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Differentiation of bovine mammary epithelial cells in the presence of linolenic acid in combination with thiazolidenediones

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ABSTRACT

Cytoplasmic lipid droplets (CLD's) formation is critical for lactation and health and so it could be detrimental in upholding the formation of CLD's in bovine mammary epithelial cells (MAC-T). We therefore, treated MAC-T cells with differentiation medium containing alpha-linolenic acid (ALA) (100 μM) and thiazolidenediones (TZD's) (10 μM) and observed CLD's formation in cellular cytoplasm with Oil-red-O staining and elution index percentage. We also observed significant up-regulation of adipogenic and down regulation of epithelial markers. In conclusion, 100 μM ALA plus 10 μM TZD's resulted in formation of CLD's and subsequent differentiation of MAC-T cells.

Key words: Alpha-linolenic acid (ALA), Cytoplasmic lipid droplets (CLD's), MAC-T bovine mammary epithelial cells, Thiazolidenediones (TZD's)

Enough evidences in the past had demonstrated that impaired CLD's formation hampers lactation period in cows, inadequate secretory activation by prolactin hormone and also inactivation of PPARγ ligands (Zhang *et al.* 2011)

Moreover, it's been now seen that lack of pregnancy hampers the differentiation and maturation of mammary cells (Russell *et al.* 2011).Later, it was presumed that maturation and differentiation of mammary epithelial cells could be achieved by introducing cytoplasmic lipid droplets (CLD's) formation with adipogenic agonists. Therefore, we designed an experiment to elucidate the possible role of linolenic acid (ALA) in combination with thiazolidenediones (TZD's) in the synthesizing CLD's mammary epithelial cell (MAC-T) and the maturation and differentiation of mammary epithelial cells (MAC-T).

MATERIALS AND METHODS

Materials: Cell culture media, antibodies, primers and other reagents used in the experiments were purchased from different companies. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), horse serum (HS), penicillin (100 IU/ml) and streptomycin (100 mg/ml) (PS) were obtained from Hyclone (Themoscientific, South Logan, Utah). The media and FBS used in this experiment were from a single lot. Primary and secondary antibodies such as K14, K18, MUC1, CEBPα, PPARγ, Donkey anti-goat IgG-FITC, Donkey anti-goat IgG-HRP were from Santacruz Biotechnology (Dallas, Texas). Opti 4 CN substrate kit was purchased from Bio-rad, cDNA synthesis kit from Toyobo Life Sciences and primers from Macrogen. DAPI (42, 6-Diamidino-2-phenylindole dihydrochloride) and other analytical grade chemicals were bought from Sigma Aldrich (Saint Louis, MO). Products used from other than above mentioned companies are mentioned in the text in bracket.

Cell culture: MAC-T, bovine mammary epithelial cells were purchased from ATCC cell line bank (ATCC, USA). The cell culture for adipogenic differentiation was followed as per Reza *et al.* (2014). After about d 2 onwards, cells were cultured in differentiation medium containing TZD's (10 μM) plus ALA (100 μM), and the medium was changed every 48 h until 8 d. Controls were cultured only in differentiation medium (DMEM medium containing 2% HS), and the entire experiment was performed in triplicate.

Immunostaining: Confocal microscopy was performed following the procedure given by Reza *et al.* (2014) in order to detect the markers such as K14, K18, MUC1, CEBPα and PPARγ.

Immunoblotting: Immunostaining was performed as per the procedure described by Reza *et al.* (2014) in order to detect the markers such as K14, K18, MUC1, CEBPα and PPARγ.

