as already described (Duplessis et al., 2014). The parenteral route was used to avoid destruction of the vitamin supplement by ruminal microorganisms. The experimental period began 24 d ± 4 before the date of calving and lasted until 8 wk after calving. Cows were fed a precalving diet during the 3 wk before calving, and a lactation diet from calving until the end of the study (Supplemental Table S1; http://dx.doi.org/10.3168/jds.2015-9623). Cows were fed a TMR once daily around 1000 h. Silages were analyzed weekly by near-infrared reflectance spectrometry to adjust for DM and, when necessary, to adjust the proportion of other dietary ingredients to maintain similar nutrient concentrations throughout the experimental period. Animals had free access to water and were fed ad libitum with allowance for up to 10% refusal. The amount of feed served and refusals were measured daily. After calving, cows were milked twice a day with a 10-h interval between the morning and evening milkings. Milk samples were collected on 2 consecutive milkings, every 2 wk, to determine milk composition. The experimental protocol was approved by the Animal Care Committee of Université Laval (authorization number: 2010022–1), and applications were reviewed in accordance with guidance provided by the Canadian Council on Animal Care (Rolfert et al., 1993).

Milk Analysis

Milk composition (fat, protein, lactose, and urea) was determined by near-infrared reflectance spectroscopy (Valacta, Sainte-Anne-de-Bellevue, QC, Canada). Milk concentrations of folates and vitamin B_{12} were analyzed as described by Preynat et al. (2009a).

Blood Sampling and Analyses

Blood samples were collected by venipuncture of the coccygeal vein always at the same time after the meal, at least 1 h after distribution of the morning meal. Samples were collected weekly during the 3 wk before the expected date of calving, twice weekly during the first 3 wk of lactation, and then weekly for the remain-

ing 5 wk. Tubes with EDTA were used for analyses of folates, vitamin B_{12}, BHBA, and NEFA, whereas heparin-containing tubes were used for glucose and urea determination. Blood was centrifuged for 20 min at 1,854 × *g* and 4°C within 1 h of sampling. Plasma was stored frozen at −20°C until analysis.

Plasma concentrations of folates and vitamin B_{12} were measured in duplicate by RIA [SimulTRAC-S Radioasssay kit, Vitamin B_{12} (^{57}Co)/Folate (^{125}I), MP Biomedicals, Diagnostics Division, Orangeburg, NY]. The interassay coefficients of variation for folates and vitamin B_{12} in plasma were 1.13 and 1.14%, respectively. Plasma urea, NEFA, glucose, and BHBA concentrations were determined using commercial kits: urea Assay kit from Diagnostic Chemicals Limited (BioPacific Diagnostic Inc., Charlottetown, PE, Canada); NEFA-C from Wako Chemicals GmbH (Neuss, Germany); glucose assay kit from Roche Diagnostics GmbH (Mannheim, Germany); and BHBA Reagent Set from Pointe Scientific Inc. (Canton, MI).

Estrous Cycle Synchronization and Collection of Granulosa Cells and Follicular Fluid

Estrous synchronization was performed to collect granulosa cells from the dominant follicle on d 57 ± 3 after calving. Each cow was treated twice with $PGF_{2\alpha}$ (cloprostenol 25 mg i.m.; Estrumate, Schering-Plough Corporation, Summit, NJ) with a 14-d interval starting on d 40 ± 3. Development of the dominant follicle was monitored by ovarian ultrasound at 40, 44, 54 ± 3 d postpartum and before ovarian pick-up (**OPU**). The functional lysis of the corpus luteum was validated by measuring progesterone in blood serum at the same time points than ultrasound. Granulosa cell populations were collected from the dominant follicle by transvaginal OPU 66 ± 2 h after the second treatment with $PGF_{2\alpha}$. Only granulosa cells from the largest follicle (with a diameter ≥12 mm measured by ovarian ultrasound) were collected. In the absence of a follicle with a diameter ≥12 mm on d 57 ± 3, OPU was performed on d 71 ± 3 after a third administration of $PGF_{2\alpha}$ on d 68 (control: n = 3; treated: n = 3). Treatments were continued as described previously until the day of OPU. Ovarian pick-up was performed under epidural anesthesia with 2% lidocaine, 0.22 mg/mL, and a vacuum pump. Granulosa cells were sorted under a stereomicroscope. Cells were isolated from the follicular fluid by centrifugation at 800 × *g* for 2 min at room temperature. The follicular fluid was then decanted, measured, and quickly immersed in liquid nitrogen. The pellet was suspended in 500 μL of PBS at 4°C. After centrifugation for 2 min at 800 × *g* at 4°C, the supernatant was removed and the granulosa cells were frozen in liquid nitrogen. Samples were stored at −80°C until use.

Analyses of Homocysteine, Cysteine, Urea, and BHBA in Follicular Fluid

Protocols used for follicular fluid analyses were similar to those used for plasma. Total homocysteine and cysteine concentrations were measured by HPLC (HP Agilent 1200 series; Agilent, Mississauga, ON, Canada) following the method described by Araki and Sako (1987). Follicular fluid urea and BHBA were analyzed using commercial kits: Urea Assay kit, Diagnostic Chemicals Limited (BioPacific Diagnostic Inc.) and BHBA Reagent Set (Pointe Scientific Inc.).

RNA Extraction and Microarray Hybridization

Granulosa cell RNA was extracted from each individual follicle and purified using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems by Life Technologies, Carlsbad, CA) with DNase treatment on the purification column according to the manufacturer's instructions. The quality and concentration of RNA were measured by microfluidic 2011 Bioanalyzer (Agilent). Samples with a RNA integrity number greater than 7 were selected for analyses. Samples were stored at −80°C until use.

