Research Note: All-*trans* retinoic acids induce adipogenic differentiation of chicken embryonic fibroblasts and preadipocytes

Dong-Hwan Kim,[*] Joonbum Lee,[*,†] Yeunsu Suh,[*] Michael Cressman,[*] and Kichoon Lee[*,†,1]

[*]*Department of Animal Sciences, The Ohio State University, Columbus, OH, USA; and* [†]*The Ohio State University Interdisciplinary Human Nutrition Program, The Ohio State University, Columbus 43210, USA*

ABSTRACT Adipocytes store excess energy in the form of lipids, whereas fat accretion contributes to feed efficiency, meat quality, and female reproduction in poultry. As a metabolite of vitamin A, all-*trans* retinoic acid (**atRA**) has been shown to have influence over metabolic functions such as lipid and energy homeostasis, as well as adipogenesis. Although atRA has been known to function as a regulating factor in mammalian adipogenesis, the effects of atRA on adipogenesis has not been studied in chickens. In this study, chicken preadipocytes isolated from leg fat tissues at embryonic day (**E**) 14 and chicken embryonic fibroblasts (**CEF**) harvested at E5 were cultured. The preadipocytes and CEF in culture with 10% chicken serum were treated with various concentrations (0 μmol, 100 μmol, or 150 μmol) of supplemented atRA for 48 h. In these cells, cytoplasmic lipid droplet accumulation and mRNA expression for adipogenic genes were analyzed by Oil-Red-O staining and quantitative real-time PCR, respectively. Analysis of the relative amount of Oil-Red-O staining (lipid accumulation) revealed that all 3 variables increased in a dose-dependent manner, in response to increasing atRA supplementation. Genes involved in adipocyte differentiation, fatty acid transport, and triacylglycerol synthesis in both E14 preadipocytes and E5 CEF were upregulated by supplementation of atRA. These data demonstrated that atRA alone promoted adipogenesis of embryonic preadipocytes and fibroblasts in vitro, suggesting that atRA has an influential role in multiple stages of adipogenesis in chicken embryos.

Key words: all-*trans* retinoic acid, preadipocytes, chicken embryonic fibroblasts, adipogenesis, chicken

2020 Poultry Science 99:7142–7146
https://doi.org/10.1016/j.psj.2020.09.006

INTRODUCTION

Adipogenesis is defined as a process by which preadipocytes differentiate into adipocytes, and an increase in the size and number of adipocytes results in increased fat tissue accumulation in body. Biological processes of adipocyte differentiation in chickens have been studied using stromal-vascular cells isolated from chicken adipose tissues (Shang et al., 2014; Zhang et al., 2015) and epithelial oviduct cells (Khuong and Jeong, 2011). In addition, our recent studies demonstrated that chicken embryonic fibroblasts (**CEF**) can serve as a new in vitro model to study processes of adipogenesis (Kim et al., 2020).

Vitamin A (**retinol**) is an essential lipid-soluble nutrient in animals. All-*trans* retinoic acid (**atRA**), a metabolite of retinol, functions as a key regulatory molecule in controlling embryogenesis and differentiation of many types of cells in mammals, including adipocytes. Previous studies using mice showed the antiadipogenic effect of atRA in vitro (Stoecker et al., 2017) and injection of atRA in vivo (Mercader et al., 2006) but increased fat deposition in cattle by retinol injection (Harris et al., 2018). In addition, our previous study using a murine 3T3-L1 preadipocyte cell line demonstrated both proadipogenic and antiadipogenic functions of supplementation of low and high atRA concentrations, respectively (Kim et al., 2019). The effect of atRA on adipogenesis in chicken cells has not been studied; therefore, in the present study, we examined whether atRA can regulate adipogenesis in CEF harvested on embryonic day (**E**) 5 and chicken preadipocytes isolated from leg fat cells on E14.

Received May 7, 2020.
Accepted September 4, 2020.