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Table 1. List of primers

Gene	Accession number	5′ sequence	3′ sequence
MUC1	AF399757	5'-CGC AGA ACT ACG CCA GTT TCC-3'	5'-AGA GCG GGT GGT CAT GGA TG-3'
EpCAM	BC014785.1	5'-CACTCATTTTCTTCCCAAGAG-3'	5'-GAACTGGATAGAGGAACGTG-3'
K8	NM_001256293.1	5'-GCGGCAGCTGCGTGAGTA-3'	5'-GCTGAGGCCGGGGCTTGTGAG-3'
K18	NG_008351.1	5'-CCTGTCCTTTCTCTCTCCCC-3'	5'-TCCCTCCTACCCCTTACCTG-3'
K14	NG_008624.1	5'-ACAAGGCACCAGCTCTGG-3'	5'TATGGGCACGCACCACTG-3'
PPARγ	NM_001100921.1	5'-ACG GGA AAG ACG ACA GAC AAA-3'	5'-GAC GGA GCG AAA CTG ACA CC-3'
C/EBPα	BC149006.1	5'-AGT CCG TGG ACA AGA ACA GC-3'	5'-GGT CAT TGT CAC TGG TCA GC-3'
Adipo Q	BC140488	5'-GATCCAGGTCTTGTTGGTCCTAA-3'	5'-GAGCGGTATACATAGGCACTTTCTC-3'
LPL	M16966	5'-TACCCTGCCTGAAGTTTCCAC-3'	5'-CCCAGTTTCAGCCAGACTTTC-3'
GAPDH	NG_006129.5	5'-TGAACGGGAAGCTCACTGG-3'	5'-TCCACCACCCTGTTGCTGTA-3'

RT-PCR: mRNA was isolated from the cell samples using TRIzol reagent (Invitrogen; Life Technologies Inc., Grand Island, NY) according to manufacturer instructions. To determine differentiation stage of the cells, various genes (both epithelial and adipogenic) were detected and quantified by RT-PCR with specific primers and QIAxcel Advanced system (QIAGEN Sample & Assay Technologies, Hilden, Germany). List of primers used are given in Table 1.

Determination of adipogenic differentiaton of MFPASC's by oil-red-O: Oil-red-O and elution index was followed as per the procedures given by Reza *et al.* (2014).

Statistical analysis: The statistical analysis was performed by ANOVA using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). Significant differences were detected (P<0.05) by Duncan's multiple range test using SAS.

RESULTS AND DISCUSSION

We demonstrated the formation of CLD's in bovine mammary epithelial cells (MAC-T) using the combination of α-Linolenic acid (ALA) and thiazolidinediones (TZD's) (Fig. 1). ALA is a omega-3 fatty acids found in plant and known as essential fatty acid and precursor of other two omega-3 fatty acids such aseicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found mainly in the sea fish, EPA and DHA and have been reported to have anti-inflamatory and anti-cancerous effects (Zheng *et al.* 2013).TZD's are also responsible for the synthesis of lipoprotein cholesterol, and binding PPARγ to activators reduces the levels of coactivators available for binding to pro-inflammatory transcription factors includng NK-$_k$B (Georgiadi and Kersten 2012). ALA and TZD's action on bovine epithelial cells (MAC-T) have demonstrated in the current study the activation of adipose specific transcripts (PPARγ, CEBPα, LPL and adipo-Q) and formation of CLD's in cytoplasm.

We received the confirmation of adipogenic activation with positive and significant (P<0.05/P<0.01) up-regulation of adipogenic genes/transcript and significant (P<0.05/ P<0.01) down-regulation of epithelial genes/transcript along with the microscopically evident cytoplasmic lipid droplets (Figs. 1, 2A) with ALA and TZD's treatment that we assume would have promoted a pre-lactation environment to the cell under treatment. Formation of cytoplasmic lipid droplets in differentiating secretory epithelial cells is a chronological ordered process regulated and we believe that ALA and TZD's treatment would have activated the switches on by increasing adipogenic gene/transcript expression and has certainly connected the development by providing fatty acid availability and that has attributed to the activation of lipid synthesis gene expression in the secretory epithelium and reduced lipid storage in mammary adipose tissue (Russell *et al.* 2007) leading to the formation of CLD's within the bovine mammary epithelial cells.

