

# Tissue distribution and developmental changes of PTEN in the immune organs of chicken and effect of IBDV infection on it

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**ABSTRACT** Phosphatase and tensin homolog (*PTEN*), a tumor suppressor gene, functions in antiviral innate immunity and regulates the development and function of T cells and B cells. However, limited information about *PTEN* is available in poultry. In the present study, quantitative real-time polymerase chain reaction and immunohistochemistry staining were used to study the tissue distribution and developmental changes of *PTEN* in the main immune organs of chicken. The effects of infectious bursal disease virus (IBDV) infection on *PTEN* mRNA expression in the bursa of Fabricius (BF) of chickens were also investigated. The results are as follows. 1) The order of *PTEN* mRNA expression levels at the 18th d of hatching (E18) was: muscle and immune organs (spleen and thymus) > visceral organs (heart, lung, kidney, and liver) > hypothalamus and digestive tracts (duodenum, jejunum, ileum, cecum, proventriculus, BF [originates from cloaca], and cecum tonsil [locates at the lamina propria of cecum]). However, at the 15th d of raising (D15), the *PTEN* mRNA expression in the heart was the highest among all the tissues, followed by those in the liver, proventriculus, and kidney. The *PTEN* mRNA expression levels in the rest

tissues were very low and were only 1.20 to 19.47% as much as that in the heart ( $P < 0.05$ ). 2) The changes in the expression of *PTEN* mRNA in the BF, spleen, and thymus from E15 to D15 had no obvious regularity. *PTEN*-immunopositive (*PTEN-ip*) cells in the BF were distributed in epithelium mucosa, bursal follicles and interfollicles before hatching, but only in bursal follicles after hatching. *PTEN-ip* cells in the spleen were expressed in the periarterial lymphatic sheath from E18 to D15. Most of *PTEN-ip* cells distributed in the thymic medulla and only a few distributed in the thymic cortex during the whole experiment. 3) Chicken with IBDV infection had a remarkable decrease in *PTEN* mRNA expression from 1 d postinfection (dpi) to 7 dpi. Although *PTEN* mRNA level was reversed at 7 dpi, it was still significantly lower than that at 0 dpi ( $P < 0.05$ ). These findings suggest that the *PTEN* of chicken might play important roles in the development of embryos and T/B lymphocytes, and the downregulation of *PTEN* in chickens infected with IBDV might be a mechanism of IBDV evasion from host immunity. Strategies designed to restore *PTEN* expression may be a therapy for preventing chickens from IBDV infection.

**Key words:** *PTEN*, tissue distribution, developmental change, IBDV, chicken

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## INTRODUCTION

Phosphatase and tensin homolog (*PTEN*) located on chromosome 10q23 was first identified as a tumor suppressor gene by two groups independently in 1997 (Li and Sun, 1997; Steck et al., 1997). *PTEN* as a tumor suppressor is a dual-specificity phosphatase, which has

lipid phosphatase and protein phosphatase activity (Myers et al., 1998; Yin and Shen, 2008). On the one hand, *PTEN* can dephosphorylate phosphatidylinositol (3,4,5)-trisphosphate (**PIP3**) and create phosphatidylinositol (4,5) bi-phosphate (**PIP2**) to further inhibit the phosphatidylinositide 3-kinase (**PI3K**)/protein kinase B (**AKT**) signaling pathway and prevent **PIP3** membrane recruitment and stimulation of **AKT** (Maehama and Dixon, 1998). On the other hand, *PTEN* plays its protein phosphatase activity to inhibit the **PI3K** signaling pathway through the dephosphorylation of phosphopeptides at tyrosine, serine, and threonine sites (Leslie and Downes, 2002). **PI3K** signaling pathway plays important roles in regulating cell metabolism,

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## MATERIALS AND METHODS

### Experimental Animals and Sample Collection

survival, proliferation, apoptosis, growth, and migration (Worby and Dixon, 2014). Thus, mutated, deleted, or methylated PTEN results in different cancers, including human brain, breast, and prostate cancer (Li et al., 1997; Podsypanina et al., 1999). Furthermore, PTEN is essential for cells, tissue, and embryo development. PTEN mutation in mice causes embryonic lethality (Suzuki et al., 1998), and PTEN loss in myogenic progenitors leads to postnatal skeletal muscle hypertrophy but age-dependent exhaustion of satellite cells (Yue et al., 2016). Moreover, PTEN plays critical roles in immunological activities. PTEN regulates the development and function of T cells and B cells (Suzuki et al., 2003; Buckler et al., 2006; Shojaee et al., 2016). PTEN also has antiviral function in hepatitis C virus (HCV) core protein-transfected HepG2 cell model and HCV-infected Huh-7.5 cell model (Zhang et al., 2014; Wu et al., 2017). Li et al. (2016) reported that PTEN functions in antiviral immunity by regulating the import of interferon regulatory factor 3 (IRF3) into the nucleus and inducing type I interferon (IFN) production. Pereiro et al. (2020) demonstrated that PTEN responds against viral infections in zebrafish by regulating the expression of numerous immune genes.

