Peptidomics Analysis Discloses That Novel Bioactive Peptides Participate in Necrotizing Enterocolitis in a Rat Model

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Necrotizing enterocolitis (NEC) is a common devastating gastrointestinal disease in premature infants, the molecular mechanisms of which have not been fully elucidated. Recently, endogenous peptides have garnered much attention owing to their role in diagnosis and treatment. However, changes in the peptide expression of NEC intestinal tissues remain poorly understood. In the present study, a comparative peptidomics profiling analysis was performed between NEC and control intestinal tissues via liquid chromatography-tandem mass spectrometry (LC-MS). In total, 103 upregulated and 73 downregulated peptides were identified (fold change ≥ 2.5, p < 0.05). Bioinformatics analysis revealed that these differentially expressed peptides were significantly associated with NEC pathophysiology, including apoptosis, the TGF-β signaling pathway, the Wnt signaling pathway, and the MAPK signaling pathway. Furthermore, two putative peptides could inhibit apoptosis and promote the migration of intestinal epithelial cells induced by lipopolysaccharide; these peptides were derived from the protein domains MT1 and EZRI, respectively. In conclusion, our study revealed that endogenous peptides are involved in the pathophysiologic mechanism of NEC; nevertheless, further exploration is required in this regard.

1. Introduction

Necrotizing enterocolitis (NEC) is one of the most common fatal diseases in preterm infants, with an incidence rate of 5–10% in very low birthweight (<1500 g) cases [1]. Some studies suggest that the administration of steroids, immunoglobulins, or Lactobacillus can reduce the incidence of NEC in neonates. However, the current treatment strategies predominantly focus on medical stabilization and the prevention of disease progression [2]. The mortality rate may rise to 50% when irreversible necrosis or perforation that requires surgery is present [3]. It is known that the disease progresses primarily via the invasion of inflammatory factors and bacteria in the gastrointestinal tract; however, the underlying molecular mechanisms remain to be elucidated completely [4].

Peptidomics, a novel branch of proteomics, has garnered considerable attention for its role as biological markers and therapies [5]. Recently, peptidomics analyses have been applied in detecting peptide changes in different diseases, including cancer [6, 7]. Increasing evidence has shown that endogenous peptides play a crucial role in digestive system diseases [8]. For instance, trefoil factor family peptide 3 (TFF3), mainly located in goblet cells, is significantly upregulated in peptic ulcer. TFF3 not only participates in maintaining and repairing mucosal integrity but also promotes the healing of intestinal ischemia-reperfusion injury in
weaned rats [9]. Therefore, an analysis of differentially expressed peptides in NEC tissues may provide new insights to explore treatment strategies.

To explore the peptide expression profile of NEC, quantitative liquid chromatography-mass spectrometry (LC-MS) was performed, and the peptide content of rat NEC intestinal tissue (NEC group) and normal control intestinal tissue (Ctrl group) was analyzed. Based on preliminary functional verification, two downregulated peptides derived from the EZRI (EDP1) and MT1 (MDP2) domains showed a significant protective effect in a lipopolysaccharide- (LPS-) induced NEC model in vitro and are worthy of further study.

2. Materials and Methods

2.1. Ethics Statement. The study was conducted with the approval of the Animal Ethics Committee of Nanjing Medical University (No: IACUC-1807004). To minimize the suffering of the animals used in this research, the procedures followed were in strict compliance with the Guide for the Care and Use of Laboratory Animals.

2.2. Establishment of the Animal Model. Eighteen neonatal Sprague–Dawley rats provided by the Animal Core Facility of Nanjing Medical University were randomly assigned to the Ctrl and NEC groups. The rats in the Ctrl group were fed by their mothers as needed; the NEC rat model was established as follows [10]: (1) hypoxia-cold stimulation: asphyxia stress via exposure to 95% nitrogen twice a day for 5 min, followed by exposure to a cold temperature (4°C) for 10 min; (2) formula feeding (ionized water (10 mL) and Esbilac (PetAg) canine milk (5 mL) (2:1) mixed with Similac Advance infant formula (Abbott Nutrition) 2 g); the animals received perfused hypertonic milk (0.08–0.1 g/kg) immediately after cold stimulation. Four days for establishing the model, the rats were sacrificed, and intestinal tissue was collected. Intestinal tissue (3 cm) from the terminal ileum was harvested and subjected to NEC model determination and peptide extraction.

2.3. Peptide Extraction and Purification. Three pairs of intestinal tissue samples from the Ctrl and NEC groups were ground into powder in liquid nitrogen and fully compatible with denaturing buffers including 8 M urea/100 mM TEAB (pH 8.0), 1 mM PMSF, 2 mM EDTA, and 10 mM DTT. The mixture was sonicated on ice for 10 min and centrifuged at 4°C (12,000 rpm) for 30 min; then, the supernatant was transferred to a fresh centrifuge tube. MWCO (10 kDa) filter tubes (Millipore, Billerica, MA, USA) were used to filter the supernatant. The filter tubes were centrifuged at 4°C (10,000 × g); flow-through containing the peptides was collected and purified using a C18 column. Finally, the flow-through was dried under vacuum and stored at -80°C.

