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Milk fat globule is an alternative to mammary epithelial cells for gene expression analysis in buffalo

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Owing to the difficulty in obtaining mammary gland tissue from lactating animals, it is difficult to test the expression levels of genes in mammary gland. The aim of the current study was to identify if milk fat globule (MFG) in buffalo milk was an alternative to mammary gland (MG) and milk somatic cell (MSC) for gene expression analysis. Six buffalos in late lactation were selected to collect MFG and MSC, and then MG was obtained by surgery. MFG was stained with acridine orange to successfully visualise RNA and several cytoplasmic crescents in MFG. The total RNA in MFG was successfully isolated and the integrity was assessed by agarose gel electrophoresis. We analysed the cellular components in MFG, MG and MSC through testing the expression of cell-specific genes by qRT-PCR. The results showed that adipocyte-specific gene (AdipoQ) and leucocyte-specific genes (CD43, CSF1 and IL1α) in MFG were not detected, whereas epithelial cell marker genes (Keratin 8 and Keratin 18) in MFG were higher than in MSC and lower than in MG, fibroblast marker gene (vimentin) in MFG was significantly lower than in MG and MSC, milk protein genes (LALBA, BLG and CSN2) and milk fat synthesis-related genes (ACC, BTN1A1, FABP3 and FAS) in MFG were higher than in MG and MSC. In conclusion, the total RNA in MFG mainly derives from mammary epithelial cells and can be used to study the functional gene expression of mammary epithelial cells.

Keywords: Buffalo, milk fat globule, mammary epithelial cell, milk somatic cell.

Along with the development of the economy and society, the requirement for milk products is increasing. It is well-known that the quantity and quality of milk are influenced by nutritional supply (Bauman & Griinari, 2003), pathologic status (Xavier et al. 2009) and genotype (Avondo et al. 2015). A large body of research shows that milk is synthesised and secreted in mammary epithelial cell (MEC) (Lu et al. 2014). Accordingly, there is an interest in analysing gene expression in mammary epithelial cells.

Currently, there are a large number of alternatives to study the functional gene expression of mammary epithelial cell in many species. The traditional and classical approach is to take advantage of mammary gland (MG) tissue via slaughtering (Cui et al. 2014) or percutaneous biopsies (Bionaz & Loor, 2008). The advantage of the method is to be able to directly reflect the real expression status of the mammary gland. However, owing to the value of dairy livestock, especially buffalo, it is difficult to obtain mammary gland without considerable expense as well as risk to the animal's well-being. In addition, mammary tissue contains fibroblasts and adipocytes, and the proportion of epithelial cells is only around 73–79% (Capuco et al. 2001), so a tissue sample maynot reflect the real expression level of functional genes in the mammary epithelial cell. Consequently, researchers have isolated total RNA from milk somatic cells (MSC) to detect gene expression in mammary epithelial cells (MEC) (Boutinaud et al. 2002; Murrieta et al. 2006; Wickramasinghe et al. 2012). This method is very convenient and has the advantage of being non-invasive, nevertheless, due to the existence of large leucocyte populations and the low proportion of mammary epithelial cell in milk (2–15%) (Lindmark-Mansson et al. 2006), the milk somatic cells are also unable to reflect the real status of the mammary epithelial cell.

Thus, it is important to gain an effective alternative to mammary gland tissue through a non-invasive, economical

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and convenient technique. Total RNA has previously been isolated from human milk fat globules (MFG) (Maningat et al. 2007) for examination of the expression profile of human MEC during lactation (Maningat et al. 2009), the gene expression changes of MEC during short-term administration of recombinant human GH (Maningat et al. 2011), gene regulatory changes of UDP-galactose synthesis and transport (Mohammad et al. 2012) and lipid synthesis and milk fat production (Mohammad & Haymond, 2013). In fact, this method has also been used in animals (Brenaut et al. 2012; Cánovas et al. 2014; Choudhary et al. 2015).

