ABSTRACT

The small intestine is the primary site of nutrient digestion and absorption, which plays a key role in the survival of neonatal calves. A comprehensive assessment of the phosphoproteomic changes in the small intestine of neonatal calves is unavailable; therefore, we used phosphopeptide enrichment coupled with liquid chromatography-tandem mass spectrometry to investigate the changes in the phosphoproteome profile in the bovine small intestine during the first 36 h of life. Twelve neonatal male calves were assigned to one of the following groups: (1) calves not fed colostrum and slaughtered approximately 2 h postpartum (n = 3), (2) calves fed colostrum at 1 to 2 h and slaughtered 8 h postpartum (n = 3), (3) calves fed 2 colostrum meals (at 1–2 and 10–12 h) and slaughtered 24 h postpartum (n = 3), (4) calves fed 3 colostrum meals (at 1–2, 10–12, and 22–24 h) and slaughtered 36 h postpartum (n = 3). Mid-duodenal, jejunal, and ileal samples of the calves were collected after slaughter. We identified 1,678 phosphoproteins with approximately 3,080 phosphosites, which were mainly Ser (89.9%), Thr (9.8%), and Tyr (0.3%) residues; they belonged to the pro-directed (52.9%), basic (20.4%), acidic (16.6%), and Tyr-directed (1.7%) motif categories. The regional differentially expressed phosphoproteins included zonula occludens 2, sorting nexin 12, and protein kinase C, which are mainly associated with developmental processes, intracellular transport, vesicle-mediated transport, and immune system process. They were enriched in the spliceosomes, adherens junctions, and tight junctions. The observed changes in the phosphoproteins in the tissues of small intestine suggest the protein phosphorylation plays an important role in nutrient transport and immune response of calves during early life, which needs to be confirmed in a larger study.

Key words: LC-MS/MS, newborn calf, phosphoproteins, phosphoproteome, phosphorylation sites

INTRODUCTION

The growth and health performance of young animals, especially for newborn calves during early life, has a direct effect on the realization of their genetic capacity in adulthood (Heinrichs and Heinrichs, 2011; Chuck et al., 2018). Colostral components, such as immunoglobulins, are transferred from the mother to calves and then absorbed through the intestine to establish passive immunity and protect against infectious diseases (Godden, 2008). Similarly, the nutritive substances in the colostrum have beneficial effects during the morphological development of the small intestine (Blättler et al., 2001; Fischer-Tlustos et al., 2020) and the functional maturation of the intestinal epithelial barrier in neonatal calves (Ghaifari et al., 2021). For example, colostrum-fed calves showed elevated serum levels of adiponectin and insulin-like growth factor 1 (Kesser et al., 2015; Mann et al., 2020), indicating that colostrum can regulate the expression of hormones. Moreover, several studies reported that colostrum proteins undergo significant changes in the small intestines of newborn ruminants. In our previous study, we employed 2-differentially expressed (DE)-based proteomics to examine the changes induced by colostrum feeding in the intestinal proteins of neonatal calves and

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discovered that several immune and membrane binding-related proteins were markedly internalized within the small intestine (Yang et al., 2019). Similar findings were observed in our other studies (Wang et al., 2020; Zhu et al., 2021). The transcriptome analysis of the intestinal tissues in neonatal calves revealed the altered expression of genes associated with nutrient ingestion and absorption, thereby contributing to intestinal health and development (Liang et al., 2016; Zhao et al., 2019). Thus, the colostral components digested and absorbed by the small intestine can affect the expression of proteins that are beneficial to the growth and development of neonatal animals.

Protein phosphorylation is an important post-translational modification in eukaryotes that facilitates various biological processes, such as cell growth, differentiation, and metabolism (Robles et al., 2017). Thousands of phosphoproteins in the rat liver, intestine, and other tissues were identified using phosphopeptide enrichment using titanium dioxide (TiO2) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The tissue-specific phosphorylation sites that modulate the protein interaction networks are essential for the functions in different tissues (Lundby et al., 2012). Enriched phosphopeptides labeled with Tandem Mass Tag and 2-dimensional LC-MS/MS were used to analyze the termination of liver regeneration in mice; proteins with dysregulated phosphorylation sites were mainly involved in metabolic and tight junction (TJ) regulation (Zhang et al., 2020). In addition, colostrum-fed newborn calves exhibited altered levels of protein phosphorylation in the liver; the phosphoproteins with altered expression were mainly involved in energy metabolism and immune response, suggesting that protein phosphorylation plays a key role in regulating liver function (Zhao et al., 2021). Therefore, protein phosphorylation could play an important regulatory role in different tissues. However, information pertaining to the changes in the phosphorylation of proteins in the intestinal tract of newborn calves is limited.