The totality of chicken *PTEN* cDNA, which was cloned and translated into a putative protein, has more than 95% sequence identity with that characterized in mammals (humans and mice) (Vaudin et al., 2006). However, limited information about chicken PTEN has been reported. How *PTEN* mRNA expression changes during the development of chicken and whether *PTEN* expression is affected when chickens are infected with virus have not been reported yet.

Infectious bursal disease virus (IBDV) is a member of family *Birnaviridae*. The virus targets IgM-bearing B-lymphocytes in the bursa of Fabricius (BF) and results in severe immunosuppression due to the loss of lymphocytes in the BF (Tsukamoto et al., 1995; Petkov et al., 2009). IBDV-induced immunosuppression predisposes chickens to secondary infections by bacteria, viruses, and parasites and results in increased mortality, growth retardation, and condemnation (Berg, 2000; Geerligts et al., 2015; Lee et al., 2015). Therefore, IBD causes serious problems for the poultry industry worldwide because of direct and indirect economic loss. Although vaccines are effective, the emergence of variant IBDV strains can escape from the protection of vaccines (Geerligts et al., 2015). Therefore, finding out new genes in chicken to improve the host disease resistance is urgent.

In the present study, immunohistochemistry staining and quantitative real-time polymerase chain reaction (qRT-PCR) were used to detect the tissue distribution of PTEN and its developmental changes in the immune organs of chicken. An IBDV-infected chicken model was used to detect the effect of IBDV on *PTEN* expression. We hope that this study will provide a basis for further research on the role of PTEN in chicken infected with IBDV.

Sixty fertile HyLine brown eggs were purchased from Datian hatchery (Xinxiang City, Henan Province, China) and incubated in an intelligent automatic incubator (Dezhou Ruike Incubation Equipment Factory, Shandong Province, China) at  $37.5 \pm 0.2^\circ\text{C}$  and  $55\% \pm 2\%$  relative humidity. Six embryos or chickens were randomly collected at the 15th, 18th, and 20th d of hatching (E15, E18, and E20, respectively) and at the 1st, 5th, 10th, and 15th d of raising (D1, D5, D10, and D15, respectively). They were euthanized under deep Nembutal anesthesia ( $45 \mu\text{g/g}$  body weight [BW], intraperitoneal injection; Shanghai Chemical Factory, Shanghai, China). Then, 16 different tissues, namely, the heart, skin, muscle, cecum, lung, jejunum, duodenum, ileum, hypothalamus, proventriculus, kidney, liver, BF, cecum tonsil, thymus, and spleen, were collected. One part of each BF, spleen, and thymus was fixed in 4% paraformaldehyde for immunohistochemistry staining. The other part of each BF, spleen, thymus and all the other tissues were frozen in liquid nitrogen and then transferred into  $-80^\circ\text{C}$  storage for mRNA isolation.

Ninety newborn specific pathogen-free (SPF) HyLine brown laying chickens from Hualan Biological Engineering, Inc. (Xinxiang, Henan Province, China) were maintained in negative-pressure isolators (GJ-1, Suzhou Fengshi Laboratory Animal Equipment Co., Ltd., Suzhou, China) at SPF animal laboratory. The feed for SPF chicken was purchased from Merial Vital Laboratory Animal Technology Co., Ltd. (Beijing, China), and sterile water was given ad libitum. At the 19th d of raising, the chickens were weighed, randomly divided into two groups ( $n = 21 \times 2$  pens), and kept in different isolators in different rooms. One group of chickens were inoculated with the virulent IBDV strain BC6/85 (China Institute of Veterinary Drug Control, Beijing, China) at a dose containing  $10^{6.23}$  EID<sub>50</sub>/0.1 mL each through ocular–nasal route. Chickens in the other group were mock treated with 0.1 mL of sterile PBS (pH 7.38) each. Three chickens were randomly taken from each pen of each group at 0, 1, 2, 3, 4, 5, and 7 d postinfection (dpi) and euthanized by cervical dislocation under deep Nembutal anesthesia ( $45 \mu\text{g/g}$  BW, intraperitoneal injection; Shanghai Chemical Factory, Shanghai, China). The BF was quickly collected from each chicken, frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  for total RNA isolation. All animal experiments in the study were approved by the scientific ethical committee of Henan Institute of Science and Technology.

