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Expression of Nerve Growth Factor (NGF) and Its Receptors TrkA and p75 in the Reproductive Organs of Laying Hens

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ABSTRACT

In order to investigate the expression levels of nerve growth factor (NGF) and its receptors (TrkA and p75) in prehierarchical follicles and oviducts of hens, five 130-day-old laying hens were examined by immunohistochemistry and RT-PCR analysis. NGF and its receptors were expressed in theca cells and granulosa cells of prehierarchical follicles, and they were also expressed in the epithelial cells of oviducts. The expression of the genes NGF, TrkA and p75 were significantly different in prehierarchical follicles ($p<0.05$ or $p<0.01$), and NGF and TrkA gene expression was significantly different in different parts of oviduct ($p<0.05$ or $p<0.01$). The expression of NGF and p75 mRNA levels was highest in large white follicle (LWF), as well as the expression of TrkA in small yellow follicle (SYF). In the oviduct, the expression of NGF was the highest in infundibulum, and lowest in isthmus. These results suggest that NGF may play an important role in the regulation of hen reproduction.

INTRODUCTION

The nerve growth factor (NGF), a 26-kilodalton (kDa) polypeptide (Andrews, 1951), belongs to a family of related target-derived proteins (Levi-Montalcini, 1987). It is required for the survival, maintenance, and development of discrete neuronal populations in the central and the peripheral nervous systems (Snider, 1994). Also, it is believed that NGF not only has effects on the nervous system, but also plays a prominent role in a variety of non-neuronal systems (Leon *et al.*, 1994). NGF initiates its biological functions by binding two different membrane-spanning receptors. One is the high affinity receptor tyrosine kinase A (TrkA), a 140-kDa transmembrane tyrosine kinase receptor encoded by members of the trk proto-oncogene family (Barbacid, 1994; Meakin and Shooter, 1992). The other receptor with low affinity is known as p75, a 75-kDa glycoprotein with a short cytoplasmic sequence, which belongs to the family of tumor necrosis receptors (Dechant and Barde, 1997).

NGF and its receptors play an important regulatory role in follicular development, ovulation, ovarian hormone synthesis and early embryonic development. The reproductive system of females in avian species is unique, as it is able of completing follicular development, ovulation, and fertilization continuously. Due to this characteristic, chickens are widely used as experimental model in follicular developmental biology research.

The chicken ovary presents many prehierarchical follicles, which are categorized by follicle diameter size. Prehierarchical follicles consist of small white follicles (SWF, diameter less than 2 mm), large white follicles (LWF, 2-5 mm in diameter) and small yellow follicles (SYF,

6-10 mm in diameter). According to their volume, prehierarchical follicles are classified from F1 to F6 (Liu and Zhang, 2008; Onagbesan *et al*., 2009). In order to maintain the level of ovulation, one prehierarchical follicle will be recruited every day before entering the ovulation. SYF is significantly important in screening into dominant follicles (Onagbesan *et al*., 2009). To our knowledge, there is no report about whether NGF and its receptors can be expressed in prehierarchical follicles and oviducts of hens. The aim of this study was to identify a new marker for chicken breeding.

MATERIALS AND METHODS

Animals and experimental procedure

Five 130-day-old hens were purchased from QingLongShan Forest Farm (Nanjing, China), and fed a standard diet. All experimental protocols were performed according to the guidelines of regional Animal Ethics Committee. Two hours after laying eggs, hens were anesthetized with ethylic ether and sacrificed. Ovaries and oviducts were removed into sterilized saline. Follicles were then classified according to diameter, and oviducts were segmented into infundibulum, magnum and isthmus. Four follicles from each prehierarchical follicle (SWF, LWF, SYF) per hen and part of oviducts were immediately fixed in 4% paraformaldehyde for immunohistochemical analysis. The other parts of prehierarchical follicles and oviducts were stored in liquid nitrogen for RT-PCR and biochemical assay. As control group, five 83-day-old hens purchased from same farm were sacrificed and their ovaries and oviducts were collected and stored in liquid nitrogen for RT-PCR.

