Molecules and Cells

The Integrins Involved in Soybean Agglutinin-Induced Cell Cycle Alterations in IPEC-J2

Li Pan[1], Yuan Zhao[1], Zhijie Yuan[1], Mohammed Hamdy Farouk[1,2], Shiyao Zhang[1], Nan Bao[1], and Guixin Qin[1,*]

[1]Key Laboratory of Animal Production, Product Quality and Security, Ministry of Education, Key Laboratory of Animal Nutrition and Feed Science, Jilin Province, College of Animal Science and Technology, Jilin Agricultural University, Changchun, P. R. China, [2]Animal production Department, Faculty of Agriculture, Al-Azhar University, Nasr City, Cairo, Egypt
*Correspondence: qgx@jlau.edu.cn
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Soybean agglutinin (SBA) is an anti-nutritional factor of soybean, affecting cell proliferation and inducing cytotoxicity. Integrins are transmembrane receptors, mediating a variety of cell biological processes. This research aims to study the effects of SBA on cell proliferation and cell cycle progression of the intestinal epithelial cell line from piglets (IPEC-J2), to identify the integrin subunits especially expressed in IPEC-J2s, and to analyze the functions of these integrins on IPEC-J2 cell cycle progression and SBA-induced IPEC-J2 cell cycle alteration. The results showed that SBA lowered cell proliferation rate as the cell cycle progression from G0/G1 to S phase ($P <$ 0.05) was inhibited. Moreover, SBA lowered mRNA expression of cell cycle-related gene CDK4, Cyclin E and Cyclin D1 ($P < 0.05$). We successfully identified integrins $\alpha2$, $\alpha3$, $\alpha6$, $\beta1$, and $\beta4$ in IPEC-J2s. These five subunits were crucial to maintain normal cell proliferation and cell cycle progression in IPEC-J2s. Restrain of either these five subunits by their inhibitors, lowered cell proliferation rate, and arrested the cells at G0/G1 phase of cell cycle ($P < 0.05$). Further analysis indicated that integrin $\alpha2$, $\alpha6$, and $\beta1$ were involved in the blocking of G0/G1 phase induced by SBA. In conclusion, these results suggested that SBA lowered the IPEC-J2 cell proliferation rate through the perturbation of cell cycle progression. Furthermore, integrins were important for IPEC-J2 cell cycle progression, and they were involved in the process of SBA-induced cell cycle progression alteration, which provide a basis for further revealing SBA anti-proliferation and anti-nutritional mechanism.

INTRODUCTION

The Soybean agglutinin (SBA) is a major anti-nutritional factor of soybean, it binds to the intestinal epithelial cells in mammals, affecting mucus secretion, digestion and absorption of nutrients, resulting in deleterious anti-nutritional effects (Li et al., 2003). The main anti-nutritional functions of SBA are inducing inflammation, destroying carcinogenic cells, influencing cell migration, signal transduction, proliferation, and division (Grant, 1989; Hart et al., 2010; Maxwell, 2011; Yau et al., 2015).

Integrins are heterodimeric transmembrane receptors, and they are the primary means for sensing and making a response to the cellular microenvironment (Naci and Aoudjit, 2014). Integrins elicit many intracellular signals to mediate cellular processes, including cell adhesion, migration, growth, differentiation, cell proliferation and apoptosis (Pan et al., 2016).

Recently, the cell proliferation, apoptosis and cell signal transduction mechanisms have become interesting research topics. The abnormality of cell proliferation in intestinal cells seriously affects the integrity and immunity of animal intestines, and causes death. Intestinal epithelial cells under-

go a continuous and rapid turnover, and they use specific integrins to regulate a wide variety of cell function (Gilcrease, 2007). In addition, the cell cycle progression of mammalian cells is strictly regulated by integrin-mediated adhesion and signaling (Assoian and Schwartz, 2001; Giancotti, 1997). Loss of the adhesion to the extracellular matrix (ECM) causes a complete G1 phase arrest (Giancotti, 1997). It has been established that, the regulations of epithelial cell growth and functional differentiation are affected by cell integration with ECM via integrins (Louvard et al., 1992). Thereby, integrins are essential for healthy intestinal function.

Studies have demonstrated that SBA can affect the integrity and permeability of cell membrane, and decline the cell proliferation in the intestinal epithelial cell line from piglets (IPEC-J2s) (Pan et al., 2013; Rhoads et al., 1994). However, the underlying mechanisms are still unclear. 18 α-subunits and 8 β-subunits of integrins have been identified in vertebrates (Barczyk et al., 2010). These subunits are expressed widely in metazoan, ranging from sponges to mammals (Burke, 1999). Nevertheless, to date, the types of integrin subunits specifically expressed in IPEC-J2s have not been identified. Integrins are involved in many cell biological processes, however, no study has reported whether integrins are also involved in SBA-induced alterations in IPEC-J2 cell cycle progression.