2.4. TMT Labeling and LC-MS Analysis. Formic acid (0.1%) was used to dissolve the peptide samples, followed by reducing in 10 mM DTT and alkylating with 50 mM iodoacetamide for 30 min. Following desalting and lyophilization, the peptides were labeled with TMT reagent (TMT 6 Relabeling Reagent, Thermo Fisher Scientific, USA). The labeled peptides were mixed and analyzed via LC-MS/MS [11]; an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) was used for this mass spectrometry analysis. In total, 2000 single emission spectra were accumulated from 10 random positions of each sample. Using isotope correction factors recommended by the manufacturer, search for MS data using MASCOT database (Matrix Science, Boston, MA, USA) and SwissProt database. Additionally, to reduce the possibility of erroneous peptide identification, only those peptides with a significance score (≥20) within a 99% confidence interval and greater than “identity” were counted via MASCOT probability analysis [12].

2.5. Bioinformatics Analysis. The characteristics of the identified peptides were analyzed using ProtParam tool. Presumed functions on peptides and protein–protein interactions were predicted using the UniProt and STRING databases. We used DAVID v6.7 (Database for Annotation, Visualization and Integrated Discovery; https://david.ncifcrf.gov/) for conducting GO and KEGG pathway enrichment analyses to investigate the potential functions of peptides and their precursor proteins.

2.6. Hematoxylin-Eosin Staining (H&E Staining). After sacrifice, intestinal tissue from the rats’ terminal ileum was

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**Figure 1:** Establishment of the NEC model: (a) H&E staining of representative intestinal tissue sections from the Ctrl and NEC groups (bar = 100 μm); (b) pathological scores of representative intestinal tissue sections from the Ctrl and NEC groups (**p < 0.001**).
Table 1: Differential peptides in the intestine tissue of NEC rat model.