In our study, we further identified that the transcript of MFG could be used to study the functional gene expression of mammary epithelial cells in animals, especially genes of milk component synthesis. This provides another alternative for the research communities to study the molecular mechanism of milk components synthesis, nutritional regulation and the development of MEC.

Materials and methods

Ethics statement

The mammary gland tissue samples were collected in compliance with the institutional guidelines and under a protocol approved by the Institutional Review Board of Guangxi University.

Animals and collection of milk sample and mammary gland tissue sample

To minimise the losses caused by trauma, six disease-free buffaloes in late lactation (467, 388, 1017, 455, 184, 239 d of milk in 1st to 4th lactationswith milk yield of 2·3 ± 1·3 litre) were selected at the farm of Guangxi Buffalo Research Institute, China. In the farm, the buffaloes were milked twice a day. Firstly, the udders of the six buffaloes were cleaned and disinfected. The milk was collected by hand. The first 500 ml of milk was discarded to avoid obtaining cistern milk and the latter 450 ml of milk was transported aseptically to the laboratory in ice within 30 min. After that, the collection site (the right or left rear quarter of the mammary gland) of the buffaloes was shaved and sterilised thoroughly with iodine tincture (Yueda, Linyi, China) and medical alcohol, and the skinwas anesthetised with 0·1 ml xylazine hydrochloride (Huamu, Changchun, China). The skin, fat and connective tissue of the site was incised with a sterile scalpel and the mammary gland was collected, snap-frozen in liquid nitrogen and then stored at −80 °C until used for total RNA extraction. After collection, 400 IU penicillin (Yuanzheng, Shijiangzhuang, China) was added into the trauma site and then the trauma site was sutured.

Collection of milk fat globule and milk somatic cell

The milk fat globules and milk somatic cells were collected as described earlier (Maningat et al. 2007; Wickramasinghe et al. 2012). Briefly, the transported milk was divided into two parts. 50 ml milk was centrifuged at 3000 ***g*** for 10 min at 4 °C, the fat layer was transferred into a new tube using a spatula, and then Trizol (Invitrogen Life Technology) was added into the tube at a proportion of 1 ml per 200 mm^3. 400 µl 0·5 M EDTA was added into other 400 mL milk at a concentration of 0·5 mM, and then the milk was centrifuged at 3000 ***g*** for 10 min at 4 °C, the fat layer and the skimmed milk were discarded, the milk fat attached to the tube was wiped with sterile gauze. The cell pellet was washed with PBS and then suspended with PBS-0·5 mM EDTA, and then was filtered into a new tube with sterile gauze to remove impurities. The suspended cell pellet was centrifuged at 1800 ***g*** for 10 min at 4 °C, and then the supernatant was discarded, 1 ml Trizol was added into the tube. The collected milk fat globule fraction and milk somatic cells were stored at −80 °C until used for total RNA extraction.

Detection of cytoplasmic crescents

A separate 45 ml sample of milk was collected from a different healthy late lactation buffalo and transported to the laboratory. The milk sample was mixed with 5 ml of 0·1% acridine orange and incubated for 5 min at room temperature. The mixture was centrifuged at 3000 ***g*** for 10 min at 4 °C, the fat layer was transferred into 60 mm petri dish and flattened with a spatula.The dish was visualised with a fluorescent microscope (Nikon, Japan) with appropriate excitation and emission filters.

Isolation and detection of total RNA

Total RNA was isolated from various samples as described (Maningat et al. 2007) in manual. The treated milk fat globule was firstly centrifuged at 12 000 ***g*** for 10 min, the fat layer was discarded and then the samples were conventionally treated to isolate total RNA. After isolation, the RNA concentration was confirmed by A260/280 using a Nanodrop spectrophotometer (Quawell 5000 UV-vis Spectrophotometer, America) and the quality of total RNA was assessed by the presence of distinct intact 28S and 18S ribosome RNA bands in an electrophoresis gel. The isolated total RNA was stored at −80 °C.