Hence, the present work aims to study the effects of SBA on cell proliferation and cell cycle progression of IPEC-J2, to identify the integrin subunits especially expressed in IPEC-J2s, and to analyze the functions of these integrins on IPEC-J2 cell cycle progression and SBA-induced IPEC-J2 cell cycle progression alteration. This research will provide new insight into the mechanisms of SBA anti-nutritional functions.

MATERIAL AND METHODS

Cell culture
IPEC-J2s were cultured in Dulbecco's Modified Eagle Media: Nutrient Mixture F-12 medium (DMEM/F12) (Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin (Sigma, USA), incubated at 37°C and in an atmosphere of 5% CO_2. The medium was refreshed every 2 d and the cells were subcultured with 0.05% trypsin (Gibco, USA). Monolayer cells were formed within 2 days after incubation, and then they were used in this study.

Cell proliferation assays
Cell counting kit-8 (CCK-8) is a highly sensitive kit for cell proliferation and cytotoxicity detection. It has the characteristic of stable effect and higher sensitivity when compared with MTT or MTS assay. The SBA cytotoxicity and the effects of SBA on IPEC-J2 cell proliferation were quantified by CCK-8 assay (Beyotime Biotechnology, China). IPEC-J2s were seeded into 96-well plates at a density of 2×10^3 cells per well for 80% completely differentiated. Then the cells were cultured in the presence of SBA (Sigma, USA) dissolved in DMEM/F12 medium at concentrations of 0, 0.125, 0.25, 0.5, 1.0 or 2.0 mg/ml for 24 h. The media were discarded and the cells were quantified by CCK-8 assay according to the

manufacturer's instructions. The plates were read using a multiplate reader (Multiskan FC, Thermo Scientific, USA) at 450 nm wavelength. Each independent experiment was performed for three times.

IPEC-J2 cell cycle analysis
Upon reaching 80% confluence, the cells were treated with 0, 0.125, 0.25, 0.5, 1.0, or 2.0 mg/ml SBA for 24 h. PI/RNase staining buffer was used to determine the cell cycle by flow cytometry (FCM). After the digestion by 0.05% trypsin, the cells were collected and fixed with 75% pre-cooling ethanol at 4°C for 18 h. After washed with PBS for three times, the cells were incubated with 0.5 ml PI/RNase staining buffer for 15 min at 37°C (according to the reagent instructions). Then samples were stored at 4°C in a cassette to protect them from light before evaluation.

Effects of SBA on mRNA expression of cell cycle-related gene
IPEC-J2 cells at 80% confluence were treated with 0 (as control) or 0.125 mg/ml of SBA for 24 h. The total RNA of IPEC-J2s was extracted using Trizol reagent (Takara, Japan) according to the manufacturer's instructions. The yield and purity of the total RNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). cDNA synthesis (1 μg of total RNA was used for cDNA synthesis) was conducted in 20 μl reaction system using cDNA synthesis kit (Takara, Japan). The real time reverse transcriptase-polymerase chain reaction (q-PCR) was used to evaluate the effects of SBA on the relative mRNA expression of CDK4 (accession: NM_001123097), Cyclin E (accession: XM_005653265) and Cyclin D1 (accession: XM_013994006) using the SYBR Premix Ex Tap II (Tli RNaseH Plus, Takara, Japan). The relative mRNA expression of CDK4, Cyclin E, Cyclin D1 and β-actin were assessed using the following primers with a concentration of 10 μM as shown in Table 1. The relative mRNA expression levels were calculated using the formula: $2^{-\Delta\Delta CT}$ (Vandesompele et al., 2002).

Cell membrane protein extraction
In order to explore the roles of integrins in the biological process of IPEC-J2s, the types of integrin subunits that are specifically expressed in IPEC-J2s should be firstly identified. IPEC-J2s were seeded at 5×10^4 cells/cm^2 in cell flasks and completely differentiated for 2 days. Membrane proteins were isolated using a native membrane protein extraction kit (Calbiochem, Germany) according to the manufacturer's instructions. The concentration of membrane proteins was measured by a BCA kit (Thermo Scientific, USA) and these protein samples were stored at -80°C.

SDS-PAGE
The SDS-PAGE consisting of 5% stacking gel and 10% separation gel was used to isolate the extraction of membrane proteins. Membrane protein samples of 1.0 mg/ml were mixed with SDS-PAGE loading buffer (CWBIO, China) at a radio of 4:1. The volume of the mixed protein sample was 10 μl per well. Pre-stained higher molecular weight protein standard (BioLab, China) was used to identify the protein

Table 1. Primer sequence used in q-PCR analysis

Gene	Forward primer	Reverse primer
CDK4	GCGGAGATTGGTGTTGGTG	CATTGGGGACTCTTACGCTCTT
Cyclin E	CCCTCCGTGTCCTACTTCAA	CCTCGCACTTCTGCTCCTC
Cyclin D1	CTCGCCACTGCCTATACTGA	GGTGCCGCTGCATAAGGT
β-actin	GGACTTCGAGCAGGAGATGG	AGGAAGGAGGGCTGGAAGAG

bands on the gels. After electrophoresis, gels were Coomassie brilliant blue-stained at room temperature.

Electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS) analysis

After separation by SDS-PAGE, the gels were scanned by a gel imaging system (LanScamTM6.0, EPSON, Japan). According to the molecular weight of integrins (80-220 kDa), the protein sample bands with a molecular weight of 80-220 kDa were collected and analyzed by Beijing Protein Institute using ESI-Q-TOF-MS. Integrin sequences were identified according to the SWISS-Prot database. Individual ion scores > 12 indicate statistically significance or extensive homology ($P < 0.05$).

Integrin functional inhibition test
Preliminary exploration of the optimal concentration of integrin inhibitors

IPEC-J2s were seeded in 96-well plates at 80% confluence. The cells were exposed to different integrin subunit functional inhibitors (α2: MAB1950Z; α3: MAB1952P; α6: MAB1378; β1: MAB1959; or β4: MAB2058, Millipore, USA) in a series dilution of 0, 5, 10, or 20 μg/ml in DMEM/F12 media containing 10% FBS for 24 h. Cell proliferation rates were quantified using CCK-8 assay according to the manufacturer's instructions. Plates were read at 450 nm wavelength using a multiplate reader (Multiskan FC, Thermo Scientific, USA), to select the optimal concentration of integrin inhibitors.

Effects of integrin inhibitors on cell cycle progression with or without SBA stimulation

Both SBA and integrin inhibitors (α2, α3, α6, β1 or β4) with their optimal concentration were used to stimulate the IPEC-J2 cells at 80% confluence. The cells were divided into twelve groups as presented in Table 2. Plates were collected at 24 h post-treatment. The cell cycle phase in different groups was measured using FCM and conducted as described before.

Statistical analysis

Each experiment was repeated at least for three times, and numerical data were presented as mean ± SEM. Student's *t*-test was used to compare data between two groups. Data among three or more groups were analyzed using ANOVA followed by the least significant difference (LSD) tests using SPSS Statistics Base 19.0 (SPSS Inc, USA; SPSS, 2010). $P < 0.05$ was considered significant.

Table 2. Structure of the divided cell groups in integrin inhibitor experiment

Treatment no.	Structure	
	SBA level	integrin inhibitor type
1(control)	--	--
2	SBA	--
3	--	α2 inhibitor
4	SBA	α2 inhibitor
5	--	α3 inhibitor
6	SBA	α3 inhibitor
7	--	α6 inhibitor
8	SBA	α6 inhibitor
9	--	β1 inhibitor
10	SBA	β1 inhibitor
11	--	β4 inhibitor
12	SBA	β4 inhibitor

RESULTS

SBA cytotoxicity and IPEC-J2 cell proliferation detected by CCK-8 assay

CCK-8 assay was used to detect the SBA cytotoxicity and IPEC-J2 cell proliferation by their capacity to reduce WST-8 to yellow formazan dye. The results indicated that SBA induced cytotoxicity in IPEC-J2 cells as shown in the decreased mitochondrial viability. Cell proliferation rates of IPEC-J2s were significantly ($P < 0.05$) lower by the increase of the SBA concentration, compared with the control group (Fig. 1). When the concentration of SBA was 2.0 mg/ml, cell proliferation rate was significantly ($P < 0.05$) lower, compared with the other SBA treatment groups (0 to 1.0 mg/ml).

Cell cycle arrest at G0/G1 phase after SBA stimulation detected by FCM

Nuclear staining with PI/RNase are indicators of the cell cycle phase. To determine the mechanism responsible for the low rate of cell proliferation in SBA treated groups, the cell cycle profile was examined. In the herein study, after application of 0, 0.125, 0.25, 0.5, 1.0 and 2.0 mg/ml SBA for 24 h, a significant ($P < 0.05$) delay in the G0/G1 to S phase transition was observed, when compared with control (Figs. 2A-
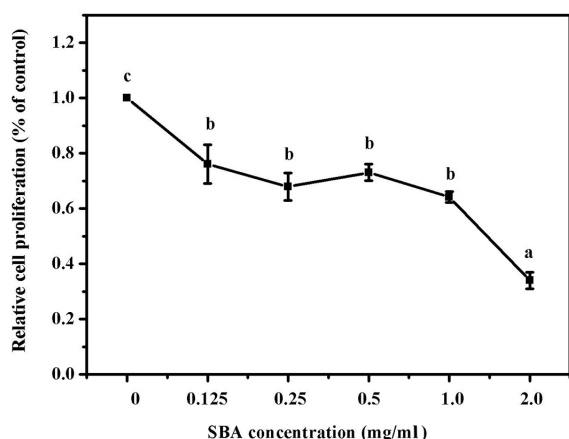
Fig. 1. Effects of SBA on IPEC-J2 proliferation rate. SBA cytotoxicity and cell proliferation was measured by CCK-8 assay at six concentrations points (0, 0.125, 0.25, 0.5, 1.0, 2.0 mg/ml) of SBA for 24 h. The absorbance was measured at 450 nm. Data are represented as mean ± SEM. Different lowercase letters are significantly different ($P < 0.05$).