| Accession | Gene       | Peptide             | Fold change | p value |
|-----------|------------|---------------------|-------------|---------|
|           |            | Upregulated peptides |             |         |
| G3V8B3    | Hist1h2bq  | AVTKYTSSK           | 57.72612    | <0.05   |
| P69897    | Tubb5      | FVFGQSGAGNN         | 31.84287    | <0.01   |
| Q10758    | Krt8       | DGKLVSSESDIMSK      | 25.55914    | <0.001  |
| Q10758    | Krt8       | LVSESSDIMSK         | 22.05736    | <0.001  |
| P15999    | Atp5a1     | VGLKAPGIIPR         | 21.49717    | <0.001  |
| O88989    | Mdh1       | VIVVGNPANTNLASK     | 12.14385    | <0.05   |
| Q66HT1    | Aldob      | ALQASALAAWWGGK      | 11.93121    | <0.001  |
| G3V8B3    | Hist1h2bq  | LLLPGELAKH          | 11.86002    | <0.01   |
| P04182    | Oat        | ALQDPNVAAF          | 11.16052    | <0.05   |
| P05197    | Eef2       | VFSGVSTGKLVR        | 10.57569    | <0.05   |
| Q10758    | Krt8       | QPGFGSVDGGSST       | 10.57101    | <0.01   |
| F1LNF1    | Hnrnpa2b1  | GFGFVFTF            | 10.55872    | <0.05   |
| K7S2S2    | Hist2h2a3  | AGLQFPVGR           | 10.3368     | <0.05   |
| Q7M0E3    | Dstn       | FLWAPEQAPLKS        | 10.01604    | <0.01   |
| Q10758    | Krt8       | GGLTSPGF           | 9.12657     | <0.001  |
| M0R7B4    | Hist1h1d   | SVGSLAALKK        | 8.945185    | <0.001  |
| Q10758    | Krt8       | QPGFGSVDGGSST       | 8.793551    | <0.001  |
| P69897    | Tubb5      | FVFGQSGAGNN         | 8.771316    | <0.05   |
| D3ZGY4    | Gapdh-ps2  | GAANQNIAPSTGAAKAVGK | 8.399639    | <0.001  |
| D3ZBN0    | Hist1h1b   | SLVSKGTQVTK         | 8.352781    | <0.01   |
| M0R7B4    | Hist1h1d   | GILVQTKGTSAGS       | 8.334644    | <0.001  |
| P02262    | NA         | VGAGAPVYL          | 8.301511    | <0.001  |
| Q10758    | Krt8       | GMSSFQPGFSGVGSST    | 8.18755     | <0.01   |
| P10719    | Atp5b      | IGLFGGAGVGK         | 7.935978    | <0.01   |
| B5DEM5    | Rpl14      | AAAIAAAAAAK         | 7.751116    | <0.01   |
| K7S2S2    | Hist2h2a3  | GLQFPVGR            | 7.629358    | <0.05   |
| P62630    | Eef1a1     | ESFSDYPPGLR         | 7.607978    | <0.001  |
| O55159    | Epcam      | QKDCVCNNYKLTSR      | 7.485879    | <0.001  |
| P15865    | Hist1h1e   | SETAPAAAPAPAEKTPIKK | 7.38236     | <0.01   |
| D3ZGY4    | Gapdh-ps2  | VKVGVNGFFGR         | 7.323894    | <0.001  |
| F1M577    | LOC100359671 | AFIAHPKLGK     | 6.954795    | <0.05   |
| Q6P725    | Des        | TFGGAPGFGSGLPSLS    | 6.720618    | <0.05   |
| G3V8B3    | Hist1h2bq  | LLLPGELAK           | 6.59403     | <0.01   |
| D3ZIE9    | Aldh18a1   | VGLGMEAKVK          | 6.321181    | <0.001  |
| Q925G0    | Rbm3       | DYSGSQGQGDREYGNN    | 6.259407    | <0.01   |
| Q10758    | Krt8       | SFQPFGSVDGGSST      | 6.237045    | <0.01   |
| Q10758    | Krt8       | SFQPFGSVDGGSST      | 6.119025    | <0.01   |
| Q9QXQ0    | Actn4      | STALYGESDL          | 5.964186    | <0.001  |
| Q10758    | Krt8       | GSLGGFAGAGVGGIT     | 5.600793    | <0.05   |
| P05065    | Aldoa      | ALQASALK            | 5.47993    | <0.001  |
| M0RA08    | Plin3      | VTVGADVTCGVK        | 5.466526    | <0.001  |
| Q6P9V9    | Tuba1b     | VAEITNACFPAN        | 5.315759    | <0.05   |
| A0A0G2K3Z9 | NA        | SSGNAKIGHPAPSFK     | 4.969645    | <0.01   |
| Q66H80    | Arcn1      | VLLAAAVCTK          | 4.865557    | <0.05   |
| D3ZGY4    | Gapdh-ps2  | ALNDFVKK            | 4.838244    | <0.05   |
| B0K031    | Rpl7       | IALTDNSLVAR         | 4.834916    | <0.01   |
| P62268    | Rps23      | KANPFQGASHAK       | 4.801351    | <0.001  |
| Accession | Gene    | Peptide                  | Fold change | p value |
|-----------|---------|--------------------------|-------------|---------|
| D3Z7Y6    | Krt20   | LGVAPSYVGGAGGHGT         | 4.677876    | <0.001  |
| Q10758    | Krt8    | KLEVDVPNQAVR             | 4.663074    | <0.001  |
| D3ZBN0    | Hist1h1b| SLVSKGTLVQTKGTSAGSF      | 4.551461    | <0.01   |
| M0R7B4    | Hist1h1d| SGVSLAALK                | 4.43164     | <0.01   |
| P05197    | Eef2    | VNFITVDQIR               | 4.41721     | <0.01   |
| V5QR27    | NA      | FPGQPQGGPGA              | 4.341792    | <0.05   |
| P63029    | Tpt1    | LDYREDGVTPF              | 4.230072    | <0.05   |
| Q5EB49    | Enol    | SFRNPLAK                 | 4.188444    | <0.05   |
| Q802E7    | Cd2ap   | GLPAGGIQPHQTK            | 3.871932    | <0.01   |
| M0R7B4    | Hist1h1d| ASGPPVSELTK              | 3.803902    | <0.001  |
| P18420    | Psm1    | LVSILIGSK                | 3.745425    | <0.05   |
| P13437    | Aca2    | GVFFIAAK                 | 3.72717     | <0.01   |
| D3ZGY4    | Gapdh-ps2| GAAQNIIPASTGAK          | 3.718082    | <0.001  |
| Q10758    | Krt8    | SLGGFGGAGVGGIT           | 3.670916    | <0.01   |
| D3ZBN0    | Hist1h1b| ALAAGGYDVKENNSR          | 3.654154    | <0.05   |
| Q9WVK7    | Hdh     | TGEGFYKK                 | 3.578854    | <0.01   |
| K7S2S2    | Hist2h2a3| AGLQQPVGRVH            | 3.386712    | <0.01   |
| A0A0G2K6S9| Myh11  | STVAALEAK                | 3.278155    | <0.01   |
| P06302    | Ptna    | SDAAVDTSSEITTK           | 3.185452    | <0.05   |
| G3V779    | Ld1     | TEVLVTPAGVASK            | 3.18017     | <0.01   |
| B2GV1     | S100a6  | LIYNEALK                 | 3.138931    | <0.001  |
| K7S2S2    | Hist2h2a3| AEILELAGNAAR          | 3.121051    | <0.01   |
| P69897    | Tub5    | YNEATGKYVPR              | 3.11043     | <0.01   |
| D3Z7Y6    | Krt20   | SLSSSQGPALOTSAGSL        | 3.061158    | <0.05   |
| P63601    | Hspa8   | SKGPAVGIDLGTT            | 3.057902    | <0.05   |
| A0A0G2JWK7| Tagln   | EFTDSQLQEGK              | 3.027585    | <0.05   |
| P66230    | Eef1a1  | QTAVGVIK                 | 3.020994    | <0.05   |
| P21913    | Sdhb    | AQTAAPAARIK              | 2.929567    | <0.01   |
| Q5RJK6    | Inpp1   | LIQSLGPLKT               | 2.890027    | <0.01   |
| P10860    | Glud1   | VVQQFGNGVGLH             | 2.760697    | <0.05   |
| P47875    | Csrp1   | GFGFQGGAGALVH            | 2.63933     | <0.001  |
| Q63191    | Mamdc4  | ADQVTLEPISTSNP           | 2.631403    | <0.01   |
| Q07936    | Anxa2   | STVHEILCK                | 2.62143     | <0.01   |
| P06302    | Ptna    | AAVDTSSEITTK             | 2.568546    | <0.01   |
| Q3MHS9    | Cct6a   | AAVKTLNPKAERV            | 2.554117    | <0.05   |
| P38552    | Lgal4   | YPSAGYNPPQ               | 2.508311    | <0.05   |
| P05197    | Eef2    | VAVEAKNPADLPK            | 2.48379     | <0.01   |
| Q5RKI0    | Wdr1    | SVADGYSENNVF             | 2.474195    | <0.001  |
| G3V8B3    | Hist1h2bq| HAVSEGTKAVTKYTTSS       | 2.468693    | <0.05   |
| Q6URK4    | Hnrnpa3 | YGGGGGNYNDFGN            | 2.394012    | <0.05   |
| Q6VPP3    | Clca4   | DTPQVLENFSR              | 2.382552    | <0.05   |
| P12346    | Tf      | LLEACTFHKS               | 2.333879    | <0.05   |
| P63029    | Tpt1    | GAIDDSLIGGN              | 2.312302    | <0.001  |
| Q10758    | Krt8    | GGFGGAGVGGIT             | 2.276436    | <0.05   |
| Q07936    | Anxa2   | STVHEILCK                | 2.247658    | <0.01   |
| P00731    | Cpa1    | ALSTDSPNAYTH             | 2.173098    | <0.05   |
| Q6URK4    | Hnrnpa3 | SGPSYGGSYGSGGGGSYGS      | 2.128543    | <0.05   |
| P38552    | Lgal4   | YPSAGYNPPQMN             | 2.11776     | <0.001  |
| Accession | Gene | Peptide | Fold change | p value |
|-----------|------|---------|-------------|---------|
| Q4FZY0    | Efhd2| ATDELASKLSR | 2.05165 | <0.05 |
| A0A0G2QC04 | Pls1 | EGITAIGGTSS | 2.004625 | <0.01 |
| P38552    | Lgals4| PAYPSAGYNPPQM | 1.905471 | <0.001 |
| Q9Z144    | Lgals2| SEKFEVTNLNMK | 1.899939 | <0.05 |
| D3ZVN3    | Bfox10| HNAEAGVDIR | 1.77112 | <0.05 |
| G3V779    | Lad1 | QQVVGAVQPQPKVE | 1.722176 | <0.01 |
| P46462    | Vcp | AVANETGAF | 1.668905 | <0.001 |
| D4AZ38    | Tmco3 | LIRRIPTQSV | 1.649045 | <0.05 |