Synthesis of cDNA

The first-strand cDNA was synthesised with Primescript™ reagent Kit (Zhao et al. 2012) (Takara, Dalian, China). The genomic DNA in total RNA was eliminated with gDNA Eraser and the reverse-transcription reaction proceeded with Primescript RT Enzyme. Then the cDNA solution was diluted 10-fold with sterile water for PCR reaction. The diluted cDNA solution was stored at −20 °C.

Quantitative real-time PCR

Total RNA was isolated and cDNA was synthesised as described above. All quality control steps conformed to the MIQE guidelines (Bustin et al. 2009). Based on the

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Table 1. Detailed information of primers used for qRT-PCR

Gene	Primers 5′–3′ (forward, reverse)	Product length
Keratin 8	AGTGGCTACGCAGGTGGACT CCGCAAGAGCCTTTCACTTG	199 bp
Keratin 18	GCAGACCGCTGAGATAGGA GCATATCGGGCCTCCACTT	144 bp
IL1-1α	GGCCAAAGTCCCTGACCTCT CTGCCACCATCACCACATTC	224 bp
CSF1	CTCCCTCTTGCCCAGAGAG ACGTCTTCCATCCCAGTGAC	186 bp
CD43	CTCGTGGCCTTAATCCTGTTG TCTACTGCCCCATTGCGTT	95 bp
AdipoQ	GATCCAGGTCTTGTTGGTCCTAA GAGCGGTATACATAGGCACTTTCTC	131 bp
Vimentin	GACTCGGTGGACTTCTCGTTG CTCTCGCATCTCCTCCTCGT	225 bp
ACC	TCCTGCTGCTATCGCTACTCCA CGCACTCACATAACCAACCAT	88 bp
BTN1A1	AGGACGGACTGGGCAATTG ATGACTCCCGAGAATGGGTTC	81 bp
FABP3	TTGTGCGGGAGATGGTTGA TGCCGAGTCCAGGAGTAGCC	147 bp
FAS	GCAAAGTGGTCATTCAGGTACG CCCAGTGATGATGTAGCTCTTG	123 bp
BLG	TCCCTGCCGTGTTCAAGATCG AAATTTCTCCAGGGCCTCGTC	176 bp
CSN2	CCATAACAGCCTCCCAC GCCATAGCCTCCTTCAC	111 bp
LALBA	CTCTGCTCCTGGTAGGCATC ACAGACCCATTCAGGCAAAC	125 bp
RPS9	CCTCGACCAAGAGCTGAAG CCTCCAGACCTCACGTTTGTTC	64 bp

sequence published on NCBI, a pair of primers was designed using Oligo 7 software; other primers were designed from literatures. All primers (Table 1) were assessed against standard curves to examine their efficiency and specificity. Acceptable efficiency was deemed between 90 and 110%. All products were cloned and subsequently sequenced. The qRT-PCR reaction system (20 μl) was as follows: 5 μl of diluted cDNA, 4 μl of sterile and nuclease free water, 10 μl of SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Takara, Dalian, China), and 0·5 μl of forward and reverse primer (10 μM). qRT-PCR amplifications were carried out using CFX-96 (Bio-Rad, USA) as follows: an initial denaturation step 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. Product purity was identified by melt curve analysis. A no-template control was also included in each run for each gene. All reactions were run in triplicate. Expression levels of each gene were normalised to RPS9 mRNA (Bionaz & Loor, 2007; Yadav et al. 2012). qRT-PCR analysis was performed by the ΔΔCT method.

Statistical analysis

Results were reported as mean ± SE. Individual differences were assessed using one-way analysis of variance ANOVA (SPSS 17.0) following LSD method. Statistical significance was defined as $P < 0{\cdot}05$.

Results

Isolation and detection of total RNA

We successfully isolated total RNA from MFG using Trizol method. Spectrophotometer displayed that the A260/280 of total RNA was from 1·80 to 2·10. Agarose electrophoresis showed that the bands of 18 and 28 s ribosomal RNA were clear and standard without obvious degradation.