Fig. 2. (A-F) SBA arrested cell cycle progression at G0/G1 phase. IPEC-J2s stimulated with different concentrations of SBA for 24 h. Cell cycle were evaluated using flow cytometry (FCM). Different cell cycle phases (G0/G1 phase, S phase and G2 phase) in different SBA treatments are shown in control (0 mg/ml) (A), SBA treated groups: 0.125 mg/ml (B), 0.25 mg/ml (C), 0.5 mg/ml (D), 1.0 mg/ml (E), and 2.0 mg/ml (F).

2F and Supplementary Fig. S1). The concentration of 0.125 mg/ml SBA was the first effective point on cell cycle progression. At this concentration, the percentage of cells at G0/G1 phase was significantly higher ($P < 0.05$), at the same concentration, the percentages of the cells at S phase and G2 phase were significantly lower ($P < 0.05$), compared with the control group. In addition, the highest percentage of the arrested cells at G0/G1 phase was in 2.0 mg/ml SBA treatment, compared with the other SBA treatments ($P < 0.05$). Thus the treatment of 2.0 mg/ml SBA was selected as an optimal concentration in integrin inhibitor experiments.

Relative mRNA expression of cell cycle-related genes detected by q-PCR

The intensity of fluorescence signal released by SYBR Green combined with DNA is an indicator of mRNA expression level. According to the selected first effective point on cell cycle progression, 0.125 mg/ml was selected as the stimulated concentration to determine the effects of SBA on cell cycle-related genes. The results indicated that the concentration of 0.125 mg/ml SBA had significantly ($P < 0.05$) lower mRNA expression at G1 phase regulated genes, including CDK4, Cyclin E and Cyclin D1, in IPEC-J2s (Fig. 3). The lower mRNA expression levels of these cycle-related genes also indicated the cell cycle G0/G1 phase arrest.

Integrin subunits identification

The membrane proteins were successfully extracted from IPEC-J2s and separated with SDS-PAGE (Fig. 4). Mass spectrometry provides important data (such as protein molecular mass and information on amino acid sequence etc.) for protein identification process (Soares et al., 2014). From the mass spectrometry results, individual ions scores >12 indicate significance or extensive homology at the level of $P < 0.05$. Owing to 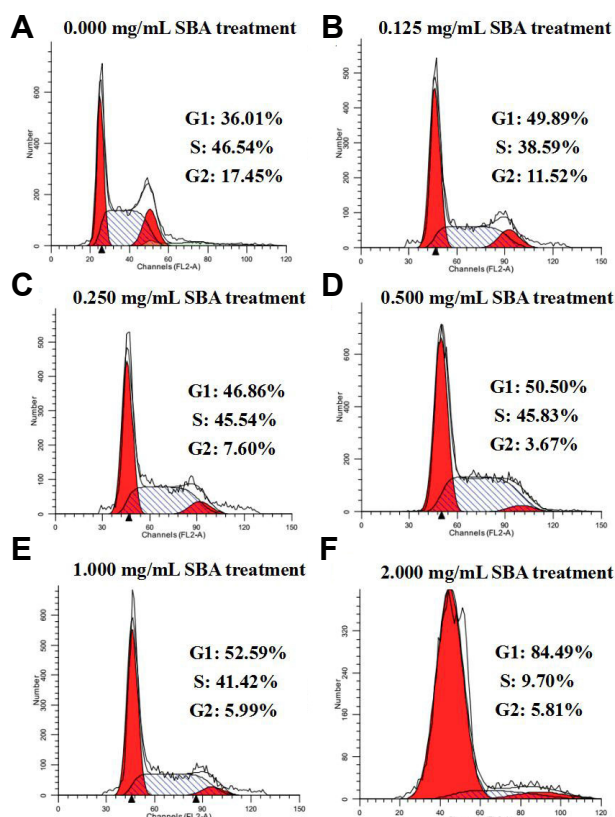the molecular weight of integrins (80-220 kDa), a total of 5 integrin subunits ($\alpha 2$, $\alpha 3$, $\alpha 6$, $\beta 1$, and $\beta 4$) were successfully identified in a range between 80 and 220 kDa gels by MS/MS (Table 3).

