Seasonal expression of extracellular signal regulated kinases in the colon of wild ground squirrels (Spermophilus dauricus)

Yue Song¹ · Xiaoying Yang¹ · Xueying Zhang¹ · Jueyu Zhu¹ · Yixin Chen² · Fuli Gao¹ · Haolin Zhang¹ · Yingying Han¹ · Qiang Weng¹ · Zhengrong Yuan¹

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Abstract

Background The purpose of the experiment was to explore the localization and seasonal expression of extracellular signal regulated kinase (ERK) in the colonic tissue of wild ground squirrels (Spermophilus dauricus).

Methods and results Hematoxylin–eosin staining, immunohistochemistry, real-time quantitative PCR and Western blotting were used in this experiment. The histological results showed that the diameter of the colon lumen enlarged and the number of glandular cells increased in the non-breeding season. It was found in the immunohistochemical results that both ERK1/2 and pERK1/2 were expressed in the cytoplasm of goblet cells and intestinal epithelial cells, while pERK1/2 was also expressed in the nucleus of them. The immune localization of both was more obvious in the non-breeding season, especially in intestinal epithelial cells. Real-time quantitative PCR and Western blotting showed that ERK1/2 and pERK1/2 were seasonally highly expressed in the non-breeding season.

Conclusions The expression of ERK1/2 and pERK1/2 was seasonal changes and had significant increases in the non-breeding season. This study revealed that ERK1/2 had potential roles in the colon to the adaptation of seasonal changes in wild ground squirrels.

Keywords Extracellular signal regulated kinase · Colon · Wild ground squirrel · Seasonal adaptation

Background

Extracellular signal regulated kinases (ERKs) are important members of the Mitogen activated protein kinases (MAPKs) family. ERK1 and ERK2 are the first reported MAPKs [1]. 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, etc. [2]. In addition to these functions, ERK1/2 is also key enzyme in the development of the immune system, nervous system, memory formation, and heart development [3–5].

ERK stimulating factors bind to receptors to activate the Ras pathway and then interact with downstream kinase Raf. Activated Raf binds and phosphorylates MAPK. Activated MAPK 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 Paneth cells reduced, and the differentiation of goblet cells increased. The results suggested that ERK signaling pathway may be involved in determining intestinal process of cell differentiation in Paneth 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 typical seasonal reproduction, which made it a good material for studying seasonal reproduction [14]. Usually their breeding season is in April and May, and their hibernation begins in October [15, 16]. Up to date, there is no related study between the expression of ERK1/2 in the colon and wild ground squirrels. Researchers have observed the seasonal changes in colon of wild ground squirrels. This study focused on the expression of ERK1/2 in the colon of the wild ground squirrels to explore the relationship between colonic ERK1/2 expression and seasonal adaptation.

Materials and methods

Animals

Wild ground squirrels were captured in Hebei Province, China. The treatment of animals was followed by the AVMA (American Veterinary Medical Association) Guidelines for the Euthanasia of Animals: 2020 Edition. 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. Colonic tissues were extracted. Half of the colons were fixed with 4% paraformaldehyde for 48 h and stored in 70% alcohol. The other half samples were preserved under −80 °C after treatment.

Antibodies

The antibodies used in this experiment were all derived from Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China. Antibodies used are listed below: rabbit polyclonal anti-ERK1/2 (BS-2637R), rabbit polyclonal anti-pERK1/2 (BS-3016R) and β-actin (BS-0061R). The dilution ratio of rabbit polyclonal antibodies was 1:200 for immunohistochemistry and 1:750 for Western blotting. The dilution of goat anti-rabbit IgG and β-actin were 1:1000 for Western blotting.

Histology

The colonic tissues were embedded in paraffin wax after dehydrated in ethanol series. The tissues were cut into 6 μm continuous sections using microtome and the sections were placed on adhesive slides (Sigma-Aldrich, USA). The tissues were stained with hematoxylin–eosin (Merck, Tokyo, Japan).

