Molecular characterization of tumor necrosis α -induced protein 6 and its human chorionic gonadotropin-dependent induction in theca and mural granulosa cells of equine preovulatory follicles

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Abstract

The preovulatory rise in gonadotropins causes an expansion of the cumulus–oocyte complex, a process requiring the induction of several genes. The objectives of this study were to clone the equine tumor necrosis factor α -induced protein 6 (TNFAIP6), and investigate its regulation in equine follicles during human chorionic gonadotropin (hCG)-induced ovulation. The isolation of the equine TNFAIP6 cDNA revealed that it contains an open reading frame of 834 bp (including the stop codon), encoding a predicted 277 amino acid protein that is highly similar (91–93% identity) to known mammalian homologs. The regulation of TNFAIP6 mRNA was studied in equine follicles isolated during estrus between 0 and 39 h post-hCG and in corpora lutea (CL) obtained on day 8 of the estrous cycle. Results from semi-quantitative RT-PCR/Southern blot showed that levels of TNFAIP6 mRNA were low in follicles obtained at 0 h, increased at 12 h, returned to basal levels at 24 h, and increased again at 36 h post-hCG ($P < 0.05$). Levels of TNFAIP6 transcripts were relatively moderate in CL, but low in non-ovarian tissues tested. Analyses performed with isolated preparations of theca and granulosa cells indicated that TNFAIP6 mRNA was regulated in both layers, with a maximal induction obtained 33–36 h post-hCG ($P < 0.05$). Immunohistochemical staining of sections of equine follicles isolated at 0 and 33 h post-hCG confirmed the induction of TNFAIP6 protein in both cell types after hCG treatment. Thus, the present study describes for the first time the gonadotropin-dependent regulation of follicular TNFAIP6 during the ovulation in a monoovulatory species. The biphasic induction of TNFAIP6 in equine theca and granulosa cells differs from the pattern observed in rodents, suggesting a distinct control of gene expression in this monoovulatory species.

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Introduction

Tumor necrosis factor α -induced protein 6 (TNFAIP6) belongs to the hyaladherin superfamily of hyaluronan (HA)-binding proteins (Wisniewski & Vilcek 2004). It is composed of 277 amino acids that give rise to a secreted product of 35 kDa, and consists of the N-terminal Link module and the C-terminal CUB domain. The Link module is known to interact with HA (Goetinck *et al.* 1987, Perin *et al.* 1987), whereas the CUB domain is found in a variety of proteins, many of which are involved in fertilization and development, but its precise physiological role remains unclear (Bork & Beckmann 1993). Expression of TNFAIP6 is usually undetectable in normal adult tissues or non-stimulated cells but is rapidly induced during inflammatory diseases (Milner & Day 2003). Indeed, high levels of TNFAIP6 have been detected in the synovial fluid of patients with rheumatoid

arthritis or osteoarthritis (Bayliss *et al.* 2001), but not in healthy joint tissues (Wisniewski *et al.* 1993). In this context, TNFAIP6 has been shown to protect against cartilage matrix destruction and to exert anti-inflammatory activities by inhibiting the migration of polymorphonuclear cells into the inflamed site (Wisniewski *et al.* 1996, Getting *et al.* 2002, Glant *et al.* 2002, Mindrescu *et al.* 2002, Szanto *et al.* 2004).

Ovulation is a complex luteinizing hormone (LH)-induced process that involves a series of biochemical and biophysical events. It is characterized by a marked formation of extracellular matrix (ECM) surround the cumulus cells that lead to the characteristic expansion of the cumulus–oocyte complex (COC; Salustri *et al.* 1999, Zhuo & Kimata 2001). The highly viscoelastic ECM is composed predominantly of HA as well as other factors essential for matrix assembly, including proteoglycans, link protein, serum-derived factor inter α -trypsin

inhibitor (I α I), and TNFAIP6 (Salustri *et al.* 1999, Zhuo & Kimata 2001, Russell *et al.* 2003a). Expression of TNFAIP6 is increased in periantral mural granulosa and cumulus cells of mouse and rat preovulatory follicles after administration of an ovulatory dose of human chorionic gonadotropin (hCG; Fulop *et al.* 1997, Yoshioka *et al.* 2000). Owing to its ability to bind HA, it has been suggested that TNFAIP6 stabilizes the ECM (Fulop *et al.* 1997, Jessen & Odum 2003). Indeed, TNFAIP6 and I α I have been detected in the HA-rich matrix surrounding cumulus cells of the expanded mouse COC, and interactions of TNFAIP6 with I α I and HA at this levels have been reported (Wisniewski *et al.* 1996, Carrette *et al.* 2001, Mukhopadhyay *et al.* 2001). Furthermore, disruption of the *TNFAIP6* gene resulted in impaired cumulus expansion and infertility in female mice (Fulop *et al.* 2003).