To demonstrate that the isolated RNA was from the cytoplasmic crescents, milk fat globule was stained with acridine orange. The results showed that DNA fluorescing green, RNA fluorescing red and several cytoplasmic crescents in milk fat globule (Fig. 1). Furthermore, the whole field of vision contained fluorescence.

In our experiment, after centrifuging 50 ml milk sample, there was 1000 mm^3 of fat layer in the sample. Spectrophotometer displayed that there was 5·090 ± 1·769 μg of total RNA in MFG from 50 ml milk sample and 2·660 ± 0·455 μg of total RNA in MSC from 400 ml milk sample (Table 2).

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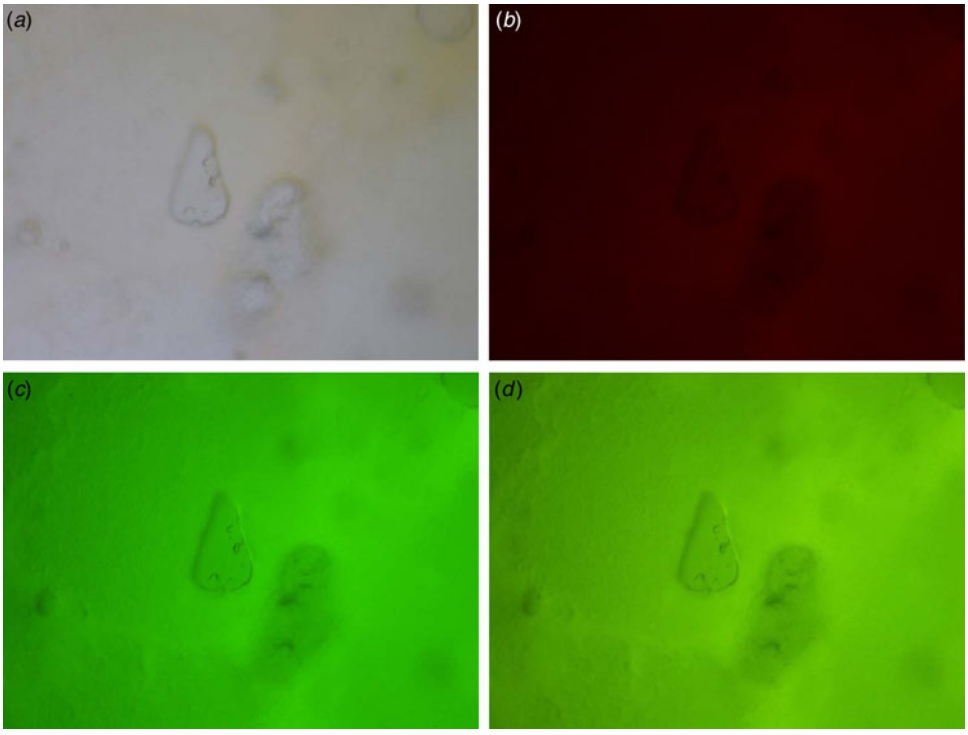

Fig. 1. Detection of cytoplasmic crescents. a: cytoplasmic crescents (bright right, ×200); b: RNA fluoresced red (×200); c: DNA fluoresced green (×200); d: RNA and DNA look yellow (×200).

Table 2. Detailed quality and quantity of total RNA in MG, MFG and MSC

Samples	A260/280	Samples	A260/280	Content (μg)	Samples	A260/280	Content (μg)
MG1	2·06	MFG1	2·09	6·564	MSC1	1·90	2·864
MG2	2·04	MFG2	1·98	3·948	MSC2	2·02	2·774
MG3	2·03	MFG3	1·86	4·468	MSC3	2·09	2·064
MG4	2·03	MFG4	1·99	2·844	MSC4	2·06	2·980
MG5	1·95	MFG5	2·07	5·028	MSC5	2·04	2·128
MG6	2·13	MFG6	2·10	7·688	MSC6	2·05	3·154

Note: The content of total RNA in MFG and MSC respectively derived from 50 and 400 ml of milk sample.

