Comprehensive Assessment of Milk Composition in Transgenic Cloned Cattle

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Abstract

The development of transgenic cloned animals offers new opportunities for agriculture, biomedicine and environmental science. Expressing recombinant proteins in dairy animals to alter their milk composition is considered beneficial for human health. However, relatively little is known about the expression profile of the proteins in milk derived from transgenic cloned animals. In this study, we compared the proteome and nutrient composition of the colostrum and mature milk from three lines of transgenic cloned (TC) cattle that specifically express human α-lactalbumin (TC-LA), lactoferrin (TC-LF) or lysozyme (TC-LZ) in the mammary gland with those from cloned non-transgenic (C) and conventionally bred normal animals (N). Protein expression profile identification was performed, 37 proteins were specifically expressed in the TC animals and 70 protein spots that were classified as 22 proteins with significantly altered expression levels in the TC and C groups compared to N group. Assessment of the relationship of the transgene effect and normal variability in the milk protein profiles in each group indicated that the variation in the endogenous protein profiles of the three TC groups was within the limit of natural variability. More than 50 parameters for the colostrum and mature milk were compared between each TC group and the N controls. The data revealed essentially similar profiles for all groups. This comprehensive study demonstrated that in TC cattle the mean values for the measured milk parameters were all within the normal range, suggesting that the expression of a transgene does not affect the composition of milk.

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Introduction

The rapid development of transgenic technology has led to the generation of a broad spectrum of transgenic cloned animals for agricultural and biomedical use [1,2]. Among numerous applications, expressing recombinant human proteins with benefits for human health and nutrition has become a reality [http://www.gtc-bio.com/; http://www.pharming.com/]. However, some aspects of the process of cloning transgenic animals, such as epigenetic reprogramming, exogenous gene insertion and pleiotropy, have increased the attention paid to the risks associated with foods produced from these animals compared with food from conventionally bred animals.

Since 2001, the FDA has conducted an intensive evaluation that includes the examination of the safety of food from cloned animals and the risks of cloning to animal health. The accumulated data indicate that the gross composition of milk and meat from cloned animals is within the normal range and is as safe as milk and meat from conventionally bred cattle [6–8]. Meanwhile, similar considerations have been given to the safety of food produced from transgenic animals. Transgenesis and cloning belong to two different risk groups because new transgenes are introduced into the genome of transgenic animals but not in cloned animals. Therefore, the analysis of the risk of consuming foods from transgenic animals should focus on characterization of the expected products of the transgene and any other unintended changes [9]. To date, very limited published information is available on the composition of food products derived from transgenic cloned animals. One study that examined the production of milk containing higher than normal levels of bovine β-casein and κ-casein revealed that the nutritional composition of the milk from the transgenic cloned cattle and conventionally bred cattle was similar [10].

However, some unanswered concerns remain. Whether the protein profiles of milk from transgenic cloned cattle are altered by the cloning technology or by the expression of exogenous human genes in bovine mammary epithelial cells, and the mechanism by which such changes might occur, is unknown. Technological advances in proteomics have allowed an increased understanding and characterization of milk proteins. Previous proteomic studies have focused on the identification of milk whey proteins and have
the advantage of being able to detect, identify and characterize a large number of milk proteins simultaneously, including low-abundance proteins [11–13]. Thus, current proteomic technology is an essential component of addressing the food safety concerns associated with transgenic technology and of making the milk from transgenic cloned animals more attractive to consumers.

Previously, we described the generation of three lines of transgenic cloned cattle that specifically express human α-lactalbumin (TC-LA), lactoferrin (TC-LF) or lysozyme (TC-LZ) in milk as a result of the integration of a specific transgene into the genome and were cloned by subsequent SCNT [14–16]. The major changes observed between milk from these animals and milk from conventionally bred animals were the high levels of human milk proteins. Thus, the milk of these transgenic animals has very different properties from that of conventionally bred animals and provides a unique model for evaluating the effects of exogenous transgenes on the profile of the endogenous milk proteins. The objective of this study was to examine differences in the milk proteome and other important milk components in the colostrum and mature milk from three transgenic cloned cattle lines. This comprehensive study provides unequivocal evidence that the expression of a transgene does not affect the composition of milk, and these results may assist with the assessment of the safety of food derived from transgenic cloned animals.

Materials and Methods

TC Cattle and Matched Breed Controls

The three different lines of transgenic cattle expressing approximately 0.01 mg/mL hLZ (n = 10), 1.5 mg/mL hLF (n = 4), or 3.5 mg/mL hLF (n = 3), as described previously [14–16], were used for the milk composition analysis, with the C (n = 3) and N animals (n = 9) used as controls. In briefly, the concentration of the recombinant proteins was assayed by ELISA using Human Lactoferrin ELISA Kit (Bethyl, Montgomery, TX, USA), Human LYZ ELISA Kit (Abnova, Taiwan) and Human LALBA ELISA Kit (Abnova, Taiwan), respectively, according to the manufacturer’s instructions. The absorbance of the product was measured at 450 nm using a model 550 microplate reader (Bio-Rad, Hercules, CA). All TC cattle lines and C cattle were produced by SCNT, with the C control cattle being from the unmodified donor nuclear cell lines that were used to produce the TC cattle following the introduction of the transgenes. In addition, the Holstein cow breed N cattle had a genetic background similar to that of the transgenic cloned cattle. The cattle were similar in age and lactation period and were housed under the same conditions.

Milk Sample Collection

The protocol was approved by the Institutional Animal Care and Use Committee of China Agricultural University (ID: SKLAB-2010-05-01). The colostrum was obtained during the initial three days of lactation and mature milk was obtained on the 30th, 60th and 90th day after lactation. Milk was collected from the cows twice daily and was pooled to form one daily sample. A portion of the samples was used for the analysis of the milk nutrient composition. The remaining portion was centrifuged at 2500×g for 30 min at 4°C to obtain the skim milk fraction and subsequently ultracentrifuged at 150,000×g for 1 h at 4°C to remove the casein micelles. The supernatant (whey fraction) of each cow per day was collected and stored at −80°C for ELISA analysis. For the proteomics analysis, three days of mature whey samples from each cow was pooled together to generate one individual sample and then individual samples in the same groups (TC-LZ, TC-LA, TC-LF, C and N) were pooled again to generate one group sample according to the equal protein mass (three days of whey samples from nine control cows were pooled and then analyzed separately for HCA and PCA analysis).