[1]Corresponding author: lee.2626@osu.edu

MATERIALS AND METHODS

Isolation of Preadipocytes and CEF

Experiments using chicken embryos are exempt from requiring University Institutional Animal Care and Use Committee approval because avian embryos are not considered live animals by the Public Health Service Policy (ILAR News, 1991). All fertile eggs of the Rhode Island Red breed of chicken were obtained from the Ohio State University poultry research farm. To isolate preadipocytes and CEF from embryos at E14 and E5, respectively, the procedures were followed by previous studies (Zhang et al., 2015; Kim et al., 2020). In short, to isolate preadipocytes, leg fat from E14 chicken embryos was minced and incubated with 3.2 mg/mL of collagenase II (#C6885, Sigma-Aldrich, St. Louis, MO) and filtered through a 70-μm cell strainer (#352350, BD Falcon, Franklin Lakes, NJ). For separation of CEF, E5 chicken embryos were sampled and trypsinized (0.05% Trypsin-EDTA, #15400, Gibco, Grand Island, NY) and then filtered through a 70-μm cell strainer.

Adipogenic Differentiation

To induce adipogenic differentiation at 80% confluency, preadipocytes and CEF were incubated in Dulbecco's Modified Eagle Medium (#11965, Gibco) with 10% chicken serum (**CS**) and 3 different concentrations (0 μmol, 100 μmol, or 150 μmol) of atRA (#R2625, Sigma-Aldrich) for 48 h. After inducing differentiation for 48 h, cells were fixed for Oil-Red-O staining and collected for total RNA isolation.

Cell Counting, Oil-Red-O Staining, and Quantification

To calculate total cell numbers after inducing adipocyte differentiation for 48 h, cells were stained with 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (**DAPI**) (#D9542, Sigma-Aldrich) after fixation with 4% normal buffered formalin for 1 h. The stained nuclei were imaged in randomly selected areas (3–4 fields per dish) using a microscope (EVOS cell imaging system, Thermo Fisher Scientific, Waltham, MA), and numbers of cells in each image were counted with the National Institutes of Health (**NIH**) ImageJ software (ImageJ, Ver. 1.52, http://imagej.nih.gov/ij) to calculate total numbers of the cells in each cell culture dish. After DAPI staining, cells were stained by Oil-Red-O (#O0625, Sigma-Aldrich) to visualize lipid droplets, as followed by a previous study (Kim et al., 2019). Cells were fixed with 4% normal buffered formalin for 10 min and washed with distilled water, and then stained with 60% Oil-Red-O solution for 1 h at room temperature. After washing with distilled water, the cells were visualized under the microscope (EVOS cell imaging system, Thermo Fisher Scientific). The Oil-Red-O was extracted by 100% isopropanol to quantify lipid accumulation, and then absorbance values were measured by a spectrophotometer at 490 nm (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA). Undifferentiated preadipocytes or CEF were used as negative controls. To estimate the relative amount of Oil-Red-O per cell, the Oil-Red-O value obtained from each cell culture dish was divided by the total cell number. The values of preadipocytes at E14 or E5 CEF supplemented with 0 M atRA were used as the control for standardization.

Analysis of Gene Expression

Total RNA was isolated from E14 preadipocytes and E5 CEF using the TRIzol reagent (#15596026, Life Technologies Inc., Grand Island, NY) according to the manufacturer's instructions. The RNA was reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (#28020513, Invitrogen, Carlsbad, CA). Quantitative real-time PCR (**qPCR**) was performed using AmpliTaq Gold polymerase (#N8080241, Applied Biosystems, Foster City, CA) and SYBR green I as a detection dye for an amplification curve on the ABI 7300 Real-Time PCR instrument (Applied Biosystems). To analyze quantitative expression levels of genes related to adipogenesis, qPCR was performed in duplicate and all primer sequences with qPCR condition in this study were described in a previous study (Kim et al., 2020) except *C/ebpβ* (NCBI reference sequence: NM_205,253.2, F: 5′-GCAAGAACAAGCCCAAGAAGTG, R: 5′-CAAGACTTTGTGCTGCGTCTCC, size:137 bp). All qPCR data were analyzed using the $2^{-\Delta\Delta CT}$ method as described in our previous study (Kim et al., 2020).