Preovulatory follicular development and ovulation in mares show several distinctive characteristics compared with rodents, such as a large diameter of the ovulatory follicle (40–45 mm), a relatively long ovulatory process (39–42 h), and the follicular rupture at a specific region of the ovary, the ovulatory fossa (Stabenfeldt *et al.* 1975, Duchamp *et al.* 1987, Ginther 1992, Sirois & Doré 1997, Kerban *et al.* 1999). In contrast to rodents in which the expansion process appears primary limited to the COC, the preovulatory rise in gonadotropins in mares causes an extensive expansion of the entire mural granulosa cell layer characterized by an abundant accumulation of ECM (Kerban *et al.* 1999). The unique and remarkable cellular modifications observed in equine follicles prior to ovulation suggest that the regulation of TNFAIP6 may differ in this species. Moreover, all studies performed thus far on the regulation of TNFAIP6 in the ovary have been limited to rodents (Fulop *et al.* 1997, 2003, Yoshioka *et al.* 2000, Carrette *et al.* 2001, Mukhopadhyay *et al.* 2001), with no reports in primates and non-primate monoovulatory species. Therefore, the specific objectives of this study were to clone and characterize the equine TNFAIP6 cDNA and to investigate its regulation in equine follicles during hCG-induced ovulation.

Materials and Methods

Cloning of the equine TNFAIP6 cDNA

The full-length equine TNFAIP6 cDNA was isolated by a combination of RT-PCR, and 5'- and 3'-RACE. RT-PCR was performed using sense and anti-sense primers (primers 1 and 2) designed from a highly conserved region identified after sequence alignments of human (GenBank accession number: NM_007115) and mouse (GenBank accession number: NM_009398) TNFAIP6 homologs, 100 ng pooled equine ovarian RNA, and a OneStep RT-PCR System (Qiagen) as directed by the manufacturer (Fig. 1Aa). Pooled ovarian RNA consists of

equal amounts of RNA prepared from a preovulatory follicle isolated before hCG, another follicle isolated 36 h after hCG, and a CL obtained on day 8 of the cycle (day 0, day of ovulation), as previously described (Boerboom *et al.* 2000). The RT-PCR product was subcloned into pGEM-T Easy vector (Promega), and sequenced (Service de Séquençage de Université de Laval, Québec, Canada). A large equine TNFAIP6 cDNA fragment was obtained and used to design the primers for 3'- and 5'-RACE reactions.

To characterize the 3'-end of equine TNFAIP6 cDNA, a 3'-RACE was performed as previously described (Boerboom *et al.* 2000) using 5 µg total RNA isolated from a preovulatory follicle obtained 36 h post-hCG. Briefly, RT was performed as directed by the manufacturer using SuperScript III RNase H-Reverse Transcriptase (Invitrogen Life Technologies) and a poly-dT oligonucleotide with anchor sequences at its 5'-end (primer 3) followed by a first PCR done with sense primer 4 and anti-sense 5, and a second nested-PCRs with sense primer 6 and anti-sense primer 7 (Fig. 1Ab). Primers 4 and 6 were designed from the 3'-end of a large equine TNFAIP6 cDNA fragment.

To obtain the missing 5'-end of equine TNFAIP6 cDNA, the 5'-RACE system version 2.0 (Invitrogen Life Technologies) was used as directed by the manufacturer. RT was performed as directed using anti-sense primer 8 and 3 µg RNA from 36 h post-hCG preovulatory follicle. The first 5'-RACE/PCR was performed with sense abridged anchor primer 9 (Invitrogen Life Technologies) and anti-sense primer 10, whereas the second 5'-RACE/PCR employed the sense abridged universal amplification primer 11 (Invitrogen Life Technologies) and anti-sense primer 12 (Fig. 1Ac). The primers 8, 10, and 12 were designed from the 5'-end of large equine TNFAIP6 cDNA fragment. PCRs consisted of 35 cycles of 94 °C for 30 s, 58 °C for 60 s, and 72 °C for 1 min. Final PCR products were subcloned into pGEM-T Easy vector and sequenced. The complete equine TNFAIP6 coding region was isolated by RT-PCR using 100 ng RNA from 36 h post-hCG preovulatory follicle, and sense primer 13 and anti-sense primer 14 (Fig. 1Ad), subcloned into pcDNA 3.1 (+) expression vector (Invitrogen Life Technologies), and sequenced. Primer sequences used are shown in Fig. 1B.

Equine tissues and RNA extraction

Equine preovulatory follicles and CL were isolated at specific stages of the estrous cycle from Standardbred and Thoroughbred mares as previously described (Kerban *et al.* 1999). Briefly, when preovulatory follicles reached 35 mm in diameter during estrus, the ovulatory process was induced by injection of hCG (2500 IU, i.v.) and ovariectomies were performed via colpotomy using an ovariectome at 0, 12, 24, 30, 33, 36, or 39 h post-hCG ($n=4-6$ mares/time point). Corpora lutea ($n=3$ mares)

