Seasonal Expression of Extracellular Signal Regulated Kinases in the Colon of Wild Ground Squirrels (Spermophilus Dauricus)

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Research Article

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Abstract

Background: The aim of this study was to explore the localization and expression of extracellular signal regulated kinase/phospho-extracellular signal regulated kinases (ERK/pERK) in the colonic tissue of wild ground squirrels (*Spermophilus dauricus*) in the breeding season and the non-breeding season.

Methods and Results: Hematoxylin-eosin staining, immunohistochemistry, real-time quantitative PCR and Western blot were used in this study. The histological results showed that the diameter of the colon lumen in the non-breeding season was larger than that in the breeding season, and the number of glandular cells in the non-breeding season was also more than those in the breeding season. The immunochemical results showed that ERK1/2 was expressed in the cytoplasm of goblet cells and intestinal epithelial cells, while pERK1/2 was expressed in the cytoplasm and nucleus of goblet cells and intestinal epithelial cells. The immunolocalization of ERK1/2 and pERK1/2 expression were more obvious in the non-breeding season, especially in intestinal epithelial cells. The results of real-time quantitative PCR and Western blot showed that the expression of ERK1/2 and pERK1/2 in the breeding season was significantly lower than that in the non-breeding season.

Conclusions: The expression of ERK1/2 and pERK1/2 in the colons of the wild ground squirrels had seasonal changes, which had significant increases in the non-breeding season comparing to those in the breeding season. This study revealed the potential role of ERK1/2 in colon to the adaptation of seasonal changes in wild ground squirrel.

Background

Mitogen-activated protein kinase (MAPK) is an important family of protein kinases in eukaryotic cells. Extracellular signal regulated kinases (ERKs) are important members of the MAPKs family. ERK1 and ERK2 are the first reported MAPKs. They share 84% in common and share many common functions[1], so usually, they are called ERK1/2. At the level of mRNA, ERK1/2 is often referred to as MAPK1/3.

ERK1/2 can regulate cell cycle progression, proliferation, cytokinesis, transcription, differentiation, cell death, migration, gap junction. In addition to these functions, ERK1/2 are also key enzymes in the development of the immune system, the development of the nervous system, memory formation, and heart development [2-5].

ERK stimulating factors bind to receptors on the cell surface to activate the Ras pathway and then interact with downstream kinase Raf. Activated Raf binds and phosphorylates MAPKK. Activated MAPKK phosphorylates threonine and tyrosine in the conserved structure of Thr-Glu-Tyr (TEY) in the activation ring of ERK1/2 and the bind to downstream substrates[6].

The target sites of ERKs vary in location and function and include cytoplasmic, nuclear, and membrane proteins that encode transcription factors, RNA-binding proteins, or signaling proteins. After receiving external stimulation, ERK, which is widely distributed in the cell, is phosphorylated to pERK and moves

toward the target. This is generally considered to be the activation of the ERK pathway. Phosphatase dephosphorylates and inactivates extracellular signal regulated kinases, thereby closing this pathway[7].

ERKs play important roles in nerve cell protection. Activation of ERKs is a pathway to induce the growth of nerve axons[8]. In tumor and cancer, aspirin can promote the enhancement of TNF-related apoptosis-inducing ligand (TRAIL) significantly, and a combination of TRAIL can significantly inhibit ERK1/2 activation and enhance TRAIL-induced apoptosis[9]. In human colon cancer cell lines Caco2 cultured in vitro, the increased level of activated phosphorylated ERK1/2 promoted cell proliferation. In addition, Caco2 tumor cells could spontaneously differentiate into intestinal epithelial cells to form clones, and the differentiation of Caco2 cells was also affected after treatment with MAPKK inhibitor[10]. ERK1/2 is also related to the differentiation of the intestinal cell population, and the activation of ERK1/2 inhibits the transcription of the sucrose-maltose gene induced by caudal-related homeobox transcription factor 2/3 (CDX2/3), thereby inhibiting the differentiation of intestinal epithelium[11]. Eric's study found that in the kirsten rat sarcoma viral oncogene (*KRAS*) mutant mice, the intestinal epithelial cells multiplied, the Panthera cells reduced, and the differentiation of goblet cells increased. The results suggested that ERK signaling pathway may be involved in determining intestinal prosses of cell differentiation in Panthera cells and goblet cells[12]. The gut microbiota can change the content of androgen in the blood of female mice through the Hypothalamic-Pituitary-Gonadal axis (HPGA), which indirectly leads to the occurrence of polycystic ovary syndrome[13].

