Milk-derived mammary epithelial cells as non-invasive source to define stage-specific abundance of milk protein and fat synthesis transcripts in native Sahiwal cows and Murrah buffaloes

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Received: 10 December 2018 / Accepted: 20 February 2019 / Published online: 26 February 2019
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Abstract

The molecular physiology of milk production of two important dairy species; Sahiwal cows (*Bos indicus*) and Murrah buffaloes (*Bubalus bubalis*) are not fully understood due to constraints in obtaining mammary tissue samples because of sacred and ethical reasons. The present study suggests the use of milk-derived mammary epithelial cells (MECs) as a non-invasive method to understand molecular aspects of lactation biology in dairy animals. A total of 76 MECs were collected from five different lactation periods viz. colostrum (0–2), early (5–20), peak (30–50), mid (90–140) and late lactation (> 215 days) stages from Sahiwal cows and Murrah buffaloes to study the transcription kinetics of milk protein, fat synthesis, and their regulatory genes. Significant changes were observed in milk composition of both dairy species with lactation stages. High mRNA abundance of all milk protein and fat synthesis genes was observed in MECs of Murrah buffaloes as compared to Sahiwal cows. The mRNA abundance of caseins (*CSN1S1*, *CSN1S2*, *CSN2*, and *CSN3*) and whey protein (*LALBA*, *LF*) were higher in early lactation stage. Similarly, the expression of milk fat synthesis genes (*SCD*, *BTN1A1*, *ACACA*, *GPAM*, *FAPB3*, *FASN*) was also high in early lactation stage. The relative abundance of 4 regulatory genes (*JAK2*, *STAT5*, *SREBF1* and *EIF4BP4I*) remained high during early lactation indicating their regulatory roles in lactogenesis process. Overall, results suggested a significant effect of lactation stages on milk composition and transcription abundance of milk protein and fat synthesis genes. The present study establishes the fact that milk-derived MECs could be utilized as a valuable source to understand mammary gland functioning of native cows and buffaloes.

Keywords Sahiwal cows · Murrah buffaloes · Lactation stages · Mammary epithelial cells · Gene expression · Milk and fat synthesis genes

Introduction

Cattle and buffaloes are the two most important livestock species that play a major role in the agricultural economy of the world. To meet future food demand and for sustainable

agriculture, it is necessary to improve the animal productivity of these native breeds by employing newer technological advancements. Amongst several factors related to animal productivity, the difference in lactation length and productivity are two major areas of concern to the animal breeders. A huge economic interest of dairy industries has been focused on improving the health benefits of dairy products by identifying the factors affecting the milk composition. Previous studies reported the milk composition changes with lactation stages (Kay et al. 2005; Stoop et al. 2009; Idamokoro et al. 2017). Additionally, there is a great inquisitiveness to determine the differences in milk production ability of animals at the genetic level.

India is home to some of the best breeds of two dairy species: zebu cattle (*Bos indicus*) and riverine buffaloes (*Bubalus bubalis*) that form an integral component of

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agriculture system in terms of milk production and draft power (Nanda and Nakao 2003; Singh et al. 2009). In recent years, few studies have been attempted to characterize candidate genes associated with milk production in Indian cattle and buffaloes (Sodhi et al. 2007; Javed et al. 2011; Kishore et al. 2013). Nowadays, there is a growing interest in understanding the physiology of lactation in indigenous dairy animals particularly to explore genes control the regulation and composition of milk. The milk composition significantly differs in breeds, species and depends upon lactation stages and other various genetic and environmental factors (German and Dillard 2006). To date, expression kinetics of important genes regulating milk production is poorly understood in Indian zebu cattle and riverine buffaloes. The major limitation had been getting the mammary biopsies from the live experimental animals due to various ethical and religious issues. To overcome this limitation, the study utilizes milk-derived mammary epithelial cells (MECs) as an alternative source to represent mammary tissue across lactation stages. As MECs are responsible for converting most precursors into milk constituents and transporting them to the mammary lumen, thus suggesting these cells could be used as a potential cellular model to unravel the mammary gland biology of cattle and buffaloes. The use of these cell types possibly would provide a better understanding of gene expression pattern covering whole lactation period starting from early to peak lactation and to late lactation stages. In past, several studies have supported the use of milk-derived MECs for transcriptional studies mainly in Holstein cows (*Bos taurus*) (Bionaz and Looor 2011; Sigl et al. 2012). Moreover, studies have linked MECs count with daily milk yield and shape of lactation curve (Capuco et al. 2003; Boutinaud and Guinard-Flament 2004; Boutinaud et al. 2008). Milk yield and its composition (caseins/whey proteins & fat content) change proportionally to lactation curve/stages that act as a determinant of milk quality (Capuco et al. 2003; Sigl et al. 2012). Hence, cellular mechanistic approach and molecular events in mammary epithelial cells could provide a better way to comprehend the milk synthesis across lactation period. Protein and fat synthesis in mammary gland and their secretion via MECs is regulated at several steps at transcriptional and translational level (Kadegowda et al. 2009; McFadden and Corl 2010; Rudolph et al. 2010). In past, regulatory roles of transcription factors like *SREBF1*, *PPAR γ* , and *STAT5 α* in milk production have also been studied in *Bos taurus* cows (Kadegowda et al. 2009; Menzies et al. 2010; Rudolph et al. 2010; Bionaz and Looor 2011), however, their pattern of expression across lactation stages has not been studied yet in zebu cows and riverine buffaloes. Considering the above issues, the present study was designed to determine the effect of lactation stages on transcription kinetics of milk proteins (caseins and whey), fat synthesis and regulatory genes in colostrum and milk-derived MECs of Sahiwal cows