2D SDS-PAGE

The whey protein was precipitated using a 2D Clean-UP kit (GE Healthcare). Whey proteins (1 mg) were loaded onto IPG strips (linear, 24 cm length, pH 4–7) by overnight rehydration at 20°C. The IEF was performed using IPGphor III (GE Healthcare) by gradually increasing the voltage. SDS-PAGE was performed using an Etlan DALT System (GE Healthcare) and the protein spots were visualized by “blue-silver” staining. The image analysis was performed using the Image Master 2D Platinum 6.0 software (GE Healthcare). Student’s t-tests were performed on the proteins showing greater than 2-fold changes, and p≤0.05 were considered to be significant.

LC-MS/MS Analysis

The in-solution tryptic digestion proteins (200 μg) were analyzed using 2D-Nano-LC-ESI-MS/MS, performed on a nano Acuity UPLC (Waters) connected to a LTQ Orbitrap XL mass spectrometer (Thermo Fisher). Each scan cycle consisted of one full MS scan in profile mode followed by five MS/MS scans in centroid mode. The acquired MS/MS spectra were used to search against a non-redundant protein Bovidae database using the SEQUEST program in the BioWorks™ 3.3.1 software suite with the following parameters: the mass tolerance of 1.4 Da for precursor ions and 1.0 Da for fragment ions, allowing two missed cleavage. The false discovery rate was less than 1%, which was calculated using a database containing reversed sequences. The peptide identifications were filtered by PeptideProphet with a confidence level of 95% and protein identifications were accepted with greater than 99% probability [17].

MALDI-TOF MS/MS Analysis

In-gel digestion was performed using standard protocols [18]. The peptides were analyzed on a 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems) operated in reflector positive mode, followed by MS/MS of eight most-intense peptide ions. The acquired MS/MS spectra were used to search against a BOVINenr database in the MASCOT software with the following parameters: a peptide tolerance of 100 ppm, a fragment tolerance of 0.6 Da, allowing one trypsin missed cleavage.

Quantitative Analysis of Major Whey Proteins

The amount of seven major bovine whey proteins (β-LG, α-LA, BSA, IgG, IgA, IgM and transferrin) in the colostrum and mature milk from the five groups of animals was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Bethyl, Montgomery, TX, USA) according to the manufacturer’s instructions. The absorbance of the product was measured at 450 nm using a model 550 microplate reader (Bio-Rad, Hercules, CA).

Nutrient Composition Analysis

The whole-milk samples were delivered to the Beijing Research Institute for Nutritional Resources, which establishes the criteria and methods for the analysis of large quantities of biochemical and nutritional components. More than 50 nutritional components were analyzed and they include 18 kinds of amino acids, 4 kinds of total fatty acids as well as 16 kinds of specific fatty acid profiles, mineral (K, Na, Ca, Mg, Fe, Zn, Se and P) and vitamin contents (vitamin A, vitamin B1, vitamin B2, Vitamin B3, vitamin B5,
vitamin B12 and vitamin C). The entire composition was measured according to the national food safety standard raw milk issued by China’s Ministry of Health (GB19301-2010). All values show standard deviation and statistically analyzed using t-test (*p<0.05).

Results

Overview of Milk Whey Proteome and Identification of Specifically Expressed Proteins in the TC Groups

The milk protein compositions of each group were compared comprehensively using 2D-Nano-LC-MS/MS. Database searches using the fragmentation spectra as experimental material identified unique proteins from distinct peptides, and 215, 258, 172, 236 and 262 proteins were identified in the TC-LZ, TC-LA, TC-LF, C and N groups, respectively. Venn diagrams of the protein profiles of the TC-LZ, TC-LA, TC-LF and C groups were created with respect to the N group. The TC-LZ had 182 proteins in common with the N group, and 33 and 80 proteins were unique to the TC-LZ and N groups, respectively (Figure 1A). A similar distribution was found for the TC-LA, TC-LF and C groups (Figure 1B–1D). Altogether, 44 proteins were identified that were exclusive to the TC groups, 7 of which had been previously confirmed to be present in milk from cattle of the background strain using similar technological strategies [19–21]. The TC and C groups had 26 proteins in common that were not detected in the N group (Figure 1D). To further examine the specifically expressed proteins, the 37 newly identified proteins were categorized according to their cellular component, biological process or molecular function as annotated in the Gene Ontology (GO) database (Table 1 and Figure 2). Our results indicate that the majority of the proteins detected were either completely or partially extracellularly localized, as expected for milk proteins. When organized according to biological activity, most proteins were found to be involved in oxygen transport. Functionally, the majority of proteins identified were involved in pattern binding, polysaccharide or carbohydrate binding.

Characterization of Differentially Expressed Proteins in the TC Groups

To assess whether the variation in milk protein expression profiles was due to the effect of the transgene or to natural variability between each group, the TC, C and N groups were investigated using a quantitative 2DE combined with MALDI-TOF/TOF. A total of 708 protein spots were detected on 2DE, and 602 protein spots that originated from unique genes were positively identified by MALDI-TOF/TOF. Many of the spots that were not identified were for proteins of extremely low abundance. ImageMaster and statistical analyses revealed that 58 spots were significantly different between the TC and N groups and 12 spots between the C and N groups, with at least 2-fold differences (p≤0.05) (Figure 3). Of these 70 spots, 46 spots, which were classified into 22 types of proteins, were identified successfully. Compared with the N group, leukocyte elastase inhibitor (SERPINB1) was changed in all four groups; 10 proteins were differentially expressed in only one group whereas 7 proteins in any two groups. Lactotransferrin (LTF) was absent in the C group and upregulated in both the TC-LA and TC-LZ groups but downregulated in the TC-LF group. Cathelicidin-1 (CATHL1) was absent in the TC-LF group but upregulated in the other three groups. A complete list of the identified differentially expressed proteins is provided in Table 2, where these proteins are grouped according to GO classifications (Figure 4). In terms of biological processes, the majority of proteins belonged to the defense response; when grouped by molecular function, the proteins most commonly had endopeptidase inhibitor activity, and the majority of proteins were components of the extracellular region.