The RNA underwent 2 rounds of amplification using the T7 RNA amplification kit RiboAmp HSplus (Molecular Devices, Sunnyvale, CA). The antisense RNA was labeled with fluorochromes (Cy3 and Cy5) using the ULS Fluorescent labeling kit (Kreatech, Amsterdam, Netherlands). The labeled products were then purified using the PicoPure RNA extraction kit (Applied Biosystems) to remove uncoupled dyes. The RNA was quantified and labeling efficiencies were determined using a spectrophotometer (NanoDrop ND-1000, Thermo Scientific, Wilmington, DE). The antisense RNA from 3 pairs of cows was hybridized (825 ng for each hybridization) on an Agilent microarray slide specifically developed for bovine granulosa cells (Custom microarray GE-G2514F-028298; Robert et al., 2011) using dye-swap design (Supplementary Figure S1; http://dx.doi.org/10.3168/jds.2015-9623). In accordance with the Agilent protocols, the arrays were sequentially washed in buffer 1 (containing 0.005% Triton X-102) for 3 min at room temperature, followed by buffer 2 (containing 0.005% Triton X-102) for 3 min at 42°C, and acetonitrile for 10 s at room temperature before addition of the drying and stabilization solution for 30 s at room temperature. Slides were scanned with

PowerSCANNER (Tecan, Durham, NC). Spots were automatically identified using Array-Pro 6.3 (Media-Cybernetics, Bethesda, MD).

Reverse Transcription-Quantitative PCR

Reverse transcription-quantitative PCR (**RT-qPCR**) was performed on 6 RNA samples from control cows and 6 RNA samples from animals receiving the vitamin supplement. Gene selection was based on differential expression, physiological relevance, and relevance to reproduction. The RNA was quantified using a spectrophotometer (NanoDrop ND-1000, Thermo Scientific). Reverse transcription of RNA samples was performed with oligo-dT and the Flex qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD). The final cDNA reverse transcription was diluted with nuclease-free water (Ambion, Life Technologies). Primers (Supplementary Table S2; http://dx.doi.org/10.3168/jds.2015-9623) were designed using the software PrimerQuestSM (Integrated DNA Technologies, Coralville, IA). Reverse transcription-qPCR was carried out on 96-well plates (Roche Applied Science, Penzberg, Germany) using the LightCycler 480 System (Roche Applied Science). The standard curve consisted of 5 purified PCR product standards diluted from 0.10 pg to 0.1fg. Each reaction contained 10 μL of qPCR SYBR Green qPCR Master Mix, 7 μL of sterile water, 0.5 μL of forward and reverse primer (10 μM), and 2 μL of cDNA (or water for the negative control). An annealing temperature of 57°C was used for RT-qPCR. Products were analyzed on a 2% agarose gel to confirm the presence and length of a single band. The normalization factor was calculated from the expression of 3 housekeeping genes: *GAPDH*, conserved helix-loop-helix ubiquitous kinase (*CHUK*), and β-actin 1 (*ACTB1*) using the software GeNORM (Vandesompele et al., 2002; http://medgen.ugent.be/~jvdesomp/genorm/).

Statistical Analyses

Two levels of B-vitamin supplementation (control, or folic acid and vitamin B_{12}) were used in a complete block design in which cows were blocked according to milk production during the previous lactation period and their expected calving date.

Production Variables and Plasma Metabolites. Production variables were analyzed as the averages for the 3 wk before calving and for the first 56 d using the MIXED procedure of SAS (ver. 9.4, SAS Institute Inc., Cary NC) according to a complete block design with blocks and treatments as main effects. Data from plasma analyses were analyzed using the MIXED procedure of SAS according to a complete block design, with blocks and treatments as main effects and repeated measures in time. Data were tested for normality using the Shapiro-Wilk test. Before calving, when the time intervals were equal, the smallest fit statistical values were obtained with the compound symmetry covariance structure. After calving, when time intervals were different, the smallest fit statistical values were obtained with the spatial power covariance structure. Results are reported as least squares means and standard errors of the mean. Means were assumed to be different at $P \leq 0.05$ and tended to differ at $0.05 < P \leq 0.10$.

Analysis of Microarray Slides. Microarray data analysis was performed using FlexArray 1.6.1 (Genome Québec, Montreal, QC, Canada) to assess the transcriptional effect of vitamin treatment in granulosa cells of the dominant follicle. As described (Robert et al., 2011), data were submitted to a background subtraction, a Loess within-array normalization, and a Quantile between-array normalization. Normalized data were assessed using e-Bayes moderated *t*-test (Limma R package) to calculate fold changes of probe intensity. A false discovery rate algorithm (Benjamini/Hochberg) was also applied (adjusted *P*-value threshold of 0.05). Molecular functions and genetic networks were studied by analyzing microarray data with Ingenuity Pathway Analysis (**IPA**) software (Ingenuity Systems, Redwood City, CA). In a second analysis, gene expression in vitamin treatment was compared with gene expression in granulosa cells collected either 6 or 22 h post-LH (Gilbert et al., 2011). In fact, Gilbert and collaborators compared gene expression patterns of granulosa cells at 6 and 22 h after LH surge with that of 2 h before LH surge (control) to identify the genes which were having an early (6 h) or late (22 h) effect of LH. The analysis we provide is a simple comparison of the genes in our list and the genes differentially expressed after LH surge produced by Gilbert et al. (2011) to estimate the LH effect on our results.

RT-qPCR. Statistical analyses for RT-qPCR results were conducted using Prism 5.00 GraphPad for Windows (GraphPad Software, La Jolla, CA). Statistical significance was assessed by the Mann-Whitney test (nonparametric test). Probabilities of $P \leq 0.05$ were considered statistically significant. All values are presented as means with their corresponding SEM.

RESULTS

Weekly treatment with folic acid and vitamin B_{12} had no effect ($P \geq 0.3$) on average daily DMI during the 3 wk before calving and during the postcalving period (Table 1). No treatment effect ($P = 0.57$) on BW losses was noted during the first 8 wk of lactation, at 12 ± 8 and 4 ± 10 kg for control and treated animals,

Table 1. Effects of intramuscular administration of folic acid and vitamin B_{12} on production performance[1]

Item	Treatment		*P*-value
	Control	Vitamin	
n	12	11	
Precalving[2]			
DMI, kg/d	13.0 ± 0.76	13.7 ± 0.78	0.33
Postcalving[3]			
DMI, kg/d	22.5 ± 1.23	23.9 ± 1.28	0.43
Milk, kg/d	39.5 ± 1.41	40.7 ± 1.44	0.29
ECM,[4] kg/d	38.1 ± 1.34	39.7 ± 1.35	0.07
Fat, g/kg	39.2 ± 1.19	40.4 ± 1.24	0.49
Protein, g/kg	31.1 ± 0.50	30.4 ± 0.53	0.40
Lactose, g/kg	45.9 ± 0.33	45.6 ± 0.35	0.61
Folates, ng/mL	108.3 ± 6.68	141.1 ± 6.97	0.007
Vitamin B_{12}, ng/mL	3.58 ± 0.406	7.41 ± 0.424	<0.0001

[1]Least squares means ± SEM. Control animals (control) were injected with a saline solution (0.9% NaCl).