(SAC) and Murrah buffaloes (MUB), the two major dairy species of India.

Materials and methods

Animals used in the study and sample collection

Healthy and multiparous animals from SAC and MUB maintained at National Dairy Research Institute, Karnal, India were included in the study. The lactation stages considered as colostrum (0–2 days, $n=5$ SAC, 5 MUB), early (5–25 days, $n=10$ SAC, 5 MUB), peak (30–50 days, $n=10$ SAC, 5 MUB), mid (90–140 days, $n=10$ SAC, 5 MUB), late (>215 days, $n=10$ SAC, 5 MUB) stages. A total of 70 milk samples (1–2 litres); 45 of SAC and 25 of MUB were collected from both the species and were immediately processed for epithelial cell isolation. All the samples were collected early morning (5.00 AM–6.00 AM) and immediately brought to the laboratory in a sterile jar. All procedures followed were in accordance with guidelines provided by the institutional ethical committee.

Milk composition analysis

Milk samples collected from 5 healthy animals each of SAC and MUB during different lactation stages (colostrum, early, peak, mid and late) were transported to the laboratory at 4 °C. The milk composition of each sample was determined by determining fat, protein, solid-not-fat (SNF) and lactose content using a milk analyser (Master-Pro). Milk yield data were also collected for both dairy species throughout the lactation period.

Isolation of MECs from fresh milk of SAC and MUB

Initially, the milk samples were filtered to remove dust particles and other contaminants with a sterile muslin cloth. The samples were centrifuged at $800\times g$ for 20 min at 4 °C to defat them. The resulting somatic cell pellets were washed twice with 1X PBS. MECs were isolated from somatic cells by immune magnetic cell binding separation technique using Dynabeads (Pan Mouse IgG, Dyna Biotech, Invitrogen) coated with anti-mouse Cytokeratin 18 (clone K8.13, Sigma-Aldrich Chimie). The detailed protocol followed to purify MECs from milk was described in our previous studies (Jatav et al. 2016). The purified cells were stored in trizol at -80 °C for RNA isolation.

RNA extraction and cDNA preparation

Total RNA was extracted from purified MEC samples ($n=70$) harvested across different lactation stages using

Trizol (Invitrogen Corp., CA). RNA was purified using RNase-free DNase treatment through silica membrane columns (RNeasy Mini Kit columns, Qiagen, Germany). The RNA samples were quantified using Nanovue (GE healthcare). First strand cDNA was synthesized using 100 ng RNA with Revert aid first strand cDNA synthesis kit as per instruction manual (Fermentas, Thermo Scientific). The mixture was incubated at 65 °C for 5 min and kept on ice for 3 min. A total of 6 µL of master mix composed of 5× enzyme buffer, 1 µL M-MuLV RT (Fermentas) was added. The reaction cycle for cDNA synthesis was performed in a

PCR cycler (Eppendorf, Germany) using the program: 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min. Before performing qPCR, the freshly prepared cDNA was diluted 1:4 (v:v) with DNase-/RNase-free water.

Quantification of target gene expression by qPCR

To facilitate the real-time PCR analysis, primers were selected from the literature. The primer details of the selected target genes were listed in Table 1. The qPCR was performed using the StepOne Plus instrument (Applied

Table 1 Details of primer sequences, amplification temperature, slope and PCR efficiency of the target and reference genes

