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Original Research

Effects of Spermidine on Cell Proliferation, Migration, and Inflammatory Response in Porcine Enterocytes

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Abstract

Background: Polyamines have been demonstrated to be beneficial to porcine intestinal development. Our previous study showed that putrescine mitigates intestinal atrophy in weanling piglets and suppresses inflammatory response in porcine intestinal epithelial cells, it is still unknown the role of spermidine in mediating putrescine function. **Objective**: The current study aimed to investigate the effect of spermidine on the proliferation, migration, and inflammatory response in porcine intestinal epithelial cells (IPEC-J2 cell line). **Methods**: The effects of spermidine on proliferation and migration of IPEC-J2 cells were measured. Difluoromethyl ornithine (DFMO) and diethylglyoxal bis (guanylhydrazone) (DEGBG) were used to block the production of putrescine and spermidine, respectively. A cell inflammation model was established with lipopolysaccharides (LPS) stimulation. Gene expression and protein abundance were determined by real-time quantitative PCR and western blotting, respectively. **Result**: Spermidine significantly enhanced cell proliferation in DFMO (or/and) DEGBG treated IPEC-J2 cells ($p < 0.05$). Pretreatment with putrescine restored cell growth inhibited by DFMO but did not prevent the decrease in cell proliferation caused by DEGBG ($p > 0.05$). Similarly, spermidine but not putrescine significantly elevated the rate of migration in DEGBG treated IPEC-J2 cells ($p < 0.05$). Spermidine deprivation by DEGBG dramatically enhanced mRNA abundance of pro-inflammatory cytokines IL-8, IL-6, and TNF-α ($p < 0.05$), and the addition of spermidine attenuated excessive expression of those inflammatory pro-inflammatory cytokines, moreover, spermidine but not putrescine suppressed the phosphorylation of NF-κB induced by DEGBG. Spermidine supplementation also significantly suppressed LPS-induced the expression of TNF-α. **Conclusions**: The present study highlights a novel insight that putrescine may be converted into spermidine to modulate cell proliferation, migration, and inflammatory response on porcine enterocytes.

Keywords: spermidine; putrescine; cell proliferation; cell migration; inflammatory response

1. Introduction

The intestine plays a critical role in the digestion and absorption of nutrients and host defense. Intestinal epithelial cells form a monolayer physical barrier to prevent the invasion of harmful substances from the intestinal lumen [1–3]. External stressors such as pathogens, toxins, and weaning affect gut health, which causes damage to the structure and function of the small intestine, resulting in intestinal diseases such as diarrhea and chronic inflammatory disorders [4–6]. The studies have demonstrated that proliferation and migration of intestinal epithelial cells were directly involved in repairing mucosal damage [7]. Therefore, it is necessary to improve and maintain intestinal health by promoting the growth and migration of enterocytes.

Biogenic amines, including monoamines, diamines, and polyamines, are important nitrogenous organic compounds that have the physiological function of signal transduction in living cells [8,9]. Polyamines are mainly bound to polyanionic molecules in cells to perform multiple beneficial functions that include anti-inflammation, antioxidation, anti-aging, and enhancing mitochondrial metabolism [10,11]. Specifically, they have an essential physiological role in cell growth, proliferation, maturation, and regeneration [12]. Spermidine, as a downstream family member of putrescine metabolites, is a biologically active polyamine whose intracellular concentration is strictly regulated by controlling the levels of the two rate-limiting enzymes in polyamine metabolism, ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) [13]. There is an inextricable relationship between spermidine and cell proliferation, migration, and inflammatory response of mammalian cells [13–15]. Our previous study found that putrescine increased the proliferation and migration of the porcine intestinal epithelial cells (IPEC-J2) and mitigated mucosal atrophy of the small intestine by suppressing inflammatory responses in weanling piglets [16]. Still, it is unknown whether putrescine must be metabolized to spermidine to exert its effects.

Therefore, the primary purpose of this study is to investigate the effect of spermidine on the proliferation, migration, and inflammatory response of porcine intestinal ep-

ithelial cells and to detect the role of spermidine in mediating the physiological functions of putrescine. We hypothesized that the conversion of putrescine into spermidine is essential for putrescine to perform its functions in cell proliferation, migration, and anti-inflammation. Difluoromethyl ornithine (DFMO), an irreversible inhibitor of ODC, can effectively block the synthesis of putrescine from ornithine, and diethylglyoxal bis (guanylhydrazone) (DEGBG), a specific inhibitor of SAMDC, can effectively block the synthesis of spermidine from putrescine and spermine from spermidine. They were used to block the production of putrescine and spermidine in the cells, respectively. Results from this study provided new insights into the mechanism of polyamine function in regulating the inflammatory response of porcine enterocytes.

2. Materials and Methods

2.1 Cell Culture

IPEC-J2 cells were from Dr. Guoyao Wu from Texas A&M University. The cells were cultured as described previously [17]. Briefly, the cells were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), 0.01% epidermal growth factor (5 μg/L, Corning Inc., Corning, NY, USA) and 0.1% ITS solution (containing 5 μg/L insulin, 5 μg/L transferrin, and 5 ng/L sodium selenite, Corning Inc., Corning, NY, USA) at 37 °C in a humidified incubator containing 5% CO_2. Culture medium was replaced every 24 h. When the cells covered about 80–90% of the bottom of the plastic culture flask (75 cm^2, Corning Inc., Corning, NY, USA), cells were passaged utilizing 0.25% trypsin-EDTA (Gibco, Grand Island, NY, USA) and subcultured for further experiments. We used passage 12–15 cells in these studies.

2.2 Cell Proliferation Assay

IPEC-J2 cells were seeded at 0.5 $\times$ 10^5 cells per well into 96-well plates (Corning Inc., Corning, NY, USA) with six replications (wells) per treatment. After the cells adhered to the plate bottom (4 h), each well was washed three times with PBS, followed by adding 100 μL culture medium (containing 2% FBS) with different concentrations of putrescine (200 μmol/L, 400 μmol/L) or spermidine (1 μmol/L, 2 μmol/L, 4 μmol/L, 8 μmol/L, 16 μmol/L, 32 μmol/L, 64 μmol/L) per well for each treatment. The concentration of putrescine (putrescine dihydrochloride, Sigma-Aldrich, Co., Saint Louis, MO, USA) was selected based on a previous report in our laboratory [16]. To determine the specific effect of putrescine and spermidine on the proliferation of the cells, 5 mmol/L difluoromethyl ornithine (DFMO, a specific inhibitor of ODC, MedChemExpression, Monmouth Junction, NJ, USA) was added in culture media for 72 h, and 1 mmol/L diethylglyoxal bis (guanylhydrazone) (DEGBG, an inhibitor of SAMDC) was added in culture media for 24 h to inhibit the production of putrescine and spermidine in cells, respectively. After the third or fourth day of cell seeding, the growth of IPEC-J2 cells was measured using the cell counting kit (CCK-8, Monmouth Junction, MedChemExpression, Monmouth Junction, NJ, USA) according to the manufacturer's instructions. Concisely, the cells were washed twice with PBS, 100 μL media containing 10% CCK-8 solution was added to each well, followed by incubation for 3 h at 37 °C. Optical density (OD) values of culture media at 450 nm were measured employing a microplate reader (BioTek Instruments, Inc., Montpellier, VT, USA). The number of cells was calculated according to the fitted standard curve from the OD value.