Identifying phosphoproteomic changes throughout the bovine small intestine is critical for understanding its regulatory role in developmental processes during the early stages of life. We hypothesized that changes in the protein phosphorylation in the small intestine are both regional and temporal and that these proteins are associated with varied functions. Therefore, in this study, we aimed to systematically identify and quantify the phosphoproteins in the small intestinal tissues of newborn calves using Ti4+ immobilized metal ion affinity chromatography (IMAC) phosphopeptide enrichment coupled with LC-MS/MS. This study would help characterize the changes in protein phosphorylation and their potential regulatory functions in the small intestine during the early life of bovine neonates.

MATERIALS AND METHODS

Animals and Sample Collection

Twelve neonatal male calves from multiparous Holstein dairy cows (40 ± 2 kg) reared at the Chuzhou dairy farm (Chuzhou, China) were used in this study. The procedures involving the care and use of animals were approved by the Animal Care Advisory Committee of the Anhui Academy of Agricultural Sciences (Hefei, China). The detailed experimental procedures are described in our previous studies (Qi et al., 2018; Zhao et al., 2018). Briefly, the first 2 milking colostrum samples were taken from healthy, multiparous Holstein cows, pooled, and then stored at −20°C. Before use, the colostrum was warmed to 40°C and then fed to newborn calves at approximately 8.0% of the animal’s BW. Three calves were not fed colostrum and were slaughtered approximately 2 h after birth (n = 3). Three calves were fed 1 colostrum meal at 1 to 2 h postpartum and were slaughtered approximately 8 h after birth (n = 3). Three calves were fed 2 colostrum meals at 1 to 2 h and 10 to 12 h postpartum and were slaughtered approximately 24 h after birth (n = 3). Three calves were fed 3 colostrum meals at 1 to 2, 10 to 12, and 22 to 24 h after birth and were slaughtered approximately 36 h postpartum (n = 3). Following the sacrifice, mid-duodenal (DU), jejunal (MJ), and ileal (IL) samples were collected from the same region, and washed thrice with pre-cooled phosphate-buffered saline (4°C) to remove the ingesta. All samples were stored at −80°C for further analysis.

Tissue Protein Extraction and Digestion

Total protein extraction and digestion using small intestine tissues were performed following a previously published method (Zhao et al., 2021). First, each sample was mixed with 10 volumes of lysis buffer (8 M urea, 2 M thiourea, 4% 3-[3-cholamidopropyl]-dimethylammonium]-1-propanesulfonate, 20 mM Tris-base, 30 mM dithiothreitol), followed by sonication on ice using a high-intensity ultrasonic processor (Ningbo Scientz Biotechnology Co. Ltd.). Second, after 3 volumes of acetone were added, the mixture was centrifuged at 13,000 × g for 20 min at 4°C to collect the precipitate. Third, the precipitate was redissolved with 40 mM ammonium bicarbonate and 5 M urea, and the protein concentration was detected using Bradford Assay Kit, according to the manufacturer’s instructions.
Fourth, the protein solution was mixed with 100 mM dithiothreitol for 1 h at 45°C and incubated with 100 mM iodoacetamide for 1 h at 25°C in the dark. Finally, trypsin solution was added (1:50; trypsin: protein mass ratio), and the sample was incubated at 37°C for overnight digestion. The resulting tryptic peptides were stored at −80°C.

**Phosphopeptide Enrichment Using Ti4+-IMAC**

The Ti4+-IMAC materials were prepared according to the method mentioned in our previous study (Zhao et al., 2021). For phosphopeptide enrichment, the tryptic peptide mixtures were incubated with the Ti4+-IMAC microsphere (5 mg) in 500 μL of binding buffer [6.0% trifluoroacetic acid (TFA), 80% acetonitrile (ACN)] at room temperature and with shaking for 120 min. The sample was centrifuged at 13,000 × g for 5 min at room temperature, and the beads containing the captured phosphopeptides were sequentially washed with 1 mL of washing buffer A (0.6% TFA, 50% ACN, and 200 mM NaCl), followed by 1 mL of washing buffer B (0.1% TFA and 30% ACN). The bound phosphopeptides were eluted twice with 100 μL of 500 mM dipotassium phosphate (pH 7.0), and the pooled elutions were desalted using Zip-Tip C18 columns (Millipore). The supernatant containing the phosphopeptides was collected and vacuum-dried for subsequent analysis.