Gene symbol	Primers 5'–3' (forward, reverse)	T_a (°C)	PCR efficiency	Slope	R^2
<i>CSN1S1</i>	ATGAACTTCTCATCTTACCTGTCTT CCAATATCCTTGCTCAGTTCATT	60	97.137	– 3.386	0.991
<i>CSN1S2</i>	AGCTCTCCACCAGTGAGGAA GCAAGGCGAATTTCTGGTAA	60	100.147	– 3.318	0.998
<i>CSN3</i>	TGCAATGATGAAGAGTTTTTTCCTAG GATTGGGATATATTTGGCTATTTGT	60	94.151	– 3.013	0.989
<i>CSN2</i>	AGAGTGAGGAACAGCAGCAAACAG AAGGCGGCACCACCACAGG	60	102.28	– 3.468	0.989
<i>LF</i>	GAACATCCCCATGGGCCT CAGCCAGGCACCTGAAAG	60	101.11	– 3.296	0.991
<i>LALBA</i>	CTTACTTCACTCCCCTTAGCA GACAGAAGCAGCAAAGACAGC	60	92.84	– 3.501	0.978
<i>LPL</i>	ACACAGCTGAGGACACTTGCC GCCATGGATCACCACAAAGG	60	96.975	– 3.397	0.980
<i>BTN1A1</i>	CAGTCACTGATGGGCTT GACAGTGGGATCTCCTTTG	60	106.139	– 3.191	0.986
<i>SCD</i>	TCCTGTGTGTGTGCTTCATCC GGCATAACGGAATAAGGTGGC	60	97.138	– 3.386	0.896
<i>ACACA</i>	CATCTTGTCCGAAACGTCGAT CCCTTCGAACATACACCTCCA	60	92.26	– 3.52	0.981
<i>FASN</i>	CCGACTAGAATCTGTCCC CCAGGGAGCTGTGAATAA	60	106.20	– 3.181	0.987
<i>GPAM</i>	GCAGGTTTATCCAGTATGGCATT GGACTGATATCTTCCTGATCATCTTG	60	102.25	– 3.26	0.989
<i>SREBF1</i>	CACGCTGGAGAAGCTCGG CTGGAGGTCACAGTGGTC	60	97.98	– 3.382	0.956
<i>FABP3</i>	GAACCTCGACTCCCAGCTTGAA AAGCCTACCACAATCATCGAAG	60	102.93	– 3.047	0.924
<i>JAK2</i>	TCTGGTATCCACCCAACCATGTCT AATCATGCCGCCACTGAGCAA	60	95.68	– 3.116	0.998
<i>STAT5</i>	GTGAAGCCACAGATCAAGCA TCGAATTCTCCATCCTGGTC	60	104.142	– 3.222	0.988
<i>EIF4EBP1</i>	GAA CTC ACC TGT GAC CAA GA CTCAAACCTGTGACTCTTCACC	60	98.56	– 3.362	0.981
<i>RPL4</i>	TTGGAAACATGTGTCGTGGG GCAGATGGCGTATCGCTTCT	60	112.86	– 3.323	0.954
<i>EEF1A1</i>	CATCCCAGGCTGACTGTGC TGTAAGCCAAAAGGGCATGC	60	102.392	– 3.266	0.994
<i>GAPDH</i>	TGGAAAGGCCATCACCATCT CCCCTTGATGTTGGCAG	60	102.91	– 3.254	0.984
<i>ACTB</i>	GCGTGGCTACAGCTTCACC TTGATGTCACGGACGATTTT	60	106.08	– 3.184	0.997

Biosystems) in a 96-well optical plate (Applied Biosystem). Each reaction was comprised of 10 µL consisting 4 µL diluted cDNA combined with 6 µL of a mixture composed of 5 µL 2× Syber Green with ROX master mix (Fermentas), 0.4 µL each of 10 µM forward and reverse primers, and 0.2 µL DNase/RNase-free water. For each gene, samples were analyzed in duplicate (technical replicates) along with a 6-point relative standard curve and the non-template control. Prior to qPCR, primer specificity was further confirmed in a 20 µL PCR using the same protocol described for qPCR except for the final dissociation protocol. The accuracy of primer pairs was also ensured by the presence of a unique peak during the dissociation step at the end of qPCR.

Statistical analysis

The geometric mean of 4 reference genes *viz.* *GAPDH*, *ACTB*, *RPL4*, and *EEF1A1* identified previously was utilized to normalize target gene data (Jatav et al. 2016). The ΔC_T values were calculated individually for each target gene as $\Delta C_T = C_T$ (reference gene) – C_T (target gene) (first derivative method) (Pfaffl 2001). Statistical analysis of MEC expression data was assessed by One-way ANOVA and Two-way ANOVA to study the effect of stage and species using SPSS V.20 software and a *p* value of <0.05 was considered significant. Effect of lactation stages on milk composition and gene expression values were determined by a general linear model (GLM) using SAS and SPSS V.20 statistical tools.

Results

For the present study, a total of MECs (*n* = 70) purified from the milk of SAC and MUB covering different stages of lactation were utilized to determine the changes at transcription level of important milk protein genes, fat metabolism-related genes, and specific regulatory genes. The PCR efficiency of

target genes varied from 90 to 108% with high linear Pearson's coefficient of *r* > 0.9 (Table 1).

Changes in milk yield and composition across lactation stages

Milk yield was increased gradually from colostral stage to peak lactation in both species and was highest (13.23 kg/day-SAC, 24.56 kg/day-MUB) at peak stage. It decreased after peak to late lactation to 3–4 kg/day in both species. Significant changes were noticed in SAC and MUB milk composition across lactation stages (Table 2). In SAC, Milk fat was highest (5.9%) at the colostral stage and then decreased gradually to peak lactation (4.18%). A similar trend was followed in MUB except that fat % was maximum at late lactation stage. Likewise, lactose (5.96% SAC; 7.46% MUB) and solid-not-fat (SNF) (10.92% SAC; 13.51% MUB) was significantly (*p* < 0.05) higher at a colostral stage in both species and decrease gradually to peak lactation. The protein content did not exhibit many changes throughout the studied lactation period in SAC; however, in MUB it was highest at early lactation stage and decreased thereafter. MUB milk was richer in fat, SNF, lactose and protein content than SAC at all lactation stages. Changes in the fat–protein ratio (FP ratio), a known indicator of negative energy balance is shown in Table 2.