Statistical Analysis

All data were expressed as means ± SEM (n = 4). All individual experiments in E5 CEF or E14 preadipocytes treated with each atRA dose (0 μmol, 100 μmol, or 150 μmol) were performed 4 times. The data were analyzed using the GraphPad Prism software, version 6.02. Multiple means were compared by one-way ANOVA followed by Tukey's multiple comparison test. $P < 0.05$ was considered as a statistically significant difference.

RESULTS AND DISCUSSION

Adipocytes play a crucial role in energy homeostasis and endocrine function during embryonic development. Recently, several hormonal and nutritional factors such as insulin, oleic acid, and linolenic acid were used for inducing adipogenesis in chickens (Khuong and Jeong, 2011; Shang et al., 2014; Kim et al., 2020) and although atRA has been studied both in vitro and in vivo as a regulatory factor in mammalian adipogenesis (Mercader et al., 2006; Kim et al., 2019), the regulation of adipogenesis in vitro by atRA has not yet been investigated in chickens.

In the present study, to investigate the function of atRA on adipogenesis in chickens, atRA was supplemented into cultured preadipocytes isolated from E14 chicken

embryos to induce adipocyte differentiation for 48 h. Morphologically, lipid droplets increased in both the number and size, responding to atRA supplementation in a dose-dependent manner (Figure 1A). Quantitative analysis of Oil-Red-O staining revealed that the degree of lipid droplet formation also responded in a dose-dependent manner to increasing supplementation of atRA (Figure 1B). The OD values for Oil-Red-O staining were 2.6-fold higher and 3.2-fold higher in the preadipocytes supplemented with 100 μmol and 150 μmol of atRA, respectively, than in the 0-μmol control group. These results suggest that atRA can promote lipid accumulation in E14 chicken preadipocytes. To analyze how atRA affects preadipocyte differentiation, relative expression levels of genes involved in adipocyte determination/differentiation and lipid metabolism were measured by qPCR. Expression levels of an adipogenic determination/differentiation marker (Gupta et al., 2010; Kim et al., 2020), *Zinc finger protein 423* (***Zfn423***), were 4-fold higher in the preadipocytes supplemented with 150 μmol of atRA than in the 0-μmol control group ($P < 0.05$). *C/ebpβ*, *Pparγ*, and *Fabp4*, well-known adipogenic factors (Kim et al., 2019, 2020), were also upregulated by atRA supplementation (Figure 1C). Genes involved in fatty acid uptake [*fatty acid transporter 4*, *acyl-CoA synthetase long-chain family 1*] and triacylglycerol (**TAG**) synthesis [*acylglycerolphosphate acyltransferase 1* and *diacylglycerol O-acyltransferase homology 2*] (Kim et al., 2020) were also significantly upregulated by supplementation of 150 μmol atRA ($P < 0.05$) (Figures 1D and 1E). In agreement with morphological and quantitative results of lipid formation, these gene expression patterns indicate that atRA alone can induce differentiation of chicken preadipocytes to adipocytes in vitro.

