Short communication: Locus-specific interrelations between gene expression and DNA methylation patterns in bovine mammary gland infected by coagulase-positive and coagulase-negative staphylococci

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ABSTRACT

Pathogens are able to alter the cell cycle program and immune response of the host by changing the transcription and epigenetics of genes responsible for cell cycle control and inflammation. In this regard, we evaluated interrelations between DNA methylation and expression of autophagy, apoptosis, and lipid metabolism-related genes in a sample set of mammary gland secretory tissue sections derived from bovine mammary glands infected with coagulase-negative and coagulase-positive staphylococci. We assessed relative transcript abundance and DNA bisulfite sequencing in loci of the *ATG5*, *IGF1R*, *TERT*, and *DGAT1* genes. Lack of DNA methylation in *ATG5* and *DGAT1* loci might be associated with maintenance of *ATG5* and *DGAT1* expression regardless of the health status of bovine mammary gland. Complete methylation of intragenic CpG regions in the *IGF1R* locus was apparently not related to the presence of its transcript in the investigated udder parenchyma samples. Detected hypermethylation of the *TERT* upstream element was associated with a small amount of *TERT* mRNA in bovine mammary gland, regardless of the presence, or absence, of the pathogen. A significant decrease in *TERT* gene expression in tissue sections of mammary gland free of bacteria and in those infected with coagulase-positive staphylococci was observed in parenchyma samples infected with coagulase-negative staphylococci. Two possible explanations are the direct involvement of the *TERT* gene in the etiology of bovine mastitis or the increase of *TERT* mRNA due to activation of the MAPK signaling pathway in response to release of exotoxins by coagulase-negative bacteria in the bovine mammary gland.

Key words: mastitis, DNA methylation, differential gene expression

Short Communication

Pathogens represent highly active environmental agents able to alter the host transcriptional program (Tran Van Nhieu and Arbibe, 2009). *Staphylococcus aureus* is one of the most frequently isolated pathogens in bovine mastitis and may cause a severe or chronic subclinical form of the disease. This coagulase-positive bacterium may lead to pathogenic induction of gene expression in infected cells of the host (Heimer et al., 2010). Moreover, pathogens may alter the epigenetic machinery of gene regulation (Tran Van Nhieu and Arbibe, 2009). Methylation is a chemical modification of DNA that can block gene activity. Methylation of regulatory sequences enriched in CpG sites is associated with compacted chromatin, an environment that impedes binding of transcriptional factors. In contrast, a lack of methylation of regulatory elements facilitates gene transcription (Deaton and Bird, 2011). Once inherited, DNA methylation patterns undergo changes throughout life under environmental influences and aging-relevant processes. As a result, alterations in methylation of regulatory sequences can indicate interactions between the environment and gene expression patterns.

With respect to mastitis-induced chronic inflammation of the mammary gland, in the current research we evaluated the importance of methylation and expression profiles of genes important for autophagy (*ATG5*; Ye et al., 2018), cell growth and apoptosis (*IGF1R*; Werner and Le Roith, 2000), and telomere functioning (*TERT*; Blackburn, 2005). In the current study, we also assessed the relevance of methylation or expression patterns of the *DGAT1* gene in studied material, polymorphisms in which are crucial for milk fat composition (Grisart

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et al., 2004). In general, DGAT enzymes modulate the inflammatory effects of fatty acids in white adipose and muscle tissues (Yen et al., 2008; Koliwad et al., 2010). However, we wanted to determine whether links exist between *DGAT1* activity and pathogen-induced inflammation related to mammary gland infection.

In this study, we evaluated RNA and DNA samples of parenchyma tissue of bovine mammary gland infected with coagulase-positive and coagulase-negative staphylococci and uninfected tissues. Inspected samples were previously tested against bacterial infection on the basis of microbiological examination. Detailed information on animals, their maintaining conditions, tissue sampling, and milk microbiological examination is presented in Kościuczuk et al. (2014, 2017). The experimental groups in the present report included 31 tissue sections obtained from 18 donor cows with confirmed infection with coagulase-positive staphylococci (**CPS** group) and 14 tissue sections infected with CNS obtained from 11 cows (CNS group; Supplemental Table S1; <https://doi.org/10.3168/jds.2020-18404>). The control group included 11 tissue sections free of bacteria obtained from 6 healthy cows (**H** group; Supplemental Table S1). Tissue sections came from different quarters of the udder, depending on the presence or lack of particular type of bacteria (as designated in the first column of Supplemental Table S1).