Immunohistochemistry

The prehierarchical follicles and oviduct sections fixed in 4% paraformaldehyde solution overnight were dehydrated in graded series of ethanol, cleared in xylene, and embedded in paraffin. Paraffin-embedded samples were serially sectioned (5μm thick), and mounted on 3-aminopropyl-triethoxysilane (APES)-coated slides. The sections were then deparaffinized with xylene and rehydrated in graded ethanol before being washed with twice-distilled water. To increase epitope exposure, the sections were heated for 10 min in sodium citrate buffer (0.01M, pH 6.0) in a microwave oven. After incubating in 3% H_2O_2 (v/v) in methanol at 32 ºC for 30 min, the sections were blocked with normal goat serum for 1h and then incubated overnight at 4 ºC with rabbit polyclonal antibodies specific for NGF (Santa Cruz Biotechnology, California, USA), diluted 1: 100 in the phosphate-buffered saline (PBS, 0.01 M, pH 7.2). Control sections were incubated with blocking serum alone. The specific protein immunoreactivity was visualized using a VECTASTAIN ABC Kit (Zhong Shan-Golden Bridge Biological Technology, Beijing, China) and a DAB kit (Sigma, San Francisco, USA). In order to identify structural components and cell morphology, the sections were counterstained with hematoxylin and mounted with coverslips. Immunostaining was evaluated in the digitalized images taken with an Olympus camera.

RNA extraction, reverse transcription (RT) and quantitative PCR

Total RNA was extracted from prehierarchical follicles and oviducts using a RNeasy Mini kit® (Qiagen, China), according to the manufacturer's procedure. The concentration and purity of the isolated total RNA was determined spectrophotometrically at 260 and 280 nm in a Nanodrop® 8000 (Thermo Fisher Scientific, Wilmington, USA). Total RNA (1 μg) was reversely transcribed to cDNA using an Omniscript® Reverse Transcription kit (Takara, China) with oligo-dT primers (Takara), according to the manufacturer's protocol.

Target genes and the housekeeping gene, beta actin (ACTB; Dori and Johnson, 2005), were quantified by real-time PCR using an ABI 7300 and a commercial kit (SYBR Premix Ex Taq, TaKaRa, Dalian, China). The gene-specific primers were designed based on chicken mRNA sequences using Primer Version 5.0 (Table 1). PCR reactions were run in triplicate in total volume of

Table 1 – Primers used for real-time PCR analysis

Genes and sequence reference	Primer sequence	Size of PCR Product (bp)	Annealing temperature (ºC)
NGF	F: 5'-ATGCCAGATGGAACAGAAGAT-3' R: 5'-CTGGCAGTCGTGTTGAGAGAG-3'	170	58.0
TrkA	F: 5'-GTCTTCGCCTCCCTCTTCCTCT-3' R: 5'-GTTGCTCTTCAGCCCGTCCAG-3'	192	60.0
p75	F: 5'-AACAACCGCCCTGTGAACCA-3' R: 5'-CTCAGCAGTTTCTCCACATCCTC-3'	200	58.0
β-actin	F: 5'-GCCAACAGAGAGAAGATGAC-3' R: 5'-CACAATTTCTCTCTCGGCTG-3'	288	58.0

20 µL (consisting of SYBR Premix Ex Taq, ROX Reference Dye, 200 nM primer, and 100 ng cDNA template). The conditions of amplification were as follows: DNA polymerase activation at 95 °C for 30 s, followed by 42 amplification cycles of denaturation at 95 °C for 5 s, annealing at 58 °C for 30 s, and extending at 72 °C for 30 s. The specificity of the PCR product was verified by a melting curve and by agarose gel electrophoresis. The relative concentration of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Ct values of the ovaries and oviducts of the 83-day-old hens were used as calibrators.

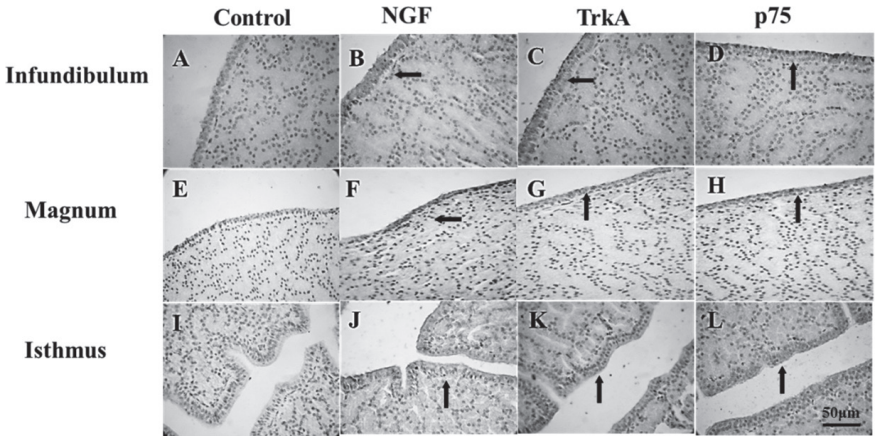

Figure 2 – Immunohistochemical localization of NGF (B, F and J), TrkA (C, G and K), and p75 (D, H and L) in the infundibulum (A-D), magnum (E-H) and isthmus (I-L) of the oviducts. A, E and I stained with normal rabbit serum (negative control). Arrows indicate epithelial cells. Bar = 50 µm. All sections were photographed using the same magnification. (200X)