Figure 1 Cloning strategy for isolating the equine TNFAIP6 cDNA. (A) The open reading frame (ORF) of equine TNFAIP6 is represented as an open box whereas the 5'- and 3'-UTR are shown as solid lines; the numbers in parenthesis indicate the size in base pairs of equine TNFAIP6 ORF. The equine TNFAIP6 cDNA was characterized by a combination of RT-PCR (a), 3'-RACE (b) and 5'-RACE (c), as described in Materials and Methods, and the complete equine TNFAIP6 coding sequence was isolated by RT-PCR; arrows and numbers refer to the orientation, relative position and identity of oligonucleotide primers used in each cloning procedure. (B) List of primers used in section A for equine TNFAIP6 cloning. The abridged anchor primer (9) and abridged universal amplification primer (11) are components of the 5'-RACE system.

were isolated on day 8 of the estrus cycle. Preovulatory follicles and CL were dissected from the surrounding ovarian tissues with a scalpel. Follicles were dissected into three cellular preparations referred to as the follicular wall (theca interna with attached granulosa cells), and isolated granulosa cells and thecal layers, as described (Sirois *et al.* 1991). The relative purity of each cellular preparation is estimated to exceed 95% based on the selective expression of P450 17 α -hydroxylase-C17–20 lyase (CYP17A1) and P450 aromatase (CYP19A1) mRNAs by theca interna and granulosa cells respectively (Boerboom *et al.* 1999). Testicular tissues were obtained from the Large Animal Hospital of the Faculté de médecine vétérinaire (Université de Montréal) following a routine castration, whereas other non-ovarian tissues were collected at a local slaughterhouse. The institutional animal use and care committee approved all animal procedures. Total RNA was isolated

from tissues with TRIzol reagent (Invitrogen), according to manufacturer's instructions using a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Montréal, Canada).

Semi-quantitative RT-PCR and Southern blot analysis

The semi-quantitative analysis of TNFAIP6 and rpl7a mRNA levels in equine tissues was performed using a OneStep RT-PCR System as directed by the manufacturer (Qiagen), and sense (5'-TAC AAG CAG CTA GAG GCA GCC-3') and anti-sense (5'-CTT CAA GGT CAT GAC ATT TCC TG-3') primers specific for equine TNFAIP6 (generating a DNA fragment of 483 bp), and sense (5'-ACA GGA CAT CCA GCC CAA ACG-3') and anti-sense (5'-GCT CCT TTG TCT TCC GAG TTG-3') primers specific for equine rpl7a (generating a DNA fragment of 516 bp). The equine rpl7a primers were designed from a published sequence (GenBank accession number: AF508309) and its transcript expression has been shown to be relatively constant during the equine ovulatory process (Brown *et al.* 2004, Sayasith *et al.* 2005). Each reaction was conducted using 100 ng total RNA, and cycling conditions were one cycle of 50 °C for 30 min and 95 °C for 15 min, followed by a 17 (for TNFAIP6) or 18 (for rpl7a) PCR cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 2 min. The number of PCR cycles used was determined by running a RT-PCR in 50 μ l. A volume of 5 μ l was pipetted after each three PCR cycles, starting from the end of the twelfth cycle of amplification. PCR products were electrophoresed on 2% Tris-acetic acid EDTA (TAE)-agarose gels, transferred to biotrans nylon membranes (ICN Pharmaceuticals, Montréal, Québec), and hybridized with corresponding radiolabeled TNFAIP6 and rpl7a cDNA fragments using QuikHyb hybridization solution (Stratagene, LaJolla, CA, USA). Membranes were exposed to a phosphor screen. Signals were quantified on a Storm imaging system using the ImageQuant software version 1.1 (Molecular Dynamics, Amersham Biosciences), and used to establish a standard curve of amplification in which the number of PCR cycles falling in linear range of amplification corresponding to optimal reactions (exponential amplification) was determined.

Cell cultures, transient transfection, and immunoblotting analysis

To validate the specificity of the antibody used against equine TNFAIP6, equine granulosa cell line was assessed in our laboratory (unpublished results) and cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 (Invitrogen Life Technologies) containing L-glutamine, non-essential amino acids, 10% fetal bovine serum, and penicillin (100 units/ml) and streptomycin (100 μ g/ml). Cultures were maintained at 37 °C

in a humidified atmosphere of 5% CO₂, and transiently transfected with 4 µg/petris TNFAIP6 expression vector using 30 µg LipofectAMINE PLUS reagent in 4 ml DMEM-F12, in accordance with manufacturer's protocol (Invitrogen Life Technologies). Three hours after transfection, cells were rinsed and incubated in culture media for 24 h. After incubation, culture medium and cells were harvested, and the whole cell protein extraction was performed as previously described (Sayasith *et al.* 2004). Protein concentrations were determined by the method of Bradford (1976; Bio-Rad Protein Assay). Samples (100 µg) were analyzed by one-dimensional SDS-PAGE (10%) and electrophoretically transferred to PVDF membranes. Membranes were incubated with anti-human TNFAIP6 polyclonal antibody (1:100 dilution; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-human TNFAIP6 polyclonal antibody (2 µg/ml; R&D Systems), or anti-mouse TNFAIP6 polyclonal antibody (2 µg/ml; R&D Systems), and immunoreactive proteins were visualized by incubation with the horseradish peroxidase-linked donkey anti-goat secondary antibody (1:5000 dilution) and the ECL detection system (Western Blotting Luminol Reagent) according to manufacturer's protocol (Santa Cruz Biotechnologies). In parallel, the follicular fluid isolated from equine preovulatory follicle after 36 h post-hCG was also assayed.