[2]Precalving = 3 wk before the actual date of calving.

[3]Postcalving = first 8 wk of lactation.

[4]This equation was derived from the NRC (NRC, 2001) equation for NE_L, taking the energy value of milk as 0.749 Mcal/kg of milk, as determined by (Tyrrell and Reid, 1965).

respectively. Average daily milk production, in addition to fat, protein, and lactose milk contents, were not different ($P \geq 0.3$) between the 2 treatments (Table 1). However, ECM yield during the first 56 d of lactation tended to be increased ($P = 0.07$) by 1.6 kg/d in cows receiving the vitamin supplement. Concentrations of folates and vitamin B_{12} were greater ($P \leq 0.007$) in milk of cows supplemented with folic acid and vitamin B_{12}.

During the 3 wk before calving, plasma concentrations of folates and vitamin B_{12} were increased ($P \leq 0.04$) by intramuscular administration of folic acid and vitamin B_{12} (Table 2). Plasma concentrations of vitamin B_{12} in control cows remained stable during these 3 wk, whereas a steady increase was observed in treated cows (interaction treatment × time, $P = 0.008$). Plasma concentrations of glucose, NEFA, and BHBA were unaffected by treatments ($P \geq 0.2$) before calving. However, urea plasma concentration was greater ($P = 0.05$) in the control group (Table 2). During the first 8 wk of lactation, plasma concentrations of folates and vitamin B_{12} were greater ($P \leq 0.002$) in cows injected with the vitamin supplement (Table 2). Plasma concentrations of BHBA tended to be lower ($P = 0.07$) in cows receiving the vitamin supplement, but no treatment effect ($P \geq 0.3$) was seen for plasma concentrations of glucose, NEFA, and urea.

The recovered volumes of follicular fluid from the dominant follicle were greater ($P = 0.03$) in treated animals than in the control group, at 2.49 ± 0.22 and 1.62 ± 0.17 mL, respectively. No treatment effect ($P \geq 0.22$) was noted on follicular fluid concentrations of homocysteine, Cys, urea, and BHBA, respectively. Average concentrations of homocysteine, Cys, urea, and BHBA were 9.92 ± 1.21 μ*M*, 165.4 ± 8.4 μ*M*, 5.54 ± 0.39 m*M*, and 0.652 ± 0.039 m*M*, respectively.

A microarray platform with transcripts covering the entire bovine genome has been used to study granulosa cell gene expression of the dominant follicle. Microarray analyses performed on 3 pairs of samples (Supplemental Figure S1; http://dx.doi.org/10.3168/jds.2015-9623) revealed that 322 genes were differentially expressed in granulosa cells. A list of the genes that were the most upregulated or downregulated is presented in Table 3. Globally, results indicated a downregulation of genes

Table 2. Effects of intramuscular administration of folic acid and vitamin B12 on plasma concentrations of nutritional metabolites[1]

Item	Treatment		*P*-value		
	Control (n = 12)	Vitamin (n = 11)	Treatment	Time	Treatment × time
Precalving[2]					
Folates, ng/mL	9.5 ± 0.66	11.8 ± 0.71	0.04	0.18	0.11
Vitamin B_{12}, pg/mL	269.0 ± 14.76	372.2 ± 16.04	0.0008	0.0008	0.0008
Glucose, m*M*	3.41 ± 0.035	3.43 ± 0.038	0.67	0.11	0.18
NEFA, μ*M*	294.0 ± 45.04	258.42 ± 48.93	0.61	<0.0001	0.86
BHBA, m*M*	0.55 ± 0.034	0.47 ± 0.037	0.19	0.04	0.90
Urea, m*M*	5.81 ± 0.222	5.07 ± 0.241	0.05	0.10	0.13
Postcalving[3]					
Folates, ng/mL	11.1 ± 0.52	14.4 ± 0.56	0.002	0.10	0.85
Vitamin B_{12}, pg/mL	222.8 ± 13.16	341.5 ± 14.30	0.0001	<0.0001	0.53
Glucose, m*M*	2.98 ± 0.044	3.00 ± 0.047	0.78	<0.0001	0.86
NEFA,[3] μ*M*	422.3 ± 18.29	426.6 ± 19.97	0.88	<0.0001	0.91
BHBA,[4] m*M*	1.10 ± 0.093	0.81 ± 0.101	0.07	0.13	0.91
Urea, m*M*	5.77 ± 0.210	5.37 ± 0.227	0.25	0.46	0.91

[1]Means ± SEM. Control animals (control) were injected with a saline solution (0.9% NaCl).

[2]Precalving = 3 wk before the actual date of calving. Blood samples were collected weekly, and data presented are an average of the samples.

[3]Postcalving = until 8 wk after calving. Blood samples were collected twice weekly for the first 3 wk and weekly thereafter. Data presented are an average of the samples.

associated with cell cycle and an upregulation of genes associated with follicle differentiation, ovulation, and steroidogenesis in granulosa cells of cows receiving the vitamin supplement. These genes have been described in Table 3.

This is well illustrated by the network generated by the IPA knowledge database, where upregulated genes are represented in red and downregulated genes in green (Figure 1). Cell cycle genes are clearly downregulated (in green) and *FOXO1* (in blue) is predicted to be a key gene in this regulation. Interestingly, genes known to be involved in granulosa cell differentiation and ovulation in response to LH, such as *ACE2*, *CLD11*, *PTX3*, *IGF2*, *SERPINA5*, *CYP19A1*, *APOA1*, *NTS*, and *CITED1*, were upregulated (in red in Figure 1 and highlighted in Table 3). Although folic acid and vitamin B_{12} are involved in the Met cycle and gluconeogenesis, the vitamin supplement had no effect on the mRNA abundance of the molecular machinery associated with these 2 cellular functions in granulosa cells of dominant follicle. Data presented in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number 16774905.