The wild ground squirrel (*Spermophilus dauricus*) has the characteristic of the typical seasonal reproduction, which made it a good material for studying seasonal reproduction[14]. Usually they enter the breeding season from April to May, followed by several months of activity, and their hibernation begins in October[15-16]. Up to the date, there is no related study between the expression of ERK1/2 in the colon and wild ground squirrels. This study focused on the expression of ERK1/2 in the colon to explore the relationship between colonic ERK1/2 expression and seasonal adaption in the wild ground squirrel.

Materials And Methods

2.1 Animals and tissues

Wild ground squirrels were captured in the breeding season and non-breeding season in Hebei Province, China. The treatment of animals was observed AVMA(American Veterinary Medical Association) Guidelines for the Euthanasia of Animals: 2020 Edition (https://www.avma.org/sites/default/files/2020-02/Guidelines-on-Euthanasia-2020.pdf). Animals were placed in a container with slow passage of carbon dioxide until the animals were in a recumbent position, and then quickly killed via decapitation. And all animal experiments were approved by the Policy on the Care and Use of Animals by the Ethical Committee of Beijing Forestry University and the Department of Agriculture of Hebei Province, China (JNZF11/2007). Colonic tissues were extracted. Half of the colons of the squirrels were fixed with 4% paraformaldehyde for 48h, washed with running water for 24h and stored in 70% alcohol. The other half

samples were quickly placed in liquid nitrogen and transferred to a -80℃ refrigerator for later extraction of RNA and proteins.

2.2 Antibodies

The primary antibodies were utilized in this study, including the rabbit polyclonal anti-ERK1/2 (BS-2637R, Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China), rabbit polyclonal anti-pERK1/2 (BS-3016R, Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China), and β-actin (BS-0061R, Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China). The dilution of ERK1/2 and pERK1/2 antibodies for immunohistochemistry and Western blot observations were respectively 1:200 and 1:750, and the dilution of β-actin antibodies for Western blot observations was 1:1000.

2.3 Histology

The colonic tissues were embedded in paraffin wax after dehydrated in ethanol series. Slides coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA) were used to set serial sections (6 μm). The serial sections were stained with hematoxylin-eosin (HE) for general histological analysis.

2.4 Immunohistochemistry

The serial sections of colonic tissues were boiled in citrate buffer and blocked with 10% normal goat serum. The sections were then incubated with the primary antibody overnight at 4°C. Subsequent incubations were utilized by the secondary antibody, goat anti-rabbit IgG conjugated with biotin and peroxidase with avidin using SP Kit (Rabbit) (SP-0023, Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China), then following by the visualization with 30 mg 3,3-diaminobenzidine (Wako, Tokyo, Japan) solution in 150 mL of 0.05 Mol Tris-HCl buffer, pH 7.6, plus 30 μL H_2O_2. The reacted sections for ERK1/2 and pERK1/2 were counterstained with hematoxylin solution (Merck, Tokyo, Japan).