Total RNA of the parenchymal tissue samples was isolated using PureLink RNA Mini Kit (Applied Biosystems/ThermoFisher Scientific, Waltham, MA) including an RNA purification stage using PureLink DNase Set (Applied Biosystems/ThermoFisher Scientific). The quantity and quality of RNA were examined using a Nanodrop spectrophotometer (Thermo Scientific, Warsaw, Poland) and 2% gel electrophoresis. Reverse transcription was performed on 500 ng of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems/ThermoFisher Scientific) according to the manufacturer's protocol. Reverse transcription (**RT**)-PCR primers were designed with the option to span at least one intron or to cover exon junctions

using Primer-BLAST software (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>; Supplemental Table S2; <https://doi.org/10.3168/jds.2020-18404>). Qualitative RT-PCR was performed for the pool of 11 equimolar cDNA samples including 4 CPS (750PP, 782LT, 842PP, 855PP), 4 CNS (662LP, 750LT, 829PP, 857LT), and free H samples (719P, 749LT, 861LP; Supplemental Table S1), with the use of Hotstart Taq DNA polymerase (Qiagen, Hilden, Germany) and 35 cycles of amplification. Obtained PCR amplicons were visualized after electrophoresis in 3% agarose gel stained with ethidium bromide. Quantitative RT-PCR reactions were run on QuantStudio 7 Flex System (Applied Biosystems/ThermoFisher Scientific) in 45 cycles, performed in triplicate for each cDNA sample. Reactions were in a total volume of 10 μ L using RT-PCR primers and Sensitive RT HS-PCR Mix EvaGreen (A&A Biotechnology, Gdynia, Poland). Quantification of mRNA levels was done using the comparative $\Delta\Delta^{CT}$ method according to Pfaffl (2001), including relative mRNA abundance of *ACTB* and *RPS15* as endogenous controls (Bionaz and Looor, 2007). Normality of the distribution was tested using the Shapiro-Wilk test, and differences (in relative quantification values) between the CPS, CNS, and H groups of samples were calculated using the Mann Whitney U-test (results included in Table 1). Differences in expression were shown in boxplots.

Prepared DNA (Sherlock A&A Biotechnology, Gdynia, Poland) was bisulfite converted (EpiTect Bisulfite Kit, Qiagen) and PCR amplified using bisulfite primers designed for converted DNA sequences in CpG islands (**CGI**) located upstream of the *ATG5*, *DGAT1*, and *TERT* genes, and inside the *IGF1R* gene sequence (Supplemental Table S2; <https://doi.org/10.3168/jds.2020-18404>). The CpG islands were defined using EMBOSS CpG Plot software (https://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/). Bisulfite (**BS**)PCR amplification was done with the use of Hotstart Taq DNA polymerase (Qiagen). The 2-stage protocol for BSPCR amplification (Supplemental Table S3; <https://doi.org/10.3168/jds.2020-18404>).

Table 1. *P*-values of fold change of expression differences between coagulase-positive staphylococci (CPS), CNS, combined CPS and CNS, and healthy (H) sample group¹

Group x versus group y	Fold change of expression differences				PM value differences
	<i>ATG5</i>	<i>DGAT1</i>	<i>IGF1R</i>	<i>TERT</i>	<i>TERT</i>
CPS vs. CNS	0.22	0.66	0.91	0.04*	1.00
CPS vs. H	0.06†	0.65	0.16	0.24	0.16
CNS vs. H	0.73	0.66	0.41	0.02*	0.17
CPS + CNS vs. H	0.12	0.91	0.09†	0.09†	0.11

¹The last column shows *P*-values of methylation differences in the *TERT* locus (Mqant method). PM = methylation percent.

†*P* < 0.10 and **P* < 0.05.

/doi.org/10.3168/jds.2020-18404), included 5 cycles at 64°C and 35 cycles at 62°C using *ATG5* primers, and 5 cycles at 60°C and 35 cycles at 58°C using *DGAT1*, *IGF1R*, and *TERT* primers. The BSPCR products were sequenced using bisulfite primers. Sanger sequencing products were separated by capillary electrophoresis on an ABI3500 xl Genetic Analyzer (ThermoFisher Scientific). Partially methylated CpG sites of inspected DNA fragments (*TERT* locus) were quantified. The first approach included calculation of percent of methylation (PM) values using the Mquant method (Leakey et al., 2008) based on Sanger sequencing reads of BSPCR. Normality of the distribution was tested using the Shapiro-Wilk test and the differences between PM values of the CPS, CNS, and H groups were calculated on the basis of the Mann-Whitney U test (results included in Table 1).