The transcriptome analysis by microarray was further studied by RT-qPCR. Six RNA samples from folic acid and vitamin B_{12}-treated cows, and 6 RNA samples from saline-treated cows were also assessed. We have chosen to validate genes involved in 2 important biological functions for granulosa cells from dominant follicles. The first biological function to be assessed was the cell cycle, whereas the second function was the follicular differentiation including ovulation and steroidogenesis. The genes were chosen from those highlighted in Table 3. Well-known cell cycle genes, such as *CCNB1*, *CCNA2*, and *PLK4*, were selected as downregulated genes. The RT-qPCR results clearly showed lower expression of the *CCNB1* and *CCNA2* genes ($P = 0.06$ and $P = 0.06$, respectively) in granulosa cells of folic acid and vitamin B_{12}-treated cows (Figure 2). The 2-fold difference in mRNA abundance for *PLK4* was not ($P = 0.18$) different between treatments (Figure 2). Furthermore, RT-qPCR results showed higher expression of genes functionally associated with granulosa cell differentiation and ovulation in the treated group, such as *ACE2* ($P = 0.0152$; Figure 2). Although not significant, a 2-fold difference in expression of *ADAMTS1* (a disintegrin and metalloproteinase domain with thrombospondin motifs-1; $P = 0.39$), insulin-like growth factor 2 (*IGF2*; $P = 0.48$), and serpin peptidase inhibitor, clade A, member 5 (*SERPINA5*; $P = 0.09$) was observed between the treated and control groups (Figure 2).

The expression level of other well-known genes that are associated with ovulation and granulosa cell differentiation functions was also investigated. Although the selected genes were not significantly different in the microarray analysis, we investigated them by RT-qPCR. Thus, *PTGS2*, *TIMP2*, and *TNFAIP6* were found significantly different after RT-qPCR analyses ($P = 0.05$, $P = 0.02$ and $P = 0.02$, respectively; Figure 3A). Because the differently expressed genes are normally

Table 3. Effects of intramuscular administration of folic acid and vitamin B12 on gene expression in granulosa cells of dominant follicle. Microarray data analyses were performed by limma (log fold change 2 × and adjusted *P*-value threshold 0.05). Highlighted genes are involved in granulosa cell differentiation, ovulation and steroidogenesis (A), and in cell cycle (B)

Gene symbol	Description	Fold change
ACE2	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	11.92391
CLDN11	Claudin 11	7.959044
ACSM1	Acyl-CoA synthetase medium-chain family member 1	7.634026
PTX3	Pentraxin-related gene, rapidly induced by IL-1 β	7.60266
ISG12(A)	Putative ISG12(a) protein	4.867317
IGF2	Insulin-like growth factor 2 (somatomedin A)	4.526353
MGST2	Microsomal glutathione S-transferase 2	4.509296
SERPINA5	Serpin peptidase inhibitor, clade A, member 5	4.302946
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	4.289193
RGN	Regucalcin (senescence marker protein-30)	4.263485
APOA1	Apolipoprotein A-I	4.243196
NTS	Neurotensin	4.197917
MGC133636	Cystatin SC	4.090635
CITED1	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 1	4.01369
ASB9	Ankyrin repeat and SOCS box-containing 9	3.839227
ITPKA	Inositol 1,4,5-trisphosphate 3-kinase A	3.704174
LOC618369	Similar to Lactosylceramide 4-α-galactosyltransferase	3.465964
APOA1	Apolipoprotein A-I	3.448941
TPM2	Tropomyosin 2 (β)	3.213195
HSD3B1	Hydroxy-delta-5-steroid dehydrogenase, 3 β- and steroid delta-isomerase 1	3.187187

Continued

Table 3 (Continued). Effects of intramuscular administration of folic acid and vitamin B12 on gene expression in granulosa cells of dominant follicle. Microarray data analyses were performed by limma (log fold change 2 × and adjusted *P*-value threshold 0.05). Highlighted genes are involved in granulosa cell differentiation, ovulation and steroidogenesis (A), and in cell cycle (B)

Gene symbol	Description	Fold change
LEPREL1	Leprecan-like 1	3.106477
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	3.103006
PPM1K	Protein phosphatase 1K (PP2C domain containing)	3.049289
VNN2	Vanin 2	3.023404
ST3GAL4	ST3 β-galactoside α-2,3-sialyltransferase 4	2.996836
TF	Transferrin	2.996612
PPM1K	Protein phosphatase 1K (PP2C domain containing)	2.938192
NULL	Novel Transcribed Region; evidence: embryonic ESTs	2.907977
TRIB2	Tribbles homolog 2 (Drosophila)	2.857066
FAM129A	Family with sequence similarity 129, member A	2.815709
NULL	Novel Transcribed Region; evidence: embryonic ESTs	2.811898
RHOBTB1	Rho-related BTB domain containing 1	2.769533
PPM1K	Protein phosphatase 1K (PP2C domain containing)	2.749173
B3GALT2	UDP-Gal:betaGlcNAc β 1,3-galactosyltransferase, polypeptide 2	2.715947
ITGB5	Integrin, β 5	2.681029
LTF	Lactotransferrin	2.674316
POSTN	Periostin, osteoblast specific factor	2.656525
LOC784270	Similar to hCG2038584	2.625171
LHCGR	Luteinizing hormone/choriogonadotropin receptor	2.622056
STC1	Stanniocalcin 1	2.601672
PDGFA	Platelet-derived growth factor α polypeptide	2.600066
COL4A1	Collagen, type IV, α 1	2.591353
LOC512293	Similar to Cyclin B1	−5.186809
SDS	Serine dehydratase	−4.942093
CCNB1	Cyclin B1	−4.92484
ESCO2	Establishment of cohesion 1 homolog 2	−4.669314
RRM2	Ribonucleotide reductase M2 polypeptide	−4.18686
BUB1	Budding uninhibited by benzimidazoles 1 homolog	−4.063824
MIS18A	Protein Mis18-α	−3.969269
ASPM	Asp (abnormal spindle) homolog, microcephaly associated	−3.878871
NULL	Novel Transcribed Region; evidence: embryonic ESTs	−3.878197
PAF	KIAA0101 protein	−3.789105
SRGN	Serglycin	−3.708358
PTTG1	Pituitary tumor-transforming 1	−3.392599
NUF2	NUF2, NDC80 kinetochore complex component, homolog	−3.38071
LOC616942	MHC class I heavy chain	−3.355623
NULL	Novel Transcribed Region; evidence: embryonic ESTs	−3.352044
TCF19	Transcription factor 19	−3.351631
NULL	Novel Transcribed Region; evidence: embryonic ESTs	−3.318365
LOC507069	Similar to Brca2	−3.285852
LOC100299171	Similar to DNA replication complex GINS protein PSF2	−3.273245
NULL	Novel Transcribed Region; evidence: embryonic ESTs	−3.204962
NUSAP1	Nucleolar and spindle associated protein 1	−3.19317
GMNN	Geminin, DNA replication inhibitor	−3.150042
CENP-A	CENP-A protein	−3.146462
PAF	KIAA0101 protein	−3.127112
PRC1	Protein regulator of cytokinesis 1	−3.104341
PBK	PDZ binding kinase	−3.100178
CENPN	Centromere protein N	−3.014372
FAM83D	Protein FAM83D	−3.012712
KIF11	Kinesin family member 11	−3.011246
PCNA	Proliferating cell nuclear antigen	−2.993303
CCNA2	Cyclin A2	−2.991263
KPNA2	Karyopherin α 2 (RAG cohort 1, importin α 1)	−2.962751
LOC615206	Similar to C6orf173 protein	−2.959559
MAD2L1	MAD2 mitotic arrest deficient-like 1	−2.958476
SGOL1	Shugoshin-like 1 (S. pombe)	−2.910892
CENPH	Centromere protein H	−2.899327
MLF1IP	MLF1 interacting protein	−2.899324
CHI3L1	Chitinase 3-like 1 (cartilage glycoprotein-39)	−2.884739
CCNB2	Cyclin B2	−2.870199
CENPF	Centromere protein F, 350/400ka (mitosin)	−2.859041
RMI2	Chromosome 16 open reading frame 75 ortholog	−2.844759
BIRC5	Baculoviral IAP repeat-containing 5	−2.831619
PLK4	Polo-like kinase 4	−2.829992