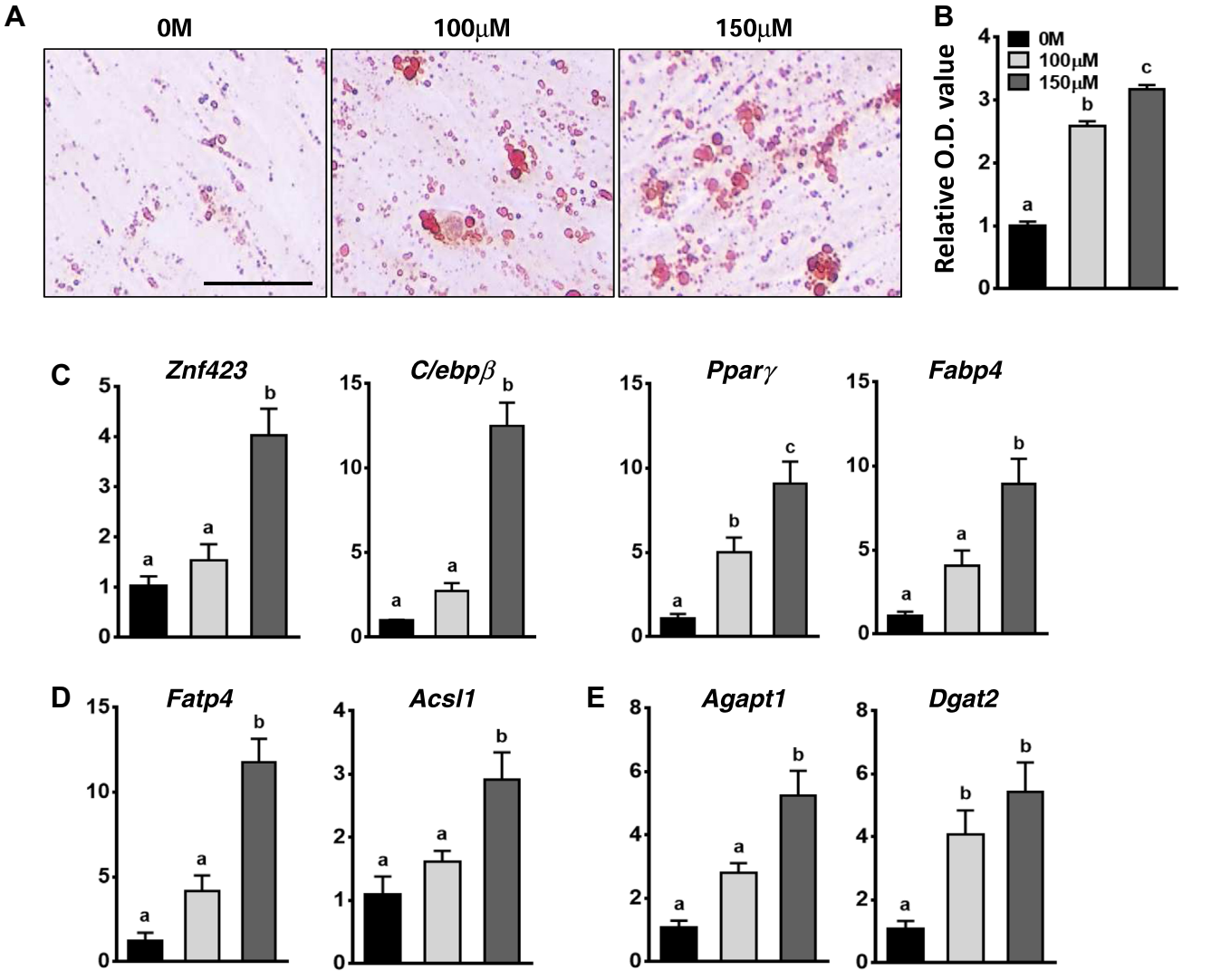

Figure 1. Effect of atRA on the preadipocyte differentiation isolated from the leg fat of an E14 chicken embryo. Oil-Red-O staining (A) and OD values (B). Preadipocytes were treated with different doses of atRA for 48 h. Oil-Red-O staining was visualized under a microscope and quantified using a spectrophotometer. The OD values were calculated by the relative amount of Oil-Red-O per cell. Undifferentiated preadipocytes were used as a blank control. Scale bar: 50 μm. Relative gene expression levels involved in adipocyte determination and differentiation markers (C) and fatty acid uptake (D), and TAG synthesis (E) by qPCR. All quantification was analyzed after inducing differentiation of preadipocyte for 48 h, and *Rps13* was used as a house keeping gene. All data are shown as mean ± SEM (n = 4). One-way ANOVA was used for statistical analysis by the GraphPad PRISM 6.02 program, $P < 0.05$. Abbreviations: atRA, all-*trans* retinoic acid; E, embryonic day; qPCR, quantitative real-time PCR.