Optimal concentration of integrin inhibitors identified by CCK-8 assay

We evaluated the effects of different concentrations (0, 5, 10, or 20 µg/ml) of integrin $\alpha 2$, $\alpha 3$, $\alpha 6$, $\beta 1$, and $\beta 4$ inhibitors on cell proliferation using CCK-8 kit. After stimulation for 24 h, IPEC-J2 cell proliferation had lower rate in a dose-dependent manner ($P < 0.05$, Fig. 5). Under the same concentrations, different integrin inhibitors had different effects on cell proliferation rate ($\beta 1 > \alpha 6 > \alpha 3 > \alpha 2 > \beta 4$). The cell proliferation rate was significantly lower ($P < 0.05$) compared with control when the first effective concentration in $\alpha 2$ treatment was 10 µg/ml; in $\alpha 3$, 10 µg/ml; in $\alpha 6$, 5 µg/ml; in $\beta 1$, 5 µg/ml and in $\beta 4$ was 10 µg/ml. To ensure the consistency of experimental conditions, 10 µg/ml was selected as the final optimal concentration for each subunit in the subsequent integrin inhibitor experiments.

Table 3. Identification of integrins in IPEC-J2s

No	Name	Accession	Score	Mw/PI	P value (t-test)	Sequence coverage (%)	Matched peptides
1	ITGB1	F1RVE7	66	91435/5.29	P< 0.05	6%	LQPED ITQIQPQQLV LQLR SLGTDLM NEMR NVLSLTDKGE VFNELVGK
2	ITGA3	F1RT62	20	118371/6.3	P< 0.05	3%	LGLPGLATF GYSLSGRMDV DENFYPDLLV GSLSDR
3	ITGA6	H0VCB5	16	116495/7.91	P< 0.05	1%	LIA TFPDTLTYSA YR
4	ITGB4	F1RVY6	39	206227/5.57	P< 0.05	1%	NVISLT EDVEEFR LLELQEM DSLLR
5	ITGA2	H0VU32	29	129679/5.25	P< 0.05	0.1%	FGIAV LGYLNR

[a]Accession number in the NCBInr database.

[b]MW, molecular weight; PI, isoelectric points.

[c]Peptide score is shown as −10log(P), where P is the probability that the observed match is a random event. Individual ions scores > 12 indicate identity or extensive homology (P< 0.05).

[d]Sequence coverage is shown with the respect to matched tryptic digest fragments.
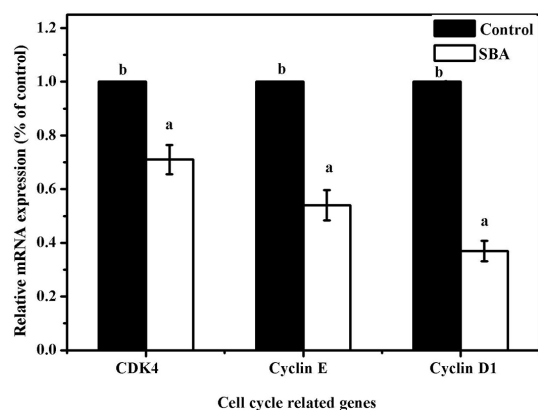

Fig. 3. Relative mRNA expression of different cell cycle genes after SBA stimulation. mRNA expression of CDK-4, Cyclin E and Cyclin D1 were analyzed after 0 (control) or 0.125 mg/ml SBA stimulation for 24 h. Data are presented as the mean ± SEM of three independent experiments and different lowercase letters are significantly different compared with their corresponding control (P< 0.05).

Fig. 4. Total membrane proteins of IPEC-J2s.

Effects of functional inhibitors of integrins on cell cycle progression

As described previously, 2.0 mg/ml of SBA, 10 µg/ml of integrin inhibitors, or both were selected in this experiment to determine the roles of integrins in IPEC-J2 cell cycle progression and SBA-induced cell cycle progression alteration.

After the incubation with 10 µg/ml inhibitors of integrin subunits (α2, α3, α6, β1 or β4) for 24 h, the percentage of the cells at G0/G1 phase in IPEC-J2s was significantly higher in the inhibitor-treated groups (β1 > α6 > α3 > α2 > β4), compared with the control group (P< 0.05, Fig. 6 A-L and Supplementary Fig. S2). The percentage of the cells both at S phase and G2/M phase were significantly lowered (P< 0.05). These results indicated that the specified five integ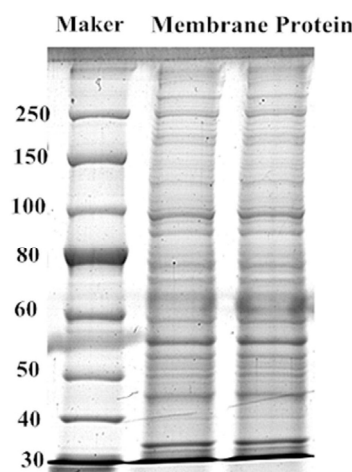rin subunits played an indispensable role in maintaining the normal cell cycle progression of IPEC-J2s, as the absence of either integrin subunits led to cell cycle arrest at G0/G1 phase. The cells percentages at G0/G1 phase in these five integrin inhibitor treatments were lower than that of 2.0 mg/ml SBA treatment group (P< 0.05), and higher than the control (Supplementary Fig. S2), revealing the inhibition ability of these five integrin inhibitors at the concentration of 10 µg/ml on IPEC-J2 cell cycle progression.