**Downregulated peptides**

| Accession | Gene | Peptide | Fold change | p value |
|-----------|------|---------|-------------|---------|
| A0A0G2K890 | Ezr | FVIKPIDKK | -1.50403 | <0.05 |
| Q9JJ19    | Slc9a3r1 | EALVEPASESPRP | -1.52587 | <0.05 |
| K7S2S2    | Hist2h2a3 | AQQGVLPNQAV | -1.53578 | <0.05 |
| P15865    | Hist1h1e | APAAPAAPAEPAPT | -1.57197 | <0.001 |
| F1M4J0    | Rictor | STELLG | -1.57875 | <0.05 |
| A0A0G2JUA5 | Ahnak | DYPDQGPDWH | -1.61943 | <0.01 |
| Q63191    | Mamdc4 | NAQVTLPESITSNP | -1.64595 | <0.05 |
| Q6P9V9    | Tuba1b | DLEPTVIDEVR | -1.66594 | <0.05 |
| B5DFA0    | Vili | EENQVITPRFL | -1.69645 | <0.05 |
| A0A0G2K3K2 | Actb | NELRVAEEHPV | -1.70874 | <0.05 |
| Q63191    | Mamdc4 | QVTLPESITSNP | -1.7079 | <0.05 |
| D3ZDZ1    | Apbb1ip | LPPPPPPP | -1.72276 | <0.01 |
| Q5EB49    | Enol | KLNVVEQIKIDQLMEMGDGENK | -1.7416 | <0.05 |
| P62630    | Eef1a1 | GYGEFEAGISKN | -1.78763 | <0.01 |
| P63269    | Actg2 | FAGIDAPAVFPS | -1.81415 | <0.001 |
| D3ZBN0    | Hist1h1b | GTGASGFLKN | -1.85091 | <0.001 |
| D3ZAU0    | Muc5b | ITFCGSCGK | -1.90446 | <0.05 |
| P06302    | Ptma | SDAVDSSEITTKDL | -1.93122 | <0.05 |
| D3ZX87    | LOC100910017 | KNQVTNYD | -1.93203 | <0.05 |
| D3ZUM4    | Glib1 | LELCTVEFVDPVF | -1.97941 | <0.01 |
| Q6P9V9    | Tuba1b | QPPTVPGGDGLAK | -2.01585 | <0.05 |
| P63312    | Tmsb10 | ADKPMGEIASFD | -2.02372 | <0.001 |
| DA4Z69    | NA | ADEIAKAOVA | -2.02826 | <0.05 |
| A0A0G2K890 | Ezr | APPPPPPPVPVEPN | -2.10935 | <0.01 |
| F1LQ48    | Hnrnp1 | DEYGPPPYPHYE | -2.13291 | <0.01 |
| P08081    | Cita | APAPOGPPALGN | -2.17074 | <0.001 |
| P06761    | Hspa5 | MKETAEAYLKK | -2.21256 | <0.01 |
| Q62803    | Spal1 | LEDDLVNTIEIV | -2.21478 | <0.05 |
| P38552    | Lgals4 | IPAPSAGYNPPQ | -2.21577 | <0.01 |
| A0A0G2K6S9 | Myh11 | AQQGQLSDDEKF | -2.22383 | <0.05 |
| P13437    | Aca2a | SAGCIGGQGSLIQQNTA | -2.23257 | <0.01 |
| P30009    | Marcks | SPEAPPPVVAE | -2.24701 | <0.001 |
| P45592    | Cfl1 | ASGVAVSDGVIK | -2.24734 | <0.01 |
| O55212    | NA | DAGAAGGPPGGPGLG | -2.30987 | <0.05 |
| D4AE06    | Fkbp15 | TPSVQSPLSHP | -2.32989 | <0.01 |
| P62630    | Eef1a1 | SGDAIVDVMVPGP | -2.36181 | <0.001 |
| P63269    | Actg2 | FAGDAPRAVPVF | -2.37939 | <0.05 |
| P62630    | Eef1a1 | FAPVNTTEVK | -2.45167 | <0.01 |
| P30009    | Marcks | CSPEAPPVVAE | -2.54825 | <0.01 |
harvested and fixed with 4% paraformaldehyde for 1 d at room temperature. Following dehydration, embedding, sectioning, defatting, rehydration, and H&E staining, the tissue sections were observed under a light microscope (Zeiss, Oberkochen, Germany).