The second approach relied on bisulfite sequencing using a cloning procedure of amplified DNA after equimolar pooling of samples representing each investigated group. Thirty nanograms of bisulfite-converted

DNA from each of 18 CPS, 12 CNS, and 10 H samples were pooled (Supplemental Table S1; <https://doi.org/10.3168/jds.2020-18404>) and subjected to BSPCR amplification as described above in a 30-μL reaction volume. Amplicons were purified using Minelute PCR purification kit (Qiagen) and cloned using TOPO TA Cloning kit for Sequencing under manufacturer recommendations (ThermoFisher Scientific). Picked bacterial colonies (45 CPS, 27 CNS, and 18 H BSPCR colonies) were amplified with T3 and T7 universal primers, purified with ExoSAP IT (ThermoFisher Scientific), and sequenced using the Sanger method. Quantification of CpG methylation and generation of methylation patterns based on clone BSPCR sequencing reads within each of investigated group were done using BISMA software (Rohde et al., 2010). Results are included in Table 2.

The relative quantification results did not reveal any significant differences in expression of *DGAT1*, *ATG5*, or *IGF1R* between the defined groups of udder secretory tissue samples (Figure 1). The Sanger bisulfite

Table 2. DNA methylation percent (PM) in *TERT* locus (gene ID: 518884) using the coagulase-positive staphylococci (CPS), CNS, and healthy (H) DNA bisulfite pooling approach for cloned bisulfite PCR sequencing¹

CpG site	Distance to TSS	PM		
		CPS	CNS	H
CpG 1	−785 bp to TSS	48.80	63.00	31.20
CpG 2	−769 bp to TSS	77.30	85.20	68.80
CpG 3	−710 bp to TSS	77.30	85.20	75.00
CpG 4	−692 bp to TSS	86.40	92.60	50.00
CpG 5	−682 bp to TSS	95.50	70.40	75.00
CpG 6	−641 bp to TSS	88.60	96.30	87.50
CpG 7	−621 bp to TSS	63.60	29.60	93.80
CpG 8	−617 bp to TSS	75.00	85.20	100.00
CpG 9	−607 bp to TSS	100.00	100.00	87.50
CpG 10	−603 bp to TSS	97.70	88.90	87.50
CpG 11	−596 bp to TSS	93.20	88.90	68.80
CpG 12	−586 bp to TSS	86.00	92.60	81.20
CpG 13	−582 bp to TSS	95.50	81.50	100.00
CpG 14	−575 bp to TSS	81.80	88.90	93.80
CpG 15	−561 bp to TSS	86.40	66.70	75.00
CpG 16	−551 bp to TSS	75.00	92.60	87.50
CpG 17	−547 bp to TSS	93.20	100.00	100.00
CpG 18	−544 bp to TSS	93.20	88.90	100.00
CpG 19	−540 bp to TSS	93.20	96.30	87.50
CpG 20	−536 bp to TSS	70.50	77.80	62.50
CpG 21	−534 bp to TSS	59.10	63.00	31.20
CpG 22	−531 bp to TSS	88.60	74.10	68.80
CpG 23	−505 bp to TSS	88.60	100.00	62.50
CpG 24	−496 bp to TSS	47.60	59.30	31.20
CpG 25	−482 bp to TSS	75.00	77.80	75.00
CpG 26	−459 bp to TSS	56.80	51.90	62.50
CpG 27	−447 bp to TSS	70.50	63.00	43.80
CpG 28	−427 bp to TSS	90.90	85.20	100.00
CpG 29	−409 bp to TSS	59.10	81.50	75.00
CpG 30	−368 bp to TSS	44.20	29.60	50.00
CpG 31	−361 bp to TSS	50.00	51.90	37.50
CpG 32	−350 bp to TSS	95.50	96.30	100.00
Average PM for CGI		78.25	78.26	73.44

¹TSS = transcriptional start site; CGI = CpG islands.

Figure 1. Box-and-whisker plots of differences in the expression of *ATG5*, *DGAT1*, *IGF1R*, and *TERT* genes among parenchymal tissue samples infected with coagulase-positive staphylococci (CPS) and CNS, as well as a healthy (H) sample group. The box represents 25 to 75% of the data, whereas the black line represents the median. Whiskers represent from 9 to 91% of the data and circles represent outliers. RQ = relative quantity. Asterisk denotes a significant difference ($P < 0.05$) between CPS, CNS, and H groups.

sequencing of these genes showed a simple pattern of either hypo- (*DGAT1* and *ATG5*) or hypermethylation (*IGF1R*) of CGI in all parenchyma samples (examples shown in Supplemental Figures S1 to S3; <https://doi.org/10.3168/jds.2020-18404>).