Based on the current results that the inhibition of integrin inhibitors on cell proliferation was also through the perturbation of cell cycle G0/G1 phase like SBA treated groups induced. Therefore, we speculate that integrins are also crucial in the SBA-induced cell cycle progression alteration, as shown in Supplementary Fig. S3. The percentage of the cells at G0/G1 phase only in the integrin inhibitor α2, α6, and β1 treatment groups had no significant difference when compared with the same integrin inhibitors + 2.0 mg/ml SBA treatment groups (P > 0.05), indicating that the addition of
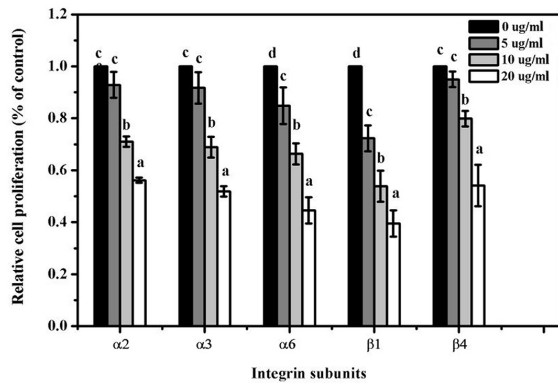
Fig. 5. Inhibitors of integrin subunits decreased cell proliferation rate in a dose-dependent manner. Cell proliferation rate was measured using CCK-8 assay at four concentrations (0, 5, 10, 20 µg/ml) of integrin subunits (α2, α3, α6, β1, β4) inhibitors for 24 h. The relative cell proliferation data are presented as mean ± SEM of three independent experiments. Different lowercase letters are significantly different for cell proliferation in the same integrin subunits inhibitor treatment group (with different concentration) compared with their control ($P < 0.05$).

integrin α2, α6, and β1 inhibitors, SBA will no longer increase the percentage of the cells at G0/G1 phase. The such treated subunits were involved in SBA-induced cell cycle progression alteration.

Despite the cell percentage in integrin α3 and β4 inhibitors + 2.0 mg/ml SBA treated groups had no significant difference with 2.0 mg/ml SBA treatment ($P > 0.05$, Supplementary Fig. S4), the proportion of the cells at G0/G1 phase in the such treated groups were significantly ($P < 0.05$) higher than the same inhibitor treatments. The results indicated that SBA still has the ability to influence the cell cycle progression, even with the addition of α3 or β4 inhibitor. Thus, integrin α3 and β4 were not involved in the process of SBA-induced cell cycle progression alteration.

In general, integrins were essential for maintaining normal cell cycle progression in IPEC-J2s, specially integrin α2, α6, and β1 were crucial for cell cycle progression alteration induced by SBA.