### Table 1: Continued.

| Accession | Gene | Peptide | Fold change | p value |
|-----------|------|---------|-------------|---------|
| A0A0G2K3K2 | Actb | DSGDGVTHTVPIYE | -2.55819 | <0.01 |
| D3ZFU9 | Mylk | AQPVGALKPIGN | -2.77191 | <0.05 |
| P15865 | Hist1h1c | SETAPAAAPAPAETPIK | -2.77847 | <0.001 |
| Q53Z83 | Mt1 | SCCSCCPVGSKCAQGCVC | -2.79709 | <0.05 |
| D3ZDZ1 | Aplb1p | NLPFPFFFPPP | -2.8025 | <0.0001 |
| P63312 | Tmsb10 | ADKPMGIEAS | -2.84338 | <0.0005 |
| Q6URK4 | Hnmpa3 | SPYGGGYSGGGSGY | -2.93019 | <0.01 |
| Q10758 | Krt8 | GFGAGVGGIT | -2.95692 | <0.01 |
| P62630 | Eef1a1 | VGEEAGIKN | -2.98559 | <0.001 |
| D3ZFU9 | Mylk | AKPAETLPVGN | -2.98675 | <0.01 |
| A0A0G2K890 | Ezr | APPPPPPVYE | -3.14377 | <0.001 |
| Q91W32 | Casp1 | KEQGAFGPSGLK | -3.15783 | <0.01 |
| Q53Z83 | Mt1 | SCCSCCPVGSKCAQGCVC | -3.27655 | <0.01 |
| P06302 | Ptna | SDAAVTSSEITDKDLK | -3.51799 | <0.001 |
| B6ID08 | Mt2a | SCCSCCPVGSKACSCQGICG | -3.56818 | <0.05 |
| Q6P9V9 | Tuba1b | LEPTVIDEVR | -3.6423 | <0.001 |
| P62630 | Eef1a1 | AQQGFAEAGIKN | -3.79477 | <0.01 |
| D3ZJF8 | Fcgbp | CVAESTAVCRAQGD | -3.88626 | <0.001 |
| A0A0G2K890 | Ezr | AELSSEGILDDR | -3.89919 | <0.05 |
| A0A0G2JUAI | Ahnak | EGPEVDVSLP | -4.05973 | <0.01 |
| Q53Z83 | Mt1 | CAQGCVGKASDKCTCCA | -4.23021 | <0.01 |
| Q63041 | A1m | AVDSVLKPKPAK | -4.3155 | <0.001 |
| D3ZBNO | Hist1h1b | GPPVSRE | -4.64319 | <0.001 |
| P13668 | Stmn1 | ASSDIQVKELE | -4.88393 | <0.01 |
| P97881 | Muc13 | GVPSQTSNYPAN | -5.02829 | <0.001 |
| Q63041 | A1m | ALVQKSDVTKPVVEPEG | -5.23966 | <0.01 |
| Q6P9V9 | Tuba1b | TVVPGGDL | -5.71525 | <0.001 |
| P15865 | Hist1h1e | SETAPAAAPAPAETP | -6.05552 | <0.001 |
| Q7M0E3 | Dstn | FLWAPEQAPL | -6.43258 | <0.01 |
| P62630 | Eef1a1 | ALPGDNVGFN | -6.78263 | <0.001 |
| Q5PQK2 | Fus | YPTQPGQGSQSN | -8.52323 | <0.001 |
| Q10758 | Krt8 | FGGAGVGGIS | -8.58147 | <0.001 |
| P62630 | Eef1a1 | APVNYTTEVK | -13.4474 | <0.001 |
| P63269 | Actg2 | GQKDSYVGDEAQSKE | -15.8726 | <0.001 |

2.7. **Cell Culture.** IEC-6 enterocytes were purchased from the American Type Culture Collection (ATCC, USA). The growth medium consisted of high glucose Dulbecco’s Modified Eagle’s medium (DMEM, Gibco BRL), 10% fetal bovine serum (FBS, Gibco BRL), 1% penicillin-streptomycin solution, and 0.1 U/mL recombinant human insulin. The cells were incubated at 37°C under 5% CO₂ and treated with 100 μg/mL LPS for 6 h to establish the NEC model in vitro.

2.8. **Wound-Healing Assay.** Cell migration was analyzed using the wound-healing assay. IEC-6 cells were seeded in 6-well plates and cultured in DMEM containing 10% FBS. On reaching confluence, the cell monolayer was scraped using sterile 1 mL pipette tips to form a uniform cell-free area. The wells were gently washed with sterile phosphate-buffered saline (PBS) to remove debris from the medium and monitored under an inverted microscope (Eclipse TS100-F, Nikon) (0 h). Next, fresh medium was added to the wells, and the plates were incubated in the presence of EDP1 or MDP2 (20 μM/L) at 37°C for 12 h and monitored under an inverted microscope (12 h). Images taken at the 0 h and 12 h timepoints were expressed as the migration area, and the distances were measured using the ImageJ software v4.16.
Figure 2: Continued.
2.9. Detection of Apoptosis. According to the manufacturer’s instructions, an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) cell apoptosis kit was used to detect the apoptosis level of the IEC-6 cells. In brief, after incubating the NEC in vitro model with EDP1 and MDP2 for 24 h, the IEC-6 cells were collected and washed with PBS, resuspended in 300 μL binding buffer at a concentration of 1 × 10⁶/mL, and mixed with 3 μL annexin V-FITC and 3 μL PI for 15 min. A BD LSRII Flow Cytometer System with the FACS Diva Software was used to analyze the mixtures within 1 h.