**LC-MS/MS Analysis**

The enriched phosphopeptides were redissolved with 100 μL 0.1% formic acid and centrifuged at 13,000 × g for 15 min at 4°C to collect the supernatant. The peptides were injected into an Easy C18 column (100 μm i.d. × 20 mm, 5 μm) using an autosampler. This procedure was repeated in triplicate for each sample. The LC-MS/MS analysis was performed using Easy-nLC 1000 System coupled with a Q Exactive Mass Spectrometer (Thermo Fisher Scientific). Chromatographic separation was carried out on a C18 column (75 μm i.d. × 15 cm, 3 μm) using buffers A (0.1% formic acid) and B (0.1% formic acid in ACN) at a flow rate of 0.35 μL/min. The gradient was set at 3 to 90% buffer B over 120 min and the remaining at 90% buffer B for 10 min. Ion signals were collected in a data-dependent mode using the following settings: resolution at 70,000; automatic gain control target, 3 × 106; maximum injection time, 20 ms; and scan range, m/z 300 to 1,800. The resolution and loop count of MS/MS was 17,500 and 10, respectively. The charge exclusion was 1 or >8 charge states, and the dynamic exclusion was 20 s. The mass spectra data were recorded using Xcalibur software (version 2.2, Thermo Fisher Scientific).

**Data Analysis**

The raw data were searched against a local database containing 46,498 protein entries for Bos taurus (downloaded from Uniprot in April 2020; [https://www.uniprot.org](https://www.uniprot.org)) using MaxQuant software (version 1.6.1.0; Cox and Mann, 2008). The search parameters were set as follows: enzyme, trypsin/P; max missed cleavages, 2; fixed modification, carbamidomethylation (C, +57.02); variable modification, oxidation (M, +15.99); and phosphorylation (Ser/Tyr/Thr, +79.96). The peptide identity was only accepted if at least 2 spectra matched the peptide sequence. Trypsin/P with up to 2 miss-cleavages was used; false discovery rate was set at 1% for the identified proteins and peptides. Decoy-target false discovery rate estimation in MaxQuant was used to determine false positives (Cox and Mann, 2008). The precursor and fragment mass tolerances were set to 20 ppm and 4.5 ppm, respectively. WebLogo software ([http://weblogo.berkeley.edu](http://weblogo.berkeley.edu)) was used to analyze the model of sequences constituting AA in specific positions of 13-mers (6 AA upstream and downstream of the site) in all protein sequences. Based on the characteristics of the surrounding residues, kinase motifs are generally grouped into 3 main classes: pro-directed, acidic, and basic. The binary decision tree was employed as follows: pro at +1 (pro-directed), ≥5 Glu/Asp at +1 to +6 (acidic), Arg/Lys at −3 (basic), Glu/Asp at +1/+2 or +3 (acidic), ≥2 Arg/Lys at −6 to −1 (basic), and otherwise (others; Villén et al., 2007).

The phosphorylation sites were identified based on the Andromeda scores of all possible sites. Only phosphorylation sites with site confidence values > 0.75 were considered. The relative abundance levels of the identified phosphorylation proteins were quantified based on the label-free approach using MaxQuant software. Triplicates of each sample were submitted to the software, and 2- and multiple-sample t-tests were performed using Perseus software (version 1.6.0.2) (Tyanova et al., 2016). Significant difference between samples was determined if the Benjamini-Hochberg FDR value was ≤0.05 and the |log2 fold change| value was ≥2.

**Gene Ontology Enrichment and Kyoto Encyclopedia of Genes and Genomes Pathway Analysis**

The biological processes related to the DE phosphoproteins were classified using Database for Annotation, Visualization, and Integrated Discovery functional annotation tools ([https://david.ncifcrf.gov](https://david.ncifcrf.gov); Huang et al., 2009). The Gene Ontology terms for the target genes of phosphoproteins were ranked using the enrichment scores. Kyoto Encyclopedia of Genes and Genomes
pathway analysis was subsequently performed to identify the associated pathways and to determine the functions of the DE phosphoproteins. The protein-protein interaction network was constructed with medium confidence (0.4) using STRING (https://string-db.org) and Cytoscape software (version 3.6.0).

