Asian-Aust. J. Anim. Sci.
Vol. 24, No. 3 : 336 - 343
March 2011

www.ajas.info

Expression of Neurotrophin 4 and Its Receptor Tyrosine Kinase B in Reproductive Tissues during the Follicular and Luteal Phases in Cows*

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ABSTRACT : The neurotrophins, required for the survival and differentiation of the nervous system, are known to be important for the development of the reproductive tissues. However, the signals initiating the growth of follicles, gamete development, and transport and the development of zygote in the reproductive system of cows remain ambiguous. The purpose of the present study was to identify the transcripts and proteins of Neurotrophin 4 (*NT4*) and its receptor tyrosine kinase B (*TrkB*) in bovine reproductive tissues. The transcripts and immunoreactivity of *NT4* and *TrkB* proteins were detected by reverse transcription polymerase chain reaction and western blot analysis. Using immunohistochemistry, the specific immunoreactivity of *NT4* and *TrkB* were detected in the oocytes of primordial follicles and in the growing primary follicles. The *NT4* and *TrkB* immunoreactivity was predominantly observed in granulosa cells, cumulus granulosa cells, cumulus oocyte complexes, theca cells of mature follicles, as well as in the oviduct epithelial cells, uterine gland cell, and epithelium cells of the uterus during the follicular and luteal phases in cows. Expressions of *NT4* and *TrkB* mRNAs were not significantly different among the ovary, oviduct, and uterus of the follicular phase. For the luteal phase, the expression of *NT4* mRNA in the ovary was significantly higher than that in the oviduct and uterus, and the expression of *TrkB* mRNA in the oviduct was significantly higher than that in the ovary and uterus, as determined by fluorescence quantitative reverse transcription polymerase chain reaction. The expression of *NT4* mRNA was significantly higher than that of *TrkB* mRNA in the ovary and uterus, whereas *NT4* mRNA expression was lower than that of *TrkB* mRNA in the oviduct during the luteal phase. The present study hypothesizes that *NT4* participates in the regulation of both gonads and extra-gonadal reproductive tissues in cows. (**Key Words :** Cows, Neurotrophin 4, Tyrosine Kinase B, Follicular Phase, Luteal Phase, Expression)

INTRODUCTION

Neurotrophins (NTs) are small, homodimeric polypeptide growth factors that regulate the survival, maintenance, and differentiation of neurons in the nervous system (Ibáñez, 1995; Anderson et al., 2002; Paredes et al., 2004). They also act on non-neural cells, such as those in the reproductive system (Spears et al., 2003; Levanti et al., 2005; Kawamura et al., 2007). They include the nerve growth factor (*NGF*), brain-derived neurotrophic factor (*BDNF*), and neurotrophins 3 and 4/5 (*NT3* and *NT4/5*), which function through two different types of receptors, namely, tyrosine kinase (Trk) (i.e., *TrkA* for *NGF*, *TrkB* for *BDNF* and *NT4*, and *TrkC* for *NT3*) and p75 receptors. When the NTs bind to their receptors, they activate a specific Trk domain, resulting in a rapid increase in the phosphorylation of second messengers and other specific cellular components (Dissen et al., 1995; Fridman et al., 1999; Dissen et al., 2000).

The development of the mammalian ovary is initiated

* This work was supported by the National High Technology Research and Development Program ("863" Program) of China (No.2008AA101003), the National Science & Technology Pillar Program of China (No.2008BADB2B09), the Project for Science and Technology Development of Jilin Province, P.R. of China (No. 20070210), and Science and Technology Development Project of Changchun City (08GH08).

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Received July 7, 2010; Accepted December 31, 2010

after migration of the primordial germ cells, which proliferate during and after migration (Witschi, 1948; Byskov, 1986). However, the factors and pathways involved are largely unknown. In the past few years, many results show that neurotrophins and their receptors play a critical role in the development of the mammalian ovary, oogenesis, folliculogenesis, and embryonic development, and in *BDNF*, *NGF*, and *NT3* (via uterine tract immunolocalization) in rodents (Bjorling et al., 2002; Krizsan-Agbas et al., 2003; Shi et al., 2006), and in *NGF* in goats (Ren et al., 2005), identified at the mRNA or protein levels. Although much information on the reproduction of a mammal has been reported, there is no any study on the expression and distribution of *NT4* and its receptor *TrkB* in the reproductive tissues of cows during the follicular and luteal phases. To elucidate further the role of *NT4* in the female reproductive system and facilitate attempts to improve understanding of the mechanisms involved in infertility and embryo development, the presence of *NT4* and *TrkB* in the ovaries, oviducts, and uteri of adult cows during the follicular and luteal phases was investigated.

