0013-7227/04/$15.00/0
Printed in U.S.A.

Endocrinology 145(3):1218–1226

doi: 10.1210/en.2003-0860

Tumor Necrosis Factor α (TNF) Increases Granulosa Cell Proliferation: Dependence on c-Jun and TNF Receptor Type 1

DEOK-SOO SON, KOJI Y. ARAI, KATHERINE F. ROBY, AND PAUL F. TERRANOVA

Center for Reproductive Sciences (D.-S.S., K.Y.A., K.F.R., P.F.T.) and Departments of Molecular and Integrative Physiology (D.-S.S., K.Y.A., P.F.T.), Anatomy and Cell Biology (K.F.R.), and Obstetrics and Gynecology (P.F.T.), University of Kansas Medical Center, Kansas City, Kansas 66160

TNFα has significant *in vitro* effects on steroidogenesis and folliculogenesis and reproductive alterations occur in TNF receptor type 1 (TNFR1) knockout mice. The present study investigated the effect of *in vitro* TNF on granulosa cell proliferation from immature mice at 28 d of age, with emphasis on intracellular signaling that regulates granulosa cell proliferation. TNF dose dependently increased granulosa cell proliferation and the proto-oncogene c-Jun protein. However, other Jun family members such as JunD was expressed constitutively and JunB was not expressed. *In vitro* TNF did not increase c-Jun and proliferation in granulosa cells from TNFR1 knockout mice. The time course of TNF-induced c-Jun revealed biphasic patterns of short-term (3 h) and long-term (24 h) induction. The time courses of Ser63- and Ser73-phospho c-Jun coincided with changes in total c-Jun. Among MAPK cascades, stress-activated protein kinase/c-Jun-NH_2-teminal kinase signaling was increased transiently in TNF-treated cells, whereas p38MAPK and ERK1 and 2 were not changed. In addition, overexpression of nuclear factor-κB and addition of ceramide and 8-bromo-cAMP did not increase c-Jun or proliferation. Antisense oligonucleotides for c-Jun blocked cell proliferation induced by TNF. In conclusion, the above results demonstrate that TNF increased c-Jun by activating stress-activated protein kinase/c-Jun-NH_2-teminal kinase signaling via TNFR1 in mouse granulosa cells, and the induced c-Jun resulted in increased cell proliferation. (*Endocrinology* 145: 1218–1226, 2004)

TNFα IS A MULTIFUNCTIONAL hormone-like polypeptide and modulates many genes involved in inflammation, infection, and malignancy. Although macrophages are a main source of TNF, oocytes, corpora lutea, and theca and granulosa cells have been reported to contain TNF or its mRNA (1–4). TNF is well known in regulating steroidogenesis (5–8), folliculogenesis (9, 10), ovulation (11), luteinization (12, 13), and fertility (14, 15) in numerous species including rodents and humans. TNF binding sites exist in granulosa cells (16, 17) and ovarian TNF signaling cascades regulating steroidogenesis act most likely via p55 TNF receptor type 1 (TNFR1) (15) rather than p75 TNF receptor type 2 (TNFR2) (10, 15). TNF exerts its function through the internal portion of TNFR1 containing three functional domains: 1) C-terminal death domain, 2) acid sphingomyelin activating domain, and 3) neutral sphingomyelin activating domain (18). Each domain binds distinct proteins and activates downstream signaling proteins including MAPK, ceramide, caspase, activation protein-1 (AP-1), and nuclear factor (NF)-κB. The Jun family (c-Jun, Jun-B, and Jun-D), a basic region leucine zipper (bZIP) family of transcription factors, forms homodimers or heterodimers among Jun family members, Fos family members (c-Fos, Fos-B, Fra-1, and Fra-2) and activating transcription factor-2 (ATF-2). The dimer binds the consensus DNA sequence (TGAC/GTCA) sites called AP-1 found in a variety of promoters (19, 20). The functional roles of AP-1 transcription factors involve several cellular processes including cell cycle, oncogenesis or apoptosis (21). In particular, c-Jun regulates differentially apoptosis, proliferation or differentiation with a cell-specific manner (20, 21). Therefore, TNF-induced c-Jun may be a key protein in explaining differential effects of TNF in granulosa cells. It is well known that TNF may affect granulosa cell apoptosis (13, 22–24), proliferation (10, 13, 25, 26) and differentiation (12, 13). Because the intracellular mechanism of TNF-induced proliferation *in vitro* is unclear, the present study focused on signaling pathways involved in TNF-induced proliferation *in vitro* of granulosa cells from immature mice and whether or not c-Jun was involved.