2.3 Cell Migration Assay

IPEC-J2 cells were seeded at 0.5 $\times$ 10^5 cells/mL (2 mL per well) into 6-well plates (Corning Inc., Corning, NY, USA) with four replications (wells) per treatment. The cells were then cultured for 48 h to fully cover the plates' bottom. Then added 2 μg/mL mitomycin C (MedChemExpression, Monmouth Junction, NJ, USA) to the culture media for 24 h to inhibit cell proliferation [18]. A 200-μL aseptic pipette tip was used to make a straight scratch, the cells were then washed twice with PBS, and the scratch area was measured (recorded as 0 h) by inverted microscopy (Axio Vert.A1, Zeiss, Jena, Thuringia, Germany). The cells were treated with putrescine (200 μmol/L) or spermidine (8 μmol/L) for 8 h in the presence or absence of DEGBG. The area of the exact location at 8 h post scratching (recorded as 8 h) was again measured by inverted microscopy (Axio Vert.A1, Zeiss, Jena, Thuringia, Germany). The scratch area at 8 h was subtracted from that at 0 h to calculate the cell migration rate (in percentage).

2.4 An in Vitro Cell Inflammation Model Induced by LPS

The cell inflammation model was established *in vitro* by adding lipopolysaccharides (LPS, Beyotime technology, Shanghai, China). IPEC-J2 cells were seeded at 0.5 $\times$ 10^5 cells/mL (1 mL per well) into 12 well plates. After being cultured for 48 h, the cells were incubated with 8 μmol/L spermidine for 24 h, and then added 100 μg/mL LPS for another 4 h. The cells were washed with PBS, and intracellular RNA was extracted for further assays.

2.5 Determination of Polyamines in Culture Media and Cells

IPEC-J2 cells were seeded at 0.5 $\times$ 10^5 cells/mL into 6 well plates. After being cultured for 48 h, the cells were treated with DMEM/F12 containing 0 μmol/L spermidine, 1 mmol/L DEGBG, 8 μmol/L spermidine, 8 μmol/L spermidine + 1 mmol/L DEGBG. Samples preparation and polyamines determination were processed according to the

method described by Dai *et al.* [19]. Briefly, the cells were harvested by centrifugation at 1000 × g for 10 min. The cells were resuspended in 300 μL pre-chilled PBS and using the sonication method to obtain cell contents. Cell-free supernatant was obtained by centrifuging the sonication mixtures at 13,000 × g for 5 min. One hundred and fifty μL of 1.5 mol/L $HClO_4$ was added dropwise to 150 μL supernatant of the cell lysis solution or culture media, and then added 75 μL of 2 mol/L of K_2CO_3 to react for 5 minutes. The supernatant solution obtained by centrifugation (13,000 × g for 5 min) was used to determine free polyamines by HPLC (Alliance e2695 HPLC system, Waters Corporation, Milford, CT, USA) using the OPA-NAC method, in which polyamines react with o-phthaldialdehyde (OPA) and N-acetyl-L-cysteine (NAC) to form relatively stable derivative products. The in-line derivatization procedure was programmed in the HPLC system to mix 10 μL of the samples with 10 μL of the OPA-NAC solution and stop for 1 min to allow a full derivatization reaction before injection of the mixtures into the system. The fluorescence of the derivative products was determined using a Waters 2475 multi λ fluorescence detector with excitation λ at 340 nm and emission λ at 450 nm. Solvent A was 0.1 mol/L sodium acetate (pH 7.2) and solvent B was HPLC-grade methanol. HPLC gradients for solvent A and B were 70/30, 35/65, 30/70, 0/100, 0/100, 70/30, 70/30 at 0, 12, 16, 18, 23, 25, 30 min, respectively.

2.6 Real-Time Quantitative PCR Analysis (qPCR)

mRNA abundance in the cells was determined by qPCR according to the procedure described by Li *et al.* [20]. In brief, total RNA was isolated from IPEC-J2 cells by using the Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA), followed by the determination of the content and quality of total RNA using an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Montpellier, VT, USA), and the first-strand cDNA synthesis by TransScript First-Strand cDNA Synthesis Kit (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. qPCR analysis was performed using the cDNA templates, primer pairs for specific genes of the target, and SYBR Green reagent (Thermo Fisher Scientific, MA, USA) on an ABI 6 flex real-time PCR instrument (Thermo Fisher Scientific, Waltham, MA, USA). The primers used for qPCR are shown in **Supplementary Table 1**, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to normalize target genes. The calculation of fold changes of the target genes relative to those of GAPDH was acquired by the comparative Ct value method.

2.7 Western Blotting Analysis

Western blot analysis was performed using a routine procedure described by Liu *et al.* [16] with slight modification. The membranes were incubated with a primary antibody overnight at 4 °C, followed by washing three times (10 min per time) with Tris-buffered saline and Tween 20 (TBST) buffer, incubated with a secondary antibody for 3 h at room temperature. The membranes were rewashed with TBST buffer before adding the reagents from Western Bright ECL Kit (Bio-Rad Laboratories Inc., Berkeley, CA, USA). Digital images were detected by the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc., Berkeley, CA, USA). The antibodies of extracellular signal-regulated kinase1/2 (ERK1/2) (1:1000), adhesion kinase (FAK) (1:1000), NF-κB (1:1000), phospho-ERK1/2 (Thr202/Tyr204, 1:1000), phospho-FAK (Tyr 397, 1:1000) and phospho-NF-κB (Ser536, 1:1000), as well as secondary antibodies (HRP-linked anti-rabbit or anti-mouse IgG, 1:2000), were obtained from Cell Signaling Technology, USA. GAPDH (1:1000, Cell Signaling Technology, Danvers, MA, USA) and α-Tublin (1:1000, Cell Signaling Technology, Danvers, MA, USA) were internal references for equality of sample loading. The list of antibodies used in western blotting is shown in **Supplementary Table 2**.