Transcript abundance of milk protein genes in MECs of cows and riverine buffaloes

The results showed varied transcript abundance of major milk protein genes in MECs harvested across different lactation stages of both SAC and MUB. The expression of caseins genes; *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* and whey protein gene; *LALBA* was significantly high (*p* < 0.05) in MECs purified during early lactation stage *vis a vis* in MECs purified during other stages of lactation (peak-, mid-, late-) (Fig. 1). The expression pattern of casein transcripts was more or

Table 2 Changes in composition and FP ratio of Sahiwal cows and Murrah buffaloes milk during different lactation stages

	Species	Colostrum	Early	Peak	Mid	Late
FAT (%)	SAC	5.9 ± 0.364 ^a	4.92 ± 0.345 ^b	4.18 ± 0.203 ^b	4.4 ± 0.344 ^b	4.52 ± 0.385 ^b
	MUB	8.82 ± 0.32 ^a	8.23 ± 0.57 ^a	7.75 ± 0.16 ^a	11.3 ± 0.5 ^b	11.27 ± 0.20 ^b
SNF	SAC	10.92 ± 0.362 ^a	9.23 ± 0.151 ^b	9.15 ± 0.154 ^b	9.47 ± 0.10 ^b	9.30 ± 0.091 ^b
	MUB	13.51 ± 0.40 ^a	11.32 ± 0.25 ^b	10.94 ± 0.32 ^b	11.05 ± 0.34 ^b	11.09 ± 0.07 ^b
Protein	SAC	3.26 ± 0.14 ^a	3.34 ± 0.05 ^b	3.3 ± 0.06 ^b	3.42 ± 0.03 ^b	3.36 ± 0.04 ^b
	MUB	5.15 ± 0.184 ^a	5.42 ± 0.15 ^a	4.66 ± 0.35 ^b	4.76 ± 0.186 ^b	4.05 ± 0.7 ^b
Lactose	SAC	5.96 ± 0.19 ^a	5.04 ± 0.08 ^b	4.98 ± 0.09 ^b	5.16 ± 0.051 ^b	5.04 ± 0.051 ^b
	MUB	7.46 ± 0.218 ^a	6.16 ± 0.12 ^b	5.86 ± 0.18 ^b	5.84 ± 0.233 ^b	6.03 ± 0.05 ^b
FP ratio	SAC	1.81	1.47	1.27	1.29	1.34
	MUB	1.51	1.52	1.75	2.37	2.78

Different letter shows significant changes (*p* < 0.05) across lactation stages in SAC and MUB

Fig. 1 Expression pattern of milk proteins caseins and whey proteins in MECs of SAC and MUB across different lactation stages. Statistical difference was determined using Two-way ANOVA by SPSS V2.0 $p < 0.05$ indicated by *, $p < 0.01$ by ** and $p < 0.001$ by ***

less similar in the two species. Further, in comparison to the colostrum stage, the expression of these transcripts was higher by 1.51- and 1.32-folds in MECs of SAC and MUB, respectively, harvested during early lactation stage (Fig. 1). Similarly, *CSN1S2* transcript showed 1.25- and 1.14-folds higher expression in early lactating MECs *vis a vis* colostrum samples of SAC and MUB, respectively (Fig. 1). The two other caseins, *CSN2* and *CSN3*, also showed higher expression in early lactating MECs of both species. The level of *CSN2* mRNA was slightly higher by 1.15- and 1.26-folds while, *CSN3* transcript expressed 1.05- and 1.33-folds higher in early lactating MECs over colostrum in SAC and MUB, respectively (Table 3).

Although the expression of protein transcripts showed a similar pattern in two species, the mRNA abundance of specific transcripts varied significantly between them, indicating the physiological differences of two major dairy species. All the four casein transcripts showed higher abundance in MUB in comparison to SAC across all lactation stages (Fig. 1; Table 3). During early lactation, *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* transcripts were found to be 1.35-, 1.44-, 1.18-, 1.77-folds higher in MUB, respectively (Fig. 1; Table 3). Similar to early lactation, mRNA abundance was higher in MUB at other lactation (peak-, mid-, late-) stages (Fig. 1; Table 3). Though all caseins showed high mRNA abundance

Table 3 Relative changes in mRNA levels of milk and fat transcripts in MECs of Murrah buffaloes and Sahiwal MECs during different lactation stages. Statistical significant analysed GLM by SPSS 20 V