Here, the *NT4* and *TrkB* transcripts and proteins were detected in the ovary, oviduct and uterus during the two phases, by using immunohistochemistry, fluorescence quantitative real-time polymerase chain reaction (FQ-RT-PCR) and Western blot.

MATERIALS AND METHODS

Sample preparation

The ovaries, oviducts and uteri of adult native yellow cows were obtained from a public slaughterhouse. The samples were collected within 15 min after slaughter, and then kept at -196°C. Groups were classified according to the follicle size or the presence of the corpus luteum. The follicular phase was defined as the period when the ovary lacks the corpus luteum, and the largest follicle size was more than 5 mm in diameter, whereas and the luteal phase was defined as the period when the ovary already has a corpus luteum. For each phase, tissue samples were collected from five animals (n = 5). Tissue pieces were excised from the ovary, uterus, oviduct, and then fixed for 24 h in Bouin's fixative, and subsequently processed for routine paraffin embedding.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from the samples using Trizol reagent (Invitrogen Life Technologies Inc., USA) and cDNA was frozen until used. Specific primers for each gene are shown in Table 1. PCR was performed in reaction mixtures with a final volume of 25 μl containing 1 μl (100 ng) of cDNA, 2.5 μl of 10×Buffer, 0.2 μl of each primer, and 0.20 units of rTaq polymerase (TaKaRa Biotechnology, Otsu Shiga Japan). RNA samples incubated without reverse transcriptase served as negative controls. PCR products were separated in an agarose gel, eluted, and then sequenced using an ABI 377 DNA sequencer (Applied Biosystems, Foster, CA, USA), with M-13F/R primers (TransGen Biotech Co., Beijing, China).

Quantitative real-time PCR

Real-time PCR was performed using the ABI PRISM 7000 (ABI, America) according to the previously reported (Li et al., 2010). The primers and probes for RT-PCR are listed in Table 1. To normalize the amount of expressed *NT4* and *TrkB* mRNAs, the internal housekeeping gene *GAPDH* was used, and each cDNA product was tested in triplicate. A standard curve was generated and used to evaluate the relative expression of the *NT4* and *TrkB* genes in terms of the ratio (fold difference) of the target gene expression to the control gene expression.

Immunohistochemistry

The tissues were embedded in paraffin, and 5 μm-thick sections were cut. Slides were blocked with normal goat serum (10%) in PBS for 30 min at room temperature. They were then incubated with a primary antibody (*anti-NT4* or

Table 1. Details of primers and probes used for FQ-RT-PCR on bovine reproductive tissues

Gene type	Primer sequence 5′- 3′	Annealing temperature (°C)	Product length (bp)	Accession number
NT4	Forward :AGTCCTACGTGCGGGCATT Reverse: CACAGGCAGTGTCAATTCGAA TAQMAN-PI:FCACCGATGCCCAGGGCCGTP	59	78	XM_583142
TrkB	Forward : GGAAAGTAAAATCAAGACAAGGTGTT Reverse: CAATGTTATGTCGCTTGATGTGC TAQMAN-PI: ：CAATGATGATGACTCTGCCAGCCCA	59	246	NM_006180
GAPDH	Forward :GGCGCCAAGAGGGACAT Reverse: GGTGGTGCAGGAGGCATT TAQMAN-PI:FTACTTCTCGTGGTTCACGCCCATCACAP	59	120	NM_001034034

F = FAM, P = TAMRA.