2.8 Statistical Analysis

Data were expressed as mean ± SE. Statistics were analyzed by one-way analysis of variance (ANOVA) using SAS v. 9.2 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple comparison test was used to determine the statistical differences among treatments. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1 Effect of Exogenous Spermidine on the Proliferation of IPEC-J2 Cells

The effect of treatment with different concentrations of spermidine on the proliferation of IPEC-J2 cells is illustrated in Fig. 1. Compared with the control treatment, the addition of 2, 4, 8, 16 μmol/L spermidine to the culture medium increased cell numbers after incubation for 24 h; cell numbers significantly increased at concentrations of 8 and 16 μmol/L when incubated with spermidine for 48 h; 1, 4, 8, 16 μmol/L spermidine supplementation increased cell numbers at 72 h; furthermore, only a low concentration of spermidine (2 μmol/L) supplementation increased the number of IPEC-J2 cells at 96 h ($p < 0.05$). In addition, the concentrations of spermidine above 16 μmol/L significantly decreased cell numbers at different processing times compared with the control ($p < 0.05$). A concentration of 8 μmol/L spermidine was used for subsequent experiments because of its positive effect on stimulating cell proliferation.

3.2 Metabolism of Putrescine into Spermidine Promoted Cell Proliferation

As shown in Fig. 2A, adding 5 mmol/L DFMO significantly inhibited cell proliferation at 72 h, and adding simultaneously exogenous putrescine at a concentration of

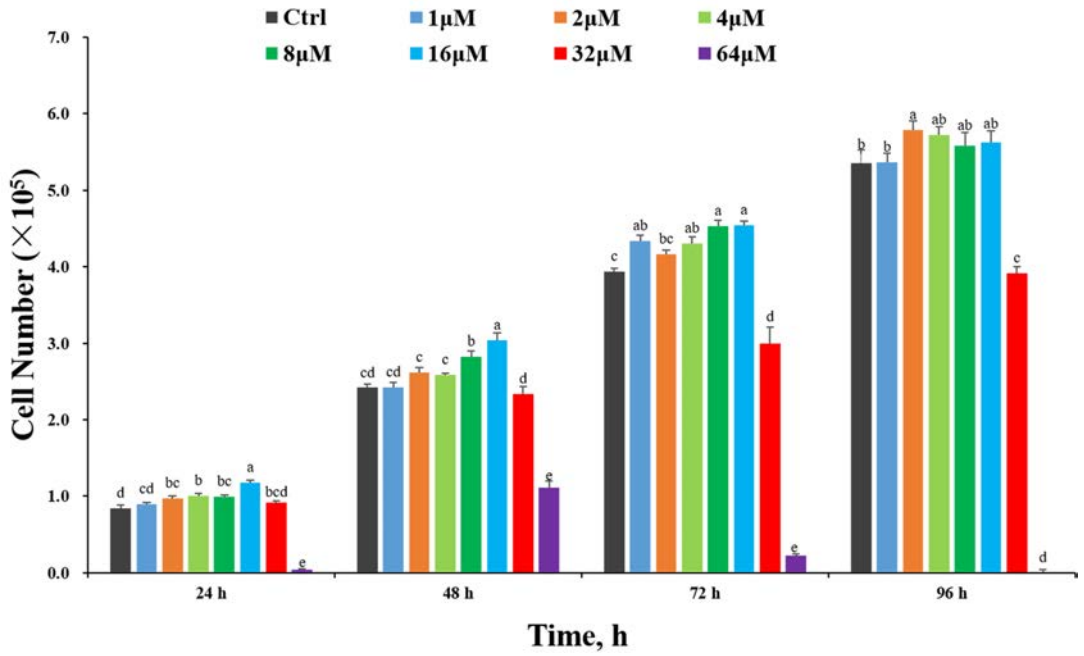

Fig. 1. Effects of different spermidine concentrations on the growth of IPEC-J2 cells. IPEC-J2 cells were seeded into 96-well plates at a number of 0.5×10^5 cells per well and then treated with 0, 1, 2, 4, 8, 16, 32, 64 μmol/L spermidine. Cell number was detected at 24 h, 48 h, 72 h, and 96 h with CCK-8 reagent. The data were expressed as the mean ± SE, n = 6. Different letters indicate a significant difference among treatments at each time point ($p < 0.05$).

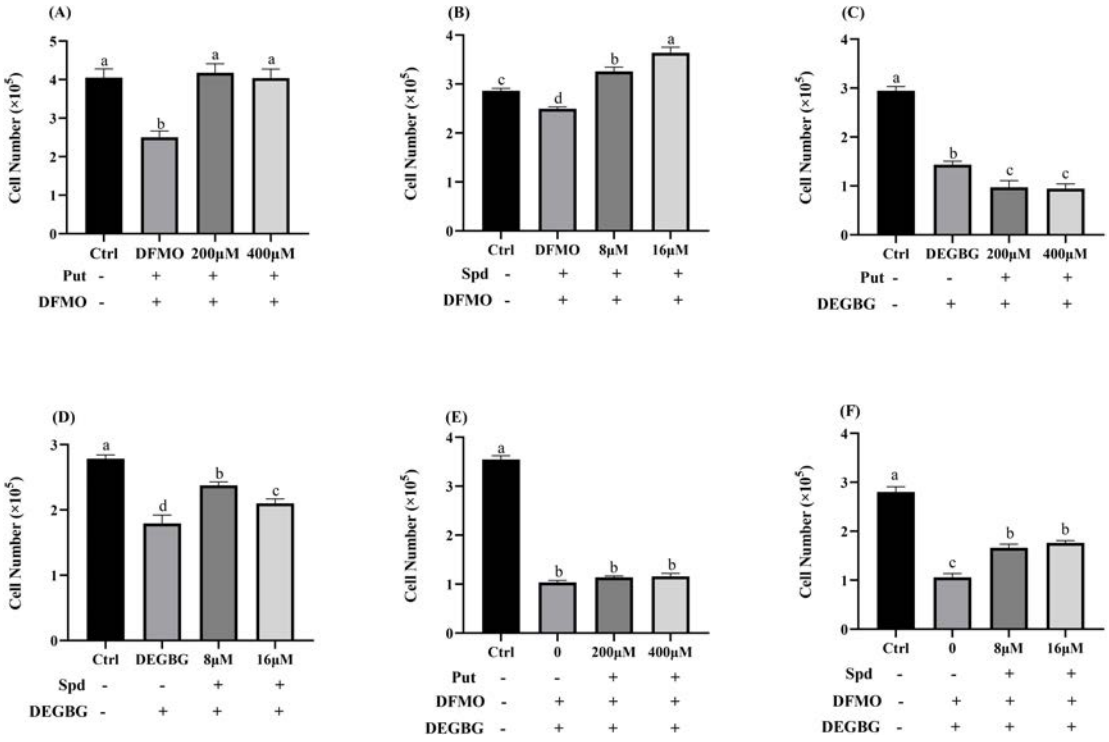

Fig. 2. Effect of polyamines (putrescine and spermidine) on IPEC-J2 cells proliferation in the presence of DFMO (or/and) DEGBG. IPEC-J2 cells were cultured with putrescine (200, 400 μmol/L) or spermidine (8, 16 μmol/L) for 72 h (with or without DFMO) (A,B) and 24 h (with or without DEGBG) (C,D); for combined addition of DFMO and DEGBG, the cells were pretreated for 48 h with or without DFMO before stimulation with DEGBG for 24 h in the presence or absence of putrescine (200, 400 μmol/L) or spermidine (8 and 16 μmol/L) (E,F). Then cell numbers were determined with the CCK-8 kit. The data are expressed as the mean ± SE, n = 6. Different letters indicate a significant difference ($p < 0.05$).