2.5 Total RNA isolation and real-time quantitative PCR

The colonic tissues were utilized to obtain Total RNA by Trizol® Reagent (Invitrogen, Carlsbad, CA, USA). About 0.1 g of each sample was thawed and immediately homogenized in 1 mL of TRIzol™ Reagent to dissociate nucleoprotein complexes. 0.2 mL of chloroform was added into the homogenate. Then, the mixture was vigorously shaken for 15 sec at room temperature and centrifuged at 12,000 g for 20 min at 4 °C. The aqueous phase was diverted to RNase free tube. 500 μL of isopropanol was added. The solutions were kept for 10 min at room temperature and centrifuged at 12,000 g for 20 min at 4°C. Then, RNA was precipitated, washed twice with 70% ethanol, and dissolved in diethylpyrocarbonate-treated water (30 μL). The concentration and quality were analyzed by spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). The mRNA concentration in different samples was adjusted to 250 ng/mL and the first-strand cDNA from total RNA was synthesized using GoScript reverse transcription system (Promega Corporation, Madison, WI, USA) and random primer according to the manufacturer's protocol. The primers sequence used for mRNA qRT-PCR were shown in Table 1. The PCR

reactions were carried out in a 10 μL volume using FastStart DNA MasterPlast SYBR green kit (Roche Molecular Systems Inc., Basel, Switzerland) and performed with ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The following listed conditions were used: pre-incubation at 95 °C for 10 min, then amplification at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, followed by PCR reaction of 40 cycles, and then melting step at 60 °C.

Table 1 The primers sequence used for mRNA qRT-PCR

Gene name	Sequence of primer	Product size (bp)
MAPK1	F:5'TGGTTCCTCCCACTCCTGAA3'	142
	R:5'TGGGCAAATAGCACACACCT3'	
MAPK3	F:5'ACTACCTGGACCAGCTCAAC3'	305
	R:5'GCTTGTTGGGGTTGAAGGTT5'	
β-actin	F:5'GACTCGTCGTACTCCTGCTT3'	223
	R:5'AAGACCTCTATGCCAACACC3'	

2.6 Protein extraction and Western blot analysis

Protein was extracted from the colonic tissues of the wild ground squirrels. Colonic tissues were diced into small pieces, solubilized in 1 mL Radio-Immunoprecipitation Assay (RIPA) lysis buffer (0.5% sodium deoxycholate, 150 mM NaCl, 1% Nonidet P 40, 50 mM Tris (pH 7.4)) with 10 μL phenylmethylsulfonyl fluoride (PMSF) (10 mg/mL), homogenized by an ultrasonic homogenizer (Scientz, Ningbo, Zhejiang, China), and then incubated on ice for 30 min. Homogenates were centrifuged at 12,000 g for 10 min at 4 °C, and then the supernatant was collected. All protein extraction processes were performed on ice or maintaining the temperature at 4 °C. Protein extracts were mixed with an equal volume of 2× LaemmLi's sample buffer, analyzed by a 15% SDS-polyacrylamide gels (SDS-PAGE) at 18 V/cm, and transferred to nitrocellulose membranes. The membranes were blocked with 2% BSA for 1 hr at room temperature followed by overnight incubation with 1:200 primary antibodies. Secondary incubation of the membranes was performed by secondary antibodies (goat anti-rabbit IgG for ERK1/2 and pERK1/2, goat anti-mouse IgG for β-actin) tagged with horseradish peroxidase for 1 hr. The membranes were stained with diaminobenzidine after being washed three times in 50 mL TTBS (Tris-buffered saline with Tween 20) buffer (0.137 M NaCl, 0.02 M Tris and 0.1% Tween-20, pH 7.6).

2.7 Statistical analysis

The quantitative data were obtained by the experiments and statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). The difference between groups was compared using the Student's t-test. Statistical values of $P<0.05$ were considered significant.

Results

3.1. Histological changes of the colons of wild ground squirrels

Histological results of colonic tissues of wild ground squirrel during the breeding season and non-breeding season were shown in Fig. 1, and details were shown in Fig. 2. The comparison between Fig. 1a and Fig. 1b showed that the lumen diameter of the colonic tissue was significantly smaller in the breeding season than those in the non-breeding season, and the number of goblet cells (GC) in the breeding season was less than those in the non-breeding season.