RESULTS

Overview of Phosphoproteins in the Small Intestines of Newborn Calves

To survey the phosphoprotein abundance, samples of 3 small intestinal segments (DU, MJ, and IL) were harvested from 2- to 36-h-old neonatal male Holstein calves. In total, we identified 1,678 phosphoproteins containing 3,080 phosphorylation sites in the small intestine tissues (Supplemental Table S1; https://doi.org/10.6084/m9.figshare.19187714.v2; Zhao, 2022). Among the identified phosphoproteins, 1,048 were detected in the 3 intestinal regions, whereas 36, 91, and 190 phosphoproteins were unique to the DU, MJ, and IL, respectively (Supplemental Figure S1A; https://doi.org/10.6084/m9.figshare.19187732.v2; Zhao, 2022a). In specific, 1,331 phosphoproteins with 2,238 phosphorylation sites were identified in the DU. Of the 2,238 phosphorylation sites, 89.9, 9.8, and 0.3% were Ser, Thr, and Tyr, respectively. In the MJ, 1,389 phosphoproteins containing 2,307 phosphorylation sites, mainly Ser (89.1%), Thr (10.5%), and Tyr (0.4%), were detected. In addition, 1,367 phosphoproteins with 2,413 phosphorylation sites were observed in the IL, in which 89.8, 9.8, and 0.4% of the identified phosphorylation sites were Ser, Thr, and Tyr, respectively (Supplemental Table S2; https://doi.org/10.6084/m9.figshare.19187741.v5; Zhao, 2022m).

Phosphoprotein Expression Patterns in the Small Intestines Across Development

We further analyzed the expression patterns of the phosphoproteins in the small intestine tissues of newborn calves across 4 developmental time points. A total of 1,016, 960, 1,102, and 898 phosphoproteins containing 1,548, 1,440, 1,720, and 1,340 phosphorylation sites were detected in the DU at 2, 8, 24, and 36 h after birth, respectively. In the MJ, we observed 1,129, 1,001, 1,011, and 1,087 phosphoproteins with 1,719, 1,506, 1,506, and 1,664 phosphosites at 2, 8, 24, and 36 h, respectively. In addition, 1,087, 1,064, 1,078, and 964 phosphoproteins contained 1,760, 1,730, 1,725, and 1,515 phosphorylation sites in the IL at 2, 8, 24, and 36 h, respectively (Supplemental Figure S1B; https://doi.org/10.6084/m9.figshare.19187768.v2; Zhao, 2022b).

To elucidate the role of peptide substrates and their corresponding kinases in the phosphorylation of proteins in the small intestines, the localized phosphosites were classified under 4 kinase motif categories, namely, prodirected, acidic, basic, or others. Notably, the phosphosites belonging to the prodirected motif category were the most abundant (54.2, 54.1, and 53.1% in the DU, MJ, and IL, respectively), followed by those in the basic and acidic motif categories. In addition, approximately 7.4, 7.0, and 8.3% of the detected phosphosites in the DU, MJ, and IL were categorized as others, which was a unique motif category (Supplemental Figure S2; https://doi.org/10.6084/m9.figshare.19187786.v2; Zhao, 2022c). In contrast, 5 kinase motifs with different amino acid residues were identified in the 3 intestinal segments, including 2 prodirected motifs (SP and TP), 2 acidophilic kinase motifs (SxE and SxD), and one unclassified motif (SxP; Figure 1).

Regional Expression of DE Phosphoproteins in the Small Intestine

We analyzed the DE phosphoproteins using principal component analyses to visualize the expression patterns in the small intestinal segments (Figures 2A). Notably, the expression patterns of the phosphoproteins in the DU and MJ clustered together and were different from that in the IL, suggesting that the expression pattern of phosphoproteins is tissue-specific. Similarly, the expression of 1,871 phosphorylated sites in the 1,084 phosphoproteins was significantly different. The DE phosphoproteins were strongly enriched in the pathways for TJ signaling, endocytosis, focal adhesion, mitogen-activated protein kinase (MAPK) signaling, and cyclic guanosine monophosphate-dependent protein kinase (cGMP-PKG) signaling (Figure 2B).

The regional expressions of the DE phosphoproteins between the DU and MJ, DU and IL, and MJ and IL regions are presented as volcano plots in Figure 3. Compared with the MJ region, 43 phosphoproteins, including tensin 1 (TNS1), zonula occludens 2 (ZO2), and eukaryotic translation initiation factor 2 subunit 2 (eIF2S2), were upregulated in the DU, whereas 152 phosphoproteins, including sorting nexin 12 (SNX12), protein kinase C (PKC), and serine/arginine repetitive matrix protein 2 (SMMR2), were downregulated (Figure 3A). Functional characterization revealed that the upregulated phosphoproteins in the DU were mainly associated with the biological process for single-organism process, developmental process, and TJ signaling was the enriched pathway. In contrast, the downregulated phosphoproteins in the DU were involved in metabolic process, cellular development,
vesicle-mediated transport, and immune response; endocytosis and insulin resistance were the enriched pathways (Supplemental Figure S3A; https://doi.org/10.6084/m9.figshare.19187816.v4; Zhao, 2022d).