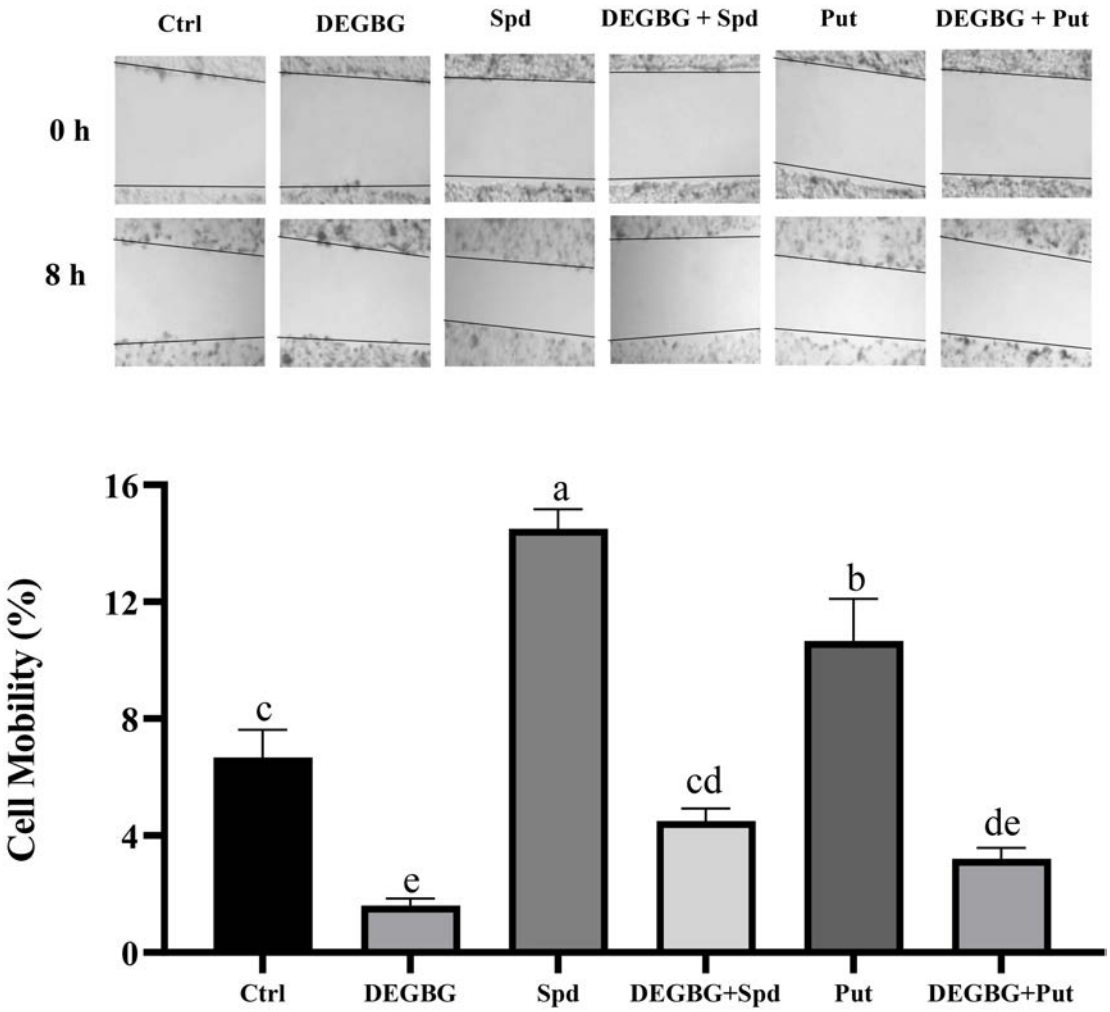

Fig. 3. Effect of putrescine and spermidine on IPEC-J2 cells migration in the presence of DEGBG. IPEC-J2 cells were seeded into 6-well plates at a concentration of 0.5×10^5 cells/mL and cultured in a medium until reaching 70–75% confluence. Then, the cells were pretreated with mitomycin C for 24 h before scratching, followed by treatment with putrescine (200 μmol/L) or spermidine (8 μmol/L) for another 8 h in the presence or absence of DEGBG. Values are means $\pm$ SE, n = 4. Different letters indicate a significant difference ($p < 0.05$).

200 or 400 μmol/L completely recovered cell growth ($p < 0.05$). Similarly, pretreatment with a concentration of 8 or 16 μmol/L spermidine significantly rescued cell growth ($p < 0.05$) (Fig. 2B).

Adding 1 mmol/L DEGBG to the culture medium significantly reduced the intracellular spermidine content and increased the intracellular putrescine concentrations ($p < 0.05$) (**Supplementary Table 3** and **Supplementary Fig. 1**). The response of 1 mmol/L DEGBG to stimulate IPEC-J2 cells for 24 h was similar to that of DFMO to stimulate IPEC-J2 cells for 72 h; the addition of putrescine failed to restore cell proliferation in enterocytes; under the same conditions, spermidine supplementation recovered cell growth in a dose-dependent manner (from 8 to 16 μmol/L) ($p < 0.05$) (Fig. 2C,D). Similarly, adding putrescine also failed to rescue cell proliferation in DFMO plus DEGBG-treated cells but adding exogenous 8 or 16 μmol/L spermidine partially recovered cell growth ($p < 0.05$) (Fig. 2E,F).

3.3 Effect of Exogenous Spermidine on the Migration of IPEC-J2 Cells

As shown in Fig. 3, compared with the control treatment, the addition of 8 μmol/L spermidine or 200 μmol/L putrescine significantly elevated the rate of cell migration at 8 h after scratching, DEGBG inhibited cell migration. Still, cell migration was partially restored in the presence of exogenous spermidine ($p < 0.05$). In addition, putrescine didn't affect this inhibition caused by DEGBG.