TrkB) at 4°C for 24 h. Following several washes with PBS, the slides were exposed to goat anti-rabbit IgG, conjugated with fluorescein isothiocyanate (FITC, 1:200; BOSTER, China) or diaminobenzidine (DAB, 1:200; BOSTER, China) for 60 min at room temperature, and were then washed with PBS. Negative controls were prepared using bovine serum albumin (BSA) instead of primary antibody diluted with PBS. Finally, the slides were examined under a confocal laser microscope (Olympus, Japan) and optical microscope (Olympus, Japan). Sections of each sample were examined in triplicate for both positive antibody staining and negative controls.

Western blot analysis

Total proteins were extracted according to the manufacturer's instructions (APPLYGEN, China). Approximately 20 μg protein from each sample was separated by SDS-PAGE on a 12% polyacrylamide gel. The separated proteins were transferred onto PVDF membranes (Millipore) using an appropriate transfer system at 60 V for 2 h. Membranes were incubated with blocking solution (5% solution of nonfat dry milk in TBST) for 2 h at room temperature, and then with primary antibody (*NT4* or T*rkB*) at 4°C overnight. The membranes were washed with TBST thrice for 15 min, and then incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:50,000 in TBST) for 2 h at room temperature. The membranes were then washed several times until the proteins were detected using Super Signal substrate (Pierce) and exposure to x-ray film.

Statistical analysis

The experiments for RT-PCR, Western blot, and Immunohistochemistry were repeated at least four times, and data are expressed here as means±SEM. Statistical analyses were performed using one-way ANOVA (as implemented in SPSS 13.0. software), and Dunnet's test was applied for multiple comparison. A value of $p<0.05$ was taken to indicate a statistically significant difference between means. The number of all tissues and cDNA samples has been described in the materials and methods.

RESULTS

Expression of NT-4 and TrkB transcripts

The *NT4* and *TrkB* mRNA were detected in the ovary, oviduct, and uterus during the follicular and luteal phases, and specific fragments of predicted sizes, 78 bp (NT4) and 246 bp (TrkB-fl) were obtained from the tissues (Figure 1). The *NT4* and *TrkB-fl* genes were cloned by PMD-18T vector and detected by an automated DNA sequencer. Sequences of these products were found completely homologous to that of the corresponding region in *NT-4* (XM_583142) and *TrkB* (NM_006180). The absence of DNA was confirmed by the amplification of a single product from *GAPDH* with the expected size for RNA (120 bp) (Figure 1), as no staining was visible in the case of negative controls wherein the template was replaced by sterile water (Figure 1).

The FQ-RT-PCR results show no significant difference in the expressions between *NT4* mRNA and *TrkB* mRNA in the ovaries, oviducts, and uteri in the follicular phase (Figure 2C). However, the expression of *NT4* mRNA (Figure 2A) in the ovaries was predominantly higher than that in the oviducts and uteri, and the expression of *TrkB* mRNA (Figure 2B) in the oviducts was significantly higher than in the ovaries and uteri for the luteal phase ($p<0.05$). In addition, the expression of *NT4* mRNA was significantly higher than that of *TrkB* mRNA in the ovaries and uteri, whereas *NT4* expression was lower than *TrkB* in the