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Data were analyzed by Kruskal-Wallis test, and means were compared by Dunn's multiple comparison test. Statistical analyses were performed using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant when the $p<0.05$.

RESULTS

Immunohistochemical analysis for NGF, TrkA and p75 in the prehierarchical follicles and oviducts

As shown in Fig. 1, NGF and its receptors were detected in thecal cells and granulosa cells of prehierarchical follicles by immunohistochemical staining. In the prehierarchical follicles, NGF and its receptors were detected in different classes of follicles including SWF (Fig. 1B, C, D), LWF (Fig. 1F, G, H) and SYF (Fig. 1J, K, L). The granulosa cells of SWF presented the strongest NGF staining. In the oviducts, positive staining was observed in the mucosal epithelial cells of infundibulum (Fig. 2B, C, D), magnum (Fig. 2F, G, H), and isthmus (Fig. 2J, K, L), but the reaction was weak.

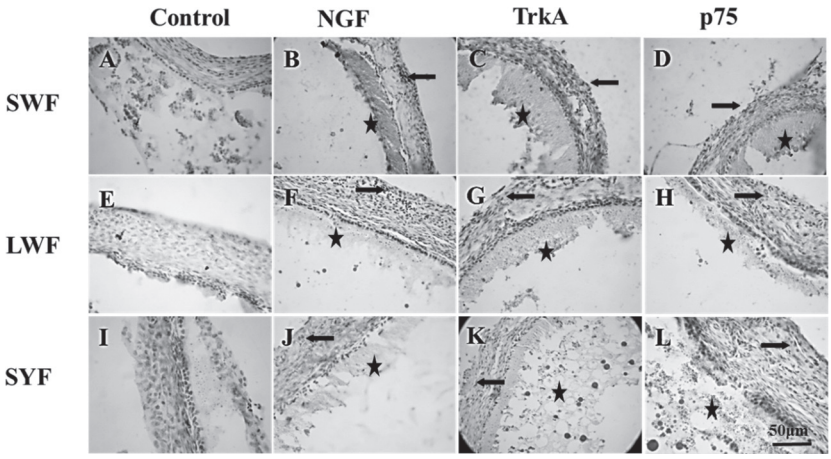

Figure1 – Immunohistochemical localization of NGF (B, F and J), TrkA (C, G and K), and p75 (D, H and L) in SWF (A-D), LWF (E-H), and SYF (I-L) prehierarchical follicles. A, E and I are stained with normal rabbit serum (negative control). Arrows indicate theca cell; stars, granulosa cells. Bar = 50 µm. All sections were photographed using the same magnification. (200X)

Real-time PCR analysis of NGF, TrkA and p75 mRNA experssion in prehierarchical follicles and oviducts

In order to determine whether expression levels of NGF, TrkA, and p75 mRNA were location-dependent, real-time PCR was performed on each kind of prehierarchical follicles (Fig. 3) and each part of oviducts (Fig. 4). NGF, TrkA and p75 gene expression was significantly different among prehierarchical follicles ($p<0.05$ or $p<0.01$), and NGF and TrkA gene expression was significantly different among different parts of oviduct ($p<0.05$ or $p<0.01$). NGF gene expression in LWF was significantly higher than in SWF ($p<0.05$), and p75 gene expression in LWF was significantly higher than in SYF ($p<0.05$). In the oviduct, NGF gene expression in the infundibulum was significantly higher than in the isthmus ($p<0.05$). TrkA mRNA in the infundibulum was reduced compared with the magnum and the isthmus ($p> 0.05$).