In the DU and IL comparison, 509 phosphoproteins [e.g., ZO2, nonspecific serine/threonine protein kinase (PAK4), and epidermal growth factor receptor pathway substrate 8 (EPS8)] were upregulated in the DU, whereas 453 phosphoproteins [e.g., occludin (OCLN), SNX12, and bridging integrator 1 (BIN1)] were downregulated (Figure 3B). The upregulated phosphoproteins in the DU were enriched in macromolecule metabolic process, response to stimulus, and developmental process; endocytosis and cGMP-PKG signaling were the enriched pathways. The downregulated phosphoproteins were mainly involved in biological regulation, developmental process, cell adhesion, vesicle-mediated transport, and focal adhesion; MAPK signaling and TJ signaling were the enriched pathways (Supplemental Figure S3B; https://doi.org/10.6084/m9.figshare.19187837.v2; Zhao, 2022e).

In the MJ and IL comparison, 918 phosphoproteins [e.g., ZO2, phosphoglucomutase 1 (PGM1), and epsin 1 (EPN1)] and 431 phosphoproteins [e.g., ZO1, formin-binding protein 1 (FNBP1), and dynein cytoplasmic 1 light intermediate chain 2 (DYNClL2)] were upregulated and downregulated, respectively, in the MJ (Figure 3C). Furthermore, the upregulated phosphoproteins in the MJ were mainly associated with response to stimulus, developmental process, and protein transport; the enriched pathways were endocytosis, insulin signaling, and TJ signaling. The downregulated phosphoproteins were involved in developmental process, cell adhesion, and intracellular transport; focal adhesion, adherens junction, and TJ signaling were the enriched pathways (Supplemental Figure S3C; https://doi.org/10.6084/m9.figshare.19187840.v2; Zhao, 2022f).

Temporal Expression of DE Phosphoproteins in the Small Intestine

In addition to the variations observed in regional expression, the phosphoproteome profiles displayed temporal changes across the small intestine regions during the first 36 h of life. These temporal changes were investigated by comparing the expression levels of phosphoproteins between proximal age groups, specifically 36 versus 24 h, 24 versus 8 h, and 8 versus 2 h. The temporal expression of the DE phosphoproteins in the small intestinal are presented as volcano plots in Supplemental Figure S4 (https://doi.org/10.6084/m9.figshare.19187852.v1, https://doi.org/10.6084/m9.figshare.19187855.v1, https://doi.org/10.6084/m9.figshare.19187861.v1; Zhao, 2022g,h,i).

In the DU, 184 DE phosphoproteins with different phosphosites were identified, in which OCLN, ZO1, and ZO2 showed downregulated expression after the extension of colostrum feeding in newborn calves, whereas PKC, Y-box-binding protein 3 (YBX3), and peptidyl-prolyl cis-trans isomerase (PIN1) had upregu-
Figure 2. (A) Principal component (PC) analysis of identified phosphoproteins across small intestine tissues. (B) Significantly enriched Kyoto Encyclopedia of Genes and Genomes pathways of the identified phosphoproteins in small intestinal tissue. DU = mid-duodenal, MJ = jejunal, and IL = ileal samples.
lated expression (Supplemental Figure S4A; https://doi.org/10.6084/m9.figshare.19187852.v1; Zhao, 2022g). Functional enrichment analysis revealed that the DE phosphoproteins were mainly related to biological regulation, cellular development, and cell adhesion; TJ signaling was the enriched pathway (Supplemental Table S3; https://doi.org/10.6084/m9.figshare.19187939.v1; Zhao, 2022n).

A total of 277 DE phosphoproteins were detected in the MJ. Notably, thyroid hormone receptor interactor 10 (TRIP10), synaptopodin 2 (SYNPO2), and apoptotic chromatin condensation inducer 1 (ACIN1) showed downregulated expression in newborn calves 2–36 h after birth, whereas YBX3, vesicle amine transport 1 (VAT1), and La ribonucleoprotein 4B (LARP4B) showed upregulated expression (Supplemental Figure S4B; https://doi.org/10.6084/m9.figshare.19187855.v1; Zhao, 2022h). Functional characterization indicated that the DE phosphoproteins were associated with macromolecule metabolic process, developmental process, and biological adhesion; RNA splicing, TJ signaling, and spliceosome assembly were the enriched pathways (Supplemental Table S4; https://doi.org/10.6084/m9.figshare.19187942.v1; Zhao, 2022o).