found in granulosa cells from post-LH surge, the results suggest that the animals from the vitamin group were exposed to an increase of LH secretion.

To validate this hypothesis, results from the present study (vitamin-treated were compared with the granulosa cell data sets obtained 6 and 22 h after an LH surge (Gilbert et al., 2011); the GEO Series accession number is GSE69247. The comparison is presented in a Venn diagram (Figure 3B); a total of 74 genes were common to all conditions (vitamin-treated, 6 and 22 h post-LH) and most of them (90%) are regulated in the same way either upregulated (24 genes) or downregulated (42 genes). A total of 106 genes were found common between 22 h post-LH and vitamin-treated (Figure 3B) and most of these genes (90%) are regulated in the same way either upregulated (19 genes) or downregulated (76 genes). Using the designed network plotted with IPA (Figure 1), we overlaid genes common between vitamin-treated and those observed 22 h post-LH (Gilbert et al., 2011) and clearly observed under both conditions a downregulation of cell cycle genes (*CCNB1*, *CCNB2*, *CCNA2* and *CDK1*; Supplementary Figure S2; http://dx.doi.org/10.3168/jds.2015-9623). In addition, genes of granulosa cell differentiation and ovulation, such as *PTX3*, *CITED1*, and *ADAMTS1*, that are upregulated after 22 h post-LH were also upregulated by the vitamin treatment (Supplementary Figure S2), which supports the fact that the granulosa cells obtained from cows receiving the vitamin treatment were probably exposed to an increase in LH secretion.

Remarkably, in Figure 3B, 122 genes were classified as specific to the vitamin treatment. To analyze this group we overlaid these 122 genes data on the same network to observe that FSH, LH, and epidermal growth factor downstream genes (mostly differentiation genes) were upregulated (red) or predicted upregulated (orange) with the vitamin treatment (Supplementary Figure S3; http://dx.doi.org/10.3168/jds.2015-9623). These last data are in accordance with the hypothesis that, in the vitamin group, genes involved in the granulosa cell differentiation and ovulation were upregulated probably because these cells were exposed to an increased LH secretion

DISCUSSION

The duration and the intensity of the negative energy balance in early lactation are closely related to the interval between calving and the first ovulation resulting from a lower LH pulse frequency (Butler, 2003). Serum concentrations of BHBA in early lactation are also negatively related to the probability of pregnancy at the first insemination (Walsh et al., 2007). In a study conducted on 805 cows in 15 commercial farms, a combined supplement of these vitamins given from 3 wk