An unsupervised hierarchical cluster analysis (HCA) was used to assess the effect of transgenes on, and the natural variability in, milk protein profiles from the TC-LZ, TC-LA, TC-LF, C and N

Figure 1. Venn diagrams representation of whey protein profiles comparison of the TC-LZ (A), TC-LA (B), TC-LF (C) and all TC (D) groups were created with respect to the C and N groups, respectively. The gray ellipse indicates the proteins reported to be present in bovine milk in previously studies. The blue, green and red circles indicate the protein sets of the TC, C and N groups, respectively.

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groups in an unbiased manner. The heat map and hierarchical tree revealed that there was no difference between any TC group and the N group or between the direct clustering of the three TC groups and the C group (Figure 5A). The TC-LZ and TC-LA groups clustered with the normal samples n6 and n8, respectively. The TC-LF group clustered with samples n1-n8, and the TC-LZ and TC-LA groups both clustered with the normal sample n9. These results indicated that each TC group was closer to the normal samples than to the other TC groups or the C group.

A principal component analysis (PCA) was then used to analyze the significant differentially expressed spots from the 2D data. Specifically, there was no visible separation of any detected spots between the TC and N samples within the first two components (Figure 5B), with 69.6% of the variation allocated to the first component (PC1) and 21.3% of the variation allocated to the second component (PC2). These data showed a pattern largely similar to that generated by the HCA analysis, in which the three transgenes and their products showed very little impact on the milk protein profiles greater than that of natural variability. Thus, these data also suggest that the insertion of the three different exogenous human genes has no major impact on the general milk protein composition.

Quantitative Comparison of Abundant Whey Proteins and Antibodies

An ELISA was used not only to obtain an accurate measurement of the concentration of the major highly abundant bovine whey proteins and immune-associated proteins (β-lactoglobulin, α-lactalbumin, serum albumin, transferrin, IgG, IgM and IgA) in the colostrum and mature milk for individual cows in the three TC groups and the N group but also to verify the quantitative proteomic results from the 2D PAGE statistical analyses.

Overall, the concentrations of these major whey proteins, particularly the three most highly abundant whey proteins (β-lactoglobulin, α-lactalbumin and bovine serum albumin), were similar in the colostrum and mature milk and were not different between the TC, C and N groups (Figure 6). Although there were some minor fluctuations in the concentration of some proteins in the colostrum and mature milk of the TC groups, the range in the concentrations of the seven major whey proteins from the TC and C groups were not significantly different from those from the N group, which were largely within the bounds reported previously for common breeds of dairy cows in China. The results showed that the expression of additional human mammary gland-specific genes (α-lactalbumin, lactoferrin and lysozyme) in bovine mam-
| No. | Protein Name                                      | Accession No. | LZ | LA | LF | MW (kDa) | pI | Cellular Component                                           | Biological Process                                           | Molecular Function                                          |
|-----|--------------------------------------------------|---------------|----|----|----|----------|----|------------------------------------------------------------|-------------------------------------------------------------|------------------------------------------------------------|
| 1   | MHC CLASS I HEAVY CHAIN (FRAGMENT)               | IP00954418.1  | N  | N  | D  | 19.3     | 4.79 | membrane; MHC class I protein complex                      | immune response; antigen processing and presentation         | U                                                        |
| 2   | SERPINA3-2                                       | IP00930024.1  | N  | N  | D  | 46.2     | 5.66 | extracellular space; cytoplasmic vesicle                   | U                                                           | serine-type endopeptidase inhibitor activity               |
| 3   | 22 kDa PROTEIN                                   | IP00970745.1  | N  | D  | N  | 22.4     | 9.71 | U                                                          | U                                                           | U                                                        |
| 4   | SIMILAR TO CYCLOPHILIN B (FRAGMENT)              | IP00838493.1  | N  | D  | N  | 11.0     | 9.74 | U                                                          | protein folding                                             | U                                                        |
| 5   | C8G PROTEIN                                      | IP00876781.1  | N  | D  | N  | 25.9     | 11.1 | U                                                          | transport                                                   | U                                                        |
| 6   | ERAP1 PROTEIN                                    | IP00867238.1  | N  | N  | D  | 10.7     | 5.91 | cytoplasm                                                 | proteolysis; positive regulation of angiogenesis            | U                                                        |
| 7   | GPR56 PROTEIN                                    | IP00847084.1  | D  | N  | D  | 77.0     | 8.80 | integral to membrane; plasma membrane                      | cell adhesion; brain development neuropptide signaling pathway | protein binding; G-protein coupled receptor activity       |
| 8   | KRT6A PROTEIN                                     | IP00845184.1  | D  | N  | N  | 60.8     | 8.28 | keratin filament                                           | U                                                           | structural molecule activity                               |
| 9   | 51 kDa PROTEIN                                   | IP00841672.1  | D  | N  | N  | 51.2     | 4.90 | keratin filament                                           | U                                                           | U                                                        |
| 10  | 13 kDa PROTEIN                                   | IP00841591.2  | N  | D  | N  | 13.2     | 8.66 | U                                                          | U                                                           |                                           |
| 11  | 39 kDa PROTEIN                                   | IP00841003.2  | N  | D  | N  | 38.8     | 8.73 | extracellular region                                       | U                                                           |                                           |
| 12  | 31 kDa PROTEIN                                   | IP00840962.2  | D  | N  | D  | 30.7     | 4.44 | extracellular region                                       | U                                                           |                                           |
| 13  | SIMILAR TO CALSNTENIN-1, PARTIAL                 | IP00838241.2  | N  | D  | N  | 107      | 4.73 | U                                                          | U                                                           |                                           |
| 14  | 174 kDa PROTEIN                                  | IP00838035.2  | N  | N  | D  | 174      | 6.52 | U                                                          | U                                                           |                                           |
| 15  | SIMILAR TO KERATIN 2                            | IP00824847.1  | D  | N  | N  | 64.3     | 8.56 | U                                                          | U                                                           |                                           |
| 16  | SIMILAR TO CDS MOLECULE LIKE                     | IP00824588.2  | N  | N  | D  | 82.7     | 5.19 | U                                                          | U                                                           |                                           |
| 17  | MHC CLASS I ANTIGEN (FRAGMENT)                   | IP00788543.1  | N  | N  | D  | 19.9     | 5.03 | membrane; MHC class I protein complex                      | antigen processing and presentation; immune response         | U                                                        |
| 18  | NAGLU PROTEIN                                    | IP00717554.3  | D  | N  | N  | 74.1     | 5.81 | U                                                          | U                                                           |                                           |
| 19  | HEMOGLOBIN SUBUNIT BETA                          | IP00716455.1  | N  | D  | N  | 16.0     | 7.01 | hemoglobin complex                                         | transport; oxygen transport                                 | metal ion binding; oxygen binding; heme binding             |
| 20  | CYSTEINE-RICH SECRETORY PROTEIN LCLL DOMAIN - CONTAINING 2 | IP00715724.4  | N  | D  | N  | 55.6     | 8.67 | extracellular matrix; transport vesicle; extracellular region | extracellular matrix organization                            | heparin binding                                            |
| 21  | ACTIN-RELATED PROTEIN 2/3 COMPLEX SUBUNIT 2       | IP00715364.1  | D  | N  | N  | 34.3     | 6.84 | cytoskeleton; cell projection                               | regulation of actin filament polymerization                  | actin binding                                              |
| 22  | HEMOGLOBIN SUBUNIT ALPHA                         | IP00710783.2  | N  | D  | N  | 15.2     | 8.06 | hemoglobin complex                                         | oxygen transport                                            | heme binding; oxygen binding; oxygen transporter activity  |
| 23  | METALLOPROTEINASE INHIBITOR 1                    | IP00709084.1  | N  | D  | N  | 23.0     | 8.46 | basement membrane                                          | erythrocyte maturation; negative regulation of catalytic activity | metal ion binding; metalloendopeptidase inhibitor activity |