Immunohistochemistry

The colon tissue sections were dewaxed and incubated in citric acid buffer at high temperature, cooled to room temperature and blocked with goat serum. The sections were incubated with primary antibody at 4 °C for 12 h, further incubated with SP kit (Rabbit) (SP-0023, Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China). 3, 3’-Diaminobenzidine Tetrahydrochloride (DAB) (Wako, Tokyo, Japan) and H2O2 solution were then used for color rendering. Sections were stained again with hematoxylin solution (Merck, Tokyo, Japan).

Real-time quantitative PCR

Colonic tissues were dissociated in TRizol® Reagent (Invitrogen, USA). Chloroform were added into the homogenate, the mixture was shaken vigorously and was centrifuged at low temperature. Isopropanol was added into the aqueous phase and stood at room temperature for 10 min, and centrifuged at low temperature and high speed. RNA was washed by 70% ethanol and dissolved in RNAase-free ddH2O. The concentration and quality of RNA were analyzed by spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific, USA). The mRNA was diluted to 250 ng/mL. cDNA was synthesized with RNA and random primers in PCR apparatus (Promega Corporation, USA). Primer sequences of mRNA Real-time quantitative PCR (qPCR) were shown in Table 1. PCR conditions were: 95 °C 10 min, 95 °C 30 s, 60°C 30 s, 72 °C 30 s, repeating for 40 cycles and at last
melting at 60°C by ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, USA).

**Western blotting**

Protein extraction from colon tissues was carried out on ice to prevent protein degradation. The colonic tissues were cut into small pieces and dissolved with 1 mL radioimmunoprecipitation analysis (RIPA) lysis buffer containing 10 μL phenylmethylsulfonyl fluoride (PMSF). Homogeneous samples and the protein extract was mixed with 4 × Laemmli sample buffer in proportion, boiled at high temperature and cryopreserved. 15% SDS–polyacrylamide gels (SDS-PAGE) was applied to transfer nitrocellulose membrane at 18 V/cm. The nitrocellulose membrane was cut in sections as required and blocked with 2% milk for 1 h, then incubated overnight with primary antibodies at low temperature. Then they were incubated with secondary antibodies for 1 h, washed three times in TTBS (Tris-Buffered Saline with Tween 20) buffer, stained by DAB and H2O2 solution.

**Statistical analysis**

The students' T-test by GraphPad Prism 5.0 (GraphPad Software Inc., USA) was used to analysis the experimental data.

**Results**

**Histological changes of the colons**

Histological results of colonic tissues of wild ground squirrels were shown in Fig. 1, and details were shown in Fig. 2. The comparison between Fig. 1a and b showed that the diameter of the colon lumen enlarged and the number of goblet cells (GC) increased in the non-breeding season.

**Immunohistochemical results of ERK1/2 and pERK1/2**

The results of immunolocalization for ERK1/2 and pERK1/2 in the colon showed respectively in Fig. 3. Both ERK1/2 (Fig. 3b, e) and pERK1/2 (Fig. 3c, f) were expressed in the cytoplasm of GC and epithelial cells (EC), while pERK1/2 was also expressed in the nucleus of them. The immune localization of both was more obvious in the non-breeding season. There was no signal shown in the negative controls (Fig. 3a, d).

**Seasonal expression of ERK1/2 and pERK1/2**

The results of mRNA and Western blotting showed respectively in Figs. 4 and 5. The MAPK1/3 expression (mRNA, Fig. 4) and the ERK/pERK expression (protein, Fig. 5) were seasonally highly expressed in the non-breeding season.