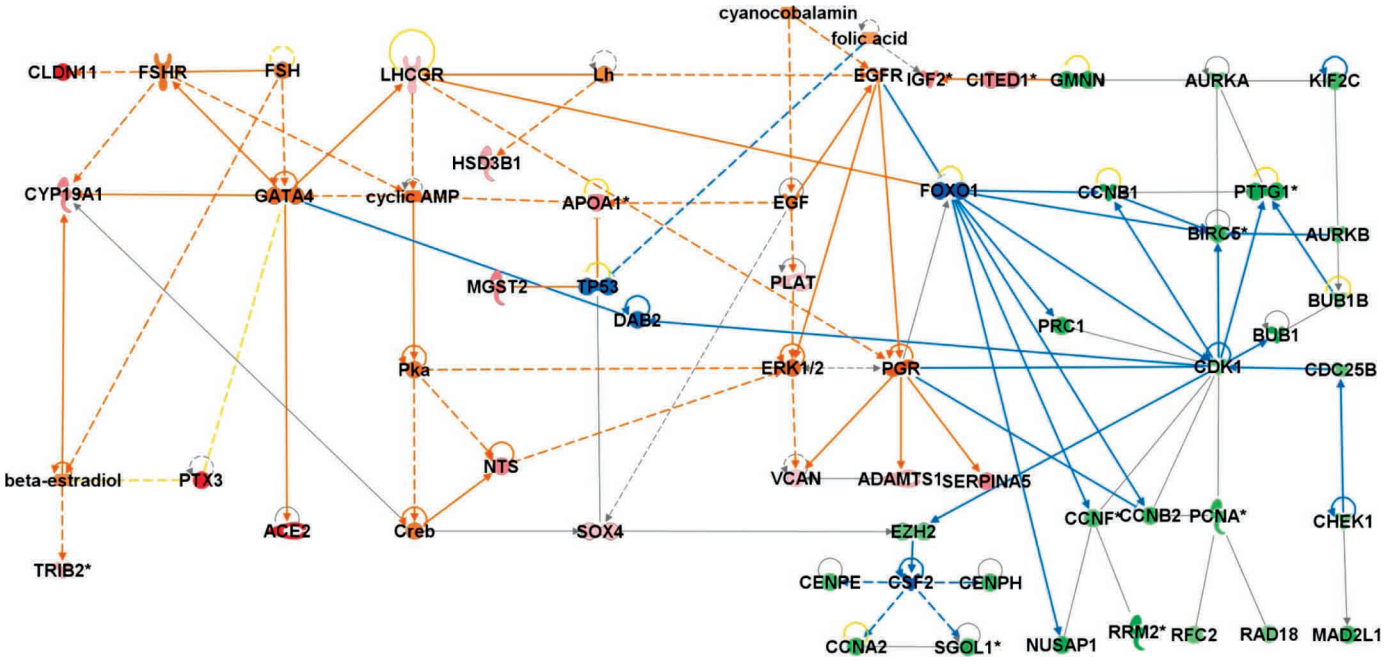

Figure 1. Ingenuity Pathway Analysis of genes associated with follicle differentiation, ovulation, and steroidogenesis, which are differentially expressed between vitamins versus control groups. Red symbols show significantly upregulated genes, orange symbols are predicted upregulated genes, green symbols the significantly downregulated genes, and the blue symbols are the predicted downregulated genes in granulosa cells of treated animals compared with granulosa cells from control animals.

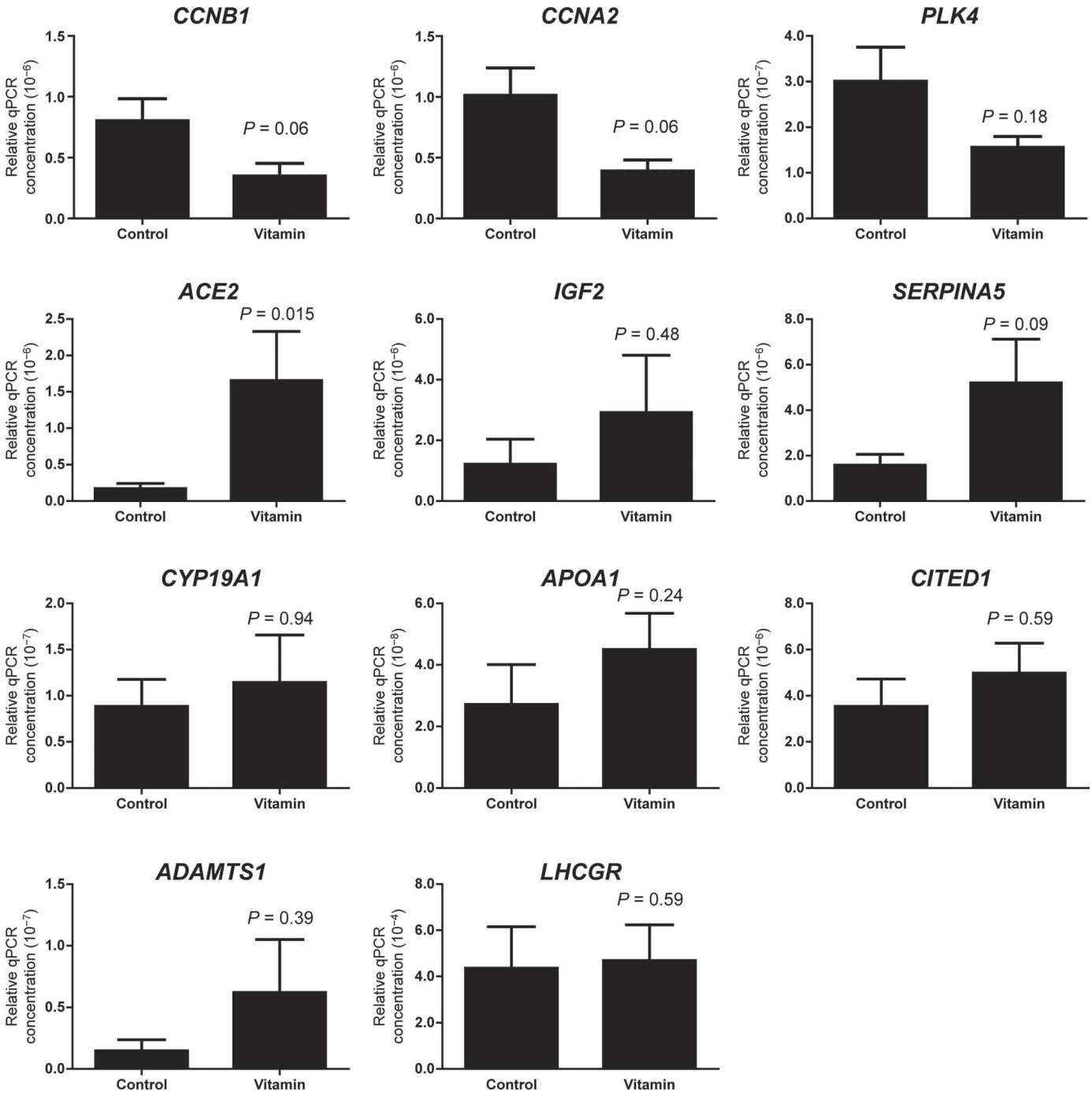

Figure 2. Effects on bovine granulosa cell gene expression measured by reverse transcription-quantitative PCR (qPCR) in response to weekly intramuscular administration of folic acid and vitamin B_{12}. The upregulated genes observed in the microarray data analysis and downregulated genes are presented as means ± SEM (n = 6). Means were compared with a Mann-Whitney test. The normalization factor was calculated from the expression of 3 housekeeping genes (*GAPDH*, *ACTB1*, and *CHUK*).

before calving until 8 wk of lactation had an effect on energy partitioning in early lactation and reduced the interval between calving and the day of the first insemination by almost 4 d in multiparous cows (Duplessis et al., 2014). In the present study, intramuscular injections of vitamin B_{12} and folic acid tended to increase ECM and decrease plasma concentrations of BHBA without an effect on DMI or BW losses, suggesting that the vitamin supplementation altered efficiency of energy metabolism. Negative energy balance during lactation