Table 1. List of the proteins specifically expressed in the transgenic groups.
Table 1. Cont.

| No. | Protein Name                          | Accession No.     | LZ | LA | LF | MW (kDa) | pl   | Cellular Component                                         | Biological Process                                      | Molecular Function                                      |
|-----|---------------------------------------|-------------------|----|----|----|---------|------|-----------------------------------------------------------|--------------------------------------------------------|----------------------------------------------------------|
| 24  | ENDOPIN 2C                            | IPI00705594.1     | D  | N  | D  | 46.7    | 5.98 | U                                                        | U                                                      | serine-type endopeptidase inhibitor activity             |
| 25  | TESTICAN 1                            | IPI00705387.2     | D  | D  | N  | 49.4    | 5.34 | proteinaceous extracellular matrix                        | signal transduction                                      | calcium ion binding                                     |
| 26  | RPE-SPONDIN                           | IPI00704612.4     | N  | D  | N  | 29.3    | 7.71 | extracellular region                                      | immune response                                          | scavenger receptor activity; polysaccharide binding      |
| 27  | RIBONUCLEASE T2                       | IPI00704364.3     | N  | D  | N  | 43.5    | 9.98 | U                                                        | U                                                      | RNA binding; ribonuclease T2 activity                    |
| 28  | VON WILLEBRAND FACTOR A DOMAIN -CONTAINING PROTEIN 1 | IPI00701880.2 | D  | N  | N  | 43.7    | 9.98 | basement membrane; interstitial matrix; ribosome          | extracellular matrix organization; translation          | structural constituent of ribosome                       |
| 29  | BIGLYCAN                              | IPI00697081.2     | N  | D  | N  | 41.6    | 6.83 | proteinaceous extracellular matrix; sarcolemma; transport vesicle | peptide cross-linking via chondroitin 4-sulfate glycosaminoglycan | glycosaminoglycan binding; extracellular matrix binding |
| 30  | OSTEOPONTIN-K                         | IPI00696774.1     | D  | N  | D  | 31.0    | 4.56 | extracellular region                                      | cell adhesion; ossification                             | protein binding                                          |
| 31  | 111 kDa PROTEIN                       | IPI00695031.5     | D  | N  | D  | 111.7   | 5.26 | U                                                        | U                                                      | ATP binding                                              |
| 32  | 83 kDa PROTEIN                        | IPI00694678.3     | D  | N  | D  | 83.2    | 6.15 | extracellular region                                      | U                                                      | endopeptidase inhibitor activity                        |
| 33  | GELSOLIN                              | IPI00694255.2     | D  | N  | D  | 80.7    | 5.54 | cytoplasm; extracellular region                          | actin filament capping; cilium morphogenesis            | actin binding; metal ion binding                         |
| 34  | ENDOPLASMIN                           | IPI00692865.2     | D  | D  | N  | 92.7    | 4.77 | cytosol; endoplasmic reticulum; melanosome                | ER-associated protein catabolic process; protein folding; anti-apoptosis | RNA binding; ATP binding; virion binding                 |
| 35  | MFG8 PROTEIN                          | IPI00689638.1     | D  | N  | D  | 47.9    | 6.80 | extracellular space                                      | cell adhesion                                           | phosphatidylethanolamine binding                         |
| 36  | Dipeptidyl Peptidase III              | IPI00686733.2     | N  | D  | N  | 82.1    | 5.09 | cytoplasm                                               | proteolysis                                             | dipeptidyl-peptidase activity                            |
| 37  | Peroxiredoxin-1                      | IPI00686092.1     | D  | D  | N  | 22.2    | 8.59 | melanosome                                              | regulation of NF-kappaB import into nucleus            | thioredoxin peroxidase activity                           |

*Proteins not detected by LC-MS/MS in the transgenic groups (LZ, LA and LF); †Proteins detected by LC-MS/MS; ‡No function annotation.
mary glands had minimal impact on the levels of the major whey proteins and immune-associated proteins, with all protein concentrations within the range of the natural variability observed in normal milk.