### Quantification of PTEN

Total RNA was extracted from different tissues with TRIzol reagent (Life Technologies, Carlsbad, CA), and cDNA was synthesized by PrimeScript RT reagent kits with gDNA eraser (TakaRa, Dalian, China) as previously

described (Yu et al., 2016). The primers (*PTEN*: gptenF, 5'-CTGAAAGACATTATGACACCGCC-3'; gptenR, 5'-CCAGTTCGTCCTTTCCAGC-3'; *Actin*: gacnF, 5'-TGTGCGTGACATCAAGGAGAAG-3'; gacnR, 5'-TACCACAGGACTCCATACCCAAG-3') were designed based on the complete nucleotide sequences of *Gallus gallus PTEN* (XM\_0152787701), and *Actin* (NM\_205518). The actin gene was used for internal standardization. The efficiency of *PTEN* and *Actin* were tested by standard curve. QRT-PCR was performed with SYBR Premix Ex TaqII (Takara, RR820A, Dalian, China) according to the manufacturer's instructions. Each quantification was repeated in triplicate. After PCR, the data were analyzed using the QuantStudio 5 (Applied Biosystems, Thermo Fisher Scientific, Marsiling, Singapore) with the  $2^{-\Delta\Delta C_t}$  method.

### Immunohistochemistry Staining

The deparaffinized tissue sections were performed to antigen retrieval in 0.01% Sodium Citrate buffer at 95 to 98°C for 15 min and were blocked with 3% peroxide-methanol at room temperature for 10min for endogenous peroxidase ablation. After that, the tissue sections were incubated with 5% normal goat serum at room temperature for 20 min, and then incubated overnight at 4°C with a mouse monoclonal PTEN antibody (1:150, Santa Cruz Biotechnology, Inc., SC-393186, Santa Cruz, CA). Then the tissue sections were rinsed in 0.01 M PBS (pH 7.4) and incubated with horseradish peroxidase-conjugated goat antimouse IgG (1:200, ZSGB-BIO, ZB-2305, Beijing, China) for 2 h at room temperature. The tissues were washed, and immunoreactivity was visualized using the DAB chromogenic reagent kit (ZSGB-BIO, ZLI-9018, Beijing, China) and hematoxylin restaining. The sections were mounted after the final rinse. The specificities of the immunostaining were verified by omitting the primary antibody from incubation.

### Statistical Analysis

The qRT-PCR results were analyzed by multiple comparison (least significant difference based on PROC ANOVA) using the SAS software (version 8.01; SAS Institute, Cary, NC) to measure the differences.  $P < 0.05$  was considered statistically significant.