3.2. Immunolocalization of ERK1/2 and pERK1/2 in the colon of wild ground squirrels

Immunohistochemical staining for ERK1/2 and pERK1/2 was performed in the colons between the breeding and non-breeding seasons respectively in Fig. 3. Both ERK1/2 and pERK1/2 were observed in the cytoplasm of the GC and epithelial cell (EC) between the breeding and non-breeding seasons. pERK1/2 was observed in the cytoplasm and nuclear of GC and EC between the breeding and non-breeding seasons. Furthermore, during the non-breeding season (Fig. 3e-f), staining signal of ERK1/2 and pERK1/2 were higher than that in the breeding season (Fig. 3b-c). No positive signal was found in the negative controls (Fig. 3a, d).

3.3. Seasonal expression of ERK1/2 and pERK1/2 in the colons of wild ground squirrels

The mRNA and protein expression patterns in the colons between the breeding and non-breeding seasons were shown in Fig. 4 and Fig. 5, respectively. The mRNA expression levels of MAPK1 and MAPK3 in the colons were significantly higher in the non-breeding season than those in the breeding season (Fig. 4). Furthermore, compared with the breeding season, the protein expression levels of ERK1/2 and pERK1/2 in the colons were significantly higher in the non-breeding season (Fig. 5).

Discussion

In this study, the localization and expression of ERK1/2 and pERK1/2 in the colonic tissue of wild ground squirrels in the breeding season and the non-breeding season were explored, using hematoxylin-eosin staining, immunohistochemistry, real-time quantitative PCR and Western blot. The experimental results showed that in the non-breeding season, the lumen diameter of the colon has increased significantly and the number of GC has increased. From the histologic results we speculated that to adapt to the energy demand of the seasonal reproduction, the colon of wild ground squirrel has enlarged. While in the non-breeding season, the energy demand of reproductive system has reduced, then the colon lumen enlarge. Using immunohistochemistry, quantitative real-time PCR and Western blot, the expression position and intensity of the ERK1/2 in the colon were preliminary explored, which laid a foundation for the subsequent study on the effect of the expression of ERK1/2 pathway in the colon on seasonal reproduction. Immunohistochemical results showed that ERK1/2 was expressed in the cytoplasm of GC

and intestinal EC, while pERK1/2 was also expressed in the nucleus of both types of cells, suggesting that ERK1/2 had migrated from the cytoplasm to the nucleus after activation of the ERK1/2 pathway. The results of Western blot showed that the expression levels of ERK1/2 and pERK1/2 in the colonic breeding season of wild ground squirrels were lower than those in the non-breeding period, and the differences were significant.

ERK1/2 can regulate a vary of cell processes and phylogeny. Research on the colon ERK1/2 signaling pathway has focused on colon cancer. The high expression of pERK1/2 in intestinal EC is a marker of early colon cancer[17]. The study on the new drug Enduo showed that the drug had a certain therapeutic effect on colon cancer by blocking the ERK signaling pathway to prevent cell migration[18]. Verticillin A increases the ratio of BIMEL/MCL-1 to overcome ABT-737-resistance in human colon cancer cells by targeting the MEK/ERK pathway[19]. PKCα activated RAS upstream of RAF, MEK and ERK, which induced the arrest of the cell cycle in intestinal cells[20]. Studies have shown that the Wnt/β-catenin signaling pathway has a synergistic effect with the ERK1/2 pathway to some extent. For example, the low expression of the long non-coding RNA Casc2c associated with gastric cancer in gastric cancer tissues may lead to decreased expression levels of the ERK1/2 pathway and the Wnt/β-catenin signaling pathway[21]. To inhibit the proliferation and migration of gastric cancer cells. In other studies on the testis of wild ground squirrels, it was found that the activin signaling pathway can also cross-interact with the ERK1/2 pathway, thus participating in the regulation of cell proliferation and apoptosis[22].