In addition, 455 DE phosphoproteins were discovered in the IL. Among these, the downregulated phospho-

Figure 3. Detected regionally differentially expressed phosphoproteins in small intestine. The X and Y axes show log2 (fold change) and −log10 false discovery rate (FDR) of each differentially expressed phosphoprotein, respectively. (A) Differentially expressed phosphoproteins identified when comparing duodenum (DU) vs. jejunum (MJ). (B) Differentially expressed phosphoproteins detected when comparing DU vs. ileum (IL). (C) Differentially expressed phosphoproteins detected when comparing MJ vs. IL. Up = upregulated; down = downregulated.
proteins were PGM1, TNS1, and protein phosphatase 1 regulatory subunit 1B (PPP1R1B), whereas the upregulated phosphoproteins included PKC, vesicle-trafficking protein SEC22b (SEC22B), and RNA-binding motif protein 15 (RBM15) (Supplemental Figure S4C; https://doi.org/10.6084/m9.figshare.19187870.v1; Zhao, 2022i). Functional classification showed that most DE phosphoproteins were related to developmental process, cell-cell adhesion, and vesicle-mediated transport; focal adhesion, TJ signaling, insulin signaling, and regulation of actin cytoskeleton were the enriched pathways (Supplemental Table S5; https://doi.org/10.6084/m9.figshare.19187993.v1; Zhao, 2022p).

Protein-Protein Interaction Network Analysis of the DE Phosphoproteins in the Small Intestine

To examine the specific interaction patterns of the proteins in the small intestines, the highly DE phosphoproteins in the DU, MJ, and IL were used to construct a protein-protein interaction network. We discovered that 39 proteins with 55 interactions were linked in the DU, among which SRRM2, nucleolar and coiled-body phosphoprotein 1 (NOLC1), and proliferation-associated protein 2G4 (PA2G4) had the most connections with 7, 6, and 6 proteins, respectively (Figure 4). Furthermore, there were 115 proteins with 256 interactions in the MJ, in which glyceraldehyde-3-phosphate dehydrogenase (GAPDH), serine/arginine-rich splicing factor 1 (SRSF1), and 40S ribosomal protein S3 (RPS3) had the most connections with 20, 15, and 15 proteins in the network, respectively (Supplemental Figure S5A; https://doi.org/10.6084/m9.figshare.19187879.v1; Zhao, 2022j). In addition, 151 proteins with 335 interactions were detected in the IL, with transforming growth factor β-1-induced transcript 1 protein (TGFB1I1), proto-oncogene tyrosine-protein kinase Src, and SRSF1 showing the most interactions with 26, 18, and 17 proteins in the network, respectively (Supplemental Figure S5B; https://doi.org/10.6084/m9.figshare.19187879.v1; Zhao, 2022k). We observed that OCLN interacted with ZO1 (Figure 4), which is associated with the TJ signaling pathway, suggesting that OCLN and ZO1 are involved in the TJ assembly formation.

**DISCUSSION**

This study aimed to identify and compare the phosphoproteome in the tissues of the small intestine of newborn calves during the first 36 h of life. The primary function of the small intestine is the digestion and absorption of colostral components (Jochims et al., 1994; Godden, 2008). Furthermore, the small intestine has a role in protecting the host from invasion of pathogenic microorganisms and maintaining intestinal health (Santaolalla and Abreu, 2012; Martens et al., 2018). In our previous studies, 2-DE-based proteomics and label-free quantitative proteomic analysis were used to explore the changes of colostral proteins in the intestines of neonatal calves (Yang et al., 2019; Wang et al., 2020). However, the phosphoproteome of the small intestines and the changes in the protein expression of newborn calves were not well-studied. Therefore, to determine the potential regulatory function of protein phosphorylation in the small intestine, a phosphopeptide enrichment method coupled with LC-MS/MS was used to map the phosphoproteomic profiles of newborn calves. In this study, 3,409 phosphopeptides with 3,080 phosphosites from 1,678 DE proteins were identified. The DE phosphoproteins were mainly involved in the pathways for response to stimulus, developmental process, vesicle-mediated transport, cell adhesion, TJ signaling, endocytosis, focal adhesion, and MAPK signaling. These results provide novel insights into the effects of phosphorylation events on small intestine function during the early life of neonatal calves.