Quantitative Comparison of Milk Whey Composition

The major nutritional components of the colostrum and milk from the first three months of lactation from the TC, C and N groups were compared. First, the proximates analysis showed that the content of five principal milk nutrients of both the colostrum and milk from the first three months of lactation was very similar in all groups and was stable, which indicated that there are no obvious changes in the major components of milk among the groups (Figure 7). For a comparison of the micro composition, more than 50 nutritional components were analyzed in the colostrum and mature milk of all the groups, and more than 90% of these components showed no significant difference among the groups (Figure S1–S5). However, there was a minor variation in 7 components compared with the N group ($p \leq 0.05$) as follows: C16:1 in the mature milk of TC-LF; C20:4n6 in the mature milk of C; Mg in the colostrum and mature milk of C and TC-LA, respectively; Se and K in the mature milk of TC-LZ; and vitamin C in the mature milk of TC-LZ and TC-LF. PCA was used for multivariate analyses of the intra- and inter-group relationships among the TC, C and N groups. In both the colostrum and mature milk, clusters were observed in the first two principal components, which accounted for 66.4% and 23.6%, 68.7% and 21.4%, respectively. This finding indicates that the nutrient levels in the TC and C groups are within the range of natural variability (Figure 8).

Discussion

Although the use of transgenic cloning technology to increase the expression of recombinant proteins in the mammary glands has been successful, changes in intermolecular interactions caused by the process of cloning transgenic animals are not yet fully understood. Traditional analytical methods used to detect specific components of transgenic food (such as nutritional substances, allergens and other nutrients) are incapable of effectively assessing potentially unpredictable changes. Some findings suggest that somatic cloning technology does not induce changes in the main nutrient content of cloned beef and milk [8,22–25]. However, thus far, there have been no reports using proteomics to comprehensively analyze milk from transgenic animals. Here, we analyzed the protein composition and nutrient components of the colostrum and mature milk from 17 transgenic cloned cows expressing one of three mammary gland-specific genes, 3 cloned cows and 9 normal cows using proteomics and metabolomics to analyze the maximum number of unpredictable changes in the milk from TC animals.

Figure 3. Differentially expressed protein spots with great than 2-fold changes ($p \leq 0.05$) in the TC and the C group compared to N group. The red and green arrows indicate the protein spots that were significantly up- and downregulated, respectively, by at least 2-fold. doi:10.1371/journal.pone.0049697.g003
The transgenes in the three transgenic cloned cattle breeds were stably integrated into random positions in the genome and specifically expressed in mammary gland tissue. The recombinant hα-LA gene and hLF gene were more highly expressed in the corresponding TC group than in the N and C groups, with an average expression level of approximately 1.5 mg/mL and 3.5 mg/mL, respectively, and the hLZ gene was expressed in transgenic cows at approximately 0.01 mg/mL. MALDI-TOF mass spectrometry showed that these three recombinant human proteins had the same molecular weight and N-terminal amino acid residue protein sequence as the endogenous homologous genes.

The merged data from previous investigations showed that an exhaustive list of 284 non-redundant annotated protein entries derived from whey proteins [26]. In this study, a total of 301 ordinary milk whey proteins were characterized in all groups. To the best of our knowledge, this study is the most comprehensive characterization of mature milk proteins in bovine whey to date. None of the 37 newly identified proteins in the TC groups was common to all three groups, which indicated that during the transgenic cloning process, potential factors, such as epigenetic errors, did not cause the specific expression of proteins in the transgenic cloned milk. In addition, we found that the 37 specifically expressed proteins identified by LC-MS were not detected in the full milk protein profile of the subsequent 2DE combined with MALDI-TOF/TOF. The most obvious difference between 2DE and the LC-MS technique is that it is more difficult to separate hydrophobic, extremely acidic or basic proteins by LC-MS [27]. However, in this experimental analysis, the milk proteins in the samples were water-soluble proteins, and their isoelectric points were distributed between pH 4 and pH 7. Consequently, when the milk proteome was isolated and identified, the only difference between the two techniques was the successful separation of low-abundance proteins [28]. In addition, the smallest detected protein spots on “blue-silver” staining gels have been demonstrated to contain 1 ng of protein; thus, this staining method approaches the sensitivity of silver staining [29]. Moreover, silver staining, which is the most sensitive protein detection technique, was also used in an attempt to identify the specifically expressed proteins, but these proteins were unfortunately still beyond the detection range [30]. Therefore, we inferred that the 37 specifically expressed proteins in the TC groups were of trace abundance.