DISCUSSION

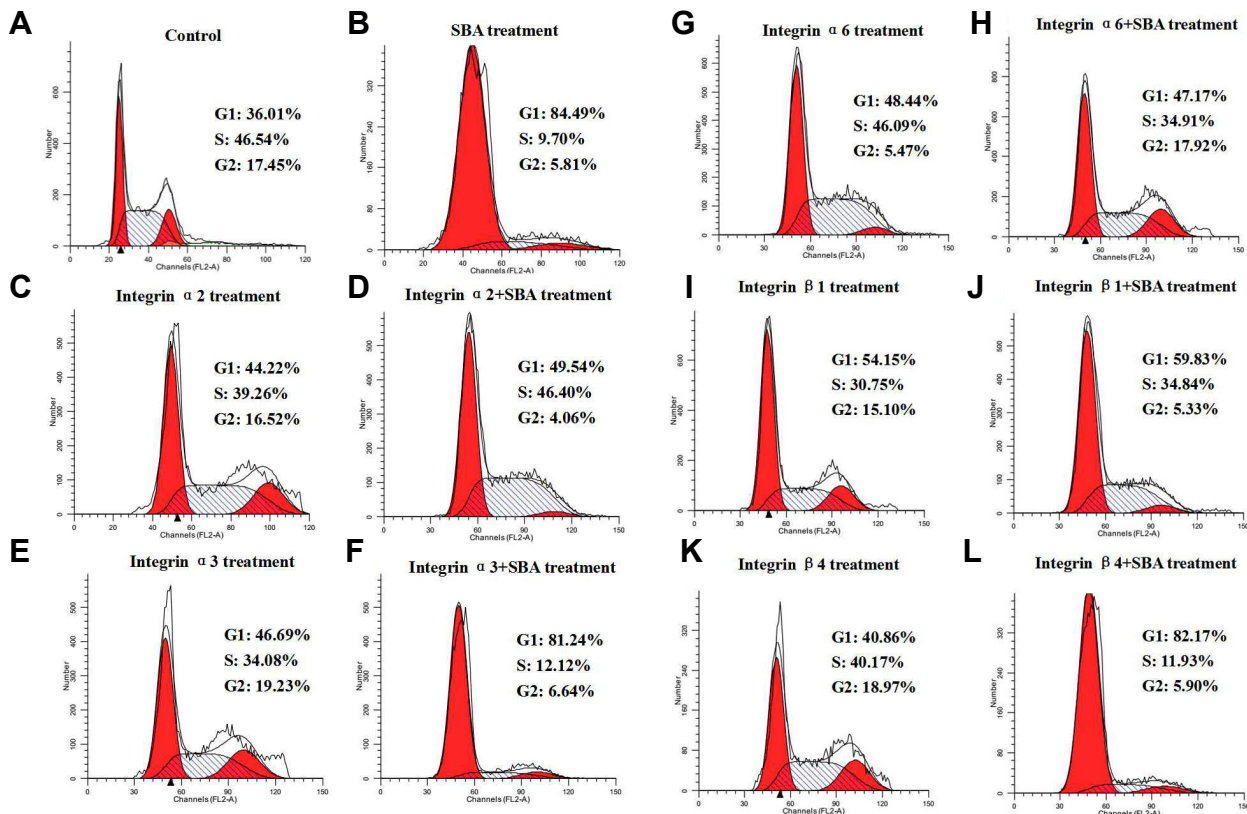The current study showed that SBA lowered IPEC-J2 cell

Fig. 6. (A-L) The effects of integrin functional inhibitions on IPEC-J2 cell cycle with or without SBA stimulation. Cell cycle from each treatment group was determined by flow cytometry (FCM). Different cell cycle phases (G0/G1 phase, S phase and G2 phase) in different treatments are shown in (A) Control, (B) Control + 2.0 mg/ml SBA treatment, (C) Control + α2 inhibitor, (D) Control + α2 inhibitor + 2.0 mg/ml SBA, (E) Control + α3 inhibitor, (F) Control + α3 inhibitor + 2.0 mg/ml SBA, (G) Control + α6 inhibitor, (H) Control + α6 inhibitor + 2.0 mg/ml SBA, (I) Control + β1 inhibitor, (J) Control + β1 inhibitor + 2.0 mg/ml SBA, (K) Control + β4 inhibitor, (L) Control + β4 inhibitor + 2.0 mg/ml SBA. The gated events are 1×10^4 cells.

proliferation rate through the perturbation of cell cycle progression from G0/G1 to S phase. Moreover, five integrin subunits ($\alpha2$, $\alpha3$, $\alpha6$, $\beta1$, and $\beta4$) were identified in IPEC-J2s. These integrins were crucial for IPEC-J2 cell proliferation and cell cycle progression, therein, $\alpha2$, $\alpha6$ and $\beta1$ were involved in the complex processes of cell cycle progression alteration induced by SBA.

SBA is an anti-nutritional factor in soybean. Our results suggested that SBA induced cytotoxicity to IPEC-J2 cells and lowered IPEC-J2 cell proliferation rate through the perturbation of cell cycle progression. Ohba and Bakalova (2003) reported that the cytotoxic effects of SBA on different cell lines take place by lossing the viability of these cells. In addition, the role of SBA in intestinal epithelial cells has been extensively studied. Research indicates that SBA decreases the IPEC-J2 cell viability (Pan et al., 2013), disrupts the brush border membrane in piglets (Zhao et al., 2011), causes the atrophy of rat small intestine microvilli and appearance of large numbers of epithelial cells in gut lumen (Pusztai et al., 1990). The cell cycle progression regulates the condition of cell proliferation (Zhang et al., 2016), and it consists of three prominent phases (G0/G1 phase, S phase and G2 phase) to maintain DNA integrity (Derheimer and Kastan, 2010). In the present research, SBA significantly lowered the IPEC-J2 cell proliferation rate and declined the percentage of the cells at both S and G2 phases, accompanied with a significant increase in the percentage of cells at G0/G1 phase ($P <$ 0.05). The increase of G1-phase cells reflects the higher number of cells in the DNA repairing process. However, the reduction of the cells at S phase indicates the inhibition in the DNA repairing process. Moreover, the lower percentage of cells at G2/M phase demonstrates the inhibition in cell mitosis (Sancar et al., 2004). These results are consistent with the review of Pan et al. (2016). Bakke-McKellep et al. (2008) also found the same trends in Atlantic salmon.