## RESULTS

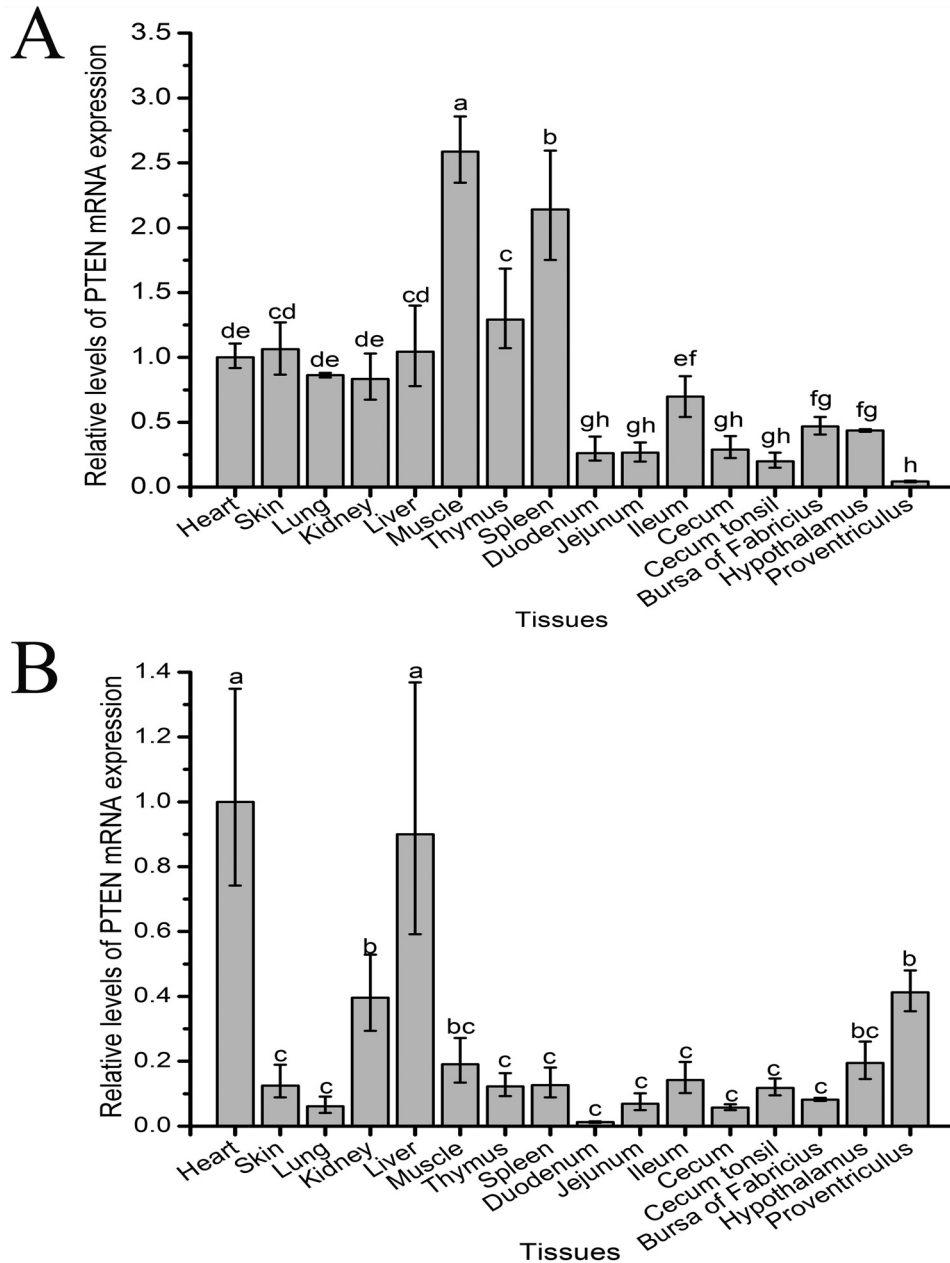
### Tissue Distribution of PTEN

The distributions of *PTEN* mRNA in various chicken tissues at E18 and D15 were investigated by qRT-PCR. The results are shown in Figure 1. The relative expression level of *PTEN* mRNA at E18 was the highest in the muscle, followed by those in the spleen and thymus. They were approximately 2.59-, 2.14-, and 1.29-fold as much as that in the heart ( $P < 0.05$ ). Low *PTEN* mRNA expression was distributed in the

hypothalamus and in various digestive tracts, such as the duodenum, jejunum, ileum, cecum, proventriculus, BF (originates from the cloaca), and cecum tonsil (locates at the lamina propria of cecum). The expression levels in these organs were 15.74 to 66.32% lower than that in the heart ( $P < 0.05$ ). The mRNA expression levels of *PTEN* in the skin and other visceral organs, such as the lung, kidney, and liver, were similar as that in the heart ( $P > 0.05$ ). Therefore, the order of *PTEN* mRNA expression levels at E18 was: muscle and immune organs > visceral organs > digestive tracts. However, at D15, the *PTEN* mRNA expression in the heart was the highest among all the tissues detected, followed by those in the liver, proventriculus, and kidney, which were 89.99%, 41.23%, and 39.63% as much as that in the heart. The *PTEN* mRNA expression levels in the rest tissues were very low and were only 1.20 to 19.47% as much as that in the heart ( $P < 0.05$ ).