There are biases in the identification of low-abundance proteins using LC-MS, and the success rate of identifying proteins of low abundance is low because the peptides that are produced enzymatically from proteins of high and low abundances cannot be completely separated by the 2D separation step in LC and the peptides derived from high level proteins may disturb the mass spectrum signal of the peptides from the low-abundance proteins.
| No. | Group ID | Protein name | Accession No. | LZ | LA | LF | C | MW (kDa) | pI | Cellular Component | Biological Process | Molecular Function |
|-----|----------|--------------|---------------|----|----|----|---|---------|----|--------------------|-------------------|--------------------|
| 1   | 32       | U            | U             | U  | U  | U  | U | U       | U  | U                  |                   |                    |
| 2   | 66       | U            | U             | U  | U  | U  | U | U       | U  | U                  |                   |                    |
| 3   | 74       | LACTOTRANSFERRIN | IPI00710664 | ↑  | ↑  | ↑  | U | U       | U  | extracellular region; secretory granule | cellular iron ion homeostasis; defense response to bacteria | heparin binding; ferric iron binding; serine-type peptidase activity |
| 4   | 80       | 26 kDa PROTEIN | IPI00906471 | ↑  | ↑  | ↑  | U | U       | U  | extracellular region | N                  | N                  |
| 5   | 84       | U            | U             | U  | ↑  | ↑  | U | U       | U  | extracellular region | N                  | N                  |
| 6   | 101      | U            | U             | U  | ↑  | ↑  | U | U       | U  | extracellular region | N                  | N                  |
| 7   | 120      | CSN2 18 kDa PROTEIN | IPI00712994 | ↓  | ↓  | ↓  | U | U       | U  | extracellular region | transport          | transporter activity; protein binding |
| 8   | 176      | U            | U             | U  | ↑  | ↑  | U | U       | U  | extracellular region | N                  | N                  |
| 9   | 179      | 26 kDa PROTEIN | IPI00906471 | ↓  | ↓  | ↓  | U | U       | U  | extracellular region | N                  | N                  |
| 10  | 199      | CATHELICIDIN-1 | IPI00717085 | ↑  | ↑  | ↑  | U | U       | U  | extracellular region | defense response   | N                  |
| 11  | 202      | CATHELICIDIN-1 | IPI00717085 | ↑  | ↑  | ↑  | U | U       | U  | extracellular region | defense response   | N                  |
| 12  | 203      | LACTOTRANSFERRIN | IPI00710664 | ↑  | ↑  | ↑  | U | U       | U  | extracellular region; secretory granule | cellular iron ion homeostasis; defense response to bacteria | heparin binding; ferric iron binding; serine-type peptidase activity |
| 13  | 204      | U            | U             | U  | ↑  | ↑  | U | U       | U  | extracellular region | N                  | N                  |
| 14  | 213      | LACTOTRANSFERRIN | IPI00710664 | ↑  | ↑  | ↑  | U | U       | U  | extracellular region; secretory granule | cellular iron ion homeostasis; defense response to bacteria | heparin binding; ferric iron binding; serine-type peptidase activity |
| 15  | 251      | 26 kDa PROTEIN | IPI00906471 | ↑  | ↑  | ↑  | U | U       | U  | extracellular region | N                  | N                  |
| 16  | 296      | CSN2 18 kDa PROTEIN | IPI00712994 | ↓  | ↓  | ↓  | U | U       | U  | extracellular region | transport          | transporter activity; protein binding |
| 17  | 302      | U            | U             | U  | ↓  | ↓  | U | U       | U  | extracellular region | N                  | N                  |
| 18  | 309      | U            | U             | U  | ↑  | ↑  | U | U       | U  | extracellular region | N                  | N                  |
| 19  | 315      | CSN2 18 kDa PROTEIN | IPI00712994 | ↓  | ↓  | ↓  | U | U       | U  | extracellular region | transport          | transporter activity; protein binding |
| 20  | 322      | U            | U             | U  | ↑  | ↑  | U | U       | U  | extracellular region | N                  | N                  |
| 21  | 327      | CSN2 18 kDa PROTEIN | IPI00712994 | ↓  | ↓  | ↓  | U | U       | U  | extracellular region | transport          | transporter activity; protein binding |
| 22  | 331      | U            | U             | U  | ↑  | ↑  | U | U       | U  | extracellular region | N                  | N                  |
| 23  | 332      | FGG 50 kDa PROTEIN | IPI00843209 | ↑  | ↑  | ↑  | U | U       | U  | extracellular region | signal transduction | protein binding |
| 24  | 333      | PIGMENT EPITHELUM-DERIVED FACTOR | IPI00716121 | ↑  | ↑  | ↑  | U | U       | U  | extracellular space; extracellular matrix; melanosome | negative regulation of angiogenesis; regulation of proteolysis | serine-type endopeptidase inhibitor activity |
| 25  | 334      | 26 kDa PROTEIN | IPI00906471 | ↓  | ↓  | ↓  | U | U       | U  | extracellular region | N                  | N                  |
| 26  | 335      | U            | U             | U  | ↑  | ↑  | U | U       | U  | extracellular region | N                  | N                  |
Table 2. Cont.