Immunohistochemical localization of TNFAIP6 in equine follicles

Immunohistochemical staining was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA), as previously described (Sirois & Doré 1997). Briefly, formalin-fixed tissues were paraffin embedded and 3 µm thick sections were prepared and then the paraffin was removed using a series of alcohol concentrations. Endogenous peroxidase was quenched by incubating the slides in 0.3% hydrogen peroxide in methanol for 30 min. After rinsing in PBS for 15 min, sections were incubated with diluted normal goat serum for 20 min at room temperature. The anti-human TNFAIP6 antibody (Santa Cruz Biotechnologies) was diluted in PBS (1:25) and applied, and sections were incubated overnight at 4 °C. Control sections were incubated with PBS. After rinsing in PBS for 10 min, a biotinylated goat anti-rabbit antibody (1:222; Vector Laboratories) was applied, and sections were incubated for 45 min at room temperature. After washing in PBS for 10 min, sections were incubated with the avidin DH-biotinylated horseradish peroxidase H reagents for 45 min at room temperature, washed with PBS for 10 min, and incubated with diaminobenzidine tetrahydrochloride (Sigma) as the chromogen substrate. Sections were counterstained with Gill's hematoxylin stain and mounted.

Statistical analysis

One-way ANOVA was used to test the effect of time after hCG on levels of TNFAIP6 mRNA in samples of theca and granulosa cells. TNFAIP6 mRNA levels were normalized with the control gene rpl7a prior to analysis. When ANOVA indicated significant differences ($P < 0.05$), the Dunnett's test was used for multiple comparisons of individual means. Statistical analyses were performed using JMP software (SAS Institute, Inc., Carry, NC, USA).

Results

Characterization of the equine TNFAIP6 cDNA

To clone equine TNFAIP6 cDNA, an equine TNFAIP6 cDNA fragment of 760 bp was first isolated by RT-PCR using primers designed by sequence alignments of human and mouse TNFAIP6 homologs (Fig. 1Aa). Sense and anti-sense primers designed from the latter cDNA fragment were used in 3'- and 5'-RACE reactions to characterize the 3'-end and to obtain the missing 5'-end of equine TNFAIP6 cDNA (fragments of 275 and 159 bp respectively; Fig. 1Ab and Ac). Results showed that the equine TNFAIP6 cDNA is composed of a 5'-UTR of 85 bp, an open reading frame of 834 bp (including the stop codon), and a 3'-UTR of 209 bp (Fig. 2A). The nucleotide sequence was submitted to GenBank with accession number AY919871.

The predicted complete coding region of equine TNFAIP6 was isolated by RT-PCR, subcloned, and confirmed by sequencing. This region encodes a 277 amino acid protein, which is similar in length with human (GenBank accession number: NM_007115), three amino acids shorter than bovine (GenBank accession number: NM_001007813), and two amino acids longer than mouse (GenBank accession number: NM_009398) TNFAIP6 (Fig. 2B). Comparative analyses revealed that the equine TNFAIP6 sequence is highly similar to its homologs, with a 91% (human and bovine) and 85% (mouse) identity observed at the nucleic acid level, and a 91% (human), 93% (bovine), and 90% (mouse) identity at the amino acid level (Fig. 2B). Moreover, structural domains putatively involved in TNFAIP6 function are conserved in the equine protein, including a Link module and a CUB domain (Fig. 2B).

Tissue distribution of equine TNFAIP6 mRNA and its regulation in mural granulosa and theca cells of preovulatory follicles and in CL

The relative expression of TNFAIP6 mRNA in various equine tissues was studied by RT-PCR/Southern blot. Results showed that levels of TNFAIP6 transcripts were highly detected in preovulatory follicle isolated 12 h after hCG, low in heart, brain, lung, adrenal gland, and uterus, and very low or undetectable in other tissues

Figure 2 Nucleotide sequence of equine TNFAIP6 and comparisons of its deduced amino acid sequence with other mammalian homologs. (A) The equine TNFAIP6 cDNA was cloned by a combination of RT-PCR and 5'- and 3'-RACE, as described in Materials and Methods, and is composed of a 5'-UTR of 85 bp (lowercase letters), an ORF of 834 bp (uppercase letters), and a 3'-UTR of 209 bp (lowercase letters). The translation initiation (ATG) and stop (TAA) codons are bolded and numbers on the right refer to the last nucleotide on that line. Overlapped Shaw-Kamen motifs are underlined and a consensus polyadenylation signal sequence is double underlined. The nucleotide sequence was submitted to GenBank with accession number AY919871. (B) The amino acid sequence of equine (equ) TNFAIP6 is aligned with the human (hum), bovine (bov), and mouse (mou) homologs. Identical residues are noted with a printed period, hyphens indicate gaps in protein sequences created to optimize alignment, the numbers on the right are for the last amino acid residue on that line, and the percentage in parentheses refers to the percentage of identity in amino acid residues when comparisons are made with equine TNFAIP6. The putative HA-binding domain, referred to as the Link module, is underlined; and the domain referred to as the CUB domain, is overlined. Asparagine (N)-linked glycosylation consensus sequences are double overlined. Signal peptide cleavage site is marked with an arrowhead.

tested (Fig. 3A). In contrast, the levels of rpL7a mRNA (control gene) remained relatively constant in these tissues (Fig. 3B).