### Table 1 The primers sequence used for mRNA qPCR

| Gene name | Sequence of primer | Product size (bp) |
|-----------|--------------------|-------------------|
| MAPK1     | F:5'TGGTTGCCCTCCTCTGAA3'  |
|           | R:5'TGGGCAAATGCACACACCT3' | 142              |
| MAPK3     | F:5'ACTACCTTGGACAGCTCAAC3'  |
|           | R:5'GCTTTGGGGTGGAAGGTATG5' | 305              |
| β-actin   | F:5'GACTCGTCTACCTCTGCTT3'  |
|           | R:5'AAGACCTCTATGCCAAACCC3' | 223              |
Fig. 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. B, the breeding season; NB, the non-breeding season; Ab, absorptive cell; cME, circular muscular is externa; EC, epithelial cell; GC, goblet cell; In, interstitial cell; lME, longitudinal muscular is externa; IG, intestinal gland; ML, mucous layer; MM, muscularis mucosa.

Fig. 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, e). Immunolocalization of pERK1/2 in the colons (c, 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). B, the breeding season; NB, the non-breeding season; NC, Negative control; ERK1/2, Extracellular signal-regulated protein kinase 1 and 2; pERK1/2, Phospho-extracellular signal-regulated protein kinase 1 and 2; EC, epithelial cell; GC, goblet cell.

Fig. 4 Expression levels of MAPK1 (a) and MAPK3 (b)
Discussion

The localization and seasonal expression of ERK/pERK in the colonic tissue of wild ground squirrels were explored, using hematoxylin–eosin staining, immunohistochemistry, real-time quantitative PCR and Western blotting. The histological results showed that the diameter of the colon lumen enlarged and the number of GC increased in the non-breeding season. On the one hand, 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 has enlarged. On the other hand, ERK pathway can down-regulate the expression of tumor suppressor genes related to cell cycle, up-regulate the expression of c-Myc, reduce cell G0 phase, accelerate G1/S phase transformation to promote cell proliferation [17]. Using immunohistochemistry, quantitative real-time PCR and Western blotting, 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 blotting showed that the expression levels of ERK1/2 and pERK1/2 in the non-breeding season were significantly higher.

ERK1/2 can regulate a variety of cell processes and phylogeny. Researches on the colon ERK1/2 signaling pathway have focused on colon cancer. The high expression of pERK1/2 in intestinal EC is a marker of early colon cancer [18]. 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 [19]. Verticillin A used the ERK pathway to overcome drug resistance in colon cancer cells [20]. PKCα activated RAS upstream of RAF, MEK and ERK, which induced the arrest of the cell cycle in intestinal cells [21]. 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 [22]. 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 cell proliferation and apoptosis [23].

Previous morphological observations showed that the diameter of colons of wild ground squirrels enlarged in the non-breeding season. This kind of change was the seasonal adaptation 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 adaptation 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 adaptation. Reproductive system generally produces seasonal changes in the process of seasonal reproduction. The weight of reproductive organs in the breeding season is higher, 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 [24]. As mentioned above, ERK1/2 can regulate cell progression, and regulate 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 adaptation.

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 [25]. 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. Studies showed that the regulation of the ERK signaling pathway by miRNAs is essential in the development of cancer [26]. 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 is significant in the formation of osteoblasts and bone metabolism [27].

This experiment found that the lumen of wild ground squirrel colons had seasonal changes between the breeding season and non-breeding season. Because of the lack of researches on ERK downstream, it can be only speculated that the seasonal expression of ERK1/2 may be related to 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 was seasonal changes of wild ground squirrels, and had significant increases in the non-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 of cells.

**Authors’ contributions** YS, XY: Participated in sample collection, Performing the experiments, Assisted with all experiments, Analyzing the data, Drafting the manuscript. XZ, JZ: Participated in sample collection. Performing the experiments. YC: Participated in sample collection. FG, HZ: Assisted with all experiments, Helped revising the manuscript. YH: Helped revising the manuscript. QW, ZY*: Designed, Supervised the study, Revised manuscript.

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

**Code availability** Not applicable.

**Declarations**

**Conflict of interest** 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.

**Ethical approval** 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** Not applicable.

**Consent for publication** 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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