Similar to the results of previous studies, we observed that the main proportion of phosphorylation site residues in the small intestine tissues were Ser
(88.4%), Thr (10.9%), and Tyr (0.7%; Lundby et al., 2012; Zhao et al., 2021). The high abundance of Ser and Thr residues may be due to the frequent occurrence of phosphorylation events in the flexible and exposed regions of the corresponding protein. For example, SRRM2 was identified as the most highly phosphorylated protein in the small intestine, in which 33 differential phosphorylation sites were involved in RNA metabolism and processing. Specifically, SRRM2 is a splicing-associated protein that plays important roles in pre-mRNA splicing and cell migration (Mukherji et al., 2006). More than 75% of multiexon genes are reportedly subjected to alternative splicing (Johnson et al., 2003), and this form of pre-mRNA post-transcriptional modification has the potential to exponentially expand the proteome, generating various proteins with different activities (Schmutz et al., 2004). Thus, investigating the splicing machinery and their associated proteins may provide new information into the biological function of the small intestine.

In this study, DE phosphoproteins with specific regional expressions were closely associated with intracellular transport, vesicle-mediated transport, and endocytosis. Colostral components, such as adiponectin and protein components, can be transported and absorbed across the small intestine (Kesser et al., 2015; Wang et al., 2020; Zhu et al., 2021). The receptor-mediated transport of IgG during the first few hours of life was corroborated by the presence of bovine clathrin in the microvillous membrane of the duodenal and jejunal enterocytes (Jochims et al., 1994). Clathrin-mediated endocytosis is a key process in vesicular trafficking that transports a wide range of cargo molecules from the cell surface to the cytoplasm (Kaksonen and Roux, 2018). We detected several phosphoproteins, including EPN1, BIN1, SNX12, and TRIP10, which are associated with the transport and endocytosis of colostral components, in the small intestines of newborn calves.

The expression of EPN1 was higher in the MJ than that in the IL. Usually, EPN1 is common expressed in all tissues at the early stages of development and is an endocytic adaptor with putative functions in clathrin-mediated endocytosis and in the internalization of specific membrane proteins (Wendland, 2002; Chen and De Camilli, 2005). The endocytic function of EPN1 is linked to Notch activation; the disruption of Notch signaling results in the absence of EPN1, leading to developmental defects in the embryos (Wang and Struhl, 2005; Chen et al., 2009). The level of BIN1 phosphorylation was higher in the DU and MJ than that in the IL. Bridging integrator 1 influences clathrin-mediated endocytosis and intracellular endosome trafficking, by binding with dynamin 1 via their SH3 domains (Pant et al., 2009). The sorting nexin proteins are a family of molecules that regulate protein trafficking and sorting, especially the selective transport of endocytic and retrograde cargos (Pons et al., 2012; Priya et al., 2017). In an RNAi-based loss-of-function study, SNX12 suppression resulted in the inhibition of cation independent mannose-6-phosphate receptor transport and changes in the morphology of the endocytic compartments (Priya et al., 2017). In addition, TRIP10, which is highly expressed in the MJ, plays tissue-specific and cell lineage-specific functions in various cellular processes (Hsu et al., 2011). In adipocytes, TRIP10 promotes glucose uptake by interacting with TC-10 to regulate the translocation of insulin-stimulated glucose transporter 4 (GLUT4) to the plasma membrane (Lodhi et al., 2007). Moreover, TRIP10 enhances GLUT4 endocytosis through bidirectional interactions with N-WASp and dynamin-2 in muscle cells, thereby inhibiting glucose uptake (Hartig et al., 2009; Feng et al., 2010). Colostrum feeding improves glucose absorption in neonatal calves (Steinhoff-Wagner et al., 2011); however, there is no evidence on the involvement of TRIP10 in intestinal glucose absorption, nor has there been evidence of TRIP10 action in adipocytes and muscle. Further studies are needed to investigate the role of TRIP10 in glucose transport and absorption in the bovine small intestine. The identified DE phosphoproteins could contribute to the regulation of nutrient transport and absorption in the DU and MJ regions of the small intestine; their molecular mechanisms should be elucidated.