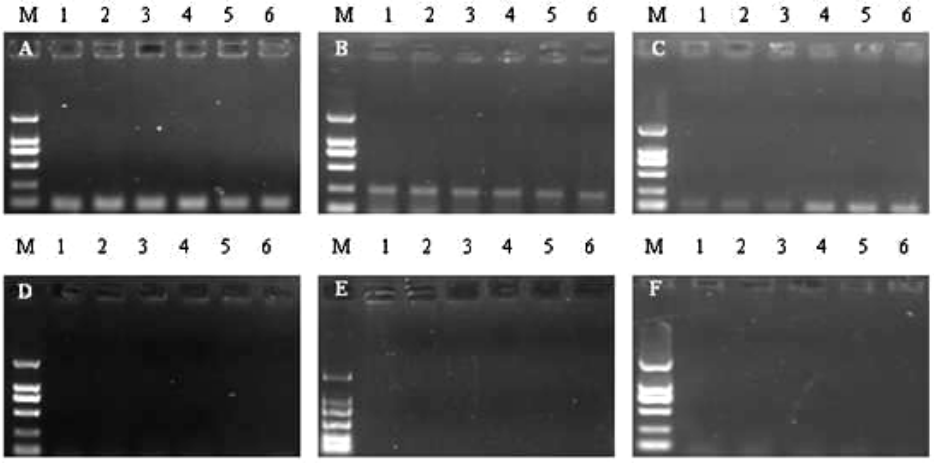

Figure 1. Results of RT-PCR analysis of *NT4*, *TrkB-fl* and *GAPDH* mRNA expression in bovine reproductive tissues. The products of *NT4* (A), *TrkB* (B), and *GAPDH* (C) were detected in the ovary (lane 1), oviduct (lane 2), and uterus (lane 3) during the luteal phase; and the ovary (lane 4), oviduct (lane 5) and uterus (lane 6) during the follicular phase. The negative controls, for which no bands were detected in *NT4* (D), *TrkB* (E), and *GAPDH* (F) (water as template).