Determination of transcript/protein expression by immunoblotting/immunostaining and RT-PCR gene expression and quantification by QIAxcel Advanced system: Immunoblotting and immunostaining profile showed significant (P<0.05/P<0.01) down-regulation of epithelial specific transcript (K18 and K14) on 8 d compared to 0 d cells. Contrastingly, the adipose specific transcript (CEBPα and PPARγ) got significantly (P<0.05/P<0.01) up-regulated at 8 d compared d 0 (Figs 2B, 2C). Keratins are the fibrous structural proteins and key structural materials of epithelial cells (Reza *et al.* 2014) and in the presence of ALA and TZD's treatment the cells might have gained the activation of adipogenic gene/transcript expression i.e. (CEBPα and PPARγ) which caused the maximum formation of cytoplasmic lipid droplets and have shown significant differentiation and maturation of MAC-T cells in the present

Table 2.Quantified relative gene expression at different days of differentiation. Significance level */#p<0.05 and **/##P<0.01. * is for up-regulation and # is for down-regulation compared to control

Gene	Day0 (Conc. ng/ul)	Day8 (Conc. ng/ul)
K18	1.1	0.0##
K8	1.0	0.0##
K 14	0.55	0.0##
CEBP-alpha	1.0	15**
PPAR-gamma	0.0	1.0**
MUC1	1.85	0.6##
EpCAM	1.75	0.7##
Adipo Q	24	28
LPL	0.4	0.85**
GAPDH	13.5	12.75

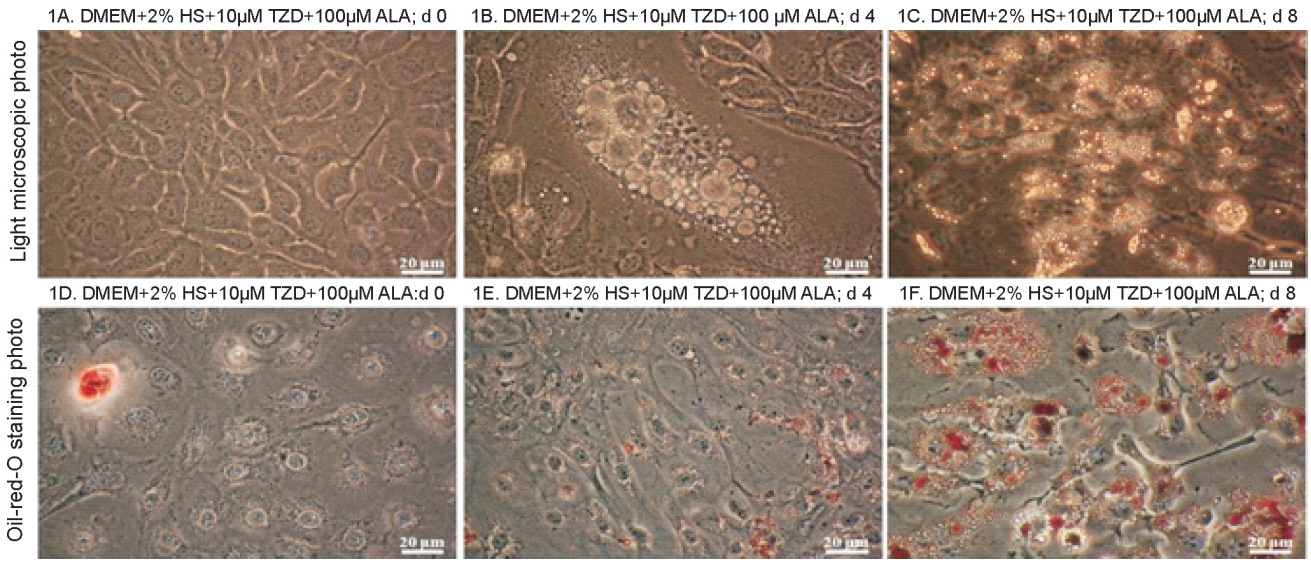
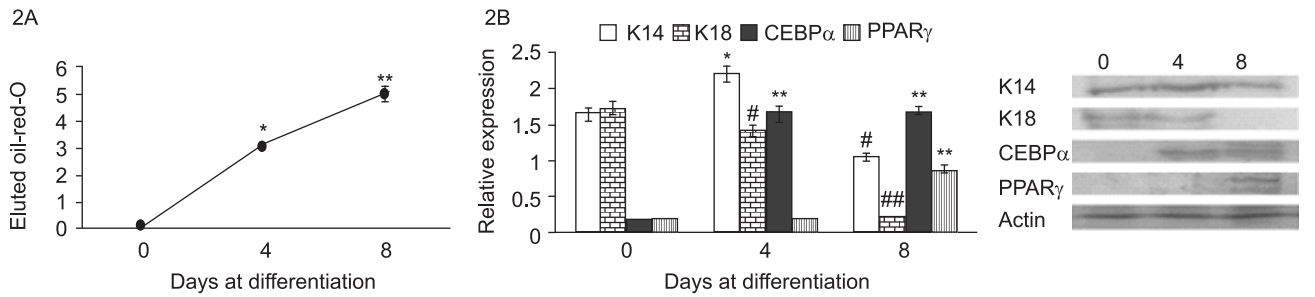