Our previous studies demonstrated the capability of undetermined CEF to be differentiated into adipocytes by insulin plus fatty acids or selenium (Hassan et al., 2014; Kim et al., 2020), and the present study showed that atRA is solely able to induce differentiation of chicken preadipocytes to adipocytes. These 2 findings led us to investigate whether undetermined CEF can be differentiated into adipocytes by atRA. Increased supplementation of atRA in CEF cultures resulted in greater abundance of lipid droplets, as well as an increased size of lipid droplets. Responding in a dose-dependent manner, the OD values for Oil-Red-O staining were 1.7-fold higher and 2.3-fold higher for CEF supplemented with 100 μmol and 150 μmol atRA, respectively, than for the 0-μmol control group ($P < 0.05$) (Figures 2A and 2B).

It is known that atRA positively regulates adipocyte determination from mouse embryonic stem cells (Dani et al., 1997), and that ZNF423 functions as a regulator for preadipocyte determination and an inducer for the expression of *PPARγ* and FABP4 in vitro and in vivo (Gupta et al., 2010). In this regard, our results showed an upregulation of *Znf423*, along with an upregulation of *Pparγ* and *Fabp4*, in atRA-supplemented CEF (Figure 2C). These results suggest that atRA favors a path toward adipogenic differentiation of undetermined CEF through upregulation of *Znf423* for preadipocyte determination and upregulation of *C/ebpβ*, *Pparγ*, and *Fabp4* for adipocyte differentiation.

Because *de novo* lipogenesis does not occur in chicken adipocytes (Hassan et al., 2014), fat accumulation in adipocytes is mainly dependent on fatty acid uptake and TAG synthesis. Therefore, expression levels of the genes involved in fatty acid uptake (*fatty acid transporter 4*), lipid biosynthesis (*Ascl1*), and TAG synthesis (*acylglycerolphosphate acyltransferase 1* and *diacylglycerol*