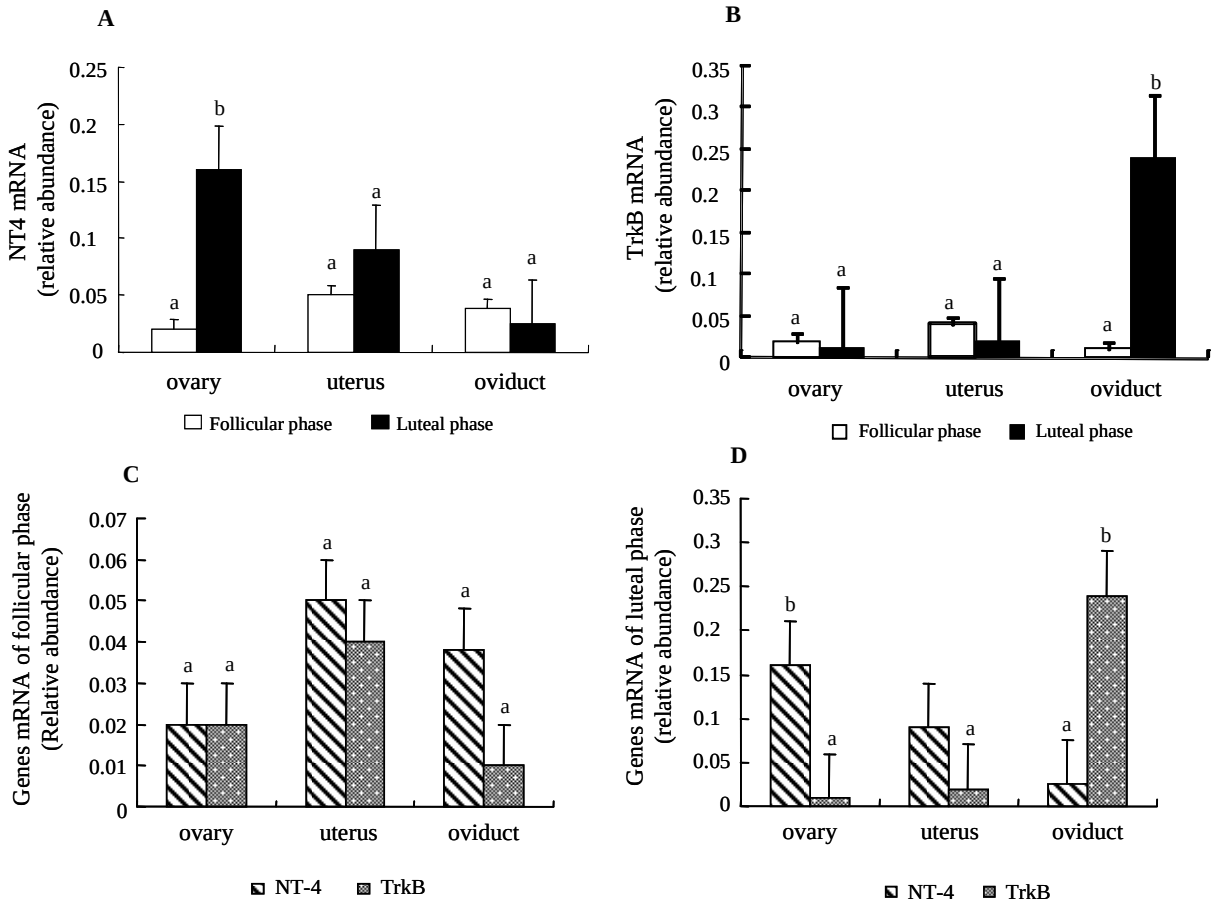

Figure 2. Relative abundance (arbitrary units) of *NT4* and *TrkB* mRNA in reproductive tissues of cows during the follicular (A) and luteal phases (B). Panels C-D show the *NT-4* and *TrkB* mRNA expression variation of the different reproductive tissues of cows during the follicular phase (C) or luteal phase (D). Different superscripts express significant differences (a and b) at $p<0.05$.

oviducts for the luteal phase (Figure 2D).

Immunolocalization of NT4 and TrkB in cow reproductive tissues

NT4 and *TrkB* immunoreactivity were observed in the specimens during both phases. *NT4* and *TrkB* are seen not only in the mature follicles of granulosa cells, cumulus granulosa cells, cumulus oocyte complexes, oocytes of primordial follicles, and growing-type primary follicles of the follicular phase (Figure 3), but also in the granulosa cells of mature follicles, single layer flat epithelials of primordial follicles, and vascular smooth muscle cells of the luteal phase (Figure 4). Their immunoreactivity were predominantly seen in the oviduct epithelium, and in the uterus mucosa epithelium cells and uterine gland at the follicular and luteal phases, respectively (Figures 3 and 4). The positive control was from the brain of adult cows. No staining was visible in the negative controls, in which primary antibodies were replaced by BSA (Figures 3 and 4).

Western blotting

The presence of *NT4* and *TrkB* proteins in the specimens in both phases in were investigated by Western blotting with rabbit anti-human *NT4* and *TrkB* polyclonal antibody. Specific bands corresponding to *NT4* and *TrkB* were detected in the extracts of the reproductive tissue proteins (Figure 5). No bands were visible in negative controls where the antibody was replaced by normal goat serum (Figure 5).

DISCUSSION

The Tyrosine kinase receptors and their ligands are present in the genital tract of several mammals (Yeh et al., 1993; Gupta et al., 1997; Krizsan-Agbas et al., 2003; Levanti et al., 2005; Ren et al., 2005; Shi et al., 2006; Buratini et al., 2007) and promote oocyte developmental competence, early embryonic development, and implantation (Kawamura et al., 2003; De Sousa et al., 2004; Kawamura et al., 2005; Betancourt-Alonso et al., 2006). *NT4* is a member of the neurotrophin protein family that plays important roles in the regulation of neuronal survival and differentiation mediated by *TrkB*. Increasing evidence has shown that neurotrophins may play a specific role in the