The regulation of TNFAIP6 mRNA during the ovulatory process was studied by semi-quantitative RT-PCR/Southern blot using total RNA extracted from follicle walls (theca interna layer with attached

Figure 3 Equine tissue expression of TNFAIP6 mRNA and its gonadotropin-dependent regulation in preovulatory follicles. Relative expression of equine TNFAIP6 (A) and rpL7a (control gene; B) were analyzed by a semi-quantitative RT-PCR/Southern blot, as described in Materials and Methods, using total RNA (100 ng) extracted from various equine tissues. The number of PCR cycles for both genes was 18 cycles and was optimized to fall within the linear range of the PCR amplification. Numbers on the right indicate the size of the PCR fragment. (C and D) RNA extracts were prepared from equine preovulatory follicles at 0, 12, 24, and 36 h after administration of an ovulatory dose of human chorionic gonadotropin (hCG) and corpora lutea (CL) were isolated on day 8 of the cycle. Samples (100 ng) were analyzed by a semi-quantitative RT-PCR/Southern blot for the expression of TNFAIP6 and rpL7a transcripts, as described in Materials and Methods. Representative results of TNFAIP6 and rpL7a mRNA levels are presented from one follicle/time point (C). The TNFAIP6 signal was normalized with the control gene rpL7a, and results are presented as ratios of TNFAIP6 to rpL7a (D). Mean $\pm$ S.E.M.; $n=4-6$ distinct follicles or $n=3$ CL (i.e. mares) per time point. Bars marked with an asterisk are significantly different from 0 h post-hCG ($P<0.05$).

granulosa cells) of equine preovulatory follicles isolated between 0 and 36 h after hCG treatment, and CL obtained on day 8 of cycle. Representative results indicated that TNFAIP6 mRNA expression was low before the hCG treatment (0 h), moderate at 12 h, low at 24 h, and high at 36 h post-hCG (Fig. 3C). Levels of TNFAIP6 transcripts were relatively moderate in CL (Fig. 3C). When results from several follicles ($n=4-6$ (e.g. each from different animal)) and CL ($n=3$) were expressed as ratios of TNFAIP6 to rpl7a, a significant biphasic pattern of the TNFAIP6 mRNA induction was observed in preovulatory follicles, with an increase in levels of TNFAIP6 transcript at 12 and 36 h post-hCG as compared with 0 h ($P<0.05$; Fig. 3D). Treatment with an ovulatory dose of gonadotropin did not affect rpl7a mRNA expression in preparations of follicle wall and CL at any time point tested.

Localization of equine TNFAIP6 in preovulatory follicles

To determine the cellular localization of equine TNFAIP6 mRNA expression in preovulatory follicles, RT-PCR/Southern blot was performed using isolated preparations of mural granulosa cells and theca layers obtained from equine preovulatory follicles collected 0–39 h after hCG treatment. Results showed that the TNFAIP6 transcript occurred in a biphasic manner in both cellular preparations, with a predominant expression of TNFAIP6 mRNA in theca layers. In mural granulosa cells, TNFAIP6 mRNA expression was low at 0, increased at 12 h, returned to basal levels at 24 and 30 h, and increased again between 33 and 39 h post-hCG (Fig. 4A). When results from several follicles ($n=4-6$) were expressed as ratios of TNFAIP6 to rpl7a, a significant increase in TNFAIP6 mRNA expression was

observed at 12, 33, 36, and 39 h post-hCG, as compared with 0 h ($P<0.05$; Fig. 4B). In theca layers, expression of the equine TNFAIP6 transcript was low at 0 h, increased at 12 h, returned to low levels at 24 h, and increased again between 30 and 39 h post-hCG (Fig. 4C). Results from multiple follicles ($n=4-6$) expressed as ratios of TNFAIP6 to rpl7a revealed an increase in TNFAIP6 mRNA levels at 12 h, and between 30 and 39 h post-hCG, as compared with 0 h ($P<0.05$; Fig. 4D).

