Research Note: Potential usage of DF-1 cell line as a new cell model for avian adipogenesis

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ABSTRACT Current research of avian adipogenesis has been dependent on primary preadipocytes culture due to the lack of commercially available immortal preadipocyte cell lines in avian species. In addition to primary stromal vascular cells, primary chicken embryonic fibroblasts (**CEF**) were suggested as new in vitro models for adipogenesis study, because CEF can be differentiated into adipocytes by a combination of fatty acids and insulin (**FI**), or all-*trans* retinoic acid (**atRA**) alone in the media containing chicken serum (**CS**). However, there are decreases in differentiation of primary cells due to diverse population of cell types and low adipogenic potential of cells after passages. In the present study, adipogenic differentiation of DF-1 cells, immortal fibroblasts derived from an embryonic chicken, was tested with 4 different medium; 10% fetal bovine serum (**FBS**), 10% CS, 10% CS with FI, and 10% CS with FI and atRA. Lipid droplets stained with Oil Red O were not shown in DF-1 cells under 10% FBS, appeared with very small sizes under 10% CS, significantly increased under 10% CS with FI, and most significantly accumulated under 10% CS with FI and atRA. In addition, expressions of markers for adipogenesis (*Znf423*, *C/ebpβ*, *Pparγ*, and *Fabp4*), fatty acid uptake (*CD36*), triglyceride synthesis (*Gpd1*, *Dgat2*), and lipid droplet stabilization (*Plin1*) were significantly upregulated by supplementation of 10% CS with FI and atRA. Morphological evidence for formation of lipid droplets and dramatic induction of adipogenic marker genes support the adipogenic potential of DF-1 cells, offering DF-1 cells as a new cell model to investigate various research studies involving avian adipogenesis.

Key words: DF-1 cell, adipogenesis, chicken, all-*trans* retinoic acid

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INTRODUCTION

The most current knowledge of adipogenesis is derived from *in vitro* and *in vivo* mice studies, and the 3T3-L1 mouse preadipocyte cell line contributes to a huge advance in understanding the cellular and molecular mechanism involved in adipocyte differentiation. However, differences between mammals and birds regarding adipocyte differentiation and lipogenesis have been revealed more and more, indicating the need of a standardized cell model for studying avian adipogenesis (Gondret et al., 2001; Bergen and Mersmann, 2005; Matsubara et al., 2005). Unfortunately, there is not a commercially available immortalized avian preadipocyte cell line, even though establishment of immortal chicken preadipocytes has been reported (Wang et al., 2017). Thus, most avian adipogenic studies are still relying on primary culture of stromal vascular cells derived from chicken adipose tissues (Matsubara et al., 2005; Shang et al., 2014). Recently, adipogenic potential of primary chicken embryonic fibroblasts (**CEF**) has been demonstrated, suggesting primary CEF as a potential cell model for studying avian adipogenesis (Kim et al., 2020a, 2020b). However, there are decreases in differentiation of primary cells due to diverse population of cell types and low adipogenic potential of cells after passages. In addition, because passaging of primary cell is losing capability of adipogenic differentiation, fresh primary cell culture is required in every study. Therefore, avian cell lines that can be used for adipogenic study need to be developed or identified.

Of the commercially available avian embryonic fibroblast cell lines, the DF-1 cell line is the most investigated and used chicken cell line (Himly et al., 1998). In addition, its continuous cell proliferation without transformation enables use of the DF-1 cell line for genome

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editing study (Bai et al., 2016). In our previous studies, primary CEF was able to be differentiated into adipocytes by supplementing a combination of fatty acids and insulin (**FI**), or all-*trans* retinoic acid (**atRA**) alone to chicken serum (**CS**)-based media (Kim et al., 2020a, 2020b). Therefore, we examined whether DF-1 cells can be differentiated into adipocytes using the previously confirmed adipogenic media and potentially serve as an in vitro model for avian adipogenesis.

MATERIALS AND METHODS

Cell Culture and Adipogenic Differentiation

DF-1 cells (ATCC number CRL-12203) were initially cultured in normal growth media containing Dulbecco's modified Eagle's medium (**DMEM**, #11965, Gibco, Grand Island, NY) with 10% fetal bovine serum (**FBS**, #F4135, Sigma-Aldrich, St. Louis, MO) and 1% antibiotics (Antibiotic-Antimycotic, #15240112, Gibco). Then, the cells were trypsinized (0.05% Trypsin-EDTA, #15400, Gibco) and moved to 12-well plates. At 80% confluency, adipogenesis was induced by replacing normal growth media to 4 different media: 1) DMEM with 10% FBS as a control, 2) DMEM with 10% chicken serum (**CS**, #16110, Gibco), 3) DMEM with 10% CS, 1:100 fatty acids, made of a 1:1 ratio of linoleic acid (#L9530, Sigma-Aldrich, 2 mol linoleic acid/mole albumin; 100 mg/mL albumin) and oleic acid (#O3008, Sigma-Aldrich, 2 mol oleic acid/mole albumin; 100 mg/mL albumin), 10 μg/mL insulin (#I0516, Sigma-Aldrich), and 4) DMEM with 10% CS, a combination of fatty acids and insulin (**FI**), and 100 μmol all-*trans* retinoic acid (**atRA**, #R2625, Sigma-Aldrich). After 48 h of adipogenic differentiation, 500 μL of fresh media were added.