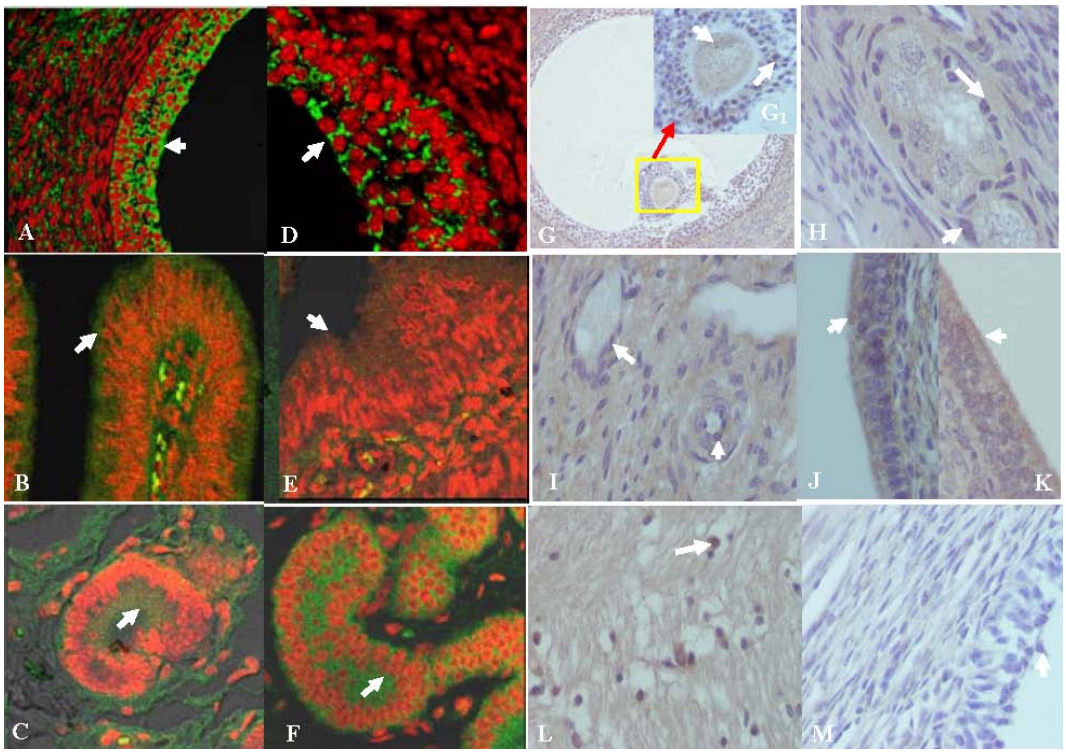

Figure 3. Detection of the *NT4* in the bovine ovary, oviduct and uterus during the follicular and luteal phases. *NT4* immunoreactivity is predominantly seen n mature follicles of granulosa cells (A), cumulus granulosa cells (G_1, long arrows), COCs (G_1, short arrows), oocytes of primordial follicles (H, short arrows), and growing-type primary follicles (H, large single arrowheads) during the follicular phase. *NT4* is detected in the granulosa cells of mature follicles (D), single-layer flat epithelial of primordial follicles (I, short arrows), and vascular smooth muscle cells (I, long arrows) during the luteal phase. *NT4* immunoreactivity is predominantly seen in oviduct epithelium during the follicular (B) and luteal phases (E), whereas it is in the uterus mucosa epithelium cells (J, C) and uterine gland (K, F) during the follicular and luteal phases, respectively. Positive control of bovine brain from adult cows (L) is also shown. Section incubated with *NT4* antibodies preadsorbed with the *NT4* peptide used to raise the antibodies (M). A-F: Original magnificationμ×40. G_1, H-M:Original magnification ×400, G: Original magnification×100.

development of reproductive tissues. Notably, *NGF* activation of *TrkA* in cultured thecal cells is reported to be involved in the disruption of gap junctions (Mayerhofer, 1996). Therefore, the possible communication between *NT4* and *TrkB* in the reproductive tissues of cows under the two phases was explored. In the present study, evidence for the presence of *NT4* and *TrkB* mRNAs and proteins in bovine ovary, oviduct, and uterus at the follicular and luteal phases, and immunological indications of their localization were obtained. In addition, *BDNF* and *TrkB* involves both autocrine and paracrine signaling within bovine oocyte-cumulus Cell Complex (COCs) (Martins et al., 2005), indicating that *NT4* and its *TrkB* receptor may have a role in the development of these cells, and that the distribution of the *TrkB* receptor may contribute to the regulation of *NT4* signaling in the immediate environment of germ and somatic cells in the bovine reproductive tract.

Oocytes are known to interact with somatic cells to form primordial follicles and survive (McLaren et al., 1991). In humans, *NT4* protein was localized to the granulosa cells by immunohistochemistry, and at the early developmental stages of epithelioid cells, the *TrkB* receptor was also localized by immunohistochemistry to the germ cells, as observed from all examined gestations in a previous study (Richard et al., 2002). Both *TrkA* and *TrkC* receptors express immunoreactivity in the bovine follicular cells (Muñoz et al., 2009), and *NGF* and *TrkA* proteins are expressed in granulosa cells, where *NGF* and its receptors play an essential role in the ovulation process (Dissen et al., 2000). The results are consistent with a previous study demonstrating granulosa cells as possible sources of *NGF* (Mattioli et al., 1999). *BDNF* plays a role in conferring oocyte cytoplasmic competence to support early embryo development, and that this may involve both autocrine and paracrine signaling within the COCs (Martins et al., 2005). This study shows that tissue specificity of *NT4* and its *TrkB* receptor was expressed in the ovary. Moreover, *NT4* and its receptor TrkB expression traits are similar to the results of other neurotrophins in the mammals, such as rats (Dissen et al., 1995), monkey (Shimizu et al., 2002), mice (Dissen et al., 2001), and cow (Dissen et al., 2000). Furthermore, *NT4* mRNA was lower in the ovary during the follicular phase