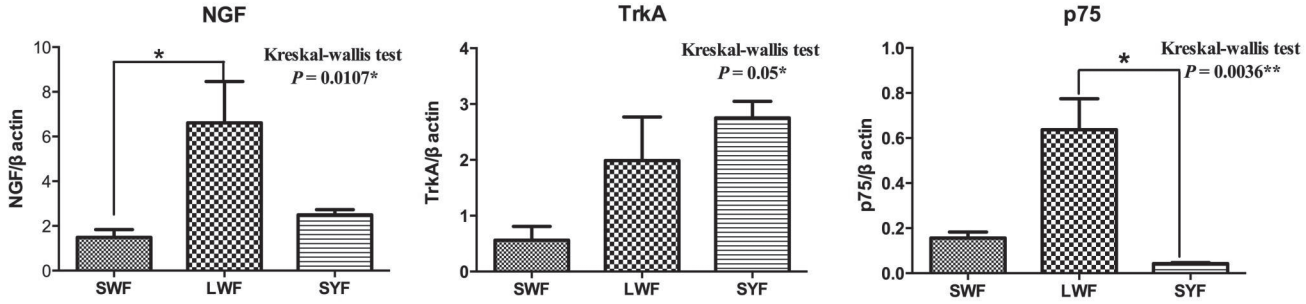

Figure 3 – Expression of NGF and its two receptors genes in each type of prehierarchical follicles. Each mRNA was normalized to β-actin mRNA expression level in the same preparation. Each column and vertical bar represents the mean and SEM (n=5). *Significantly different from the values at SWF (p<0.05, Kruskal-Wallis test, followed by Dunn's multiple comparison test), p<0.05 (*) and p<0.01 (**)

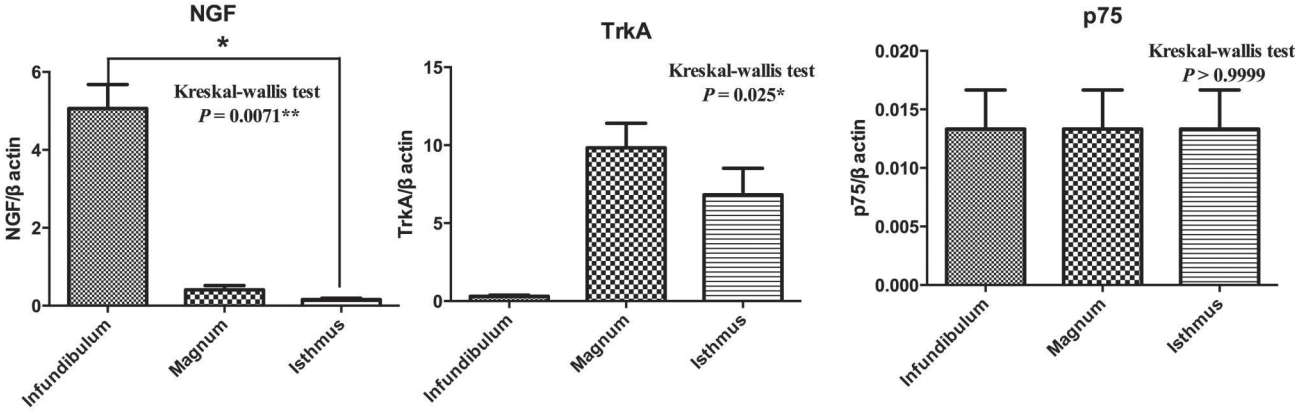

Figure 4 – Expression of NGF and its two receptors genes in different parts of oviducts. Each mRNA was normalized to β-actin mRNA expression level in the same preparation. Each column and vertical bar represents the mean and SEM (n=5). *Significantly different from the values at infundibulum, (p<0.05, Kruskal-Wallis test, followed by Dunn's multiple comparison test), p<0.05 (*) and p<0.01 (**).

DISCUSSION

Our study firstly shows that NGF and its two receptors TrkA and p75 are expressed in reproductive organs of laying hens, which suggests that NGF may be involved in the regulation of reproductive functions in chickens. The location of the expression of NGF and its receptors was also reported in cattle (Levanti *et al*., 2005), golden hamsters (Shi *et al*., 2004), and shiba goats (Ren *et al*., 2005). In the bovine ovary, NGF, TrkA and p75 mRNA are present in the theca cells. NGF and TrkA protein are also expressed in granulosa cells, where NGF and its receptors play an essential role in the ovulatory process (Dissen *et al*., 2000). Interestingly, NGF and TrkA were only found on the walls of preantral and antral follicles in women (Seifer *et al*., 2006). In rats, however, both proteins were present in granulosa layers (Dissen *et al*., 1996). In the present study, mRNA expression levels of NGF, TrkA and p75 were detected in prehierarchical follicles and oviducts. The results showed that *NGF*, as well as its receptor *TrkA* and *p75*, presented different expression levels in the theca and granulosa cells among SWF, LWF and SYF, which are in agreement with a report on porcine ovaries (Jana *et al*., 2011). These results suggest that the localization of NGF and its receptors TrkA and p75 were species-specific.