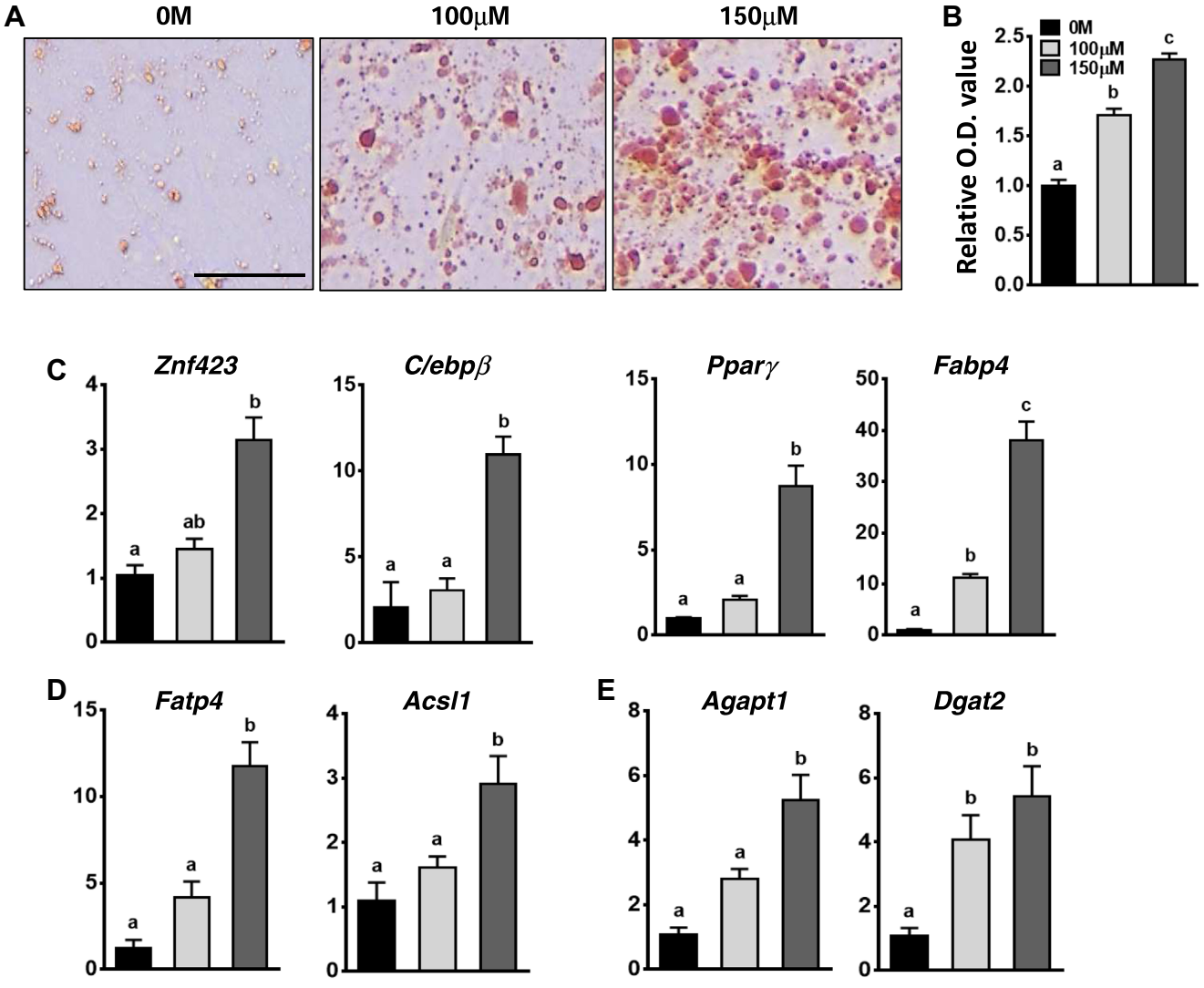

Figure 2. Effect of atRA on the differentiation of E5 CEF. Oil-Red-O staining (A) and OD values (B). Chicken embryonic fibroblasts were treated with different doses of atRA for 48 h. Oil-Red-O staining was visualized under a microscope and quantified using a spectrophotometer. The OD values were calculated by the relative amount of Oil-Red-O per cell. Undifferentiated CEF were used as a blank control. Scale bar: 50 μm. Relative gene expression levels involved in adipocyte determination and differentiation markers (C) and fatty acid uptake (D), and TAG synthesis (E) by qPCR. All quantification was analyzed after inducing differentiation of CEF for 48 h, and *Rps13* was used as a house keeping gene. All data are shown as mean ± SEM (n = 4). One-way ANOVA was used for statistical analysis by the GraphPad PRISM 6.02 program, $P < 0.05$. Abbreviations: atRA, all-*trans* retinoic acid; CEF, chicken embryonic fibroblasts; E, embryonic day; qPCR, quantitative real-time PCR.

O-acyltransferase homology 2) were measured. These genes are significantly upregulated by 150-μmol atRA ($P < 0.05$) (Figures 2D and 2E), suggesting upregulation of these genes is likely associated with increased fat accumulation in CEF by atRA.