3.4 Effect of Spermidine on the Phosphorylation of ERK1/2 and FAK Protein

Compared with the control treatment, DEGBG supplementation decreased the phosphorylation of ERK1/2, but this passive effect was reversed by the simultaneous addition of spermidine ($p < 0.05$) (Fig. 4D); however,

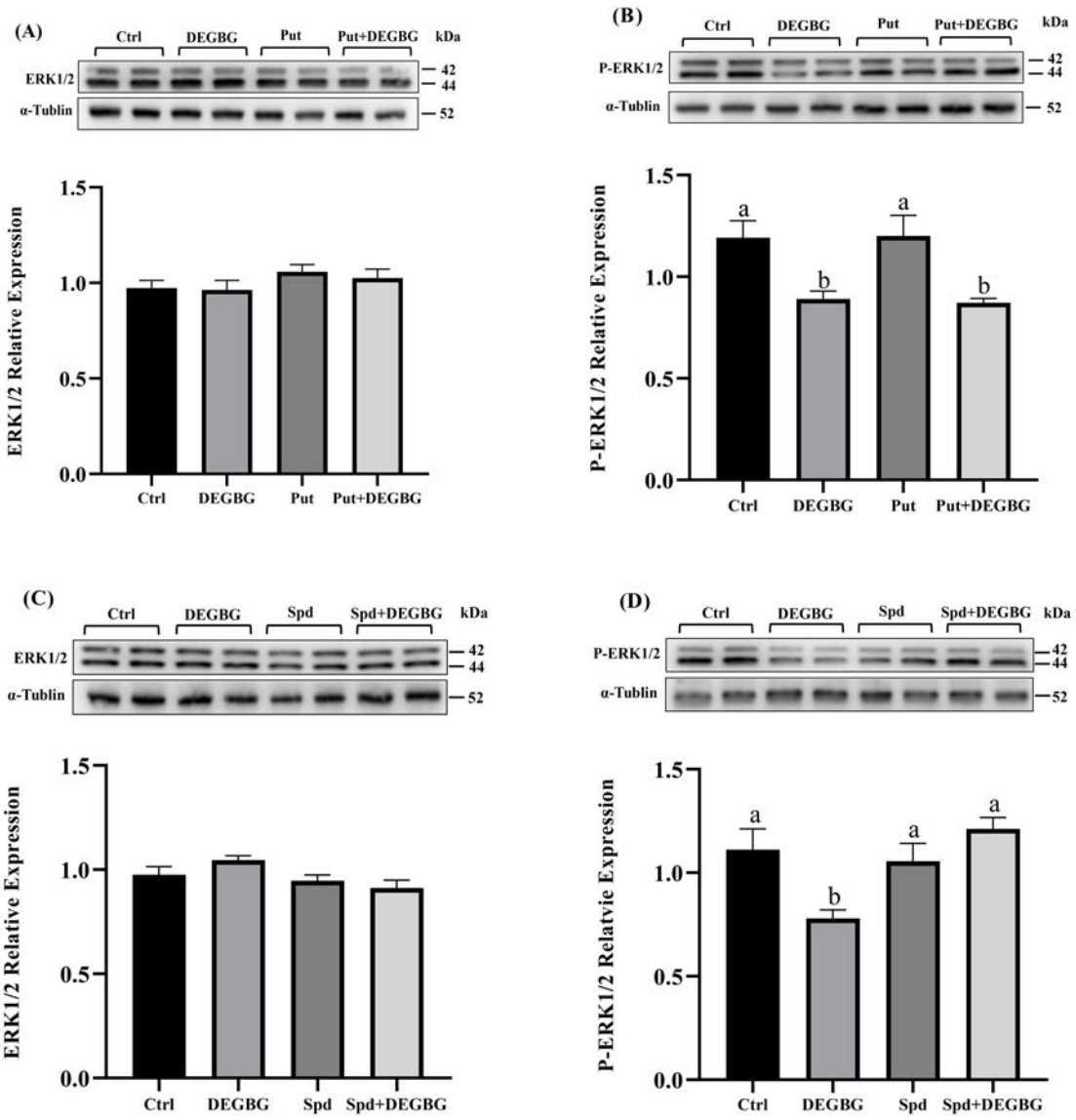

Fig. 4. Effect of putrescine and spermidine on activation of the ERK1/2 protein in IPEC-J2 cells. IPEC-J2 cells were incubated with putrescine or spermidine for 24 h in the presence or absence of DEGBG. Then, the protein abundance of ERK1/2 and P-ERK1/2 proteins were detected by western blotting. (A) ERK1/2 protein abundance and (B) the phosphorylated ERK1/2 protein abundance at 24 h after being pretreated with 200 μmol/L putrescine in the presence or absence of 1 mmol/L DEGBG. (C) ERK1/2 protein levels and (D) the phosphorylated ERK1/2 protein levels at 24 h after being pretreated with 8 μmol/L spermidine in the presence or absence of 1 mmol/L DEGBG. The data presented as the mean $\pm$ SE, n = 6. Different letters represent a significant difference ($p < 0.05$).

putrescine supplementation had no significant effects on ERK1/2 phosphorylation compared with the DEGBG stimulation ($p > 0.05$) (Fig. 4B); moreover, the addition of DEGBG, putrescine, and spermidine did not alter the abundances of ERK1/2 in cells compared with the control treatment ($p > 0.05$) (Fig. 4A,C). In Fig. 5, the combined addition of DEGBG and putrescine or spermidine remarkably increased FAK protein abundance ($p < 0.05$), but DEGBG, putrescine, or spermidine supplementation alone did not affect FAK protein abundance in IPEC-J2 cells, compared with the control treatment ($p > 0.05$) (Fig. 5A,C). In addition, extracellular putrescine decreased protein levels of phosphorylated P-FAK protein abundance in DEGBG-treated cells. Adding spermidine in the presence of DEGBG numerically increased FAK phosphorylation but was not significant compared with that treated with DEGBG alone (Fig. 5B,D).

3.5 Effect of Spermidine on the Inflammatory Response of IPEC-J2 Cells

Detection of the mRNA abundance of pro-inflammatory cytokines in IPEC-J2 cells was conducted by q-PCR, and the results were shown in (Fig. 6). Compared with the control treatment, a significant augment