Previous morphological observations showed that the diameter of colons of wild ground squirrels in the non-breeding season was larger than those in the breeding season. This kind of change was the seasonal adaption of colonic tissues to the environment. The wild ground squirrel distributes its energy through seasonal reproduction to ensure that the animals reproduce in the most favorable living environment and the best physical condition. At present, most of the researches on seasonal adaption is focused on the reproductive system and its neuro-humoral regulation. There is little research on the influence of various systems, especially the digestive system on seasonal adaption. Studies have shown that the reproductive system generally produces seasonal changes in the process of seasonal reproduction. The weight of reproductive organs in the breeding season is higher than that in the non-breeding season, and sperm generation can only occur in the testis of males during the breeding season[16]. In females, all types of follicles and luteum exist only in the ovary during the breeding season[23]. As mentioned above, ERK1/2 can regulate cell progression, and regulates phylogeny. These roles may provide new ideas for the explanation of seasonal changes in the colon. This study initially analyzed the relationship between the expression of the ERK1/2 pathway in the colonic tissue and seasonal adaption.

In recent years, studies related to the upstream and downstream regulation of the ERK pathway have also been developed in different tissues and cells. Calcium-sensing receptors (CaSR) were involved in the induction of kidney calculi related proteins. Calculi related proteins and ERK were upregulated in the kidney of rats treated with Calcium oxalate and cell apoptosis may lead to crystal adhesion on the surface of kidney cells to form calculi[24]. MicroRNAs (miRNAs) are important regulatory factors of gene

expression, which play a key role in the occurrence and development of tumors and can also regulate ERK signal, which is usually overexpressed in cancerous tissues. Study showed that the regulation of the ERK signaling pathway by miRNAs plays an important role in the development of cancer[25]. Venezuelan equine encephalitis virus (VEEV) can infect primary astrocytes by upregulation of Early Growth Response 1 (EGR1) induces cell apoptosis and viral transcription. ERK1/2 gene knockout significantly reduced the expression of EGR1, and the ERK1/2 pathway plays a key role in astrocyte apoptosis. ERK pathway is an important link in the formation of osteoblasts and bone metabolism[26].

The experiment verified the lumen of wild ground squirrel colons had seasonal changes by the results of immunohistochemistry and Western blot which can significantly observe the differences of colon breeding season. Because of the lack of research on ERK downstream in this study, it can be speculated that there may be a certain correlation between the seasonal expression of ERK1/2 and seasonal reproduction, which may provide basic data for the study of the relationship between the colon and seasonal reproduction. The specific regulatory mechanism needs further research.

Conclusion

The expression of ERK1/2 and pERK1/2 in the colons of the wild ground squirrels had seasonal changes, which had significant increases in the non-breeding season comparing to those in the breeding season. ERK1/2 was expressed in the cytoplasm of GC and EC, while pERK1/2 was also expressed in the nucleus of both types cells.

Declarations

Funding (information that explains whether and by whom the research was supported)

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Conflicts of interest/Competing interests (include appropriate disclosures)

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and material (data transparency)

All data generated or analyzed during this study are included in this published article.

Code availability (software application or custom code)

Not applicable.

Authors' contributions

Yue Song: Participated in sample collection, Performing the experiments, Assisted with all experiments, Analyzing the data, Drafting the manuscript.

Xiaoying Yang: Participated in sample collection, Performing the experiments, Assisted with all experiments, Analyzing the data.

Xueying Zhang: Participated in sample collection, Performing the experiments.

Jueyu Zhu: Participated in sample collection, Performing the experiments.

Yixin Chen: Participated in sample collection.

Fuli Gao: Assisted with all experiments, Helped revising the manuscript.

Haolin Zhang: Assisted with all experiments, Helped revising the manuscript.

Yingying Han: Helped revising the manuscript.

Qiang Weng: Designed, Supervised the study, Revised manuscript.

Zhengrong Yuan*: Designed, Supervised the study, Revised manuscript.