To determine if the induction of the TNFAIP6 mRNA was associated with the expression of its protein, immunohistochemistry was performed on sections of equine preovulatory follicles isolated before and after hCG. As anti-equine TNFAIP6 antibody is not available commercially, an anti-mouse TNFAIP6 polyclonal antibody obtained from R&D Systems (Fig. 5A) and two anti-human TNFAIP6 polyclonal antibodies obtained from R&D Systems (Fig. 5B) and Santa Cruz Biotechnologies (Fig. 5C) were primarily tested for their specificity against equine TNFAIP6 protein by immunoblotting using the follicular fluid isolated from preovulatory follicle after 36 h post-hCG (lane 1) and the culture medium (lane 2) and protein extracts (lane 3) isolated from granulosa cells overexpressing equine TNFAIP6. Results revealed that multiple protein bands were recognized by the antibodies in follicular fluid and culture medium (Fig. 5, lanes 1 and 2), situation also observed in other studies (Rugg *et al.* 2005, Sanggaard *et al.* 2005): the lower band appears in the range of 38 kDa (a) corresponding to the monomer of full-length TNFAIP6 protein in the absence of post-translational modifications; the band indicated by c letter most likely corresponds to dimeric form of TNFAIP6; the protein bands (b) between monomeric and dimeric forms may represent the TNFAIP6 proteins with different extents of glycosylation, as suggested previously (Wisniewski *et al.*

Figure 4 Cellular localization of TNFAIP6 mRNA in equine preovulatory follicles. Total RNA was separately extracted from granulosa and theca cells isolated from equine preovulatory follicles between 0 and 39 h post-hCG, and samples (100 ng) were analyzed for TNFAIP6 and rpl7a content by a semi-quantitative RT-PCR/Southern blot, as described in Materials and Methods. Representative results of TNFAIP6 and rpl7a mRNA levels are presented from one granulosa (A) and one theca (C) sample per time point. (B and D) The TNFAIP6 signal was normalized with the control gene rpl7a, and results are presented as ratios of TNFAIP6 to rpl7a (mean $\pm$ s.e.m.; $n=4-5$ samples (i.e. mares) per time point). Bars marked with an asterisk are significantly different from 0 h post-hCG ($P<0.05$).

Figure 5 Validation of the antibody specificity against equine TNFAIP6 protein. The follicular fluid isolated from preovulatory follicle after 36 h post-hCG (lane 1) and the culture medium (lane 2) or protein extracts isolated from granulosa cells overexpressing equine TNFAIP6 (lane 3) were analyzed by one-dimensional SDS-PAGE and immunoblotting using anti-mouse TNFAIP6 polyclonal antibody (A) and anti-human TNFAIP6 polyclonal antibody (B) obtained from R&D systems, and anti-human TNFAIP6 polyclonal antibody obtained from Santa Cruz Biotechnologies (C), as described in Materials and Methods. Markers on the left indicate the migration of molecular weight standards. Protein bands indicate multiple forms of TNFAIP6 in the absence or presence of the complexes: the lower band appears in the range of 38 kDa (a) corresponding to the monomer of TNFAIP6 protein; the band indicated by c letter most likely represents a dimeric form of TNFAIP6; the protein bands marked by b letter may correspond to TNFAIP6 proteins with different extents of glycosylation; the bands noted by d letter most likely correspond to the complexes of TNFAIP6 and heavy chains (HC1 and HC2) of $\text{I}\alpha\text{I}$; and the upper band (e) may represent a TNFAIP6. $\text{I}\alpha\text{I}$ complex.

1994, Sanggaard *et al.* 2005); the bands noted by *d* letter most likely correspond to the complexes of TNFAIP6 and heavy chains (HC1 and HC2) of $\text{I}\alpha\text{I}$; and the upper band (e) may corresponds to a TNFAIP6. $\text{I}\alpha\text{I}$ complex. Interestingly, antibodies originated from different suppliers or species recognize the same protein bands with different extents in similar samples (Fig. 5, lanes 1 and 2). Moreover, a single band of TNFAIP6 (a) was observed in protein extracts prepared from granulosa cells overexpressing equine TNFAIP6 (Fig. 5, lane 3), whereas, no band was detected with a mock transfection (data not shown). In any cases, further studies will be needed to establish the exact identity of TNFAIP6-binding proteins.

Using anti-human TNFAIP6 polyclonal antibody (Santa Cruz Biotechnologies), immunohistochemical analyses revealed an increase in equine TNFAIP6 staining in preovulatory follicles after hCG treatment. Indeed, TNFAIP6 immunochemical staining was low in follicles with a very compact granulosa cell layer obtained before hCG treatment (0 h; Fig. 6A), but highly increased in mural granulosa cells and theca layer of preovulatory follicles obtained at 12, 33, and 36 h post-hCG (Fig. 6B–E). This increase is accompanied by a progressive loosening (i.e. expansion) of the granulosa cell layer induced by hCG, and the presence of TNFAIP6 protein in the cytoplasm of granulosa cells as well as

outside (arrows) of these cells, in keeping with the high levels of TNFAIP6 mRNA detected during these times. As control of a staining, the primary antibody was replaced with PBS, and no staining was observed (33 h; Fig. 6F).