Fig. 1. Light photomicrograph of MAC-T cells at different days of differentiation before and after oil-red-o staining. 1A) Control cells at 0 d, photograph showing no CLD's formation and normal morphology of MAC-T cells. 1B) Photograph at 4 d of differentiation, few numbers of cells showing CLD's formation, with bulged and very large appearance. 1C) Photograph at 8 d of differentiation showing formation of CLD's in almost all cells. 1D) Oil-red-o stained cells at 0 d, the cells did not retain oil-red-o and there is no red colour formation.1E) Oil-red-o staining at 4 d of differentiation, some cells retained oil-red-o stain in their cytoplasm and most of the cells did not retained oil-red-o. 1F) Oil-red-o staining at 8 d of differentiation, almost all cells retained oil-red-o stain and oil-red-o deposited as a cluster of droplets.



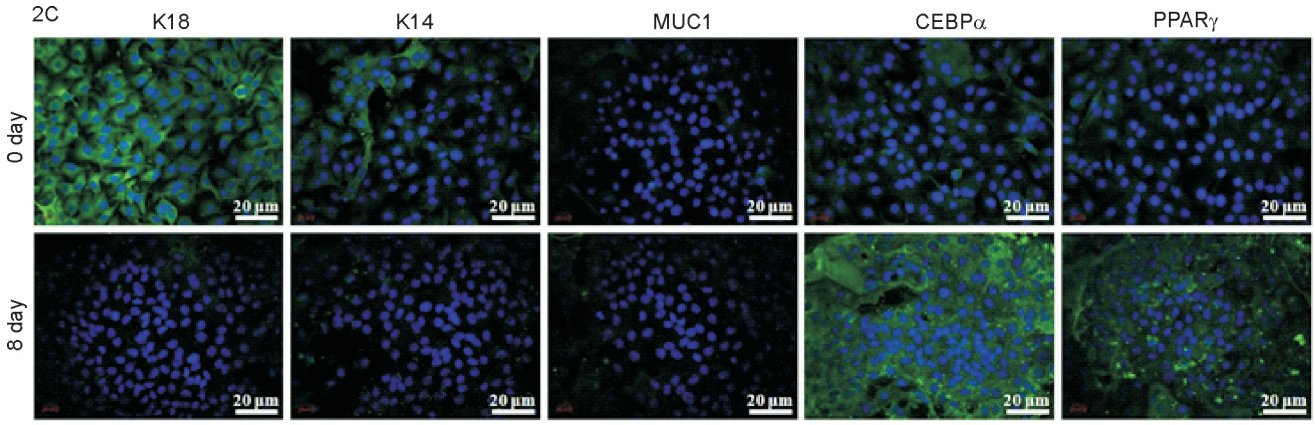


Fig. 2. Quantification of oil-red-o stain retention and confirmation of transcripts/protein expression pattern through immunostaining and Western blotting at different days of differentiation. 2A) Quantification of oil-red-o stain retention, graph showing a sharp steady increase in oil-red-o stain retention from 0 d onward to 8 d. 2B) Western blot profiling of protein expression pattern. 2C) Confocal microscopic photograph of immunostained cells, the adipogenic transcript (CEBPα and PPARγ) got up-regulated at 8 d of differentiation comparative to d 0. Significance level $^{*,\#}P<0.05$ and $^{**,\#\#}P<0.01$. * is for up-regulation and # is for down-regulation compared to control