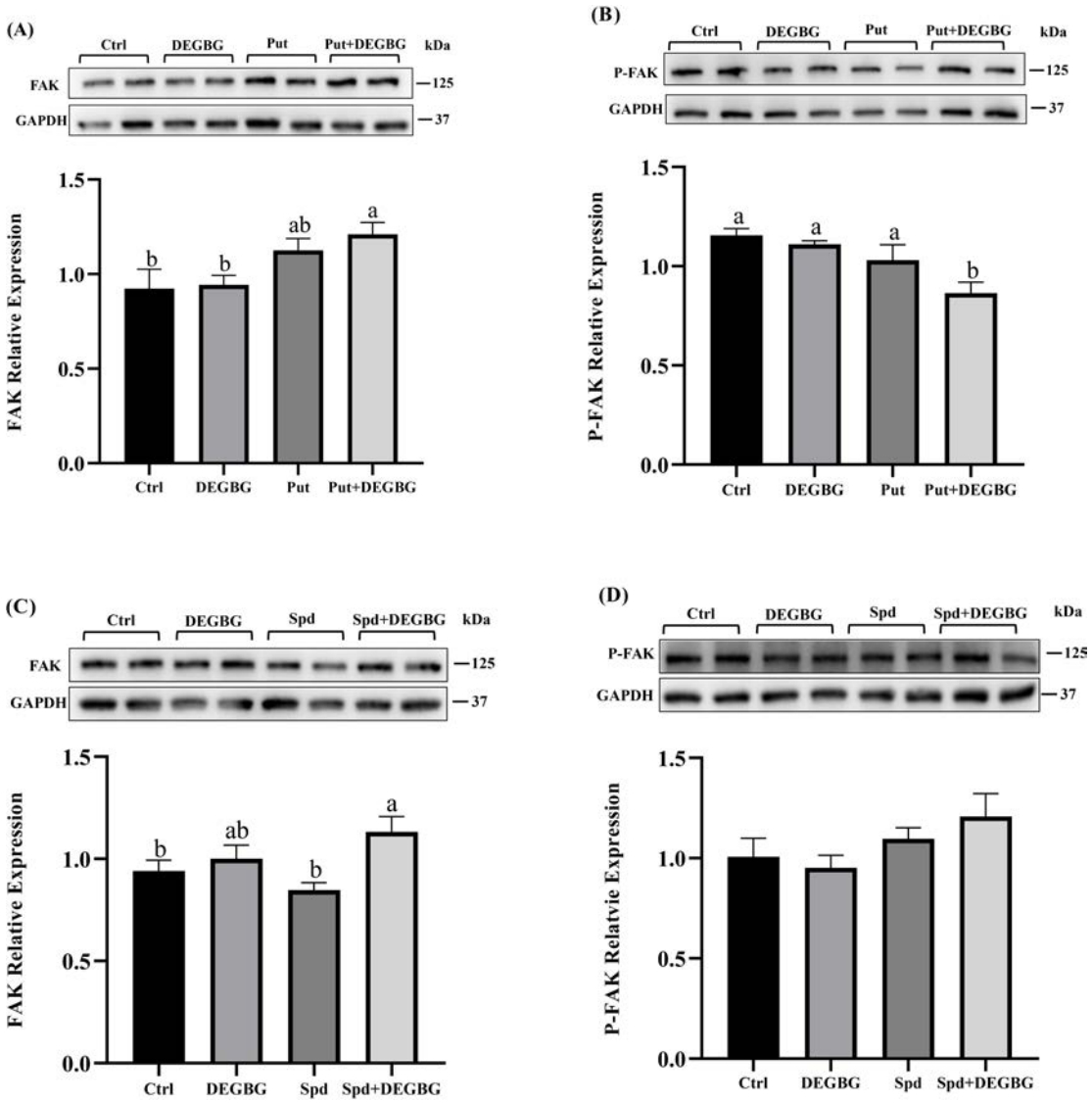

Fig. 5. Effect of putrescine and spermidine on activation of FAK protein in IPEC-J2 cells. IPEC-J2 cells were incubated with putrescine (200 μmol/L) or spermidine (8 μmol/L) for 24 h in the presence or absence of DEGBG. Then, the protein abundance of FAK and P-FAK was determined by western blotting. (A) FAK protein abundance and (B) the phosphorylated FAK protein abundance at 24 h after incubation with 200 μmol/L putrescine in the presence or absence of 1 mmol/L DEGBG. (C) FAK protein abundance and (D) the phosphorylated FAK protein abundance at 24 h after incubation with 8 μmol/L spermidine in the presence or absence of 1 mmol/L DEGBG. The data are presented as the mean ± SE, n = 6. Different letters represent a significant difference ($p < 0.05$).

in the abundance of *IL-8*, *IL-6*, *TNF-*α, and *IL-1*β genes was obtained in DEGBG-treated cells ($p < 0.05$), the addition of exogenous spermidine was found to suppress the expression of *IL-8*, *IL-6*, and *TNF-*α induced by DEGBG ($p < 0.05$) (Fig. 6A–C). However, no significant effect on the expression of *IL-1*β was found ($p > 0.05$) (Fig. 6D). DEGBG, spermidine, or putrescine addition alone did not change the total protein abundance of NF-κB compared with the control treatment ($p > 0.05$) (Fig. 7A–C). However, DEGBG significantly increased NF-κB protein phosphorylation, adding spermidine to the culture media completely abrogated phosphorylated NF-κB ($p < 0.05$) (Fig. 7D), whereas putrescine failed ($p < 0.05$) (Fig. 7B). In addition, the addition of spermidine attenuated the expression of *TNF-*α induced by LPS ($p < 0.05$) (**Supplementary Fig. 2C**).

4. Discussion

Weaning stress is one of the important factors which cause small intestinal mucosal damage in piglets. The repair of intestinal mucosal injury is mainly divided into two independent processes. Firstly, mucosal epithelial cells make up the damaged site through migration, while the cells do not proliferate in this process. Then, the cells supplement the lost cells through proliferation and differentiation [21]. Our previous study found that dietary supplementa-