Discussion

The aim of this report was to study the regulation of TNFAIP6 in theca and mural granulosa cells of equine preovulatory follicles during the ovulatory process induced by hCG (ovulation occurs around 42 h post-hCG in this species). TNFAIP6 has been shown to play important roles for completion of COC expansion during ovulation, since targeted disruption of the *TNFAIP6* gene caused a defect in the COC expansion and infertility in female mice (Fulop *et al.* 2003). Studies on the regulation of TNFAIP6 in the ovarian physiology have been limited to rodents, with no report on its expression in large monoovulatory species, including mares. In contrast to other species, the preovulatory rise of LH in mares induces a unique and remarkable expansion associated with extensive synthesis and assembly of a viscoelastic ECM within the granulosa cell layer in follicles prior to ovulation (Kerban *et al.* 1999). Previous studies have shown that the spatial and temporal regulation of several genes involved in steroidogenesis and ovulation in equine preovulatory follicles is also different from that observed in the rodent ovary (Boerboom *et al.* 1999, 2000, 2003). Therefore, the present study identified other key differences. Indeed, the gonadotropin-dependent induction of TNFAIP6 in equine preovulatory follicles was prolonged and biphasic, differing with its rapid and monophasic induction in rodents (Yoshioka *et al.* 2000). The biphasic induction of equine TNFAIP6 clearly occurred in both mural granulosa and theca cells, and was not limited to granulosa cells as observed in rodents (Fulop *et al.* 1997, 2003, Yoshioka *et al.* 2000, Carrette *et al.* 2001, Mukhopadhyay *et al.* 2001). These findings further identify marked differences in the pattern of ovarian gene expression across species, and underscore value of using different animal models.

The viscoelastic ECM is thought to play multiple roles in ovulation and fertilization, including its capacity to keep the cumulus cells together with the oocyte; extrusion of the detached complex from the follicle during ovulation; facilitating its capture by, and transport in the oviduct; and as a physical barrier for the selection of highly functional and motile spermatozoa (Mahi-Brown & Yanagimachi 1983, Neuwinger *et al.* 1991, Aitken *et al.* 1992, Salustri *et al.* 1999). Our study establishes that the ECM of equine follicles contains TNFAIP6. As demonstrated with other species, the ECM of an expanded COC contains, at least, HA, proteoglycans, $\text{I}\alpha\text{I}$, and TNFAIP6 (Chen *et al.* 1992, Salustri *et al.* 1999, Russell *et al.* 2003a, 2003b). Moreover, the co-localization of TNFAIP6, $\text{I}\alpha\text{I}$, and HA in the ECM of

Figure 6 Immunohistochemical localization of TNFAIP6 protein in equine preovulatory follicles. Immunohistochemistry was performed on formalin-fixed sections of preovulatory follicles isolated before and after hCG, as described in Materials and Methods. Results show low TNFAIP6 staining in follicles obtained at 0 h (A), but a marked immunoreactivity (staining) was detected in mural granulosa and theca cells of follicles isolated 12 (B), 33 (C and D), and 36 h (E) post-hCG, in keeping with high levels of TNFAIP6 mRNA detected at these times. (F) Control staining from the follicular tissue presented in C and D was negative when the primary antibody was replaced with PBS. The limits of the mural granulosa cell (GC) layer and theca interna (TI) are indicated (A and B). The region above the granulosa cell layer is for the antrum, whereas the region immediately beneath corresponds to thecal layers, including the limits of TI. TNFAIP6 protein is present in the cytoplasm of granulosa cells as well as secreted outside of cells (C–E; arrows). Arrowheads indicate vascular structures with circulation cells (B). Magnification, A, B, and D–F (×400), and C (×200).

murine COC has been established, and evidence showed that TNFAIP6 can covalently link to the heavy chains of $\text{I}\alpha\text{I}$, facilitating covalent links of the latter to HA, whereas the addition of a monoclonal anti-TNFAIP6 antibody impeded COC expansion in rodents (Chen *et al.* 1996, Carrette *et al.* 2001, Fulop *et al.* 2003, Ochsner *et al.* 2003, Rugg *et al.* 2005, Sanggaard *et al.* 2005, Wisniewski *et al.* 2005). Interestingly, the present study indicates that the expression of equine TNFAIP6 coincides with the induction of HA synthase-2 and the production of HA, detected from 12 h after hCG in equine preovulatory follicles, as previously reported (Stock *et al.* 2002). Of interest, further studies will be needed to verify the presence of $\text{I}\alpha\text{I}$, and the co-localization of TNFAIP6, $\text{I}\alpha\text{I}$, and HA in the equine preovulatory follicles, as observed in rodent ovaries (Carrette *et al.* 2001). As HA is present in equine preovulatory follicles, functional roles of TNFAIP6 through binding to HA are likely present in equine preovulatory follicles, including the inhibition of CD44+ leukocyte adhesion in COC expansion (Lesley *et al.* 2003, Wisniewski *et al.* 2005) and plasmin activity, and consequently inhibition of matrix metalloproteinases required to degrade ECM during ovulation as reported in rodents (Wisniewski