Oil Red O Staining and Quantification

To calculate total cell numbers after 72 h of adipogenic differentiation, cells were fixed with 10% normal buffered formalin for 1 h and stained with 4′, 6-diamidino-2-phenylindole, dihydrochloride (#D9542, Sigma-Aldrich), and then the stained nuclei were randomly visualized and counted using a microscope (EVOS cell imaging system, Thermo Fisher Scientific) (3-4 fields per well). After counting cell nuclei, the cells were stained with Oil Red O (**ORO**) (#O0625, Sigma-Aldrich). For ORO staining, the cells were washed with distilled water for 3 times and stained with 60% ORO solution for 1 h at room temperature. After, cells were washed with distilled water for 3 times and stained lipid droplets were visualized using a microscope (EVOS cell imaging system). For quantification of lipid droplet accumulation, absorbance values of ORO were measured by a spectrophotometer at 490 nm (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA) after elution of ORO using 100% of isopropanol. To estimate the relative amount of ORO per cell culture dish, the ORO value from each cell culture dish was divided by the total cell number. The value of DF-1 cells cultured in DMEM with 10% FBS was used as the control for standardization.

Analysis of Gene Expression

Total RNA was isolated from DF-1 cells at 48 h and 72 h after inducing of adipogenic differentiation using Trizol reagent (#15596026, Life Technologies Inc. Grand Island, NY) as per the manufacturer's instructions. Synthesis of cDNA from RNA and quantitative real-time PCR (**qPCR**) were performed as following our previous studies (Kim et al., 2020a, 2020b). Quantitative real-time PCR was performed in duplicate and all primer sequences with qPCR condition in this study were described in a previous study (Kim et al., 2020a, 2020b), except *Peroxisome proliferator–activated receptor gamma* (***Pparγ***, NCBI Reference Sequence: NM_001001460.1, F: 5′-TGCCAAGCATTTGTATGACTC, R: 5′-TGCGAATTGCTACTTCTTTGTT, size: 200 bp), *Cluster of differentiation 36* (***CD36***, NCBI Reference Sequence: NM_001030731.1, F: 5′-AGGTACTGCGCTTCTTCTCC R: 5′-TCCAGCCAGTGTGCAGTT, size: 194 bp), *glycerol-3-phosphate dehydrogenase* (***Gpd1***, Ensembl Reference Sequence: ENSGALT00000076926.2, F: 5′-GGCTTTTGCCAAGACTGGGAA, R: 5′-GGTTTGCCCTCATAGCAGATCTG, size: 177 bp). *Diacylglycerol O-acyltransferase 2* (***Dgat2***, NCBI Reference Sequence: XM_419374.6, F: 5′- GAACGAAGTGTACAAGCAGGTGA, R: 5′- ATCGATCTTTGGGATGGTGATGG, size: 202 bp), and *Perilipin 1* (***Plin1***, NCBI Reference Sequence: NM_001127439.1, F: 5′-AAGAAGGAGGACAGCCATGC, R: 5′-CTGCCTCGTCCTTGGACT, size: 289 bp). *Glyceraldehyde 3-phosphate dehydrogenase* (***Gapdh***, NCBI Reference Sequence: NM_204305.1, F: 5′-CTCTGTTGTTGACCTGACCTG, R: 5′-CAAGTCCACAACACGGTTGCT, size: 262 bp) was used as a housekeeping gene. All qPCR data were analyzed using the $2^{-\Delta\Delta CT}$ method (Kim et al., 2020a).

Statistical Analysis

All data were expressed as means ± SEM (n = 4). Multiple means were compared by one-way ANOVA for quantification of ORO and expression levels of genes followed by Tukey's multiple comparison test using GraphPad Prism software, version 6.02. *P*-value, $P < 0.05$, was considered statistically significant.