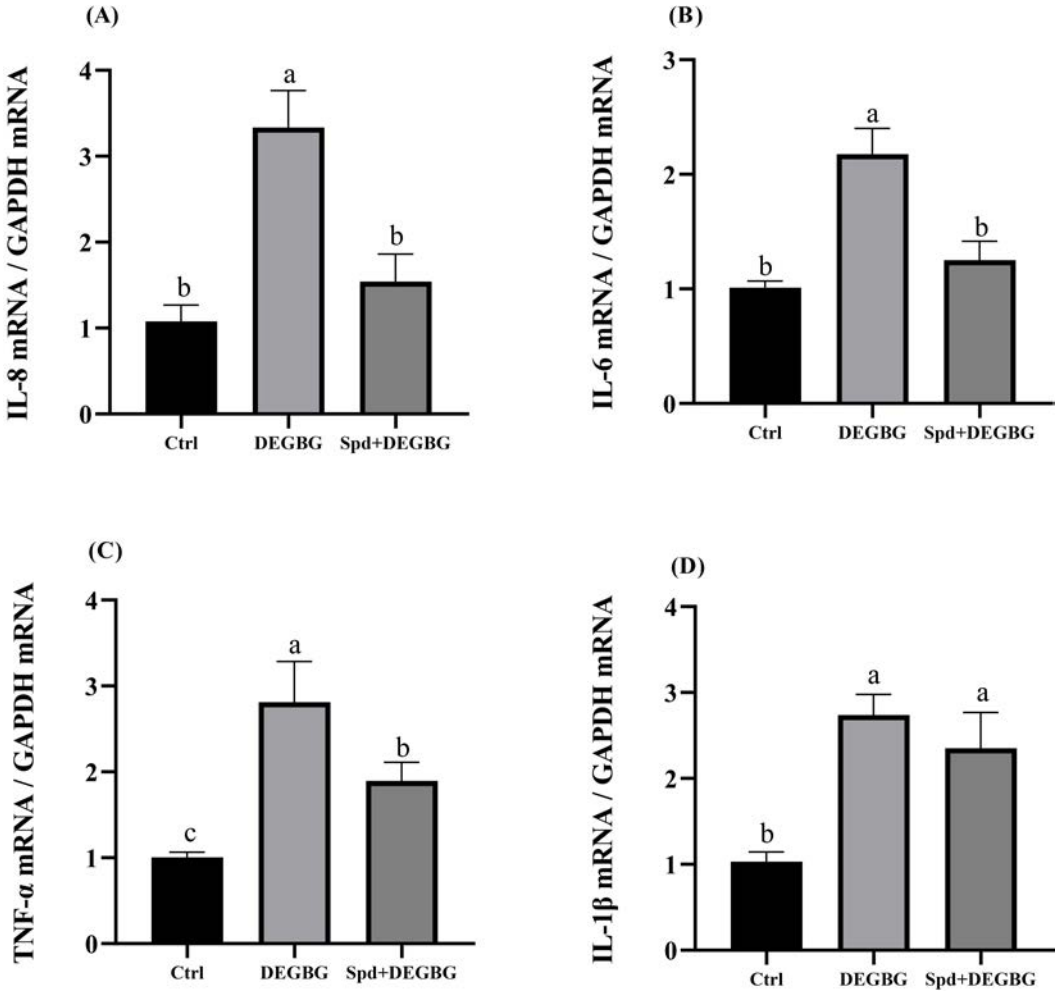

Fig. 6. Effect of spermidine on the expression of inflammatory genes in IPEC-J2 cells induced by DEGBG. IPEC-J2 cells were incubated with spermidine (8 μmol/L) for 24 h in the presence or absence of DEGBG. Then, the expression levels of *IL-8* (A), *IL-6* (B), *TNF-α* (C), and *IL-1β* (D) were quantified by qPCR. The data are presented as the mean ± SE, n = 6. Different letters represent a significant difference ($p < 0.05$).

tion with 0.2% putrescine promoted the repair of intestinal mucosal injury and reduced the diarrhea rate of piglets [16]. Moreover, adding 200 μmol/L putrescine to the culture media promoted the growth and migration of IPEC-J2 cells [16]. But it is unclear whether putrescine needs to be metabolized into spermidine to perform its function.

In the present study, we found that spermidine supplementation stimulated cell proliferation in a dose-dependent manner. This result is consistent with the previous report in which murine erythroleukemia cells proliferated faster and had higher spermidine levels than normal cells [14]. Considering the mutual conversion of polyamines during synthesis and catabolism, DFMO and DEGBG were used here to inhibit the activities of ODC and SAMDC, two key enzymes of polyamines metabolism, respectively [13]. Interestingly, supplementation with exogenous putrescine or spermidine alone completely recovered the impaired growth of IPEC-J2 cells induced by DFMO, which indicated that both putrescine and spermidine played a critical role in regulating cell proliferation. However, putrescine did not alleviate the growth inhibition induced by DEGBG, but spermidine effectively mitigated this inhibition, which meant that putrescine could not rescue cell proliferation in the case of blocking the synthesis of spermidine from putrescine. Similarly, the combined addition of DFMO and DEGBG also remarkably inhibited cell growth; spermidine partly recovered the growth of porcine enterocytes, whereas exogenous putrescine did not. These results demonstrated that putrescine exerted its function through converting to spermidine; similar results have been found in IEC-6 cells [22]. Available evidence suggests that the extracellular signal-regulated kinase1/2 (ERK1/2) signaling pathway is tightly involved in regulating cell proliferation, maturation, and differentiation [23,24]. We therefore investigated the activation of the ERK1/2 protein and found that DEGBG treatment significantly decreased ERK1/2 phosphorylation in the IPEC-J2 cells. However, the addition of spermidine restored the phosphorylation of ERK1/2. On the contrary,