et al. 1996). The present study indicates the expression of TNFAIP6 in theca and mural granulosa cells. Since the animal physiology and technical difficulties to isolate the COC from equine preovulatory follicles, the expression of TNFAIP6 in equine cumulus cells could not verify. However, as the latter expression in mural granulosa cells appear similar to that occurred in cumulus granulosa cells in rodents, we can suggest that a similar tendency may be observed in equine preovulatory follicles. As the expression of TNFAIP6 is predominant in theca layers, this suggests, in addition to cumulus expansion, other roles of TNFAIP6 in equine preovulatory follicles. For an example, at theca layers, TNFAIP6 may involve during the alteration of follicular basement membrane or in major vascular changes that highly occurred in this compartment during the ovulatory process (Kerban *et al.* 1999). In addition, theca layer may represent an important site to provide TNFAIP6, together with granulosa cells, required for a successful expansion of COC.

Studies on the molecular control of TNFAIP6 induction are very limited. Previous reports have shown that the transactivation of TNFAIP6 promoter was stimulated by TNF- α interacting with the NF-IL6 *cis*-acting element

(Klampfer *et al.* 1994), and the induction of TNFAIP6 mRNA was coincident with the increase in TNF- α concentration and COX-2 expression in gonadotropin-treated immature rats (Rice *et al.* 1998, Yoshioka *et al.* 2000), suggesting the modulation of a marked increase in TNFAIP6 mRNA after hCG treatment by a transcription activation event. COX-2 and prostaglandin E2 (PGE2) receptor (EP2) are required for optimal expansion and ovulation (Davis *et al.* 1999, Hizaki *et al.* 1999, Matsumoto *et al.* 2001). Several studies have suggested the involvement of the COX-2/PGE2/EP2-signaling pathway in the gonadotropin-dependent stimulation of TNFAIP6 in cumulus expansion of rodents, since mice lacking COX-2 or EP2 gene display altered TNFAIP6 mRNA and protein expression in cumulus cells, but not in mural granulosa cells (Ochsner *et al.* 2003). In addition, rats treated with indomethacin (a non specific COX inhibitor) exhibited a significant reduction of TNFAIP6 transcripts prior to ovulation (Yoshioka *et al.* 2000). Our study revealed the increase in levels of TNFAIP6 message in a biphasic pattern in both granulosa and theca cells of equine preovulatory follicles, but the precise reasons for this biphasic induction remain unknown. It is tempting to speculate that the initial increase of TNFAIP6 may result from the first instance of gonadotropin surge, such as the induction of TNF- α , IL-1 β , and the accumulation of HA (detected from 12 h post-hCG; Stock *et al.* 2002), whereas the second increase coincided with the COX-2 induction (detected from 30 h post-hCG; Sirois & Doré 1997, Boerboom & Sirois 1998) may result from COX-2 activity, thereby supporting the role of prostaglandins in the increase of TNFAIP6.

Ovulation is a complex process that has been characterized as an acute inflammatory reaction (Espey 1980). Our results indicate that follicles collected prior to ovulation were by far the tissues that most highly expressed TNFAIP6 mRNA, differing from very low or undetectable levels observed in other non-ovarian tissues tested. This difference may be due to the fact that TNFAIP6 expression is an integral part of the cascade of inflammatory-like changes that occur in preovulatory follicles, while the latter expression is usually undetectable in normal tissues (Milner & Day 2003).

Lastly, the present study describes for the first time the cloning and characterization of equine TNFAIP6. Its protein sequence is highly similar (90–93%) to that of other species, and identical in length with human (Lee *et al.* 1992), three amino acids shorter than bovine (GenBank accession number: NM_001007813) and two amino acids longer than mouse (Fulop *et al.* 1997) homologs. More importantly, all putative structural and functional domains are conserved in equine TNFAIP6 protein, including the signal peptide of 19 amino acid residues (Lee *et al.* 1992), two asparagine N-glycosylation consensus sequences, one site for chondroitin sulfate linkage, one distinct structural domain, referred to as a Link module, responsible for HA binding, and one

characteristic region, referred to as a CUB domain, whose function remains unclear (Goetinck *et al.* 1987, Perin *et al.* 1987).

In summary, this study reports the primary structure of equine TNFAIP6, its transcript induction, and the presence of its protein in equine follicles during the ovulatory process *in vivo*. In contrast to rodents, the induction of equine TNFAIP6 is prolonged and biphasic in granulosa and theca cells. This induction is coincident with the increase of HA production and vascular changes in preovulatory follicles (Kerban *et al.* 1999, Stock *et al.* 2002), suggesting that TNFAIP6 may involve in the equine ovulatory process, including alteration of follicular basement membrane, and COC stabilization and expansion. Further studies are needed to determine the precise molecular mechanism behind the gonadotropin-dependent regulation of TNFAIP6 and its functional roles involved in this important physiological process in mares. Considering its large size and a long ovulatory process, the equine preovulatory follicle provides a valuable model to elucidate these issues and to further study the ovarian function of TNFAIP6 in a monoovulatory species.

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