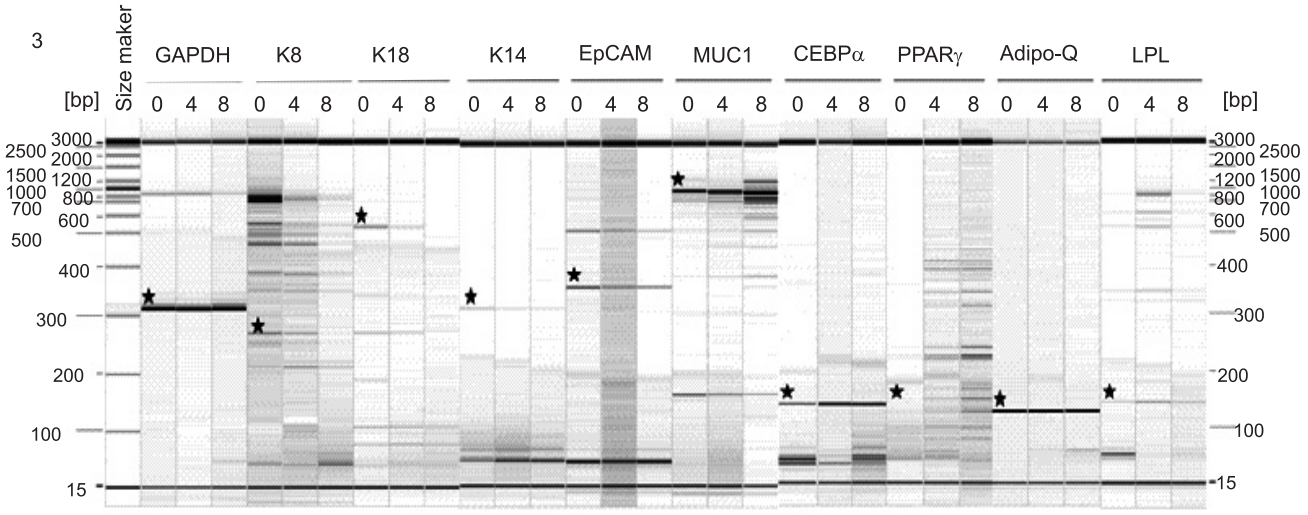


Fig. 3. Quantification of relative gene expressions during differentiation by using RT-PCR with specific primers and QIAxcel Advanced system for automatic electrophoresis and visualization. The automatically visualized band showing up-regulation of adipose specific genes and subsequent down-regulation of epithelial specific band. The star (*) indicate the band with desired base pair size.

study. Down-regulation of K18, K8, K14, MUC1 and EpCAM gene expressions when quantified on real time basis (Fig. 3 and Table 2) and their protein signatures were also similarly observed in the immunoblot and immunostain methodologies. In our previous research, we showed that TZD's alone and TZD's plus ALA both can accumulate cytoplasmic lipid in mammary adipose cells, however, TZD's alone failed to accumulate the lipid in the form of droplets, but the combination of TZD's and ALA had successfully resulted in the lipid accumulation in the form of CLD's in mammary adipose cells (Reza *et al.* 2013).

ALA and TZD's have been shown that the duo drug in combination could induce the formation of CLD's in the mammary epithelial cells efficiently without the formation of any lesions or inflammations. Therefore, we now presume that ALA and TZD's could cause safe differentiation of mammary epithelial cells and differentiating them to maturity would make them less vulnerable to carcinogenesis compared to the immature undifferentiated cells (Britt*et al.* 2007).

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