The presence of clear RT-PCR product of *DGAT1* of pooled cDNA samples of secretory tissue (Figure 2) was concordant with the lack of methylation of the *DGAT1* upstream DNA sequence (Figure S1). Unaltered hypomethylation in *DGAT1* might be associated with constant transcription of this gene, which has an embryonic background. The *DGAT1* gene is crucial for mammary gland development, as found in model organisms, and its transcriptional activity is further maintained in the ontogeny, similarly to somatic tissues (Cases et al., 2004).

The hypomethylated state of the *ATG5* upstream element (Supplemental Figure S2) coincided with the lowest amount of *ATG5* transcript (Figure 2) compared with the other genes, probably because the investigated tissue sections came from lactating cows (Kościuczek et al., 2014, 2017). Zielniok et al. (2017) reported higher expression of *ATG5* in the dry period due to the autophagy required for regeneration of mammary glandular tissue.

Complete methylation of the investigated element of bovine *IGF1R* (Supplemental Figure S3) located in the intronic sequence did not correspond to the presence of clear PCR product of *IGF1R* of pooled cDNA samples of mammary gland (Figure 2). In this case, the inspected DNA sequence might not be functionally relevant for the regulation of *IGF1R* expression, being

Figure 2. Qualitative reverse transcription-PCR results of pooled cDNAs of mammary gland secretory tissue samples. Lane 1 shows a 100-bp DNA ladder; lanes 2 to 6 show amplification products with the use of primers specific to *ATG5*, *DGAT1*, *IGF1R*, *TERT*, and *ACTB* mRNA (used as endogenous control for relative quantification).

fully methylated like most other DNA regions in mammalian genomes (Deaton and Bird, 2011).

The hypermethylation of CGI (>70% of methylation) of the upstream element of *TERT* gene in single and pooled DNA samples of parenchyma tissue (Figure 3 and Supplemental Figure S4; <https://doi.org/10.3168/jds.2020-18404>) might be linked to low transcript abundance of *TERT*. This could be observed in the form of the weaker amplification product of pooled cD-

NAs compared with results of qualitative RT-PCR for *DGAT1*, *IGF1R*, and *ACTB* (Figure 2). A small amount of *TERT* mRNA has been reported in the majority of noncancerous somatic tissues (Kim et al., 1994). However, the results of quantitative RT-PCR showed a significant increase in expression of *TERT* in CNS samples compared with CPS samples ($P = 0.04$) and H samples ($P = 0.02$; Figure 1). Direct BSPCR sequencing and clone BSPCR sequencing results of the *TERT* upstream element did not show substantial differences between PM values of the CNS, CPS, and H groups of parenchyma samples (Figure 4). It is interesting that a whole-genome differential methylation study using methylated DNA immunoprecipitation (MeDIP-chip) microarray of mononuclear lymphocytes (PBMC) of *Staph. aureus*-infected cows revealed significant differential methylation of the bovine *TERT* locus between *Staph. aureus*-infected and control groups of animals, albeit with no difference in *TERT* mRNA abundance (Song et al., 2016). The mentioned MeDIP-chip enriched region encompassed an intragenic CpG site with a bivariate methylation state (Song et al., 2016). In our study hypermethylation of *TERT* upstream element detected in infected as well as noninfected samples does not correspond to significant *TERT* upregulation in CNS samples in comparison to CPS and H sample group.

The *TERT* gene encodes a telomerase responsible for the maintenance of chromosome ends (telomeres) in the majority of somatic cells. In the pathological state, telomere shortening is associated with cellular senescence,

Figure 3. Methylation pattern at 32 CpG sites of upstream element of *TERT* for coagulase-positive staphylococci (CPS), CNS, and healthy (H) group after Sanger sequencing of cloned bisulfite PCR (BSPCR) products of pooled DNA samples after conversion. Methylated cytosines are marked in red, whereas blue denotes lack of methylation in particular CpG position. White boxes denote lack of data due to sequencing or capillary electrophoresis difficulties.