| No. | Group ID | Protein name | Accession No. | LZ | LA | LF | C | MW (kDa) | pI | Cellular Component | Biological Process | Molecular Function |
|-----|----------|--------------|---------------|----|----|----|---|----------|----|--------------------|-------------------|-------------------|
| 27  | 337      | CSN2 18 kDa PROTEIN | IPI00712994   | ↓  |    |    | U | 18.3    | 5.13 | extracellular region | transport          | transporter activity; protein binding |
| 28  | 338      | U            | U             |    |    |    | U | 21.2    | 6.42 | Golgi apparatus; extracellular space; nuclear membrane | prostaglandin biosynthetic process; regulation of circadian sleep/wake cycle, sleep | prostaglandin-D synthase activity; fatty acid binding |
| 29  | 339      | PROSTAGLANDIN-H2 D-ISOMERASE | IPI00709683   | ↑  | ↑  |      | U | 78.1    | 8.68 | extracellular region; secretory granule | cellular iron homeostasis; defense response to bacteria | heparin binding; ferric iron binding; serine-type peptidase activity |
| 30  | 340      | CSN2 18 kDa PROTEIN | IPI00712994   | ↓  |    |    | U | 18.3    | 5.13 | extracellular region | transport          | transporter activity; protein binding |
| 31  | 362      | LACTOTRANSFERRIN | IPI00710664   | ↓  |    |    | U | 21.2    | 6.42 | Golgi apparatus; extracellular space; nuclear membrane | inflammatory response; complement activation, alternative pathway | endopeptidase inhibitor activity |
| 32  | 403      | U            | U             |    |    |    | U | 6.41    | 6.41 | extracellular space; extracellular region | proteolysis; digestion | serine-type endopeptidase activity; metal ion binding |
| 33  | 420      | COMPLEMENT C3 | IPI00713505   | ↑  |    |    | U | 187.3   | 6.41 | extracellular space; extracellular region | inflammatory response; complement activation, alternative pathway | endopeptidase inhibitor activity |
| 34  | 427      | CATIONIC TRYPsin | IPI00706427   | ↑  |    |    | U | 260     | 8.40 | extracellular space | lipid transport; lipoprotein metabolic process | lipid binding |
| 35  | 429      | COMPLEMENT C3 | IPI00713505   | ↑  |    |    | U | 339     | 5.13 | extracellular region; membrane; MHC class I protein complex | immune response; antigen processing and presentation | N |
| 36  | 430      | U            | U             |    |    |    | U | 37.5    | 6.27 | membrane | immune response | N |
| 37  | 434      | U            | U             |    |    |    | U | 6.41    | 6.41 | extracellular space; extracellular region | proteolysis; digestion | serine-type endopeptidase activity; metal ion binding |
| 38  | 435      | ZINC-ALPHA-2-GLYCOPROTEIN | IPI00698993   | ↑  |    |    | U | 430     | 5.29 | chylomicron; high density lipoprotein particle | lipid transport; lipoprotein metabolic process | N |
| 39  | 438      | BOLA-NC1     | IPI00710100   | ↑  |    |    | U | 23.2    | 5.61 | extracellular space | regulation of immune system process | N |
| 40  | 442      | U            | U             |    |    |    | U | 23.2    | 5.99 | extracellular space | regulation of immune system process | N |
| 41  | 455      | CATIONIC TRYPsin | IPI00706427   | ↑  |    |    | U | 23.2    | 5.99 | extracellular space | regulation of immune system process | N |
| 42  | 459      | APOLIPOPROTEIN A-IV | IPI00695965   | ↓  |    |    | U | 23.2    | 5.61 | extracellular space | regulation of immune system process | N |
| 43  | 469      | ALPHA-1-ACID GLYCOPROTEIN | IPI00691212   | ↑  |    |    | U | 23.2    | 5.61 | extracellular space | lipid transport; lipoprotein metabolic process | lipid binding |
| 44  | 470      | U            | U             |    |    |    | U | 23.2    | 5.61 | extracellular space | lipid transport; lipoprotein metabolic process | lipid binding |
| 45  | 474      | ORM1 23 kDa PROTEIN | IPI00903510   | ↑  |    |    | U | 23.2    | 5.61 | extracellular space | lipid transport; lipoprotein metabolic process | lipid binding |
| 46  | 478      | ALPHA-1-ACID GLYCOPROTEIN | IPI00691212   | ↑  |    |    | U | 23.2    | 5.61 | extracellular space | lipid transport; lipoprotein metabolic process | lipid binding |
| 47  | 480      | U            | U             |    |    |    | U | 23.2    | 5.61 | extracellular space | lipid transport; lipoprotein metabolic process | lipid binding |
| No. | Group ID | Protein name                            | Accession No. | LZ   | LA   | LF   | C     | MW (kDa) | pl   | Cellular Component | Biological Process                                      | Molecular Function                                      |
|-----|----------|-----------------------------------------|---------------|------|------|------|-------|---------|------|-------------------|--------------------------------------------------------|--------------------------------------------------------|
| 48  | 483      | ALPHA-1-ACID GLYCOPROTEIN               | IPI00691212   | ↑    |     |     |       | 23.2   | 5.61 | extracellular space | regulation of immune system process                    | N                                                      |
| 49  | 484      | CATHEPSIN B                             | IPI00692061   | ↑    |     |     |       | 36.7   | 5.68 | lysosome; mitochondrion | proteolysis; regulation of catalytic activity | cysteine-type endopeptidase activity                   |
| 50  | 497      | U                                       | U             | U    |     |     |       | 42.2   | 5.70 | cytoplasm         | regulation of proteolysis                            | serine-type endopeptidase inhibitor activity            |
| 51  | 503      | SERPIN1 LEUKOCYTE ELASTASE INHIBITOR    | IPI00710789   | ↑    | ↓   |     |       | 23.2   | 5.61 | extracellular space | regulation of immune system process                    | N                                                      |
| 52  | 505      | U                                       | U             | U    |     |     |       | 42.2   | 5.70 | cytoplasm         | regulation of proteolysis                            | serine-type endopeptidase inhibitor activity            |
| 53  | 506      | U                                       | U             | U    |     |     |       | 42.2   | 5.70 | cytoplasm         | regulation of proteolysis                            | serine-type endopeptidase inhibitor activity            |
| 54  | 527      | ALB PROTEIN                             | IPI00708398   | ↓    |     |     |       | 69.5   | 5.82 | extracellular space | transport                                             | N                                                      |
| 55  | 529      | ALB PROTEIN                             | IPI00708398   | ↓    |     |     |       | 69.5   | 5.82 | extracellular space | transport                                             | N                                                      |
| 56  | 545      | ALB PROTEIN                             | IPI00708398   | ↓    |     |     |       | 69.5   | 5.82 | extracellular space | transport                                             | N                                                      |
| 57  | 561      | U                                       | U             | U    |     |     |       | 42.2   | 5.70 | cytoplasm         | regulation of proteolysis                            | serine-type endopeptidase inhibitor activity            |
| 58  | 565      | U                                       | U             | U    |     |     |       | 42.2   | 5.70 | cytoplasm         | regulation of proteolysis                            | serine-type endopeptidase inhibitor activity            |
| 59  | 587      | U                                       | U             | U    |     |     |       | 42.2   | 5.70 | cytoplasm         | regulation of proteolysis                            | serine-type endopeptidase inhibitor activity            |
| 60  | 663      | ALPHA-2-HS-GLYCOPROTEIN                 | IPI00707101   | ↓    |     |     |       | 38.4   | 5.25 | extracellular space | acute-phase response; osmification; regulation of inflammatory response | cysteine-type endopeptidase inhibitor activity |
| 61  | 669      | ALPHA-2-HS-GLYCOPROTEIN                 | IPI00707101   | ↓    |     |     |       | 38.4   | 5.25 | extracellular space | acute-phase response; osmification; regulation of inflammatory response | cysteine-type endopeptidase inhibitor activity |
| 62  | 674      | 26 kDa PROTEIN                          | IPI00906471   | ↓    |     |     |       | 26.0   | 8.41 | extracellular region | N                                                      | N                                                      |
| 63  | 679      | HSPA8 HEAT SHOCK COGNATE 71 kDa PROTEIN | IPI00708526   | ↓    |     |     |       | 71.2   | 5.37 | cell surface; nucleolus; melanosome | response to stress; regulation of cell cycle | ATP binding; ATPase activity, coupled |
| 64  | 700      | ALPHA-1B-GLYCOPROTEIN                   | IPI00692686   | ↓    |     |     |       | 53.6   | 5.29 | extracellular region | N                                                      | N                                                      |
| 65  | 715      | SEROTRANSFERRIN                         | IPI00690534   | ↓    |     |     |       | 77.8   | 6.75 | extracellular region | cellular iron ion homeostasis                         | ferric iron binding                                    |
| 66  | 723      | SEROTRANSFERRIN                         | IPI00690534   | ↓    |     |     |       | 77.8   | 6.75 | extracellular region | cellular iron ion homeostasis                         | ferric iron binding                                    |
| 67  | 729      | PIGR ISOFORM LONG OF POLYMERIC IMMUNOGLOBULIN RECEPTOR | IPI00696714 | ↑    |     |     |       | 82.4   | 7.07 | extracellular region; plasma membrane | N                                                      | N                                                      |
| 68  | 737      | LACTOPEROXIDASE                         | IPI00716157   | ↓    |     |     |       | 80.6   | 8.83 | extracellular space | defense response to bacteria; hydrogen peroxide catalytic process | heme binding; peroxidase activity                       |
| 69  | 744      | COMPLEMENT C3                           | IPI00713505   | ↑    |     |     |       | 187.3  | 6.41 | extracellular region | inflammatory response; complement activation, alternative pathway | endopeptidase inhibitor activity                       |
| 70  | 799      | CATIONIC TRYSIN                         | IPI00706427   | ↓    |     |     |       | 26.0   | 8.40 | extracellular space | proteolysis; digestion                               | serine-type endopeptidase activity; metal ion binding |