Relative expression levels of cell marker genes

To confirm that the total RNA in MFG was mainly from mammary epithelial cells, we detected relative expression levels of some cell marker genes in mammary gland tissue. The results showed that the expression of AdipoQ gene in MG was detected, in contrast, the expressions in MFG and MSC were not detected (Fig. 2a). The expressions of Keratin 8 and Keratin18 in MFG were lower than in MG, but higher than in MSC ($P > 0{\cdot}05$) (Fig. 2a). The expression of vimentin in MFG was significantly lower than in MSC and MG ($P < 0{\cdot}05$) (Fig. 2a). The expressions of CD43, CSF1 and IL1α in MFG were not detected and their expressions in MG were significantly lower than in MSC ($P < 0{\cdot}05$) (Fig. 2b).

Relative expression level of milk fat synthesis-related genes and milk protein genes

To demonstrate that the total RNA in MFG could be used as an alternative to the total RNA in MG and MSC, we detected relative expression levels of milk fat synthesis-related genes and milk protein genes. The results showed that the expressions of ACC, BTN1A1, FABP3 and FAS in MFG were higher ($P > 0{\cdot}05$) or significantly ($P < 0{\cdot}05$) higher than in MG and MSC, and their expressions in MG were higher than in MSC ($P > 0{\cdot}05$) (Fig. 2c). Furthermore, the expressions of LALBA, BLG and CSN2 in MFG were higher ($P > 0{\cdot}05$) or significantly ($P < 0{\cdot}05$) higher than in MG and MSC, and their expressions in MG were higher than in MSC ($P > 0{\cdot}05$) (Fig. 2d).

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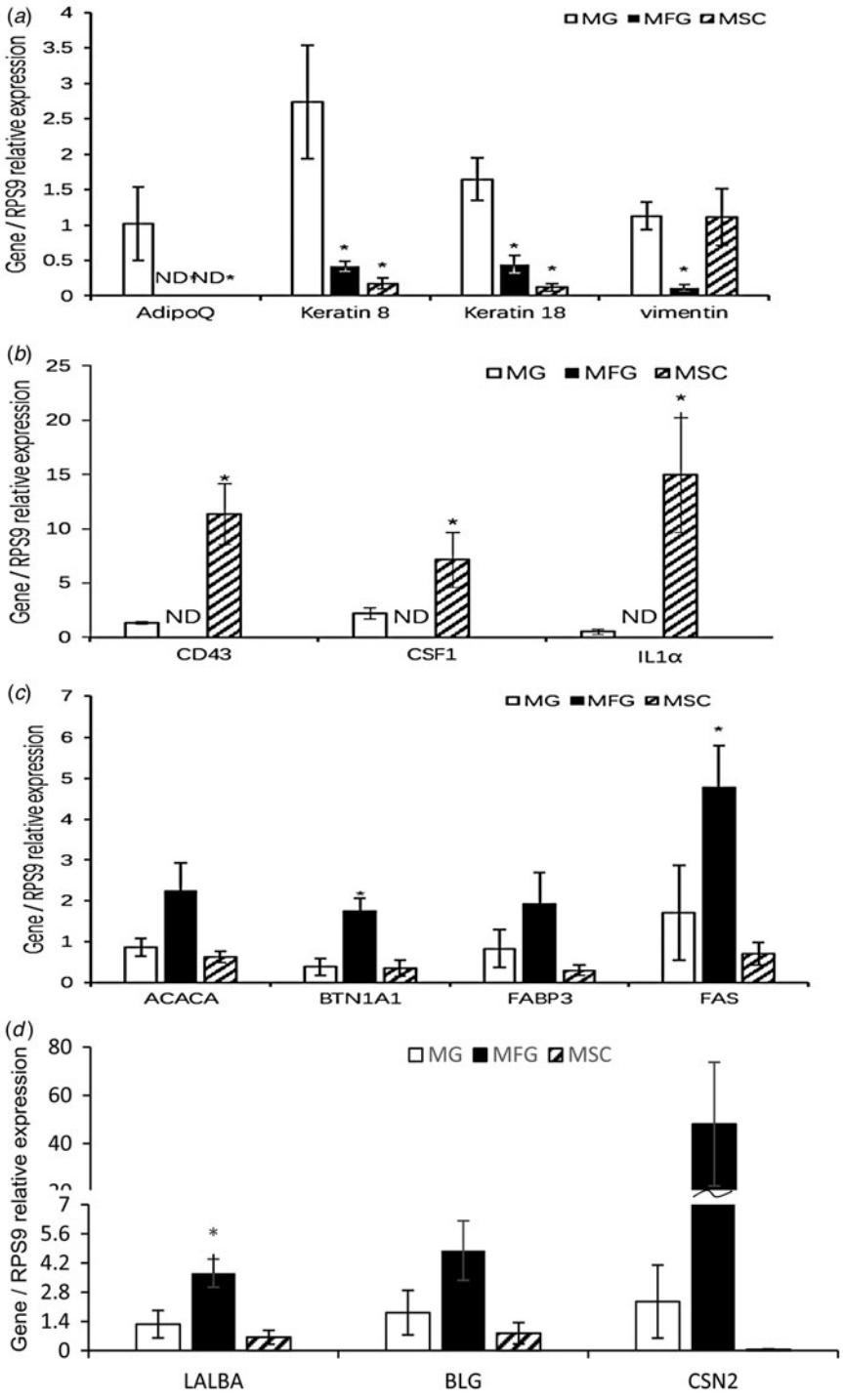