### Distribution and Location of PTEN-Immunopositive (PTEN-ip) Cells in BF, Spleen, and Thymus

Immunohistochemistry staining was used to investigate the distribution and location of PTEN-ip cells in BF, spleen and thymus. The results showed that no PTEN-ip cells were detected on the sections of negative control (Figure 2A; Figure 3A and Figure 4A). In BF, PTEN-ip cells were mainly distributed in the epithelium mucosa and the interfollicle, while a few PTEN-ip cells were distributed in the bursal follicles at E15 at the low magnification of microscope. At the high magnification of microscope, immune positive PTEN was mainly expressed in the nucleus of columnar cells in the epithelium mucosa, macrophages, reticular cells and endothelium in the interfollicle. Only a few lymphocytes showed PTEN immune positive reaction in the bursal follicles (Figure 2B). At E18, E20 and D1, immune positive PTEN were expressed not only in the nucleus of columnar cells in the epithelium mucosa, but also most of lymphocytes in bursal follicles and macrophages, reticular cells and endothelium in the interfollicle (Figure 2C–E). While immune positive PTEN were expressed mainly in the macrophages, reticular cells, lymphocytes in bursal follicles at D5, D10, and D15. Nearly no PTEN-ip cells were detected in the epithelium mucosa and in the interfollicle of BF (Figure 2F–H). In spleen, the immune positive PTEN was expressed in splenic epithelial cells, macrophages, and a few lymphocytes at E15 (Figure 3B). At E18, E20, D1, D5, D10, and D15, PTEN-ip cells were distributed in periarterial lymphatic sheath at the low magnification of microscope. At the high magnification of microscope, immune positive PTEN were found to express mainly in the interdigitating cells, endothelium, and some of the lymphocytes in periarterial lymphatic sheath (Figure 3C–H). In the thymus, during the whole experiment time, most of PTEN-ip cells were distributed in the thymic medulla and only a few were distributed in the thymic cortex at the low magnification of



**Figure 1.** Expression changes in *PTEN* mRNA in various tissues of chicken embryo or chicken. (A) E18; (B) D15. The transcriptional level in the heart was normalized to 1, and the transcription levels are expressed as ratios with the transcription level in the heart. Differences were considered significant at  $P < 0.05$ . The expression levels with the same letter represents have no significant difference, whereas those with different letters have significant difference.

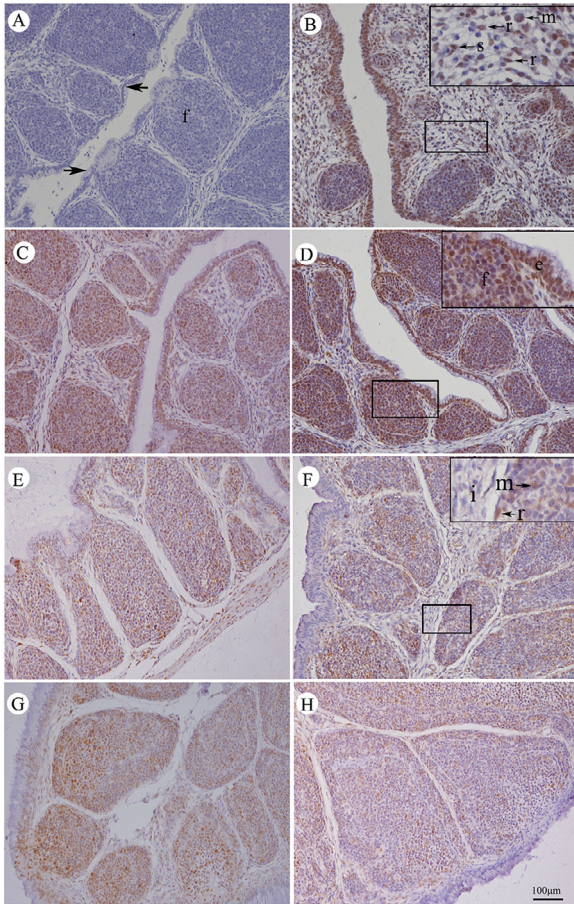
microscope. At the high magnification of microscope, immune-positive PTEN was expressed mainly in thymic epithelial cells and macrophages, and nearly no PTEN was located in thymocytes in the thymic cortex. While PTEN was expressed in thymic epithelial cells, macrophages and T lymphocytes in thymic medulla (Figure 4).

### Expression Changes of *PTEN* mRNA in BF, Spleen, and Thymus

The relative expression of *PTEN* mRNA in the BF, spleen, and thymus was investigated, and the level of *PTEN* mRNA expression at E15 was termed as "1" in these three organs. In the BF, around 3.02-, 3.11-, and

1.3-fold increase in *PTEN* mRNA expression were observed at E18, E20, and D1, respectively, and the expression levels at E18 and E20 were substantially different compared with that at E15. The level of *PTEN* mRNA expression at D5 increased abruptly and was 7.4-fold as high as that at E15, thus, the expression level at this time point was the highest during the whole experiment. However, the *PTEN* mRNA expression levels decreased substantially at D10 and D15 compared with that at D5 but were slightly higher than that at E15 (Figure 5A). The *PTEN* mRNA expression in the spleen changed slightly from E15 to D15. Interestingly, the *PTEN* mRNA expression decreased quickly at D10 and increased abruptly at D15. The expression levels at D10 and D15 were 0.37- and 1.75-fold as high as that at