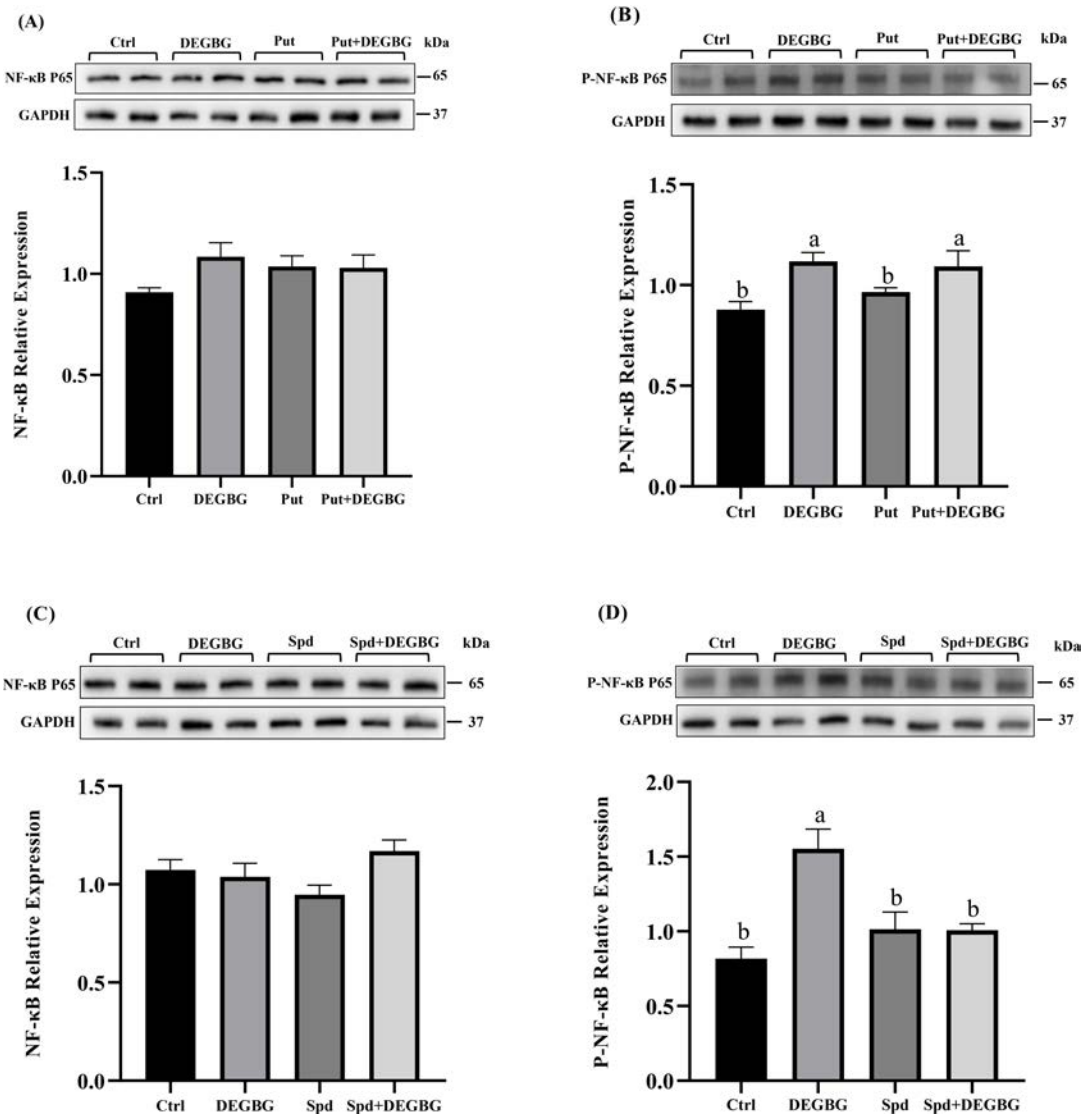

Fig. 7. Effect of spermidine on the expression of inflammatory genes in IPEC-J2 cells induced by DEGBG. IPEC-J2 cells were incubated with putrescine or spermidine for 24 h in the presence or absence of DEGBG. Then, the abundance of NF-κB and P-NF-κB proteins was detected by western blotting. (A) NF-κB protein abundance and (B) the phosphorylated NF-κB protein abundance at 24 h after incubated with 200 μmol/L putrescine in the presence or absence of 1 mmol/L DEGBG. (C) NF-κB protein abundance and (D) the phosphorylated NF-κB protein abundance at 24 h after incubation with 8 μmol/L spermidine in the presence or absence of 1 mmol/L DEGBG. The data are presented as the mean $\pm$ SE, n = 6. Different letters represent a significant difference ($p < 0.05$).

putrescine failed to recover the phosphorylation of ERK1/2 caused by DEGBG. Taken together, these results indicated that spermidine played a vital role in regulating the proliferation of porcine intestinal epithelial cells, the metabolism of putrescine to spermidine was essential for putrescine to perform its functions, and this beneficial effect of spermidine and putrescine was at least partly mediated by the activation of the ERK1/2 pathway.

Evidence showed that the level of ODC and SAMDC rapidly increased at the wound edge, which indicates that polyamines play a vital role in the acceleration of wound healing [13]. Cell migration is involved in repairing mucosal damage [25]. The current study used a model with scratching porcine intestinal epithelial cells to mimic wound healing after intestinal mucosa damage *in vivo*. This study showed that treating cells with DEGBG caused depletion of spermidine and accumulation of putrescine in cells, resulting in inhibiting the migration of porcine enterocytes. Exogenous spermidine supplementation partly restored cell migration caused by DEGBG. Consistent with our results, it has been reported that the rate of scratch wound closure of mouse embryo fibroblasts is significantly improved in the presence of spermidine [26]. However, the administration of putrescine failed to restore the migration of IPEC-J2 cells, which suggested that putrescine itself cannot rescue migration in DEGBG-treated cells. These results corrob-

orate the previous study in which putrescine itself is incapable of functioning to restore cell migration in IEC-6 cells [22]. Focal adhesion kinase (FAK) is a critical member of integrin-mediated signal transduction, mainly involved in regulating extracellular matrix signal transduction dependent on protein tyrosine kinase (PTK) activity, thus affecting cell adhesion, movement, and migration [27,28]. Previous studies have revealed that polyamines enhanced cell migration by upregulating the phosphorylation levels of FAK protein in DFMO-treated cells [16]. The present study showed that adding putrescine downregulated the levels of phosphorylated P-FAK in DEGBG-treated cells, which partially suggested that putrescine cannot activate FAK phosphorylation when the amount of spermidine is deficient, excess putrescine due to the inhibition of its metabolism even reduced FAK phosphorylation. Moreover, DEGBG alone did not downregulate FAK phosphorylation and adding spermidine in DEGBG-treated cells numerically increased FAK phosphorylation but was not significant indicated that it might need to take a longer time to affect FAK phosphorylation by DEGBG and spermidine, the treating time is 24 h for present study while treating with DFMO for 72 h has been found to reduce FAK phosphorylation [16]. Collectively, these findings demonstrated that putrescine itself might not be essential for the migration of IPEC-J2 cells, but its metabolite spermidine enhanced cell migration. The mechanism of regulating cell migration by spermidine may be through activation of the FAK signaling pathway. Future studies are warranted to test this hypothesis.

There was accumulating evidence showing that the increased inflammatory response is closely related to mucosal damage [29,30]. Spermidine has been demonstrated to perform anti-inflammatory effects *in vitro* and *in vivo* via regulating immune responses [31,32]. The current study found that spermidine depletion by DEGBG remarkably increased the mRNA expressions of the inflammatory mediators IL-8, IL-6, and TNF-α in porcine intestinal epithelial cells. However, exogenous spermidine supplementation suppressed pro-inflammatory cytokine gene expression in the DEGBG-stimulated cells. Likewise, spermidine has been reported to downregulate the expression of chemokines IL-6 in LPS-stimulated macrophages [33]. Previous studies have reported that polyamine depletion causes inflammation by rapidly activating the NF-κB pathway in IEC-6 cells [34], and spermidine could protect against LPS-induced inflammatory response by abrogating the activation of the NF-κB pathway [11,35]. Consistent with these results, our data suggested spermidine treatment significantly inhibited the phosphorylation of NF-κB in porcine enterocytes stimulated by DEGBG. In comparison, the administration of putrescine to the media cannot suppress phosphorylated NF-κB. Additionally, we also found spermidine inhibited TNF-α gene expression in the LPS-stimulated cells, which implied spermidine mitigates the LPS-induced inflammatory response by downregulating the expression of pro-inflammatory cytokines. In line with our results, other studies also suggested that spermidine inhibits the production of pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells [35]. To our knowledge, this is the first study to indicate that spermidine plays an important role in regulating the immune response of IPEC-J2 cells. The anti-inflammatory effect of spermidine has implications for anti-aging, which may be mediated by autophagy [11]. Moreover, it has been shown that spermidine attenuated acute colitis by suppressing inflammation in mice, which makes spermidine a promising candidate for inflammatory bowel disease intervention [36].

5. Conclusions

In conclusion, the current study demonstrated that putrescine needs to be converted into spermidine to efficiently promote IPEC-J2 cell growth and migration, which is associated, at least in part, with the activation of ERK1/2 and FAK signaling pathway. More importantly, our results highlight new insights into spermidine in immunomodulation. Thus, exogenous spermidine supplementation may constitute an attractive strategy to improve intestinal health and treat inflammation-related diseases.

Author Contributions

ZW and XL conceived and designed this study; ZW and LC performed the experiments; XZ and XJ analyzed the data; ZW and LC prepared the figures; ZW, LC, and XL interpreted the results of the experiments; LC and ZW drafted the initial manuscript; XJ and XL guided and revised the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2706194.

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