	Colostrum	Early	Peak	Mid	Late
CSN1S1	1.44*	1.35	1.15	2.05***	1.79*
CSN1S2	1.57**	1.44*	2.02***	2.53***	1.96*
CSN2	1.07	1.17	1.72	1.88***	1.38
CSN3	1.41*	1.77***	2.44***	2.94***	2.56***
LALBA	0.90	1.2	1	0.73	0.27***
LF	1.13	0.74	1.01	1.58**	1.60**
FASN	2.53**	1.06	0.91	1.22	1.71**
ACACA	2.36***	2.06**	2.4**	1.32	1.63**
SCD	1.45*	1.67**	1.74**	1.41	1.40*
BTN1A1	1.58***	1.37*	1.50**	1.71***	1.45*
LPL	1.15	2.49***	1.67***	1.29	1.31
FABP3	1.97	1.32	1.89**	3.64***	2.04***
GPAM	0.76*	1.26	2.45***	2.15***	1.73***

*Change is significant at the $p < 0.05$ level

**Change is significant at the $p < 0.01$ level

***Change is significant at the $p < 0.001$ level

in early lactation, their individual abundance varied between SAC and MUB. Among all lactation stages: mid-lactation displayed the highest difference between the two species with significant ($p < 0.05$; $p < 0.01$) high caseins mRNA levels, i.e., *CSN1S1*: 2.05-folds; *CSN1S2*: 2.53-folds; *CSN2*: 1.88-folds; *CSN3*: 2.94-folds in MUB (Table 3). Among all caseins, *CSN1S1* mRNA was most abundant during early lactation stage followed by *CSN2*, *CSN1S2* and *CSN3* transcripts (Fig. 1).

Likewise, *LALBA* (alpha-Lactalbumin) mRNA encoding one of the two main whey protein also showed significantly ($p < 0.05$) higher abundance during early lactation in comparison to peak and mid-lactation (Fig. 1). The mRNA transcript for another important whey protein, *LF* (Lactoferrin) that is known for its functional benefits, particularly antimicrobial activities was also assessed across lactation. Its mRNA abundance was significantly ($p < 0.05$) high in colostrum as compared with other lactation stages with the consistent pattern though in both the species. (Fig. 1). However, its mRNA abundance increased significantly ($p < 0.05$) again at late lactation stage. The overall abundance of whey proteins (*LALBA* and *LF*) was significantly higher in MUB than SAC (Fig. 2), nonetheless no correlation was found between mRNA transcript levels of these whey proteins in both species.

Transcript abundance of milk fat synthesis genes in MECs of cows and riverine buffaloes

The mRNA abundance of several major milk fat synthesis genes like, *FASN* (fatty acid synthase) *ACACA* (Acetyl-coenzyme A carboxylase alpha), *SCD* (Stearoyl-CoA desaturase), *FABP3* (Fatty acid binding protein 3), *GPAM* (Glycerol-3-phosphate O-acyltransferase), *BTN1A1* (Butyrophilin) and *LPL* (Lipoprotein lipase) were also evaluated in colostrum

and MECs during different stages of lactation (Fig. 3). The results showed that genes involved in milk fat metabolism including fatty acid uptake, *denovo* fatty acid synthesis, fatty acid desaturation, fatty acid esterification and milk fat secretion occurring in mammary gland were also expressed in colostrum and MECs at high abundance in both the dairy species (Fig. 2). A significant effect of lactation stage on fat and protein percentage in milk and mRNA expression of protein and fat metabolic genes was observed in both SAC and MUB (Table 4). Similar to milk proteins, the transcripts related to fatty acid metabolism-related genes also showed maximum abundance during early lactation.

The mRNA abundance for most of the milk fat synthesis gene was higher (non-significantly) in MUB MECs. The changes in expression values for individual fatty acid genes analyzed in colostrum and MECs across different lactation stages in MUB compared to SAC is summarized in Table 3. The mRNA abundance of *SCD*, *BTN1A1* and *ACACA*, showed relatively higher abundances 1.66, 1.36, and 2.06, respectively, at $p < 0.001$ in early lactating MECs of MUB in comparison to SAC MECs. The mRNAs of *FASN* and *ACACA* key genes regulating *de novo* fatty acid synthesis showed maximum abundance in early lactation in both zebu cows and riverine buffaloes, however, their expression declined with progression of lactation stage (Fig. 3). Between the two genes, *FASN* mRNA abundance was relatively more in comparison to *ACACA* across all stages of lactation (Table 3). In our data set, *ACACA* mRNA was significantly ($p < 0.05$; $p < 0.01$; $p < 0.001$) more abundant in buffalo's colostrum and MECs at all lactation stages in comparison to SAC (Fig. 3; Table 3). However, *FASN* mRNA abundance in two dairy species was found to be comparable. Similar to *FASN* and *ACACA*, *SCD1* the major enzyme associated with synthesis of mono-unsaturated fatty acid synthesis showed

Fig. 2 Comparative mRNA abundance of milk protein genes in SAC and MUB MECs. Statistical significance was determined using One-way ANOVA by GraphPad Prism. $p < 0.05$ indicated by *, $p < 0.01$ by ** and $p < 0.001$ by ***