Cell cycle-related genes experiments were conducted to support the theory of the inhibitory effects of SBA on cell proliferation rate by arresting the cells at G0/G1 phase. The cell cycle is tightly regulated by series of cyclins and cyclin dependent kinase (CDKs), which are the checkpoints for cell cycle progression at each stage (Moreno-Layseca and Streuli, 2014). G0/G1 phase is regulated by CDK4, Cyclin E and Cyclin D1. In the present study, a G0/G1 phase cell arrest was detected in SBA treated group, which was accompanied with the lower mRNA expression of CDK4, Cyclin E and Cyclin D1 when stimulated with SBA.

Studies show that the distributions of some integrin subunits are cell-type, tissue-type and animal species dependent (De Arcangelis and Georges-Labouesse, 2000; Pozzi and Zent, 2014; Stepp et al., 1990; Tervo et al., 1991). In this research, five integrin subunits, including $\alpha2$, $\alpha3$, $\alpha6$, $\beta1$, and $\beta4$, were successfully identified in IPEC-J2s. These results were different from the distribution of integrin subunits in different intestinal cells from human intestinal and *Drosophila*. There are six subunits, including $\alpha2$, $\alpha3$, $\alpha6$, $\alpha7$, $\beta1$, and $\beta4$ in human intestinal cells (Beaulieu, 1997; 1999), while in *Drosophila*, there are five α subunits (αPS1, αPS2, αPS3, αPS4 and αPS5) and two β subunits (βPS and βv) (Lin et al., 2013). The variations among the integrin subunits expressed in IPEC-

J2 cells, human intestinal cells, and *Drosophila* cells might be due to the differences in animal species, cell-type or both.

Integrin-mediated adhesion is crucial to cell proliferation (Pan et al., 2016). In leiomyoma cells, the inhibition of integrin $\beta1$ leads to a significant decrease in cell proliferation (Malik et al., 2012). In the present study, the stimulated IPEC-J2 cells with different concentrations of integrin inhibitors had dose-dependent lower proliferation rates compared with controls. Additionally, different integrin-subunit-inhibitors at the same concentrations had different impacts on the cell proliferation rate ($\beta1 > \alpha6 > \alpha3 > \alpha2 > \beta4$). Such different rate may be depending on their expression levels, and the ability to form heterodimers with other subunits. Integrin $\beta1$ is ubiquitously expressed and has a higher mRNA expression level in human embryonic stem cells (Lee et al., 2013). It combines with multiple α subunits (Cox et al., 2010; Pan et al., 2016). Integrin $\alpha6$ also has a high expression level, but lower than $\beta1$ (Lee et al., 2013), and binds with two β subunits ($\beta1$ and $\beta4$), while, the $\alpha2$, $\alpha3$, and $\beta4$ have a weak expression ($\alpha3$ and $\alpha2 > \beta4$), as they can only bind with one integrin subunit (Lee et al., 2013). This may explain why the integrin $\beta1$ inhibitor had the most effects on cell proliferation than the other four subunits inhibitors in IPEC-J2s, and explains the order of the effects of these five subunits on IPEC-J2 cell proliferation rate.

Interestingly, the inhibition of integrin inhibitors on cell proliferation was also through the perturbation of cell cycle progression like SBA treated groups induced. The absence of any of these subunits led to cell cycle arrest at G0/G1 phase. Integrin-mediated signals regulate the G0/G1 phase cell-cycle progression (Assoian and Schwartz, 2001). Metazoan cells will not commit to enter cell cycle progression and do not proliferate in the absence of integrin-mediated cell adhesion (Moreno-Layseca and Streuli, 2014), which was consistent with the present results. Another intriguing observation in this study is that integrin $\alpha2$, $\alpha6$, and $\beta1$ subunits were also involved in SBA-induced cell cycle G0/G1 phase arrest. The restrain of $\alpha2$, $\alpha6$, and $\beta1$ inhibited the SBA-induced cell cycle progression alteration.

In this study, IPEC-J2s were used as our *in vitro* model. Such *in vitro* model has similar structures and functions to those of porcine intestinal epithelial cells. Although the IPEC-J2s will not receive neural regulation and body fluid regulation under *in vitro* settings, they can simulate the state of the cells *in vivo*, which are useful for studying the functional mechanism of the intestinal tract.

In conclusion, SBA lowered IPEC-J2 cell proliferation rate through the perturbation of cell cycle progression. Moreover, integrins were crucial for IPEC-J2 cell cycle progression and they were involved in the complex processes of cell cycle progression alteration induced by SBA. This provides a basis for revealing SBA anti-proliferation effects. Integrins give a new viewpoint on the prevention of SBA induced cytotoxicity and anti-proliferation function. It is unclear, however, whether integrins are the only protein-group in these processes, and still needs further investigation.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

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