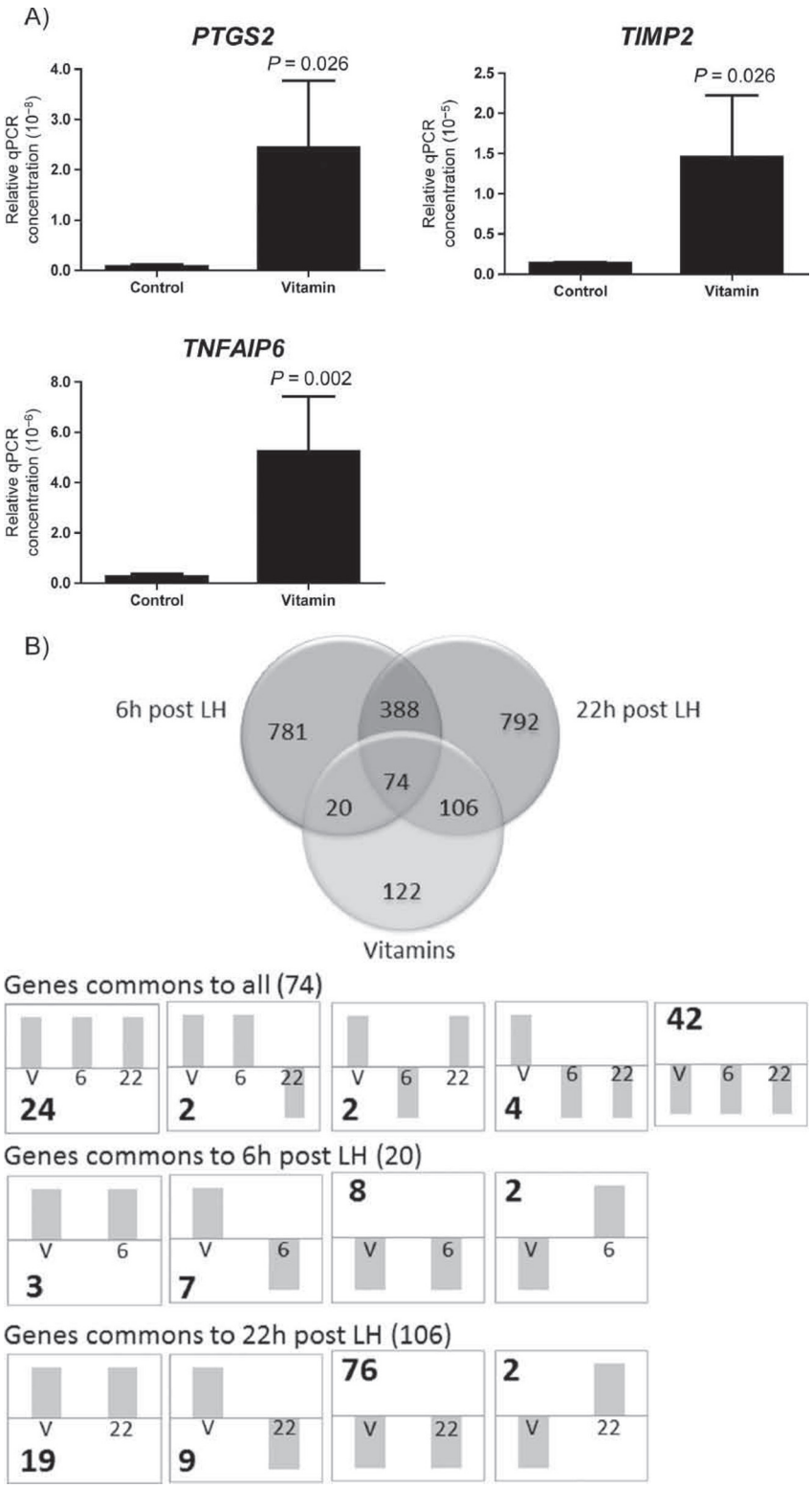

Figure 3. (A) Reverse transcription-quantitative PCR (qPCR) analysis of genes (*PTGS2*, *TIMP2*, and *TNFAIP6*) involved in granulosa cell differentiation and ovulation. Data are presented as means ± SEM (n = 6). Means were compared with a Mann-Whitney test. The normalization factor was calculated from the expression of 3 housekeeping genes (*GAPDH*, *ACTB1*, and *CHUK*). (B) Comparative analysis of vitamin (V)-regulated genes in granulosa cells with 6- (6) and 22-h (22) post-LH surge genes from the study of Gilbert et al. (2011). The Venn diagram shows the number of genes commonly and differently expressed between each group. Genes common to vitamins were further analyzed as common to all treatments (74 genes), common to 6-h post-LH (20 genes), and common to 22-h post-LH (106 genes).

reduced the size of the dominant follicle (Butler, 2000). The collected volume of follicular fluid is correlated with the size of the dominant follicle (Nataprawira et al., 1992), and in the present study the recovered volumes of follicular fluid were greater in the animals treated with vitamins than in the control group. Indeed, the growth of the dominant follicle is influenced by glucose, the IGF system, LH pulsatility, estradiol-17β, and growth factors (Diskin et al., 2003). Therefore, in the present study, it is possible that changes in energy efficiency in cows treated with folic acid and vitamin B_{12} could promote a more rapid growth of the dominant follicle, although a direct effect on the follicle or the hypothalamic-pituitary-ovarian axis cannot be ruled out.

Changes in plasma metabolite concentrations may alter the biochemical composition of the dominant follicle follicular fluid. Indeed, significant correlations have been observed between the composition of blood serum and follicular fluid obtained from follicles >10 mm in diameter ($r = 0.85$ for BHBA and $r = 0.95$ for urea; Leroy et al., 2004); however, in the present study, no significant differences were observed for follicular fluid urea and BHBA levels in treated and control animals (data not shown) in spite of a trend for a treatment effect (decrease) on plasma BHBA concentrations postpartum and plasma urea concentrations prepartum. Folic acid and vitamin B_{12} are involved in Met metabolism and enable the regeneration of Met from homocysteine, which can also be catabolized into Cys. As methionine adenosyltransferase 1A is not expressed and expression of the cystathionine β-synthase, the rate-limiting enzyme for the catabolism of homocysteine into Cys, is very low in bovine granulosa cells (Kwong et al., 2010), concentrations of homocysteine and Cys in the follicular fluid are dependent of their concentrations in blood. Intramuscular administration of folic acid either alone or in combination with vitamin B_{12} tended to decrease plasma concentrations of homocysteine in dairy cows (Preynat et al., 2009b), but in the present study, homocysteine and Cys concentrations in follicular fluid on the day of OPU did not differ between treated and control animals (data not shown).