which manifests as chronic inflammation (caused by the impairment of immune cells) that is often observed in aged individuals (Shlush et al., 2011). Substantial telomere shortening is also associated with the occurrence of viral and bacterial infections, indicating that certain pathogenic microorganisms are able to alter the cell cycle process of the host (as reviewed by Kordinas et al., 2016). This phenomenon is usually associated with a decrease in telomerase activity (Kordinas et al., 2016). In the present report, we observed that the presence of coagulase-negative bacteria can induce *TERT* expression in host tissue. Based on previous articles, we hypothesize that the increase of telomerase activity caused by CNS in infected parenchyma samples might be induced by activation of genes of the MAPK signaling pathway in the cells of this secretory tissue. It is well

known that *Staph. aureus* can increase mRNA expression of genes belonging to the MAP kinase pathway in epithelial cells due to the release of bacterial exotoxins (Heimer et al., 2010). It has been reported that *TERT* is one of the genes induced via MAPK signaling in a model of estradiol-treated human endometrial cancer cells (Zhou et al., 2013). Moreover, in previous transcriptomic studies related to CPS- and CNS-infected mammary glands, upregulation of particular loci of the MAPK signaling pathway was shown in udder secretory tissue infected with coagulase-negative (*FOS* and *EGR1* genes) and coagulase-positive bacteria (*MAP3K*; Kościuczek et al., 2014, 2017). Interestingly, our results revealed that only the CNS group of bacteria could promote *TERT* expression in infected mammary gland. We confirmed that bacterial infection may modulate

Figure 4. Average methylation percent (PM) across 32 CpG sites of *TERT* upstream CpG islands (CGI) in sections of mammary gland infected with coagulase-positive staphylococci (CPS) and CNS, and in uninfected control samples (healthy, H). Visible variability of PM values of parenchyma samples does not indicate the presence or lack of bacteria; rather, it indicates the hypermethylation of the *TERT* upstream element. BSPCR = bisulfite PCR.

telomerase activity during inflammation via changes in *TERT* expression (Kordinas et al., 2016).

We conclude that regulation of expression the *ATG5*, *DGAT1*, *IGF1R*, and *TERT* genes through DNA methylation during inflammation induced by bacterial infection of bovine mammary gland is negligible. The question remains as to whether significant alterations of gene expression of *TERT* are associated with the molecular mechanisms underlying the etiology of bovine mastitis or are a side-effect induced primarily by bacterial metabolites.