2.10. Statistical Analysis. All statistical analyses were performed using the SPSS statistical software. Student’s t-test was used to assess significant differences between the two groups. Statistical comparisons between multiple groups were analyzed using ANOVA. Statistical significance was determined as p < 0.05.

3. Results

3.1. Establishment of the NEC Model. Hypoxia-cold stimulation combined with hypertonic milk gavage was used to establish the NEC rat model. An optical microscope was used to observe stained intestinal tissue sections and evaluate the pathological score. The histopathological results showed shedding of the intestinal villi, submucosal edema, and inflammatory cell infiltration in the NEC group. In contrast, the intestinal mucosa epithelium was intact, the glands were arranged regularly, and edema and inflammatory cell infiltration were not evident in all layers of the intestinal wall in the Ctrl group (Figure 1(a)). In the NEC rat pups, the pathological score [13] was significantly higher than that in the Ctrl rat pups (Figure 1(b)). These findings revealed that the NEC model was successfully established.

3.2. Differentially Expressed Peptides in Intestinal Tissue Samples. TMT-labeled LC-MS/MS analysis was performed on peptides in the intestinal tissue samples from both the Ctrl and NEC groups. A total of 480 nonredundant peptides were identified, with 176 differentially expressed peptides derived from 91 protein precursors (fold change ≥ 1.5, p < 0.05).

Detailed information on these peptides is shown in Table 1. Upregulated or downregulated differentially expressed peptides were visualized in a hierarchical clustering heat map (Figure 2(a)). The number of upregulated or downregulated differentially expressed peptides is shown in Figure 2(b).

3.3. Characteristics of the Differentially Expressed Peptides. Some specific characteristics of the peptides were detected in this study. Among these characteristics, the amino acid numbers are mainly focused on 9–14 (Figure 3(a)), the peptides range largely from 800–1800 (Figure 3(b)), and the pI values are evenly distributed on both sides of acid and basic (Figure 3(c)). In addition, the distribution of MW relative to pI (MW/pI) was investigated based on the amino acid composition and MW distribution specific to a pI (Figure 3(d)). As the generation of peptides mainly depends upon the enzymatic cleavage of precursor proteins [6], the cleavage site patterns of the identified peptides were analyzed (Figures 3(e) and 3(f)). Among the upregulated peptides, lysine (K), arginine (R), alanine (A), and serine (S) were the four dominant amino acids, whereas among the downregulated peptides, alanine (A), lysine (K), asparagine (N), and glutamic acid (E) were the four dominant amino acids. In particular, we found that multiple peptides are derived from same precursor proteins. Among these, the precursor proteins Krt8, Eef1a1, Hist1h1b, and NA generated a total of 31 peptides (Figure 3(g)).

3.4. GO and KEGG Pathway Analyses of the Differently Expressed Peptides. GO and KEGG pathway analyses were performed to determine the potential roles of these differentially expressed peptides and their corresponding precursor proteins. For biological processes, cellular developmental process, response to oxygen levels, regulation of cell development, NF-κB import into the nucleus, regeneration, and Wnt-activated receptor activity had a high enrichment score (Figure 4(a)). Extracellular exosome, myelin sheath, intracellular ribonucleoprotein complex, cell–cell adherens junction, membrane raft, and brush border as cellular components were found to be closely related to the identified peptides (Figure 4(b)). Regarding molecular function, Wnt-activated receptor activity, RNA polymerase II transcription factor
| Amino Acid | Peptide number |
|------------|----------------|
|            | 7–8            |
|            | 9–10           |
|            | 11–12          |
|            | 13–14          |
|            | 15–16          |
|            | 17–18          |
|            | 19–20          |
|            | >20            |

| Amino Acid | Peptide number |
|------------|----------------|
|            | 0              |
|            | 10             |
|            | 20             |
|            | 30             |
|            | 40             |

| Molecular Weight (MW) | Peptide number |
|-----------------------|----------------|
| <800                  |                |
| 800–1000              |                |
| 1000–1200             |                |
| 1200–1400             |                |
| 1400–1600             |                |
| 1600–1800             |                |
| 1800–2000             |                |
| >2000                 |                |

| Isoelectric point (IP) | Peptide number |
|------------------------|----------------|
| 0                      |                |
| 1                      |                |
| 2                      |                |
| 3                      |                |
| 4                      |                |
| 5                      |                |
| 6                      |                |
| 7                      |                |
| 8                      |                |
| 9                      |                |
| 10                     |                |
| 11                     |                |
| 12                     |                |
| 13                     |                |

| Amino Acid | Peptide number |
|------------|----------------|
|            | ACDE FGH I KLM NPQR S TV W Y |

| Amino Acid | Peptide number |
|------------|----------------|
|            | ACDE FGH I KLM NPQR S TVY |

**Figure 3:** Continued.
binding, peptide hormone binding, peptide receptor activity, and calcium-dependent protein binding were identified as the most enriched subcategories (Figure 4(c)). Further, KEGG pathway analysis indicated that the potential function of these differentially expressed peptides was related to the TGF-β signaling pathway, riboflavin metabolism, axon guidance, apoptosis, the Wnt signaling pathway, and the MAPK signaling pathway (Figure 4(d)).