* Proteins not identified by MALDI-TOF/TOF; † Upregulated proteins showing greater than 2-fold changes in the TC (LZ, LA and LF) and C groups compared to N group (p < 0.05); ‡ No function annotation; ‡ Downregulated proteins showing greater than 2-fold changes in the TC and C groups compared to N group (p < 0.05).
In this study, the MS identification of five whey samples was repeated 3 times under the same conditions and using the same instrument to improve the sensitivity, comprehensive coverage and reproducibility of detection. In addition, the replicates were also made to counterbalance the extreme complexity and large dynamic range of the protein components of milk. However, there were still 7 proteins that were specifically expressed in the TC groups and were also identified in the previous report of the profile of milk from conventionally bred cattle, indicating the inevitable inability to identify low-abundance milk proteins. This observation demonstrates that not all these proteins can be considered to be truly specifically expressed in the TC groups owing to the limitations on identifying proteins of trace abundance, which are beyond the detection range.

For the differentially expressed proteins, the successfully identified 46 protein spots were classified into 22 types of proteins as one protein can be present in different spots because of glycosylation or degradation. The concentration of SERPINB1 was significantly changed in all four groups. It has been categorized as a peptidase inhibitor and is thought to reduce protein digestibility and allow proteins to reach the intestinal tract. Most of the differentially expressed proteins are involved in biological processes with the partial function of defense response, providing protection against infection. This function includes 6 out of 13 identified proteins that have significantly higher expression levels, mostly in the C group, such CATHL1, complement C3, α-1-acid glycoprotein (AGP), zinc-α-2-glycoprotein (AZGP1), BOLA-NC1 and ORM1. Our data found no difference in the expression of immunological proteins, such as IgG, IgA and IgM, in milk between the different groups. A small number of the parameters had significant changes among the groups, but the difference among the groups was not greater than...
that within the groups, indicating that they may be due to individual differences, not genetic modification.

In summary, this study is the first attempt to use proteomic approaches to provide more comprehensive information on the milk proteome. The expression of exogenous proteins did not significantly change the milk whey protein profile, and the mean values for the majority of the measured parameters were all within the normal range. The differences among the groups were not greater than those within the groups, i.e., the differences were within the scope of intragroup variability, indicating that the differences were due to individual differences between cattle, not genetic modification. It is important to note that this study was conducted with a small number of transgenic

Figure 6. Comparisons of the major whey proteins and immune-associated proteins in the colostrum and mature milk from the TC, C, and N animals. Values are means ± SD and * indicate significant difference between the TC, C and N groups.
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Figure 7. Comparisons of the proximates in the colostrum and mature milk from TC, C, and N animals. (A) The proximates of the colostrum from each group. (B) The proximates of the mature milk from each group. Values are means ± SD and * indicate significant difference between the TC, C and N groups.
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cloned cattle. The information obtained from these results, however, will improve the understanding of the bovine milk proteome and provide data for the assessment of the food safety of transgenic cloned animals, which is expected to increase the acceptance of consumers.

Supporting Information

Figure S1 Comparisons of the amino acid content of the colostrum and mature milk from TC, C, and N animals. Values are means ± SD and * indicate significant difference between the TC, C and N groups. (TIF)

Figure S2 Comparisons of the fatty acid content of the colostrum from TC, C, and N animals. Values are means ± SD and * indicate significant difference between the TC, C and N groups. (TIF)

Figure S3 Comparisons of the fatty acid content of the mature milk from TC, C, and N animals. Values are means ± SD and * indicate significant difference between the TC, C and N groups. (TIF)

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Author Contributions

Conceived and designed the experiments: NL. Performed the experiments: CG TY SS. Analyzed the data: RZ CG TY SS JW. Contributed reagents/materials/analysis tools: NL. Wrote the paper: RZ CG.

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