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REFERENCES

- Bionaz, M., and J. J. Loo. 2007. Identification of reference genes for quantitative real-time PCR in the bovine mammary gland during the lactation cycle. *Physiol. Genomics* 29:312–319. <https://doi.org/10.1152/physiolgenomics.00223.2006>.
- Blackburn, E. H. 2005. Telomeres and telomerase: Their mechanisms of action and the effects of altering their functions. *FEBS Lett.* 579:859–862. <https://doi.org/10.1016/j.febslet.2004.11.036>.
- Cases, S., P. Zhou, J. M. Shillingford, B. S. Wiseman, J. D. Fish, C. S. Angle, L. Hennighausen, Z. Werb, and R. V. Jr. Farese. 2004. Development of the mammary gland requires *DGAT1* expression in stromal and epithelial tissues. *Development* 131:3047–3055. <https://doi.org/10.1242/dev.01158>.
- Deaton, A. M., and A. Bird. 2011. CpG islands and the regulation of transcription. *Genes Dev.* 25:1010–1022. <https://doi.org/10.1101/gad.203751.1>.
- Grisart, B., F. Farnir, L. Karim, N. Cambisano, J. J. Kim, A. Kvasz, M. Mni, P. Simon, J. M. Frère, W. Coppieters, and M. Georges. 2004. Genetic and functional confirmation of the causality of the *DGAT1* K232A quantitative trait nucleotide in affecting milk yield and composition. *Proc. Natl. Acad. Sci. USA* 101:2398–2403. <https://doi.org/10.1073/pnas.0308518100>.
- Heimer, S. R., A. Yamada, H. Russell, and M. S. Gilmore. 2010. Response of corneal epithelial cells to *Staphylococcus aureus*. *Virulence* 1:223–235. <https://doi.org/10.4161/viru.1.4.11466>.
- Kim, N. W., M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich, and J. W. Shay. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science* 266:2011–2015. <https://doi.org/10.1126/science.7605428>.
- Koliwad, S. K., R. S. Streeper, M. Monetti, I. Cornelissen, L. Chan, K. Terayama, S. Naylor, M. Rao, B. Hubbard, and R. V. Farese Jr.. 2010. *DGAT1*-dependent triacylglycerol storage by macrophages protects mice from diet-induced insulin resistance and inflammation. *J. Clin. Invest.* 120:756–767. <https://doi.org/10.1172/JCI36066>.
- Kordinas, V., A. Ioannidis, and S. Chatzipanagiotou. 2016. The telomere/telomerase system in chronic inflammatory diseases. Cause or effect? *Genes (Basel)* 7:60. <https://doi.org/10.3390/genes7090060>.
- Kościczuk, E. M., P. Lisowski, J. Jarczak, J. Krzyżewski, L. Zwierzchowski, and E. Bagnicka. 2014. Expression patterns of β -defensin and cathelicidin genes in parenchyma of bovine mammary gland infected with coagulase-positive or coagulase-negative staphylococci. *BMC Vet. Res.* 10:246. <https://doi.org/10.1186/s12917-014-0246-z>.
- Kościczuk, E. M., P. Lisowski, J. Jarczak, A. Majewska, M. Rzewuska, L. Zwierzchowski, and E. Bagnicka. 2017. Transcriptome profiling of staphylococci-infected cow mammary gland parenchyma. *BMC Vet. Res.* 13:161. <https://doi.org/10.1186/s12917-017-1088-2>.
- Leakey, T. I., J. Zielinski, R. N. Siegfried, E. R. Siegel, C. Y. Fan, and C. A. Cooney. 2008. A simple algorithm for quantifying DNA methylation levels on multiple independent CpG sites in bisulfite genomic sequencing electropherograms. *Nucleic Acids Res.* 36:e64. <https://doi.org/10.1093/nar/gkn210>.
- Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:45e. <https://doi.org/10.1093/nar/29.9.e45>.
- Rohde, C., Y. Zhang, R. Reinhardt, and A. Jeltsch. 2010. BISMA—fast and accurate bisulfite sequencing data analysis of individual clones from unique and repetitive sequences. *BMC Bioinformatics* 11:230. <https://doi.org/10.1186/1471-2105-11-230>.
- Shlush, L. I., K. L. Skorecki, S. Itzkovitz, S. Yehezkel, Y. Segev, H. Shachar, R. Berkovitz, Y. Adir, I. Vulto, P. M. Lansdorp, and S. Selig. 2011. Telomere elongation followed by telomere length reduction, in leukocytes from divers exposed to intense oxidative stress—Implications for tissue and organismal aging. *Mech. Ageing Dev.* 132:123–130. <https://doi.org/10.1016/j.mad.2011.01.005>.
- Song, M., Y. He, H. Zhou, Y. Zhang, X. Li, and Y. Yu. 2016. Combined analysis of DNA methylome and transcriptome reveal novel candidate genes with susceptibility to bovine *Staphylococcus aureus* subclinical mastitis. *Sci. Rep.* 6:29390. <https://doi.org/10.1038/srep29390>.
- Tran Van Hieu, G., and L. Arbibe. 2009. Genetic reprogramming of host cells by bacterial pathogens. *F1000 Biol. Rep.* 1:80.
- Werner, H., and D. Le Roith. 2000. New concepts in regulation and function of the insulin-like growth factors: Implications for understanding normal growth and neoplasia. *Cell. Mol. Life Sci.* 57:932–942. <https://doi.org/10.1007/PL00000735>.
- Ye, X., X.-J. Zhou, and H. Zhang. 2018. Exploring the role of autophagy-related gene 5 (*ATG5*) yields important insights into autophagy in autoimmune/autoinflammatory diseases. *Front. Immunol.* 9:2334. <https://doi.org/10.3389/fimmu.2018.02334>.
- Yen, C. L., S. J. Stone, S. Koliwad, C. Harris, and R. V. Farese Jr.. 2008. Thematic review series: Glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. *J. Lipid Res.* 49:2283–2301. <https://doi.org/10.1194/jlr.R800018-JLR200>.
- Zhou, C., T. A. Stepkowski, H. K. Dickens, K. M. Malloy, P. A. Gehrig, J. F. Boggess, and V. L. Bae-Jump. 2013. Estrogen induction of telomerase activity through regulation of the mitogen-activated protein kinase (MAPK) dependent pathway in human endometrial cancer cells. *PLoS One* 8:e55730. <https://doi.org/10.1371/journal.pone.0055730>.
- Zielniok, K., A. Sobolewska, and M. Gajewska. 2017. Mechanisms of autophagy induction by sex steroids in bovine mammary epithelial cells. *J. Mol. Endocrinol.* 59:29–48. <https://doi.org/10.1530/JME-16-0247>.

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