Materials and Methods

Reagents

Recombinant murine TNFα was obtained from R&D Systems (Minneapolis, MN). SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), BAY 11-7082 (NF-κB inhibitor) and C2 ceramide (N-acetylsphingosine) were purchased from Biomol (Plymouth Meeting, PA). PD98059 (MAPK inhibitor) was obtained from Calbiochem (La Jolla, CA). The following reagents were purchased from Sigma (St. Louis, MO): dexamethasone (DEX), 8-bromo-cAMP (cAMP), penicillin G/streptomycin, and fibronectin. Lipofectamine Plus and all liquid culture media were acquired from Invitrogen (Grand Island, NY). The following antibodies were purchased from Cell Signaling Technology (Beverly, MA): ATF-2, c-Jun, phospho-c-Jun (Ser63), phospho-c-Jun (Ser73), stress-activated protein kinase/c-Jun-NH_2-teminal kinase (SAPK/JNK), phospho-

Abbreviations: AP-1, Activation protein-1; ATF-2, activating transcription factor-2; cAMP, 8-bromo-cAMP; DEX, dexamethasone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF, nuclear factor; SAPK/JNK, stress-activated protein kinase/c-Jun-NH_2-teminal kinase; TNFR1 or TNFR2, TNF receptor type 1 or 2.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

SAPK/JNK (Thr183/Tyr185) and phospho-p44/42 MAPK (Thr202/Tyr204). The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): JunB, JunD, β-actin, c-Fos, p38, and phospho-p38. Oligonucleotides for c-*jun* antisense (5′-TTC CAT CTT TGC AGT CAT-3′) (27) and its scrambled oligonucleotides (5′-TCT CCT ATT GAT CGT TCA-3′) were obtained from Integrated DNA Technologies (Coralville, IA). NF-κB luciferase vector was obtained from BD Biosciences (Palo Alto, CA). p65-pRc/RSV and p50-pcDNAI were kindly provided by Dr. Tom Maniatis (Harvard University, Cambridge, MA). ERK1/2 and its phospho-antibodies and the Luciferase Reporter Assay System were obtained from Promega (Madison, WI). Dimethylsulfoxide was purchased from Fisher Scientific (St. Louis, MO).

Animals

C57BL6 mice from Harlan, Inc. (Indianapolis, IN) and TNFR1 null mice on a C57BL6 background from Immunex (Seattle, WA) were established as breeding colonies in our laboratory. All mice were given commercial pellet feed and drinking water *ad libitum* and housed with controlled 12-h light, 12-h dark cycle under pathogen-free conditions. All handling of animals and procedures conformed to the guidelines set forth by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Granulosa cell culture and treatments

Ovaries were collected under a laminar flow hood from mice at age of 28 d and placed in cold DMEM/F12 supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). The ovaries were cleaned of all connective tissues and fat. Follicles were punctured using a 27-gauge needle attached to a 1-ml syringe to extrude granulosa cells. Granulosa cells (~2×10^5 cells/ml) were cultured at 37 C in a water-saturated atmosphere of 95% air and 5% CO_2 in fibronectin-coated 96-, 12-, or six-well culture plates with serum-free DMEM/F12 medium containing penicillin/streptomycin. After overnight culture to allow cellular attachment to the plate, the medium was removed and fresh medium was added. Treatments were initiated as outlined in *Results*.

Western blot

Total cell lysates were prepared, fractionated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes according to established procedures (28). Blocking of nonspecific proteins was performed by incubation of the membranes with 5% nonfat dry milk in Tris-buffered saline Tween-20 [TBST containing 10 mM Tris, 150 mM PBS, 0.05% Tween 20 (pH 8.0)] for 2 h at room temperature. Blots were incubated with primary antibodies at 1:1000 dilution in blocking solution overnight at 4 C. The membranes were washed three times with TBST for 10 min and followed by incubation for 1 h with horseradish peroxidase-conjugated secondary antibody according to primary antibody used at 1:2500 in 5% milk/TBST. The membranes were then rinsed three times with TBST for 10 min, and the bands were visualized by enhanced chemiluminescence. After membrane stripping for 10 min with methanol containing 3% H_2O_2, β-actin was detected immunologically to serve as an internal loading control.