In addition to transport and endocytosis, several phosphoproteins were found to be associated with TJ. Previous study found that OCLN is one of the transmembrane proteins expressed in the small intestine and is highly phosphorylated on Ser and Thr residues, which are commonly related to the assembly and disassembly of TJ (Raleigh et al., 2011). Tight junctions in the intestinal epithelium with normal barrier function may still form in the absence of OCLN (Saitou et al., 2000). However, multiple abnormalities, including growth retardation, chronic gastritis, and impaired basal ganglia calcification, were observed in OCLN-KO mice (Saitou et al., 2000; Kitajiri et al., 2014). In addition, the primary cytoplasmic actin-binding proteins in the TJ are the ZO proteins (Campbell et al., 2017), which can connect with the transmembrane proteins in the cytoskeleton and allow protein complexes to aggregate into TJs in the intracellular domains (Mandon and Cyr, 2015). Notably, ZO1 and ZO2 were discovered to be essential in TJ formation because ZO1/2-deficient cells failed to form TJ (Phua et al., 2014). Similarly, our results showed that OCLN
interacts with both ZO1 and ZO2 in the constructed protein-protein interaction network. Recent findings report that the ZO protein family can form phase-separated droplets that recruit multiple TJ components, including OCLN and claudin (Beutel et al., 2019; Schwayer et al., 2019). Consequently, the recruitment of the ZO protein family to primordial adherens junctions may trigger the phase separation of these proteins, resulting in the recruitment of TJ-associated membrane proteins to facilitate TJ formation (Beutel et al., 2019). A previous study demonstrated that the interaction between OCLN and ZO1 can regulate the TJ; the binding sites were identified as the hinge region and GuK domain near the C-terminus of OCLN (Schmidt et al., 2004). The expression of TJ-related phosphoproteins (e.g., OCLN, ZO1, and ZO2) were downregulated in the small intestine with the extension of colostrum (e.g., OCLN, ZO1, and ZO2) were downregulated in the small intestine with the extension of colostrum feeding, which could be attributed to the maturation of intestinal barrier function. However, further research is required to confirm this hypothesis.

We detected several phosphoproteins involved in immune response, including EPS8 and PKC. As a substrate of receptor and nonreceptor tyrosine kinases (Huang et al., 2018), EPS8 can reportedly be induced by bacterial lipopolysaccharide (LPS) in macrophages to facilitate the toll-like receptor 4 (TLR4)/myeloid differentiation primary response 88 (MyD88)-mediated activation of Src, focal adhesion kinase, and p38 MAPK, subsequently promoting the phagocytic activity and bacterium-killing ability of macrophages. However, EPS8 knockdown resulted in the impairment of LPS-induced TLR4/MyD88 complex formation and inhibited the intracellular killing of *Escherichia coli* in macrophages (Chen et al., 2012). It has been shown that PKC is important for both innate and adaptive immunity (Lim et al., 2015), and PKC was previously confirmed as an important protein in the TLR4 signaling pathway during macrophage activation (Loegering and Lennartz, 2011). The role of PKC in host defense against bacterial infection was revealed through a study in PKC-deficient mice, which showed a diminished response to LPS stimulation and expressed low levels of tumor necrosis factor α and interleukin 1 β (Castrillo et al., 2001). Furthermore, LPS stimulation facilitates the PKC recruitment to TLR4 and the phosphorylation on S367 and S388 via MyD88 (Faisal et al., 2008), leading to the binding with 14-3-3β. This suggests that the phosphorylation of PKC is required for subsequent binding to 14-3-3β and complex formation with TLR, MyD88, and 14-3-3β to regulate gene expression (McGettrick et al., 2006). Therefore, colostrum feeding induced alterations in the phosphorylation of several proteins, including EPS8 and PKC, which are closely related to the innate immunity of newborn calves; they could play an important role in protecting calves from pathogenic microorganisms in the early stages of life. However, further studies are needed to confirm their specific role in the innate immunity of newborn calves. This is the first study to evaluate how the proteins in the small intestinal of neonatal calves are influenced by colostrum feeding.

### CONCLUSIONS

This is the first study investigating the phosphoproteome of the small intestine in neonatal calves at the early stage of life using a Ti⁺⁺-IMAC enrichment method coupled with LC-MS/MS. In total, 3,080 phosphorylation sites on 1,678 proteins were identified in the 3 small intestine regions. We also examined the regional and temporal expression of highly DE phosphoproteins in the small intestine tissues across developmental time points. Despite the small sample size (n = 3), our results provide novel insights into proteins in the small intestine, involved in vesicle-mediated transport, TJ formation, and immune response, indicating the potential importance of protein phosphorylation in the small intestine of newborn calves. Our results warrant follow-up in a larger-sized study.

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