3.5. Bioactive Peptides Potentially Related to NEC. The functions of the precursor proteins largely determine the functions of the bioactive peptides [14]. We used the UniProt and STRING databases to analyze the protein interactions of the differentially expressed peptides and the protein–protein interaction of their protein precursors. It is well known that bioactive peptides can be derived from their precursor domain. Hence, we used the UniProt database to analyze peptides derived from their precursor protein functional domain (Table 2). Among the downregulated peptides, the protein precursor Ezrin (Ezr) plays a vital role in the apical integrity of the intestinal epithelium, and its severe defects can lead to incomplete villi morphogenesis and neonatal death [15]. In addition, Metallothio (MT1) has a protective effect on intestinal mucosal inflammation. Therefore, we attempted to detect bioactive peptides with potential NEC protective effects from the downregulated peptides.
| Gene Accession | Peptide | Peptide domain | p value |
|---------------|---------|----------------|---------|
| Hist1h2bq G3V8B3 | AVTKYTSSK | Histone H2B | <0.05 |
| Tubb5 P69897 | FVFGQSGAGNN | Tubulin/PtsZ family, GTPase domain | <0.01 |
| Mdh1 O88989 | VIVGVNPANTC | Domain in histone families 1 and 5 | <0.01 |
| Aldob Q66HT1 | ALQASALAAWWGK | Glycolytic | <0.001 |
| Hist1h2bq G3V8B3 | LLLPGELAK | Histone H2B | <0.001 |
| Eef2 P05197 | VFGVSVGAVGL | GTP_EFTU_D2 | <0.05 |
| Hnrrpa2b1 F1LNF1 | GGFGVTF | RNA recognition motif | <0.05 |
| Hist1h1d M0R7B4 | SGVLSAALK | Domain in histone families 1 and 5 | <0.001 |
| Tubb5 P69897 | FVFGQSGAGN | Tubulin/PtsZ family, GTPase domain | <0.05 |
| Gapdh-ps2 D3ZGY4 | GAANQNIIPASTGAALKAVGK | Gp_dh_C | <0.001 |
| Hist1h1b D3ZBN0 | SLVSGKTLVQTK | Domain in histone families 1 and 5 | <0.01 |
| Hist1h1d M0R7B4 | GLVQTKGTGASGS | Domain in histone families 1 and 5 | <0.001 |
| NA P02262 | VGAGAPVYL | Histone 2A | <0.001 |
| Atp5b P10719 | IGLFQSQGAGV | ATPases associated with a variety of cellular activities | <0.01 |
| Gapdh-ps2 D3ZGY4 | VKGVGNQGFGR | Glyceraldehyde 3-phosphate dehydrogenase, NAD binding domain | <0.001 |
| Hist1h2bq G3V8B3 | LLLPGELAK | Histone H2B | <0.01 |
| Aldh18a1 D3ZIE9 | VGLGGMEAKVK | AA_kinase | <0.001 |
| Actn4 Q9QXQ0 | STLYESLD | Ca2+ insensitive EF hand | <0.01 |
| Krt8 Q10758 | GGSQFGGAGVTVG | Keratin_2_head | <0.05 |
| Aldoa P05065 | ALQASALK | Glycolytic | <0.01 |
| Plin3 M0RA08 | VGTAVDVTGAVK | Perilipin | <0.001 |
| NA A0A0G2K3Z9 | SSGNAKIHAPSF | | <0.01 |
| Gapdh-ps2 D3ZGY4 | ALNDFNFK | Gp_dh_C | <0.05 |
| Rps23 P62268 | KANPFGGASHAK | Ribosom_S12_S23 | <0.001 |
| Krt8 Q10758 | KLEVPNDNQAVR | Keratin_2_head | <0.001 |
| Hist1h1b D3ZBN0 | SLVSGKTLVQTKGTGASGS | Domain in histone families 1 and 5 | <0.01 |
| Hist1h1d M0R7B4 | SGVLSAALK | Domain in histone families 1 and 5 | <0.01 |
| Tpt1 P63029 | LDYREDGVTIP | TCTP | <0.05 |
| Enolase, C-terminal TIM barrel domain | Q5EB49 | SFRNPLAK | <0.05 |
| Psm1 P18420 | LYSLLGSK | Proteasome | <0.05 |
| Aca2 P13437 | GVFIVAAK | Thiolase_N | <0.01 |
| Gapdh-ps2 D3ZGY4 | GAAQNIIASTGAALKAVGK | Gp_dh_C | <0.001 |
| Krt8 Q10758 | SLVSGKTLVQTKGTGASGS | Keratin_2_head | <0.001 |
| Hadh Q9WVK7 | TEGFHYKY | 3HCDH | <0.01 |
| Myh11 A0A0G2K6S9 | STVAALEAK | | <0.01 |
| Ptma P06302 | SDAVTDTSSEITTK | Prothymosin | <0.05 |
| Hist2h2aa3 K7S2S2 | AEIELAGNAAR | Histone 2A | <0.01 |
| Tubb5 P69897 | YNEATGKKYVPR | Tubulin/PtsZ family, GTPase domain | <0.05 |
| Hspa8 P63018 | SKGPAGVALDTHT | HSP70 | <0.05 |
| Eef1a1 P62630 | QTVAVGV | GTP_EFTU_D3 | <0.05 |
| Glud1 P10860 | VVQGPAGVNLX | Glutamate/leucine/phenylalanine/valine dehydrogenase | <0.05 |
| Ptma P06302 | AAVDTSSEITTK | Prothymosin | <0.01 |
| Eef2 P05197 | VAVEAKNPADLPK | EFG_II | <0.01 |
| Wdr1 Q5RJK0 | SVADGYSNENVF | WD40 repeats | <0.001 |
| Hist1h2bq G3V8B3 | HAVSEGTKAVT | Histone H2B | <0.05 |
| Tpt1 P63029 | GAIDSSLIGGN | TCTP | <0.001 |
| Krt8 Q10758 | GGFQGAVGGIT | Keratin_2_head | <0.05 |
| Efhd2 Q4FZY0 | ATDELASKLSR | Metallothio | <0.05 |
3.6. Protective Effects of EDP1 and MDP2. To further analyze the function of the differentially expressed peptides, we randomly selected downregulated peptide 1 derived from EZRI (EDP1) and downregulated peptide 2 derived from MT1 (MDP2) to investigate its NEC protective ability. Interestingly, EDP1 treatment could partially reverse the inhibition of cell migration caused by LPS (Figures 5(a) and 5(b)). In addition, MDP2 treatment not only reversed the inhibition of cell migration in the NEC cell model but also decreased the apoptosis level (Figures 5(a) and 5(b)).