To our knowledge, this study is the first to report a proadipogenic effect of atRA in chicken preadipocytes and CEF. The present study suggests that atRA alone is involved in both commitment and differentiation of preadipocytes, as shown by the accumulation of lipid droplets and upregulation of marker genes representing developmental steps of adipogenesis. Further investigation is still needed to clarify the molecular mechanisms underlying the proadipogenic effect of atRA in multiple stages of adipogenesis in chicken embryos and to validate the proadipogenic functions of atRA *in ovo*. In addition, it will be interesting to investigate whether atRA can affect posthatch adipogenesis in vivo and lead to changes in amounts of carcass fat and associated production efficiency.

ACKNOWLEDGMENTS

This research was funded by the United States Department of Agriculture National Institute of Food and Agriculture Grant (Project No. 2018-09301).

Conflict of Interest Statement: The authors declare no conflict of interest.

REFERENCES

Dani, C., A. G. Smith, S. Dessolin, P. Leroy, L. Staccini, P. Villageois, C. Darimont, and G. Ailhaud. 1997. Differentiation of embryonic stem cells into adipocytes in vitro. J. Cell Sci. 110:1279–1285.

Gupta, R. K., Z. Arany, P. Seale, R. J. Mepani, L. Ye, H. M. Conroe, Y. A. Roby, H. Kulaga, R. R. Reed, and B. M. Spiegelman. 2010. Transcriptional control of preadipocyte determination by Zfp423. Nature 464:619–623.

Harris, C. L., B. Wang, J. M. Deavila, J. R. Busboom, M. Maquivar, S. M. Parish, B. McCann, M. L. Nelson, and M. Du. 2018. Vitamin A administration at birth promotes calf growth and intramuscular fat development in Angus beef cattle. J. Anim. Sci. Biotechnol. 9:1–9.

Hassan, A., J. Ahn, Y. Suh, Y. M. Choi, P. Chen, and K. Lee. 2014. Selenium promotes adipogenic determination and differentiation of chicken embryonic fibroblasts with regulation of genes involved in fatty acid uptake, triacylglycerol synthesis and lipolysis. J. Nutr. Biochem. 25:858–867.

ILAR News. 1991. Office of Laboratory animal Welfare, the Public Health Service Responds to Commonly Asked Questions, Question #1. Accessed Sep. 2020. https://grants.nih.gov/grants/olaw/references/ilar91.htm.

Khuong, T. T. T., and D. K. Jeong. 2011. Adipogenic differentiation of chicken epithelial oviduct cells using only chicken serum. Vitr. Cell. Dev. Biol. - Anim. 47:609–614.

Kim, D. H., J. W. Lee, and K. Lee. 2019. Supplementation of all-trans-retinoic acid below Cytotoxic levels promotes adipogenesis in 3T3-L1 cells. Lipids 54:99–107.

Kim, D. H., J. Lee, Y. Suh, M. Cressman, S. S. Lee, and K. Lee. 2020. Adipogenic and Myogenic Potentials of chicken embryonic fibroblasts in vitro: Combination of fatty acids and insulin induces adipogenesis. Lipids 55:163–171.

Mercader, J., J. Ribot, I. Murano, F. Felipe, S. Cinti, M. L. Bonet, and A. Palou. 2006. Remodeling of white adipose tissue after retinoic acid administration in mice. Endocrinology 147:5325–5332.

Shang, Z., L. Guo, N. Wang, H. Shi, Y. Wang, and H. Li. 2014. Oleate promotes differentiation of chicken primary preadipocytes in vitro. Biosci. Rep. 34:51–57.

Stoecker, K., S. Sass, F. J. Theis, H. Hauner, and M. W. Pfaffl. 2017. Inhibition of fat cell differentiation in 3T3-L1 pre-adipocytes by all-trans retinoic acid: Integrative analysis of transcriptomic and phenotypic data. Biomol. Detect. Quantif. 11:31–44.

Zhang, J., Y. Suh, Y. M. Choi, P. R. Chen, M. E. Davis, and K. Lee. 2015. Differential expression of cell Cycle regulators during Hyperplastic and Hypertrophic growth of Broiler Subcutaneous adipose tissue. Lipids 50:965–976.