Microarray analyses performed on granulosa cells from dominant follicle revealed a lower expression of genes associated with cell cycle and a higher expression of genes associated with granulosa cell differentiation, ovulation, and steroidogenesis in treated animals. Elevated expression of genes associated with FSH (*LHCGR*, *CYP19A1*, *HSD3B1*, *TRIB2*, *GATA4*) steroidogenesis (*HSD3B1*, *CYP19A1*), progesterone receptor (*SERPINA5*, *VCAN*, *ADAMTS1*, *CYP19A1*, *APOA1*), GATA binding protein 4 (GATA4) pathway (*LHCGR*, *CYP19A1*, *ACE2*, *PTX3*, *VNN2*), and tissue remodeling (*SERPINA5*, *ADAMTS1*, *PTX3*, *VCAN*) in granulosa cells (Figure 1) suggest that these cells from the dominant follicle would be differentiating toward ovulation (Christenson et al., 2013; Wissing et al., 2014).

The result of *CYP19A1* gene expression, involved in the synthesis of estradiol, may be inconsistent with the suggestion that cells from the dominant follicle would be differentiating toward ovulation. It is well established that the expression of *CYP19A1* is highly regulated in granulosa cells during folliculogenesis (Stocco, 2008); its expression level increases in dominant follicle (Luo et al., 2011) and LH induces a rapid decrease of *CYP19A1* mRNA level (Hickey et al., 1990; Ndiaye et al., 2005; Nimz et al., 2010). The region of its proximal promoter contains several elements of regulation, such as cyclic adenosine monophosphate-responsive element (Hickey et al., 1990), a binding site for nuclear receptor 5A transcription factors (Fitzpatrick and Richards, 1993), GATA4 (Stocco, 2004), and adaptor protein-3 (Stocco et al., 2007). These *cis*-elements in the promoter region argue for a high number of factors involved in the transcription of *CYP19A1* underlining the high level of complexity of this regulation. In the present study, microarray data showed an upregulation of 4.2 fold of *CYP19A1* with the vitamin treatment (Table 3). However, the RT-qPCR result was not significantly different between treatments (Figure 2), which suggests the limitations of the approaches used. We suppose that a larger sample size from multiple animals could denote the underlying complexity of *CYP19A1* gene expression regulation.

The ovulation process involves a cascade of highly orchestrated changes in gene expression in response to LH (Espey and Richards, 2002). However, granulosa cells that are not yet under the influence of an increase of LH secretion are still involved in cell division and proliferation of the follicular cells (Gilbert et al., 2011; Christenson et al., 2013; Wissing et al., 2014). Reduced expression of genes associated with the cell cycle, such as those validated by RT-qPCR for *CCNB1* and *CCNA2* (Wissing et al., 2014; Figure 2), suggests that the granulosa cells in folic acid and vitamin B_{12}-treated cows were in the process of responding to an increase of LH (Gilbert et al., 2011; Christenson et al., 2013; Wissing et al., 2014). However, the cells seem only to begin their response to LH, as some genes that should be downregulated by LH were not yet decreased, such as *TRIB2* (Ndiaye et al., 2005), *VNN2* (Sayasith et al., 2013c), and *CYP19A1* (Hickey et al., 1990; Table 3). Some genes that should be upregulated by LH were not validated by RT-qPCR, such as *IGF2* (Nivet et al., 2013), *SERPINA5* (Hayashi et al., 2011), *APOA1* (Sriraman et al., 2010), *CITED1* (Sriraman et al., 2010),

and *ADAMTS1* (Christenson et al., 2013; Sayasith et al., 2013b; Wissing et al., 2014; Figure 2). However, genes specific to granulosa cell differentiation were validated by RT-qPCR, such as *PTGS2* (Sayasith et al., 2013a; Wissing et al., 2014), *TIMP2* (Wissing et al., 2014), and *TNFAIP6* (Sayasith et al., 2013a; Figure 3A).

The time frame of blood collection used in the present study did not allow us to further validate this hypothesis by measuring LH concentration in blood, but the level of *LHCGR* was not different between the 2 groups (Figure 2). Moreover, comparative analysis of gene profiling further supports that the treated animals may have been subjected to an increase of LH secretion. Indeed, when comparing the results with 6-h post-LH gene expression profile, most of the cell cycle genes were predicted downregulated (data not shown). The same comparison with 22-h post-LH gene expression profile showed downregulation of cell cycle genes, but most of the FSH, LH, and EGF downstream genes were predicted to be downregulated (Supplementary Figure S2; http://dx.doi.org/10.3168/jds.2015-9623). Remarkably, as seen in Figure 3B, 122 genes were classified as specific to vitamin treatment. In this last group, genes involved in cell cycle were downregulated. But, interestingly, genes known to be critical for granulosa cell differentiation were upregulated (Supplemental Figure S3; http://dx.doi.org/10.3168/jds.2015-9623), such as *ACE2A* (Tonellotto dos Santos et al., 2012), *IGF2* (Nivet et al., 2013; Wissing et al., 2014), *SEPRINA5* (Hayashi et al., 2011; Christenson et al., 2013), *APOA1* (Sriraman et al., 2010), and *VCAN* (Russell et al., 2003; Richards, 2005), and most if not all of FSH, LH, and EGF downstream genes of the plotted network were predicted to be upregulated. This IPA analysis further supports our hypothesis that the effect of vitamin supplementation seems to extend the differentiation process of granulosa cells toward an effect of LH.

In conclusion, weekly intramuscular administration of folic acid and vitamin B_{12} from 3 wk before until 8 wk after calving increased plasma and milk concentrations of folates and vitamin B_{12} and altered energy partitioning. The volume of recovered follicular fluid was greater in treated animals than in the control group. Microarray data analysis, IPA gene network, and results from RT-qPCR all suggest that the effects of the vitamin B_{12} and folic acid supplementation may be the result of an increase in LH secretion promoting cellular differentiation and an arrest of cell division in granulosa cells. As recently reported in commercial dairy herds, the first breeding postpartum for multiparous cows occurs 3.8 d earlier in cows receiving a vitamin supplement according to a protocol similar to the one used in the present study (Duplessis et al., 2014). Although the effects of intramuscular injections of folic acid and vitamin B_{12} on lactational performance and metabolic status of animals were limited, IPA of gene expression in granulosa cells suggests a stimulation of cell differentiation in vitamin-treated cows, which may be the result of an increase in LH secretion. Further investigations are required to better characterize the physiological effect of this vitamin supplementation on female reproduction and granulosa cell function.

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