4. Discussion

NEC is the most common gastrointestinal complication in premature infants [16]. Although extensive research has been conducted to investigate this disease, the underlying molecular mechanisms have not yet been fully elucidated. Peptidomics is an innovative branch of proteomics, which has been used to explore the mechanisms of various diseases [17, 18]. In our study, we detected differentially expressed peptides in intestinal tissues that were further analyzed for their potential association with NEC. Moreover, the bioactive peptides EDP1 and MDP2 showed a protective effect in the NEC model in vitro.

In total, 176 differentially expressed peptides, including 103 upregulated and 73 downregulated, were identified. GO analysis of precursor proteins revealed that the differentially expressed peptides were mainly involved in the response to oxygen levels, NF-κB import into the nucleus, extracellular exosome, intracellular ribonucleoprotein complex, cell–cell adherens junction, peptide hormone binding, and peptide receptor activity. Among these, accumulation of oxygen free radicals and low antioxidant capacity result in intestinal epithelial cell apoptosis and intestinal inflammation, which are the known causes of NEC [19]. Moreover, the aberrant activation of NF-κB has been shown to be associated with many inflammatory bowel diseases, including NEC, and the inhibition of NF-κB can reduce intestinal damage [20]. Further,
exosomes partially assumed the function of the local and long-distance communication of cells. These results revealed that the differentially expressed peptides are involved in the pathological mechanism of NEC.

KEGG analysis revealed that the TGF-β signaling pathway, apoptosis, and the Wnt signaling pathway had a high enrichment score. It is well known that the main feature of NEC is hemorrhagic and necrotizing inflammation within all layers of the intestinal wall [21]. As a pleiotropic cytokine, TGF-β can regulate multiple cellular functions and suppress immune responses [22]. In intestinal immunity, TGF-β plays a role in suppressing the strong inflammatory response in the intestinal cavity and promotes the construction of immune tolerance, thereby exerting a protective effect in inflammatory bowel diseases [23]. The destruction of the dynamic barrier between the intestinal epithelial layer and the substances in the intestinal cavity is an important process in NEC pathogenesis [24]. Widespread intestinal epithelial cell death is the key mechanism leading to this damage. Studies have reported high levels of intestinal cell apoptosis in the established NEC animal model; moreover, the effect of caspase inhibitors on apoptosis prevents NEC progression. The renewal of intestinal stem cells is a protective response of the intestine to acute injury. Intestinal renewal as well as stem cell maintenance depends mainly on the Wnt/β-catenin pathway [25]. Studies have found that Wnt/β-catenin signaling decreases during NEC development [26, 27]. This leads to impaired intestinal stem cell activity and poor intestinal regeneration ability. However, the administration of Wnt can maintain intestinal epithelial homeostasis and avoid the intestinal injury observed in NEC [28]. These results indicate that the differentially expressed peptides may be potentially involved in the mechanism underlying NEC.

Recently, an increasing number of peptides have been found to exert a protective effect in intestinal diseases. For instance, vasoactive intestinal peptides (as effective anti-inflammatory agents) exhibited the ability to regulate homeostasis of the intestinal epithelial barrier. It serves a therapeutic role by reducing inflammation and destroying tight junctions in NEC [29]. In addition, a peptide derived from Porphyra yezoensis was able to promote IEC-6 cell proliferation through the activation of insulin-like growth factor I receptors [30]. However, there are no reports about the treatment of NEC with endogenous active peptides.

In our study, peptides derived from the precursor proteins EZRI and MT1 were significantly downregulated in the NEC group. We initially synthesized two peptides EDP1 and MDP2 from the functional domains of Ezrin and Metallothio, respectively. We found that the peptide EDP1 promotes cell migration after acting on the NEC model in vitro, whereas the peptide MDP2 promotes cell migration and inhibits apoptosis in the NEC model in vitro. These results indicate that bioactive peptides are involved in the progression of NEC; however, the mechanisms underlying the peptide functions need to be further investigated.
5. Conclusion

In conclusion, we initially screened the differentially expressed peptides in the intestinal tissues of the NEC rat model based on TMT markers combined with LC-MS/MS analysis. Next, we investigated the pathological mechanism of NEC and explored the potential treatment strategies. Although the peptides EDP1 and MDP2 were confirmed to exhibit protective effects in NEC, we need to explore their therapeutic mechanisms further.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors’ Contributions

Yiwen Liu, Changlin Wang, Renqiang Yu, and Jianfeng Fan contributed equally.

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