Progesterone, which is produced by ovarian granular cells, can be transformed into androgen, and androgen will be transformed into estrogen by aromatase in granular cells (Liu and Hsueh, 1986). The expression of follicle-stimulating hormone (FSH) and its receptor (FSHR) plays a significant role in the maintenance of the activity of prehierarchical follicles, as well as follicle recruitment (Jia *et al*., 2010; McElroy *et al*., 2004). FSH, which has a major role in immature follicle granulosa cells as well as small follicles, can promote the synthesis of progesterone and estrogen, respectively (McElroy *et al*., 2004). FSH also has a dose-response effect on granulosa cells and greatly stimulated the proliferation of granulosa cells (Jin *et al*., 2006; McElroy *et al*., 2004; Schmierer *et al*., 2003). Estrogen may induce granulosa cells to produce more FSHR and cooperate with FSH to enhance the expression of luteinizing

hormone (LH). LH, in turn, enhances the activity of aromatase, and promotes further synthesis of estrogen (Parrott and Skinner, 1998; Skinner and Coffey, 1988). The activity of aromatase in granulosa cells increases as follicles develop. As a result, the amount of estrogen increases and follicles grow faster. NGF may induce the expression of functional FSHR in newly-formed follicles in rat ovaries (Romero *et al*., 2002). Similar results were found in ovarian granulosa cells before ovulation in humans (Seifer *et al*., 2006). Our results show that NGF and its receptors TrkA and p75 were expressed in thecal and granulosa cells of prehierarchical follicles of 130-day-old hens. Taken together, these findings suggest that NGF may act on thecal and granulosa cells of prehierarchical follicles, and influence the levels of estrogen and progesterone production. Consequently, it may increase the expression of FSHR in the granulosa cells, and ultimately cooperate with FSH to promote follicle maturation and with LH to promote ovulation.

During the follicular development of laying hens, a single follicle is selected from the pool of small yellow follicles per day into the preovulatory hierarchy to begin rapid growth and finally differentiation (Jia *et al*., 2010). Our results showed that the expression of TrkA mRNA in SYF was significantly higher than in other prehierarchical follicles, but p75 mRNA presented lower expression. This may due to the fact that TrkA is a high affinity receptor and p75 is a low affinity receptor. The expression of NGF mRNA was higher in LWF and SYF than in SWF. Our results suggest that NGF and its receptors may participate in the recruitment and selection of dominant follicles.

During lay, oviducts are longer and more winding, and walls are thicker compared with immature hens. The oviduct epithelial cells are composed of ciliated cells and secretory cells. Each part of oviduct has its special function. During the daily laying sequence, a new ovum is generally ovulated at about 15–75 min post-oviposition. The ovum is captured by the infundibulum, and after approximately 15 min, the ovum moves through the infundibulum and enters the magnum. It takes 3-4 h for an ovum to pass through the magnum, where the albumen proteins are secreted, and 1–2 h to move down the white isthmus, where the bilayered membranes are built around the "egg" (Liu and Li, 2013). Tubal liquid is a complex mixture composed of plasma and secreting protein (Verhage *et al*., 1988; Willis *et al*., 1994). The oviduct secretes many growth factors, such as epidermal growth factor (EGF), transformation growth factor (TGF), insulin-like growth factor-1 (IGF-1), and activin (Sahlin *et al*., 2001), indicating that both autocrine and paracrine embryo growth factors are present in the oviduct. In the present study, immune reactions to NGF and its two receptors were detected in epithelial cells of infundibulum, magnum and isthmus. Ren *et al*. (2005) provided evidences that the immunoreactivity to NGF, TrkA, and p75 was confined to muscle cells and epithelial cells in the ampulla and the isthmus of the oviduct. The present study is the first to report the expression of NGF and its receptors in the oviduct of laying hens, indicating that NGF may be necessarily involved in the oviduct function.

In conclusion, our study demonstrated by immunohistochemistry that NGF and its receptors, TrkA and p75, are present in prehierarchical follicles and oviducts of laying hens. These results suggest that NGF may play an important role in the regulation of reproduction of chickens.

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