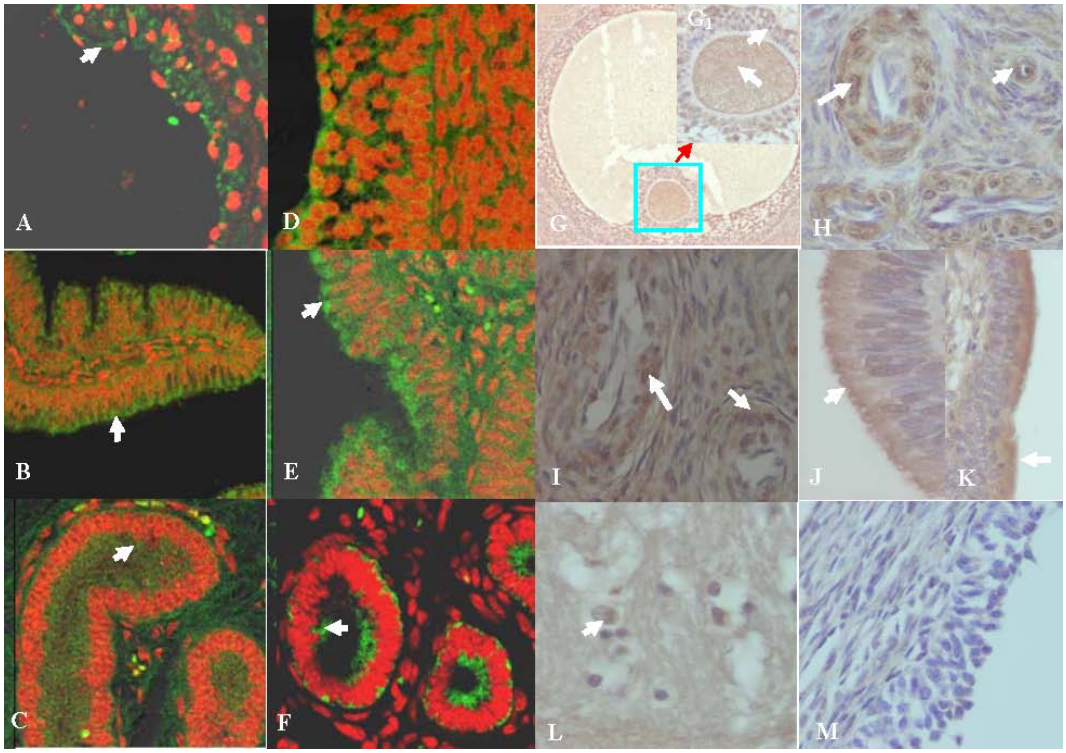

Figure 4. Detection of the *TrkB* in the bovine ovary, oviduct, and uterus during the two phases. *TrkB* immunoreactivity is seen in the granulosa cells of mature follicles (A), oocyte (G_1, long arrows), cumulus granulosa cells, COCs (G_1, short arrows), oocytes of primordial follicles (H, short arrows), and in growing-type primary follicles (H, long arrow) during the follicular phase. *TrkB* is detected in the granulosa cells of mature folli.cles (D), single layer flat epithelial of the primordial follicles (I, short arrows), and vascular smooth muscle cells (I, long arrows) during the luteal phase. *TrkB* immunoreactivity is predominantly seen in the oviduct epithelium during the follicular (B) and luteal phases (E). It is also found in the uterus mucosa epithelium cells (J, C) and uterine gland (K, F) during the follicular and luteal phases, respectively. Positive control of bovine brain from adult cows (L) is also shown. Section incubated with *TrkB* antibodies preadsorbed with the *TrkB* peptide used to raise the antibodies is shown in (M). A-F: Original magnification×40. G1, H-M: Original magnification ×400, G: Original magnification ×100.