Ethics approval (include appropriate approvals or waivers)

All animal procedures were approved by the Policy on the Care and Use of Animals by the Ethical Committee, Beijing Forestry University and the Department of Agriculture of Hebei Province, China (JNZF11/2007).

Consent to participate (include appropriate statements)

Not applicable.

Consent for publication (include appropriate statements)

Submission of an article implies that the work described has not been published previously, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

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Figures

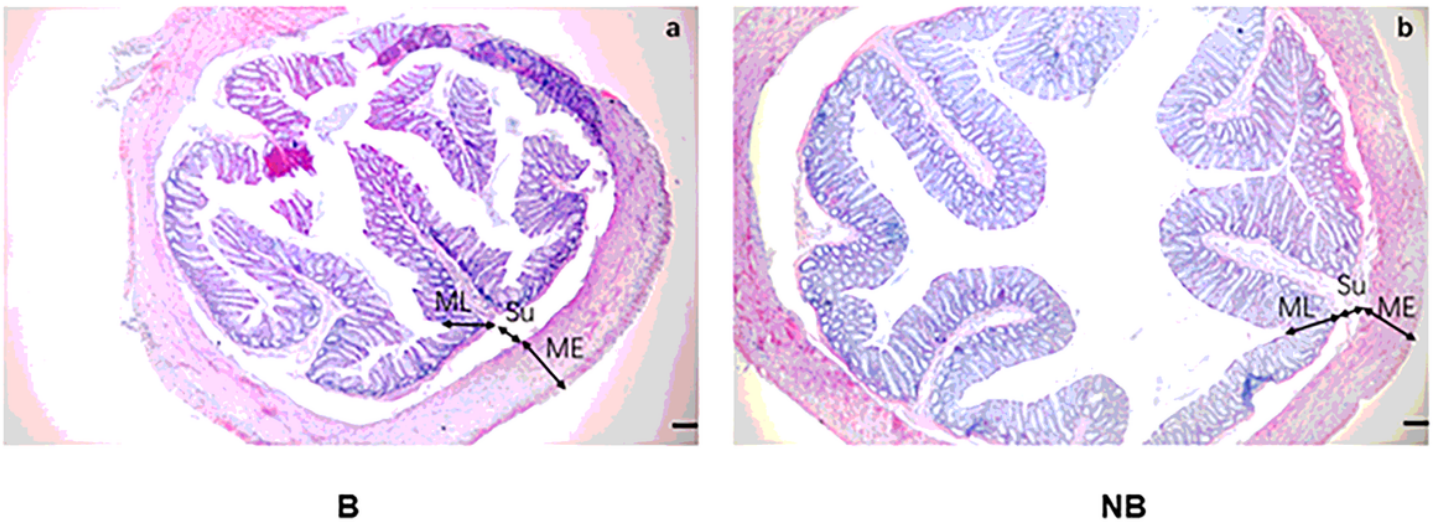

Figure 1

Histological structure of the colon by hematoxylin-eosin (HE). Histological observations of the colons in the breeding season (a) and the non-breeding season (b). Scale bars =100 μm. Abbreviations: B, the breeding season; NB, the non-breeding season; ME, muscular elements; ML, mucous layer; Su, submucosa