RESULTS AND DISCUSSION

Like our previous study demonstrating adipogenic differentiation of CEF (Kim et al., 2020a), DF-1 cells supplemented with FI to the media containing 10% CS showed more lipid droplet accumulation and significantly higher O.D. value for ORO staining than DF-1 cells cultured in the media containing 10% FBS and 10% CS only (Figures 1A and 1B). Moreover, DF-1 cells

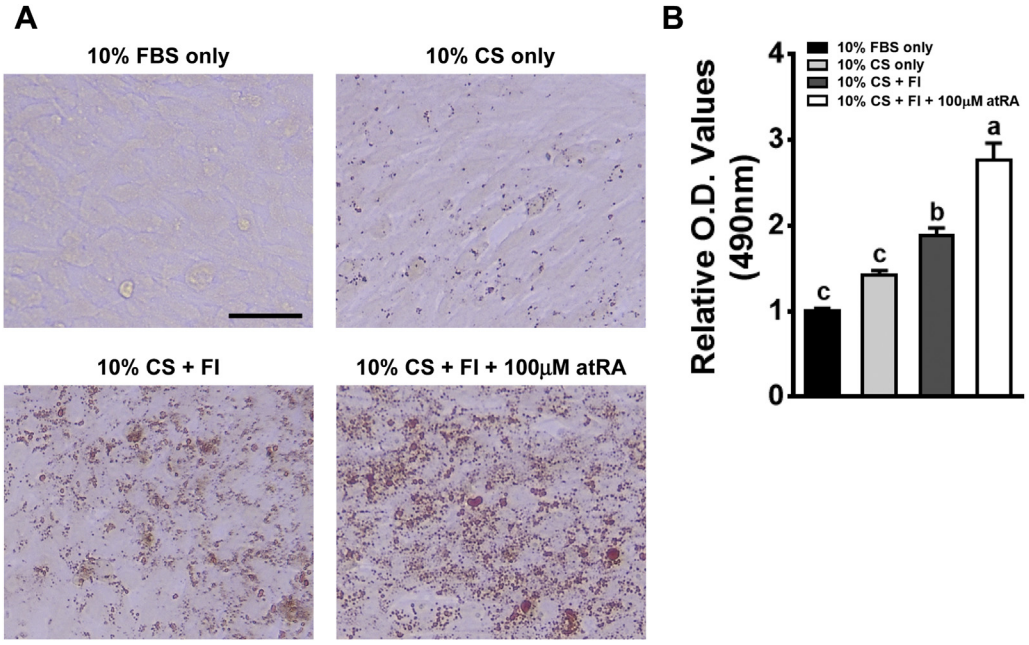

Figure 1. Effect of various media on the lipid accumulation in DF-1 cells. Oil Red O (ORO) staining (A) and O.D. values (B). Adipogenic differentiation of DF-1 cells were induced by different combinations of 10% fetal bovine serum (FBS), 10% chicken serum (CS), fatty acids and insulin (FI), and all-*trans* retinoic acid (atRA) for 72 h. The ORO-stained cells were visualized under a microscope and quantified by a relative amount of ORO per cell which is normalized by a group of 10% FBS only. Scale bar: 50 μm. All data were shown as mean ± SEM (n = 4). One-way ANOVA was used for statistical analysis by the GraphPad PRISM 6.02 program and statements of significance noted by a, b, or c are based on testing at $P < 0.05$.

supplemented both FI and 100 μmol atRA to the media containing 10% CS showed the most lipid droplet accumulation and highest O.D. value (Figures 1A and 1B). These data indicated the positive effect of atRA with other adipogenic reagents on lipid droplet accumulation. Although there was no significant difference in O.D. values (Figure 1B), there was an absence of lipid droplet formation under 10% FBS, but a relatively small size of lipid droplets were formed under 10% CS (Figure 1A), indicating CS might contain factors somewhat favoring