than the luteal phase. The behavior of *TrkB* mRNA was contrary to that of *NT4*, by FQ-RT-PCR. BDNF is secreted by the granulosa and cumulus cells as an ovarian factor stimulated by the preovulatory LH surge, which enhances the first polar body extrusion of oocytes (Kawamura et al., 2005). Hence, *NT4* may play a different regulatory role in the follicular development and ovulation through autocrine and paracrine pathways in bovine ovarian development.

The oviduct can secrete many growth factors, such as EGF, TGF, IGF, and activin (Schell et al., 1994; Gandolfi et al., 1995). In this experiment, the immunoreactions for *NT4* and *TrkB* were also detected in the epithelium of the oviduct. This is the first report of the expression of *NT4* in the cow oviduct under the two phases. *BDNF* significantly increased the proportions of MII oocytes at both 10 ng/ml in *in virto* mature (Hong et al., 2009). In addition, *BDNF* plays a role in conferring oocyte cytoplasmic competence to support early embryo development, independently of nuclear maturation. This may involve both autocrine and paracrine signaling within the COCs (Martins et al., 2005). The

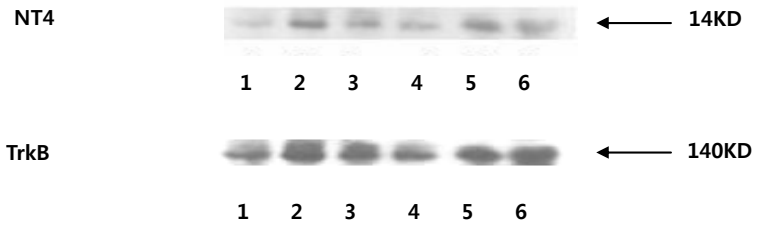

Figure 5. Western blot analysis of the *NT4* and *TrkB* protein. Protein extracts from bovine uterus (lane1), ovary (lane2), and oviduct (lane 3) during the follicular phase; and the uterus (lane 4), ovary (lane 5), and oviduct (lane 6) during the luteal phase.

present results indicate that *NT4* may play a role in the capacitation of spermatozoa and early embryonic development in the the oviduct during the follicular phase. They have shown that *BDNF* promotes the *in vitro* development of zygotes into preimplantation embryos (Kawamura et al., 2005). However, there no statistically significant difference in the blastocyst development in *in vitro* culture was found (Hong et al., 2009). In this study, the low expression of *NT4* during the letual phase, suggests that *BDNF* is not the only factor that functions during the transport and early embryonic development, but also *TrkB* has another ligand, *NT4*, that participates in the regulation of the bovine oviduct during the luteal phase.

In the present study, immunoreactivity for *NT4* and *TrkB* were observed in the endothelial and uterine gland cells of the uterus, which is similar to *NGF* and *TrkA* responses in the Shiba goat (Ren et al., 2005). The identification of *NT4* in the uterus raises the question of its role in the function of this organ. The receptivity detected in the endometrium is the main factor that influences embryo implantation. However, the state of receptivity detected in the endometrium prior to embryo implantation is coordinated and regulated by estrogen, progesterone, and other reproductive regulation factors. Given that *NT4* primarily acts as a target-derived neurotrophic factor, one of its possible roles is to attract and maintain the sympathetic and sensory innervation of the uterus. In this study, the *NT4* and *TrkB* mRNA expression diversity in the bovine uterus under the follicular and luteal phases, indicate that *NT4* may play a role in the embryonic development, transport, and embedding implantation by autocrine or paracrine signaling. Other autocrine/paracrine functions suggested for the neurotrophins in the other tissues (Zettler et al., 1991; Donovan et al., 1995) should be considered as well.

In conclusion, *NT4* and its receptor are expressed in the ovary, oviduct and uterus of the native cow during the follicular and luteal phases. The information provided in this study may strongly aid in understanding the potential roles of *NT4/TrkB* in the mammalian reproductive mechanism.

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