Fig. 3 Expression pattern of milk fat synthesis and metabolism genes in MECs across different lactation stages in SAC and MUB. Statistical difference was determined using Two-way ANOVA $p < 0.05$ indicated by *, $p < 0.01$ by ** and $p < 0.001$ by ***

maximum abundance in colostrum and MECs from early lactating zebu cows and riverine buffaloes. However, its expression declined along with the course of lactation. Again the transcript abundance of *SCD1* was significantly high ($p < 0.05$; $p < 0.01$; $p < 0.001$) in buffaloes across all stages of lactation (Fig. 3). The mRNA abundance of *GPAM* that encode enzyme for fatty acid esterification showed a similar pattern to *FASN*, *ACACA*, and *SCD1* mRNA. Its expression was maximum in colostrum and early lactating MECs in both the species. Interestingly, its abundance was higher ($p < 0.05$) in colostrum of SAC than that of MUB. Though, at all other lactation stages its

level was relatively higher in buffaloes than SAC (Fig. 3). Conversely, to expression pattern shown by other milk fat metabolism genes, *FABP3* mRNA that encodes fatty acid binding proteins showed maximum abundance during peak and late lactation stages in both cows and buffaloes (Fig. 3). Similar to other milk fat metabolism genes, the relative abundance of *FABP3* mRNA was higher ($p < 0.01$) in buffaloes than cows. The mRNA abundance of *LPL* that is responsible for uptake of lipoprotein fatty acids from circulation into the mammary gland and their transfer into milk was 2.49-fold higher ($p < 0.001$) during early lactation in MUB MECs in comparison to SAC

Table 4 Effect of lactation stages on fat and protein percentage and on mRNA abundance of milk protein and fat related genes in MECs of Sahiwal cows and Murrah buffaloes

Dependent Variable	SAC (<i>F</i> values)	Sig.	MUB (<i>F</i> values)	Sig.
Fat %	5.490	0.001	9.599	0.001
FASN	13.595	0.000	8.568	0.045
ACACA	6.897	0.000	64.950	0.000
SCD	2.555	0.054	24.547	0.000
GPAM	31.900	0.000	6.846	0.057
BTN1A1	12.121	0.000	32.730	0.000
FABP3	14.426	0.000	5.571	0.319
LPL	3.809	0.010	16.163	0.002
Protein%	20.774	0.000	10.042	0.000
CSN1A1	6.684	0.000	9.091	0.000
CSN1A2	9.776	0.000	7.839	0.001
CSN2	14.733	0.000	2.421	0.086
CSN3	9.901	0.000	2.341	0.094
LALBA	3.615	0.014	12.326	0.000
LF	42.375	0.000	7.676	0.001

MECs. This is in line with a higher abundance of fatty acids in milk in early stages of lactation.

Transcript abundance of regulatory genes in MECs of cows and riverine buffaloes

The relative mRNA abundance of four regulatory genes (*JAK2*, *STAT5*, *SREBF1*, and *EIF4BP41*) were also assessed across different lactation stages (Fig. 4). *STAT5* (Signal transducer and activator of transcription 5) is a mediator of prolactin signalling and play a major role in lactogenesis. Its mRNA abundance started increasing significantly ($p < 0.05$) during early lactation and reached a maximal level at peak- and mid-stages. Its abundance was significantly ($p < 0.05$) low in colostrum of both the species (Fig. 4). The *JAK2* (Janus kinase 2) mRNA level showed no major shift from colostrum to other lactation stages in both the species. Its mRNA abundance remained unchanged across lactation in both species (Fig. 4). Compared with other regulatory genes, *SREBF1* (Sterol Regulatory Element Binding Transcription Factor 1) mRNA showed a different expression pattern in two species. Regardless of the stage, its mRNA level was significantly ($p < 0.05$) on the lower side in MUB in comparison to SAC. Interestingly, no change in SAC was observed at different time points though, it decreased only slightly in buffaloes as the lactation progressed (Fig. 4). The mRNA abundance of *EIF4BP1* (also known as *4EBP1*) gene was also high during early- and peak-lactation stages in SAC (Fig. 4).

Discussion

Lactation cycle is a continuous process and several developmental and physiological changes occur in mammary gland with its progression that leads to a change in its functional ability, milk yield and composition. In dairy cattle, milk yield and milk components are two of the most important economic traits. To study lactation biology, it is important to collect mammary tissue samples which are quite challenging from live animals in India. Therefore, the present study was conducted to assess, the effect of lactation stages using MECs from two major dairy species to mimic the transcriptional changes occurring in the mammary gland.

Several factors have been identified to play an important role in influencing the composition of milk in ruminant animals, which include age, breed, parity, nutrition, lactation stage, and management, among others (Al-Saiady 2006; Oravcová et al. 2007; El-Tarabany and El-Bayoumi 2015). Our results clearly exhibit the changes in milk protein and fat metabolic gene expression and milk composition across lactation stages in both dairy species. Caseins are the most abundant proteins in milk and found to be maximally expressed at early lactation stages in both species- SAC and MUB and gradually decline towards the mid to late-stages of lactation.