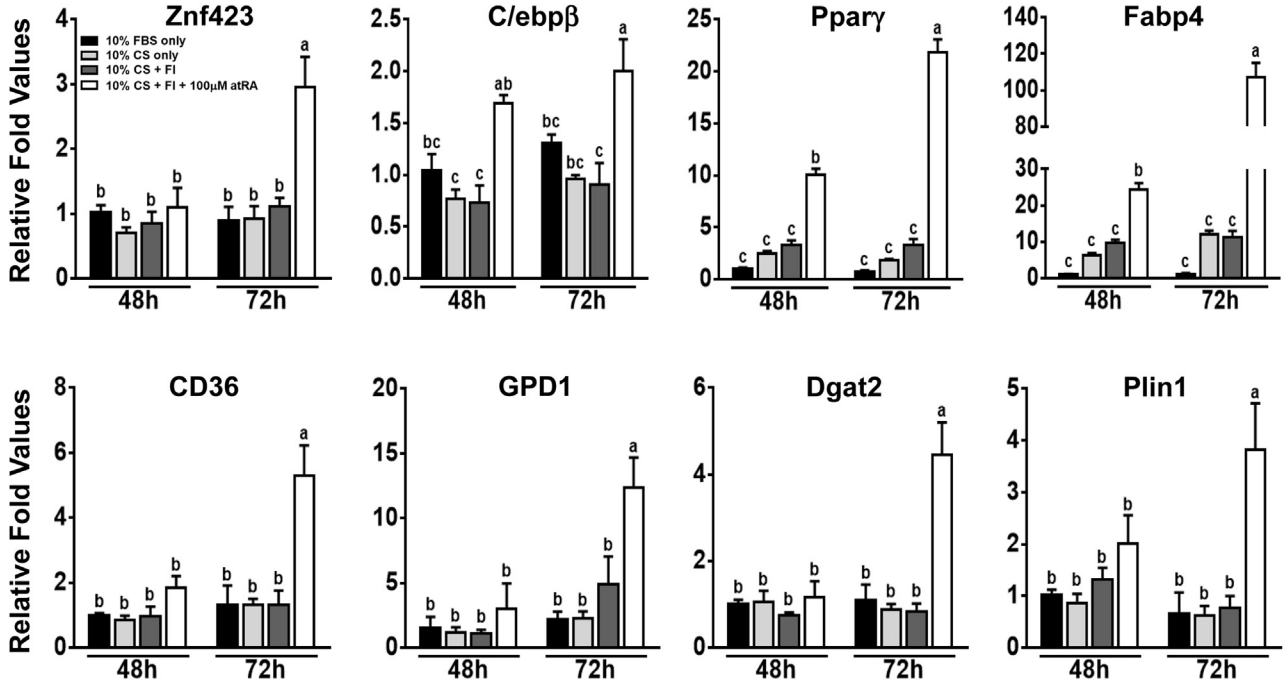

Figure 2. Relative gene expression levels during adipogenic differentiation in DF-1 cells by quantitative real-time PCR (qPCR). Expression levels of genes involved in adipogenesis (*Znf423*, *C/ebpβ*, *Pparγ*, and *Fabp4*), fatty acid uptake (*CD36*), triglyceride synthesis (*Gpd1*, *Dgat2*), and lipid droplet stabilization (*Plin1*) were analyzed by qPCR at 48 h and 72 h after inducing adipogenic differentiation. *Gapdh* was used as a housekeeping gene and all expression levels of genes are normalized by the levels of a group of 10% FBS only. All data were shown as mean ± SEM (n = 4). One-way ANOVA was used for statistical analysis by the GraphPad PRISM 6.02 program and statements of significance noted by a, ab, b, bc, or c are based on testing at $P < 0.05$.

adipogenic differentiation. In fact, serum triglyceride levels in CS are about 5 times higher than those of cattle, and thus, CS can support adipogenesis by providing sources of lipid accumulation (Khaki et al., 2010).

Subsequently, adipogenesis-related gene expression was further measured by qPCR to analyze adipogenic differentiation of DF-1 cells. All genes involved in adipocyte determination and differentiation, *zinc finger protein 423* (***Znf423***), *CCAAT/enhancer binding protein beta* (***C/ebpβ***), *Pparγ*, and *fatty acid–binding protein 4* (***Fabp4***), were significantly upregulated only by supplementation of both FI and atRA; and the value was highest at 72 h after treatment (Figure 2). In agreement with adipogenic marker genes, genes involved in fatty acid uptake, *CD36*, triglyceride synthesis, *Gpd1* and *Dgat2*, and lipid droplet stabilization, *Plin1*, were significantly upregulated only by supplementation of both FI and atRA at 72 h after treatment (Figure 2). These data indicated that induced adipogenic differentiation and enhanced lipid metabolism of DF-1 cells were attained by supplementing both FI and atRA to the media containing 10% CS. Moreover, significant upregulation of *Pparγ* and *Fabp4* at 48 h after treatment and further upregulation of all genes that were tested at 72 h after treatment indicated adipogenesis of DF-1 cells was further enhanced during the period of 48 h to 72 h after treatment. In addition, similar to our previous study (Kim et al., 2020b), atRA can be an inducer of adipogenic differentiation in DF-1 cells through upregulation of genes involved in adipogenesis and lipid metabolism.

To our knowledge, this is the first report investigating adipogenic potential of DF-1 cells through supplementing FI and atRA in 10% CS-based media. Currently, there is not a commercially available preadipocyte cell line in avian species, and thus, DF-1 cells can be considered as the first commercially available cell model for research to further study avian adipocyte determination and developmental processes. In addition, studies on effect of potential nutritional factors on adipogenesis in avian species using DF-1 cells can be conveniently performed and provide consistent results compared with the primary cell culture, considering batch-to-batch variability of primary preadipocytes or CEF culture. Importantly, it is also possible to generate stable expression or knockout of target genes in DF-1 cells to investigate its function in avian adipogenesis. Future experiments using DF-1 as a cell model for the study of avian adipogenesis is expected to contribute to the understanding of avian adipogenesis and benefit the poultry industry by identifying potential nutritional and genetic factors affecting fat accretion.

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DISCLOSURES

The authors declare that they have no competing interests.

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