Fig. 2. Relative mRNA levels of gene. a: adipocyte-specific gene (AdipoQ) epithelial cell marker genes (Keratin 8 and Keratin 18), fibroblast marker gene (vimentin); b: leucocyte-specific genes (CD43, CSF1, IL1α); c: milk fat synthesis-related genes (ACC, BTN1A1, FABP3, FAS); d: milk protein genes (LALBA, BLG, CSN2). ND, not detected. Values represent means ± SE (standard error) ($n = 6$). *$P < 0{\cdot}05$ compared with the mRNA levels in MG.

Discussion

Total RNA could be isolated from milk fat globule

Milk fat is secreted as milk fat globules which are formed in the ER membrane and then transported to the apical plasma membrane and released by the mammary epithelial cell (Keenan & Mather, 2006). The type of secretion is known as apocrine which brings with it mammary epithelial cell cytoplasmic material, including ribosomes, mitochondria, and other organelles (Huston & Patton, 1990; Thompson

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et al. 1998). The presence of cytoplasmic crescents of epithelial cell origin in buffalo milk fat explains why RNA can be isolated from MFG (Choudhary et al. 2015). Therefore, we detected the existence of RNA and cytoplasmic crescents in MFG using acridine orange staining. It has been demonstrated in human that the transcription product in the MFG could reflect those of MEC (Maningat et al. 2007, 2009). In our study, the buffaloes were milked twice a day and the collected milk was alveolar milk rather than cisternal milk. It has been reported that alveolar milk represents about 95% of total milk owing to the absence or small size of the udder cistern in buffalo (Thomas et al. 2004).We successfully isolated total RNA from MFG material and showed that the volume of milk for isolating the same amount of total RNA from milk somatic cell was about 15 times more than was required for isolation from MFG. This may be associated with the small amounts of milk somatic cell and large amounts of milk fat globule in buffalo (Cerón-Muñoz et al. 2002).