The higher mRNA abundance of casein genes during early lactation stages in both species were in accordance with findings reported in taurine cattle (Colitti and Pulina 2010; Bionaz and Loo 2011; Sigl et al. 2012). In milk somatic cells of Holstein cows, higher transcript abundance of milk proteins during early lactation was observed using RNA seq data in comparison to mid (90 days) and late (250 days) stage (Wickramasinghe et al. 2012). The results were also in accordance with the expression profile of milk protein genes in the mammary gland of sheep and goat (Colitti and Pulina 2010; Suárez-Vega et al. 2015). Similar to the present study, few reports also showed that milk protein transcripts abundance get affected by stage of lactation in Holstein Frisian cows (Kadegowda et al. 2009; Sigl et al. 2012). A greater increase in expression for different caseins and whey protein genes during early lactation compared with other lactation stages in cows and sow mammary gland was reported in a previous study (Sigl et al. 2012). The casein expression was higher in MUB than SAC at all lactation stages was well correlated with higher protein content in buffalo milk as compared to cows. The high abundance of milk casein mRNA in buffaloes compared with cows was supported by the fact that buffalo milk contains 4.0 g of casein in 100 g of milk, whereas almost half less of casein in cow milk, 2.46–2.80 g/100 g (Zicarelli 2004). One of the important reason that could be put forth for the high abundance of casein transcripts during early lactation is about

Fig. 4 Expression pattern of transcriptional and translational regulators in MECs across different lactation stages in SAC and MUB. Statistical difference was determined using Two-way ANOVA. $p < 0.05$ indicated by *, $p < 0.01$ by ** and $p < 0.001$ by ***

the role that milk caseins play in sequestering calcium in milk thereby preventing mammary gland calcification (Holt et al. 2013). Another explanation for higher mRNA abundance could be linked to higher transcription rate for casein proteins during early lactation (onset of lactation). However, it would be interesting to note here that unlike changes observed in casein mRNA transcripts, in the present study, no major difference in caseins proteins level was observed along the course of lactation. This discrepancy may possibly be explained by the fact that both caseins and whey proteins get cleaved onto smaller bioactive peptides (Schanbacher et al. 1997). Due to this reason, the actual concentration of individual protein in milk is difficult to measure. Therefore, mRNA abundance and content of individual casein protein may sometime not derive a true picture of correlation. Silva and Malcata (2005) reported that the bioactive peptides cleavage of caseins and whey proteins are higher during the

initial stages of lactation which could be linked to higher transcription rate of genes encoding caseins and whey proteins. A study by Bevilacqua et al. (2006) put forth another explanation about the discrepancy in quantities of casein proteins in milk and suggested that the four caseins translate with different efficiency. *CSN1S1* and *CSN2* transcripts translate with higher efficiency as compared with *CSN1S2* and *CSN3* genes. Therefore, in spite of active transcription of genes for caseins and whey protein during early lactation, the efficiency of translation machinery might get compromised during the early postpartum period due to negative energy balance that could be the possible reason for lack of many major changes in milk proteins along the course of lactation. Similarly, high FP ratio in SAC showed negative energy balance at early lactation exhibiting the physiological stress could be due to parturition-based changes in the mammary gland.

Similar to caseins, *LALBA* mRNA was higher in MUB compared to SAC, though its pattern of expression across lactation stages was similar in both the species. *LF*, a glycoprotein member of transferrin family act as a multifunctional protein secreted by epithelial cells in mammary tissue and has an important role to play in autocrine regulation (Ntambi and Miyazaki 2003). Higher abundance in colostrum may be due to its contribution in transferring iron into the colostrum milk, and act as an important immune-proteins specialized to combat various infections in newborn calves (Siqueiros-Cendón et al. 2014). Whereas, the higher expression in late lactation stage could be due to its role in the developmental process and help mammary gland to be prepared for involution stage. Similar results were reported in bovine mammary epithelial cells providing evidence of the regulatory action of *LF* in mammary cells development and monitoring casein expression (Riley et al. 2008).