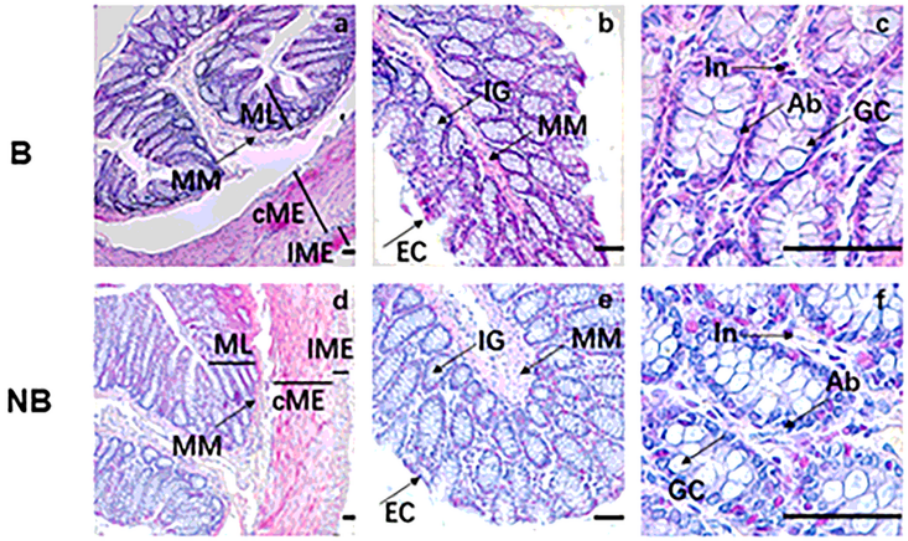

Figure 2

Detailed histological structure of the colon by hematoxylin-eosin (HE). Histological observations of the colons in the breeding season (a-c) and the non-breeding season (d-f). Scale bars = 50 μm. Abbreviations: B, the breeding season; NB, the non-breeding season; Ab, absorptive cell; cME, circular muscular elements; EC, epithelial cell; GC, goblet cell; In, interstitial cell; lME, longitudinal muscular elements; IG, intestinal glandML, mucous layer; MM, muscularis mucosa

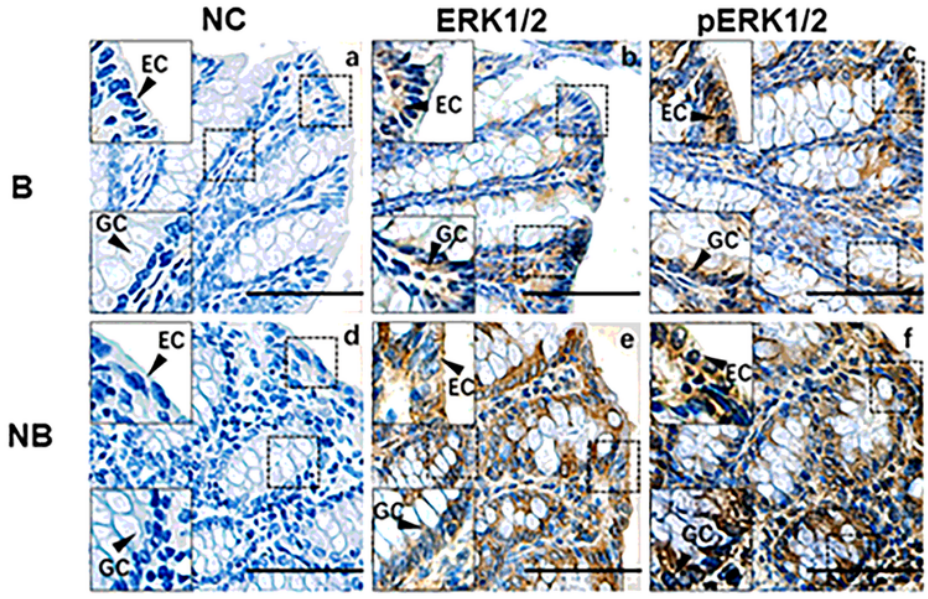

Figure 3

Seasonal immunolocalization of ERK1/2 and pERK1/2 in the colons of the wild ground squirrels between the breeding and non-breeding seasons. Immunolocalization of ERK1/2 in the colons (b-c). Immunolocalization of pERK1/2 in the colons (e-f). Negative control (a, d). The first row (a-c) represents staining in the breeding season. The second row (d-f) represents staining in the non-breeding season. Scale bars represent 50 μm (a-f). Abbreviations: B, the breeding season; NB, the non-breeding season; NC, Negative control; ERK1/2, Extracellular signal-regulated protein kinase1 and 2; pERK1/2, Phospho-extracellular signal-regulated protein kinase1 and 2; EC, epithelial cell; GC, goblet cell