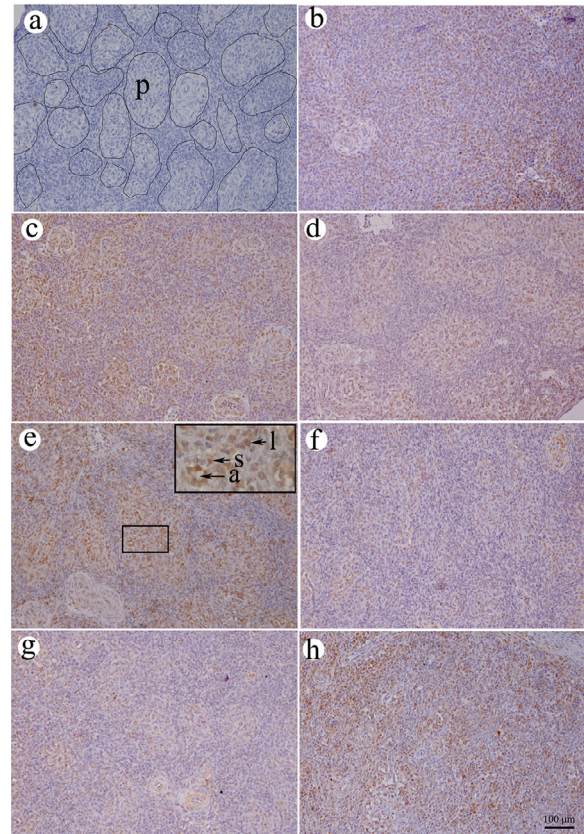


**Figure 2.** Expression of PTEN-ip cells in the BF at different ages. (A) Negative control; (B) at E15; (C) at E18; (D) at E20; (E) at D1; (F) at D5; (G) at D10; (H) at D15. Abbreviations: e, epithelial cells; f, bursal follicle; i, interfollicle; m, macrophage; r, reticular cell; s, endothelial cell.

E15, respectively ( $P < 0.05$ ; Figure 5B). The *PTEN* mRNA level in the thymus had no obvious change at E18 compared with that at E15, but it increased remarkably before and after the hatching of chicken embryos and reached the highest level at D1, which was about 3.80-fold as high as that at E15 ( $P < 0.05$ ). The *PTEN* mRNA level at D5 decreased to the level of E15 and then climbed quickly from D10 to D15. They were 2.12- and 2.77-fold as high as that at E15 ( $P < 0.05$ ; Figure 5C).

### Effect of IBDV Infection on Chicken *PTEN*

The bursa of chickens was collected at 1, 2, 3, 4, 5, and 7 dpi, and their *PTEN* mRNA expression levels were determined to further investigate the effect of IBDV on *PTEN* expression levels in chicken. The results showed that IBDV infection led to a significant decrease in *PTEN* expression at 1 to 5 dpi, which was only 13.5 to 34.3% as high as that at 0 dpi ( $P < 0.05$ ). Although *PTEN* expression was reversed at 7 dpi, it was still significantly lower than that at 0 dpi ( $P < 0.05$ ; Figure 6).

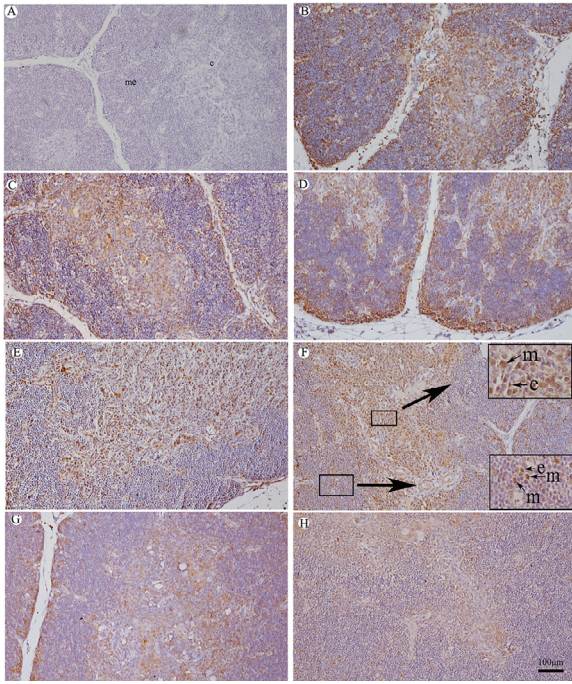


**Figure 3.** Expression of PTEN-ip cells in the spleen at different ages. (A) Negative control; (B) at E15; (C) at E18; (D) at E20; (E) at D1; (F) at D5; (G) at D10; (H) at D15. Abbreviations: a, central artery; l, lymphocyte; p, periarterial lymphatic sheath; s, splenic interdigitating cells.