MECs are responsible for the synthesis and secretion of lipids, proteins, and carbohydrates for milk (Lemay et al. 2007). Milk fat droplets are secreted via an apocrine mechanism and bring with it a crescent of the MEC cytoplasm enveloped in the plasma membrane. Milk fat is synthesized by four major pathways: denovo synthesis, the breakdown of blood lipids, from the diet, by bio-hydrogenation (Chilliard et al. 2000; Stoop et al. 2009). Change in milk composition with lactation progression affect these pathways that further leads to a change in energy status of animals. In the present study, fat synthesis and metabolic genes were maximally expressed at early lactation stage and gradually decreased to mid-lactation, before rising again at late lactation. Denovo synthesis is activated by acyl-CoA synthetase and is reduced at negative energy balance state in animals. *SCD* is known to synthesize different isomeric forms of conjugated linolenic acid that determine the nutritional quality of milk towards human health (Dhiman et al. 2005). Higher abundance of *SCD* might be linked with differences in fat content in milk across lactation stages. Activity of *SCD1* is regulated by breed, diet and seasonal variations in ruminants (Dhiman et al. 2005; Slots et al. 2009). Changes in its mRNA abundance are consistent with another studies in Holstein cows showed a decrease in conjugated linolenic acids in mid-lactation stage (Slots et al. 2009). Similar trend of fat synthesis and metabolic genes was reported in somatic cells of Holstein Frisian cows (Wickramasinghe et al. 2012). *GPAM* and *BTN1A1* that are involved in lipid droplet formation, followed the same pattern showing higher abundance in early lactation and declined thereafter as the lactation progressed (Fig. 3). Similar expression pattern of fat/lipid synthesis genes were reported in mammary gland and somatic cell transcriptome analysis (Bionaz and Looor 2011; Sigl et al. 2012; Wickramasinghe et al. 2012). *LPL* responsible for uptake and release of long chain fatty acids from digestive tract or mobilization of body fat. In the present study, the

LPL was higher at early lactation (MUB) and at late lactation in both species. Higher expression of *LPL* could be because of increase in the rate of mobilization of fat in negative energy balance state (Bauman and Griinari 2003). Mammary gland undergoes several developmental changes in early/late lactation and this change in fat content and associated genes could reflect their dependency on lactation stage. Higher fat content in milk and *LPL* mRNA abundance level at earlier lactation stages, could be as a result of the process called lipolysis caused by natural or bacteria enzymes in the mammary glands at the later stages of lactation (Strzałkowska et al. 2009).

The *FABP3* is also referred to as mammary-derived growth inhibitor (*MDGI*) because of its high abundance in the mammary gland and is mainly involved in fatty acid uptake and intracellular metabolism of long chain fatty acids. It has a higher affinity for long chain fatty acids (LCFA), particularly saturated LCFA and subjects them towards esterification into milk triglycerides (Bionaz and Looor 2008). This gene might be playing a dual role in the mammary gland for the synthesis of milk fat, and transfer of LCFA during triacylglycerol synthesis. Higher mRNA abundance of *FABP3* gene in MUB appears to be supported by the fact that buffalo's milk has substantially higher fat content in comparison to cow's milk. Its higher expression observed in MECs at late lactation stages could be related to higher fat content normally seen during late lactation of dairy animals (Mech et al. 2008). Similar findings were observed in mammary gland tissues from different lactation period (Bionaz and Looor 2008).

The higher induction of *STAT5* during lactation in response to stimulus from lactogenic hormones is a well-established fact. Being regulator of a prolactin—a major inducer of milk production—*STAT5* has also been recognized as a major candidate gene for milk production trait in cattle (Wheeler et al. 2001; Yang et al. 2016). Liu and co-workers showed that *STAT5A* deficient mice failed to undergo lactation and their mammary development was impaired (Liu et al. 1998). Our findings also support the earlier evidence stating that *STAT5* as the transcriptional regulator of milk proteins in mammary gland tissues and showed a negative correlation with fat metabolic genes in both species (Paten et al. 2015). *JAK2* is known to activate *STAT5* and regulate expression of insulin-like growth factor I (*IGF-I*) in the mammary gland. Recently, one mutation in the *JAK2* gene has shown to be associated with higher milk, protein and fat yield in cattle (Szewczuk 2015). It is important to note that *SREBF1* is an important member of a transcriptional network that regulates the lipogenic genes. Similar findings of higher abundance of *SREBF1* mRNA in lactation stage than involution in buffalo mammary gland was observed in one of our previous study (Sharma et al. 2014). The other members of this network include *PPARG*,

SREBF1, and *SREBF2*. The *PPARG* expression in bovine mammary during the onset of lactation has been linked to concomitant upregulation of *SREBF1* and *SREBF2* (Piantoni et al. 2010). Although *SREBF1* is the key member in activating the lipogenic machinery, it is interesting to realize that its expression remained stable even during the late stages of lactation. Though the present study did not include *SREBF2*, still, we believe that both these transcription factors have overlapping function in regulating the milk fat synthesis in MECs. A study has also reported *EIF4EBP1* higher abundance in the mammary gland during lactation than non-lactation period (Toerien and Cant 2007). This is somewhat intriguing due to the fact that *EIF4EBP1* is known to encode a translation repressor protein that inhibits translation initiation, part of the core of mTOR signaling (Bionaz and Loor 2011). In conclusion, our study has successfully utilized milk-derived MECs to unfold the mRNA expression pattern of milk and fat related genes in native cows and riverine buffaloes. The present findings suggest the MECs could be utilized as an alternate non-invasive source to understand the mammary gland functioning in dairy species.

Acknowledgements The work was supported by Indian Council of Agriculture Research, New Delhi under the National Agriculture Innovation Project. The authors duly acknowledge Director, National Bureau of Animal Genetic Resources for providing the research facilities to carry out this study.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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