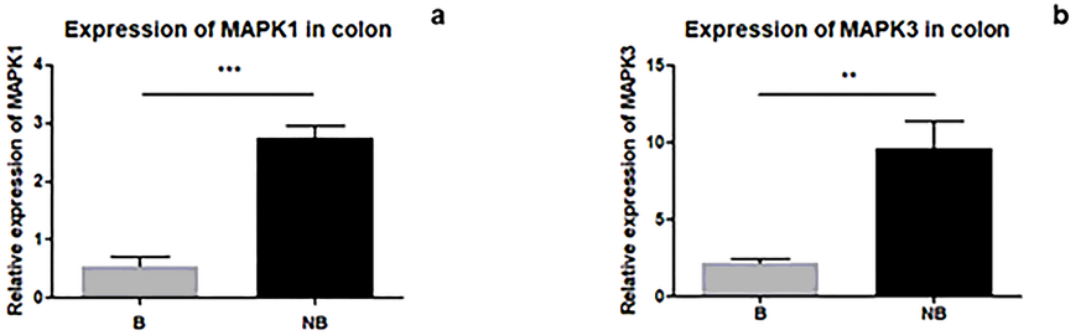

Figure 4

Expression levels of MAPK1 (a) and MAPK3 (b)

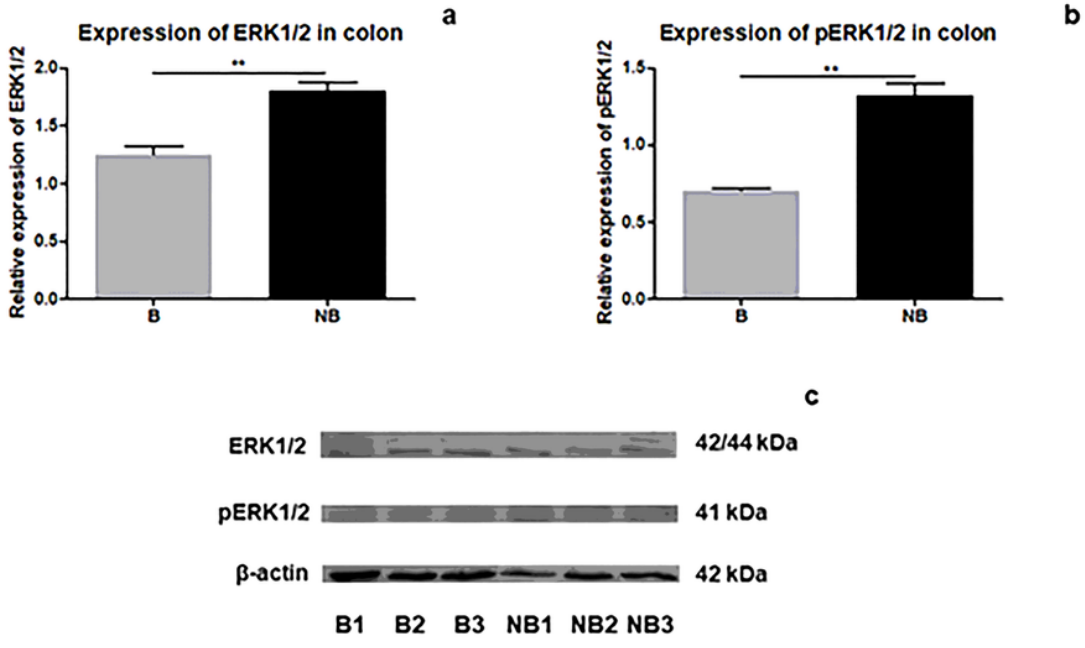

Figure 5

Expression levels of ERK1/2 (a) and pERK1/2 (b)

Supplementary Files

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