Total RNA in MFG derives from mammary epithelial cells

Mammary gland tissue consists primarily of mammary epithelial cells, fibroblasts and adipocytes (Capuco et al. 2001). Since adipocytes have the ability to synthesise fat, they are a main contaminant to study the synthesis of mammary epithelial cells. In the present study, we didn't detect the expression of AdipoQ (adipocyte-specific gene) in MFG. Additionally, a previous study has shown that AdipoQ was not expressed in human MFG RNA (Maningat et al. 2009). Cytokeratin, a type of cytoskeletal protein, is assembled into intermediate filament around the nucleus (Stewart, 1993). This explains why the expressions of keratin 8 and keratin 18 (the marker of mammary epithelial cell) in MG were significantly higher than in MFG and MSC, and their expressions in MFG were higher than MSC. In addition, we detected expression of vimentin (fibroblast marker) in MFG, but at a lower level than in MSC and MG. This was consistent with the previous study that vimentin protein was expressed in mammary epithelial cell line from Chinese Holstein cow (Hu et al. 2009). In summary, it would appear that the total RNA in MFG mainly comes from epithelial cells.

To avoid trauma and decrease economic losses, some studies on gene expression in mammary epithelial cells of animal have used somatic cells isolated from milk by centrifugation (Boutinaud & Jammes, 2002; Wickramasinghe et al. 2012). Milk somatic cells comprise lymphocytes, neutrophils, macrophages and exfoliated epithelial cells (Lindmark-Mansson et al. 2006). In the healthy mammary gland, macrophages (35–79%) are the predominant cell type, followed by leucocytes, lymphocytes (16–28%), polymorphonuclear neutrophils (PMNs) (3–26%) and mammary epithelial cells, which only accounted for 2–15% (Lindmark-Mansson et al. 2006). We did not detect the expression of CSF1 (macrophage marker gene), CD43 (leucocyte marker) or IL1α (leucocyte-specific gene) in MFG, providing further reassurance that our analysis was indicative of epithelial expression. A previous study has also reported the absence of CSF1 mRNA expression in the transcription product of MFG (Maningat et al. 2007).

In conclusion, expression of mammary epithelial cell marker genes was present in MFG, but expression of other cell marker genes was not, demonstrating that the majority of the total RNA in MFG arose from mammary epithelial cells.

The transcription product of MFG could be used as an alternative to those of MG

Milk fat synthesis and milk protein synthesis are important research areas in mammary physiology. We detected the expression of milk fat synthesis-related genes and milk protein genes in MFG. Acetyl-coA carboxylase (ACC), fatty acid synthase (FAS) and fatty acid binding protein 3 (FABP3) play crucial roles in fatty acid synthesis and transport (Bionaz & Loor, 2008; Jeong et al. 2009; Liang et al. 2014). Butyrophilin subfamily 1 member A1(BTN1A1) is a major protein which regulates the amount of lipids and size of droplets (Jeong et al. 2009). In our study, the expressions of ACC, FAS, FABP3 and BTN1A1 in MFG were higher than in MG and MSC. Milk protein genes, alpha lactalbumin (LALBA), beta lactoglobulin (BLG) and beta casein (CSN2), are the most highly expressed genes in both mice MG and human MFG (Rudolph et al. 2003, 2007; Maningat et al. 2009). Similarly, we found that the expressions of LALBA, BLG and CSN2 in MFG were higher than in MG and MSC. In all, these results suggested that total RNA isolated from MFG could be used as an alternative to RNA obtained from MG to study the expression of functional genes in mammary epithelial cells.

In conclusion, we successfully isolated total RNA from MFG and demonstrated that it could be used to study the expression of functional genes in mammary epithelial cells. There was no expression of adipocyte-specific gene and leucocyte-specific genes and lower expression of fibroblast marker gene in MFG, furthermore, expression of epithelial cell marker genes in MFG was higher than in MSC. In addition, the expressions of milk fat synthesis-related genes and milk protein genes in MFG in MFG were higher than in MG and MSC. Analysis of milk-derived MFG may be a promising technique for further studies on the function of mammary epithelial cells.

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