## DISCUSSION

*PTEN* was first identified as a tumor suppressor gene with an important role in cell metabolism, cell proliferation, apoptosis, and cell migration (Tamura et al., 1998; Leslie and Foti, 2011; Ciuffreda et al., 2014). Therefore, its role in development has attracted more attention. However, its tissue distribution in chicken embryo or chicken has not been well reported. In the present study, *PTEN* mRNA expression levels at E18 were in the order: muscle and immune organs (spleen and thymus) > visceral organs (heart, lung, kidney, and liver) > hypothalamus and digestive tract (duodenum, jejunum, ileum, cecum, proventriculus, BF, and cecum tonsil). However, at D15, *PTEN* was expressed the highest in the heart among all the tissues detected. Presumably, this distribution is related with the developmental extent of different organs during the different stage of chicken development. During the late stage of chicken embryos, muscle and immune organs begin to develop quickly to prepare for hatching, whereas the yolk digestive system maintains the capabilities of absorption necessary for sustaining life in the shell. After hatching, the growth rate of the heart is more variable than other organs during the first 2 wk, peaks at about 5 to 6 d posthatching, and then plateau to a rate similar to body growth at about 10 d (Phelps et al., 1987). Therefore, *PTEN* mRNA expression levels at E18 and D15 may be



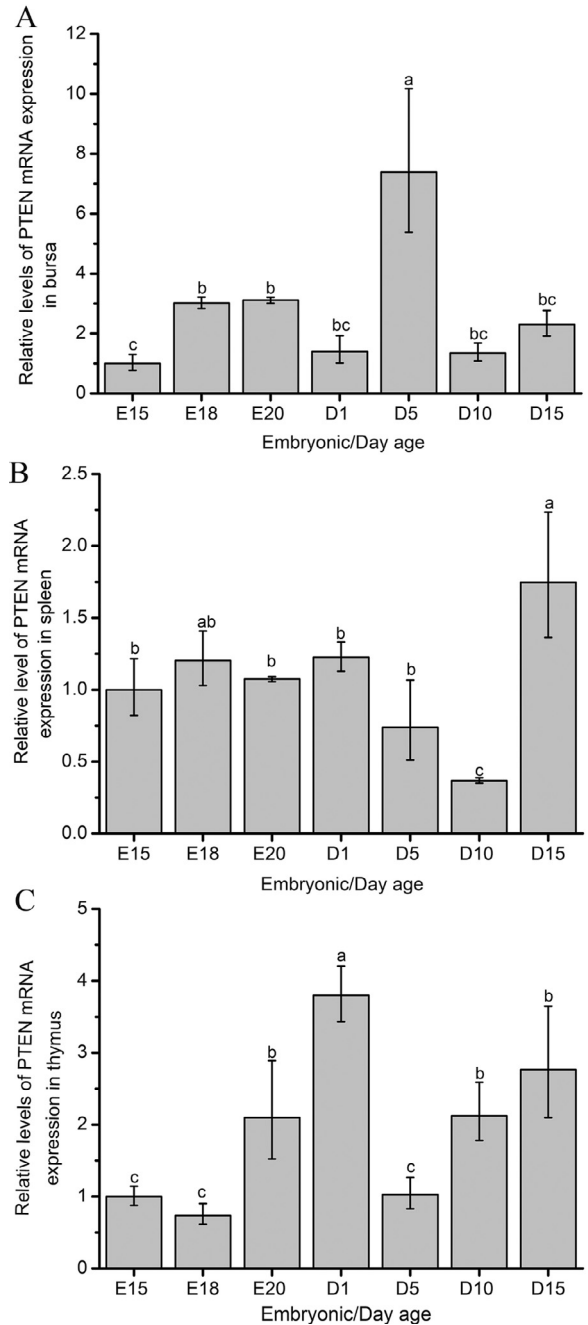


**Figure 4.** Expression of PTEN-ip cells in the thymus at different ages. (A) Negative control; (B) at E15; (C) at E18; (D) at E20; (E) at D1; (F) at D5; (G) at D10; (H) at D15. Abbreviations: c, thymic cortex; e, thymic epithelial cell; m, macrophage; me, thymic medulla.

consistent with the developmental extent of different tissue organs, which suggested that chicken PTEN has the function of regulating embryos development.

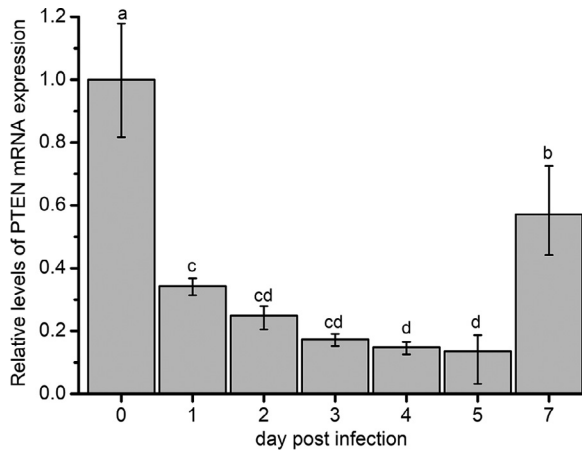
PTEN plays an important role in T-cell activation, T-cell immune tolerance, and the survival and proliferation of developing cells in the thymus (Suzuki et al., 2001; Hagenbeek et al., 2004; Buckler et al., 2006). PTEN also exerts effects on the proliferation, activation, and survival of B cells through negatively regulating AKT signaling pathways. It also participates in suppressing negative selection and promotes the oncogenic transformation of pre-B cells (Suzuki et al., 2003; Shojaee et al., 2016). In the present study, immune positive PTEN were mainly expressed in the reticular cells, thymic epithelial cells, splenic epithelial cells, macrophages in BF, thymus, and spleen. They were all necessary for T/B lymphocytes development. The results further suggested that chicken PTEN plays an important role in T/B lymphocytes development. However, the changes in *PTEN* mRNA expression have no regularity. Presumably, it was related with that PTEN expressed in more different types of cells.

PTEN, as a tumor suppression factor, governs myriad cellular processes and serves a central role in tumor suppression (Li et al., 1997; Steck et al., 1997; Myers et al., 1998). PTEN also functions in antiviral innate immunity. Li et al. (2016) reported that PTEN plays a pivotal role in the induction of type I IFN by controlling the import of IRF3, a master transcription factor responsible for IFN- $\beta$  production, into the nucleus. In zebrafish, PTEN is involved in the regulation of the expression of numerous immune genes and plays differential roles in immunity and response against viral infections (Pereiro et al., 2020). In the present study, the *PTEN*



**Figure 5.** Expression changes in *PTEN* mRNA in immune organs at different ages. (A) Bursa, (B) Spleen, (C) Thymus. The transcription levels are expressed as ratios with the transcription level at E15. Differences were considered significant at  $P < 0.05$ . The expression levels with the same letter have no significant difference, whereas those with different letters have significant difference.

mRNA level in the bursa was downregulated after chickens were infected with IBDV. This downregulation was also observed in the studies of Spring viremia of carp virus (SVCV)-infected zebrafish model and HCV core protein-transfected HepG2 cell model (Zhang et al., 2014; Pereiro et al., 2020). Subsequently, the antiviral function of PTEN in SVCV-infected zebrafish models and HCV-infected Huh-7.5 cell model was confirmed through PTEN overexpression (Wu et al., 2017; Pereiro et al., 2020). Therefore, the downregulation of *PTEN* in chicken infected with IBDV might be a mechanism of IBDV evasion from host immunity. However,



**Figure 6.** Expression changes in *PTEN* mRNA in the BF of chickens infected with IBDV. Differences were considered significant at  $P < 0.05$ . The expression levels with the same letter represents have no significant difference, whereas those with different letters have significant difference.

how IBDV utilizes PTEN to evade host immunity needs further investigation. Clearing this mechanism will benefit the design of strategies to restore the expression of PTEN, which is promising as a therapies therapy for the protection of chickens from IBDV infection.

In conclusion, this study further demonstrated the chicken PTEN was essential for the development of chicken and T/B lymphocytes through exploring the expression levels of *PTEN* mRNA in different tissues at E18 and D15 and location of immune positive PTEN in different immune organs during the development of chicken. Meanwhile, this study also demonstrated that chicken with IBDV infection led to the down-regulation of *PTEN*, which will promise as a therapies therapy for the protection of chickens from IBDV infection.

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## DISCLOSURES

All authors declare no conflict of interest.

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