Immunofluorescence

Granulosa cells grown on eight-chambered slides were treated with TNF (10 ng/ml) for 24 h. The cells in the chamber slides were washed three times in PBS and fixed for 10 min in cold absolute methanol at −20 C. The methanol-fixed slides were dried completely and rinsed quickly with PBS. The slides were incubated with 10% normal goat serum in PBS for 30 min to suppress nonspecific binding. After removing the serum solution, the slides were incubated overnight at 4 C with rabbit antibodies for c-Jun and JunD at 1:200 in PBS with 1.5% normal goat serum. The slides were then washed three times with PBS and then incubated with fluorescein isothiocyanate-conjugated secondary antibody at 1:400 in PBS containing 1.5% normal goat serum for 1 h in a dark room. Finally, the slides were washed three times with PBS, mounted with a coverslip and viewed using a fluorescence microscope.

Transient transfection and the luciferase assay

Granulosa cells at approximately 50% confluency in a 12-well plate were washed once with fresh DMEM/F12 (without additives) and were transiently transfected with vectors indicated in *Results* for 3 h according to manufacturer's instructions by using Lipofectamine Plus. Transfected cells were treated as outlined in *Results* and incubated for 24 h. Cell lysates were prepared according to manufacturer's instructions and luciferase activity was determined using a microplate luminometer. Luciferase activity expressed as relative light units was normalized to the protein level.

Cell proliferation assay

The cell proliferation assay was performed by using the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a colored product as previously described (3). Briefly, after 24 h of culture in a 96-well plate, each well was washed twice with phenol red-free media. After removing the media, 100 μl of MTT solution (1 mg/ml of phenol red-free media:PBS = 4:1) was added and the plates were incubated for 3–4 h at 37 C under 5% CO_2 in air with protection from light. The MTT solution in each well was removed and 100 μl of isopropanol was added. The plates were placed on a shaker for 10 min at room temperature to thoroughly dissolve the MTT color product. The OD was measured at 570 nm using a spectrometer.

Statistics

Where appropriate, as determined by repetition of the experiments at least three times, data were analyzed by Student's *t* test and one-way ANOVA. If statistical significance ($P \leq 0.05$) was determined by ANOVA, the data were further analyzed by the Student-Newman-Keuls method to detect specific differences between treatments or groups. If experiments were repeated only twice because of the use of limited numbers of primary cells, a mean and range is given.

Results

Effects of TNF on granulosa cell proliferation: requirement of TNFR1 (Fig. 1)

In the absence of TNF, relative cell density of granulosa cells at 24 h from C57BL6 mice increased compared with that at 0 h (*left panel*). TNF at 5 and 10 ng/ml further increased the relative number of granulosa cells from normal mice (+/+). In contrast, this effect of TNF was not observed in cells from TNFR1 null mice (−/−).

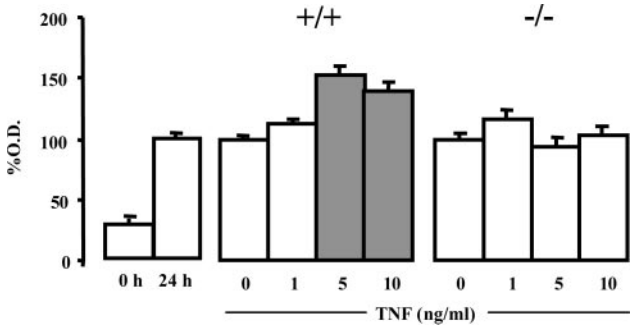

FIG. 1. Relative granulosa cell density at 0 and 24 h in C57BL6 mice (*left panel*) and effects of *in vitro* TNF (0–10 ng/ml) on relative granulosa cell density at 24 h in C57BL6 (+/+) and TNFR1 knockout (−/−) mice. Data represent the mean and range from eight determinations in two (0 and 24 h) and mean ± SEM of four (TNF) experiments. *Different color bars* indicate significance ($P \leq 0.05$) compared with 0 using the Student-Newman-Keuls method within a group.

Effects of TNF on AP-1 components in granulosa cells: specific expression of c-Jun protein (Figs. 2 and 3)

TNF increased c-Jun protein in mouse granulosa cells at 1 h after treatment without affecting c-Fos and ATF-2 that were constitutively expressed (Fig. 2A). Among Jun family members, only c-Jun was increased by TNF; JunB was not expressed and JunD was constitutively expressed (Fig. 2B). Cellular immunofluorescence of c-Jun confirmed the increase in c-Jun protein by TNF at 24 h (Fig. 3, A and B) and even within 2 h (*inset*) as well as the constitutive expression of JunD (Fig. 3, C and D). TNF increased c-Jun as evidenced by intensely concentrated nuclear fluorescence, whereas JunD was expressed uniformly throughout the entire nucleus.

Dose-dependent effects of TNF on c-Jun: involvement of TNFR1 (Fig. 4)

TNF increased c-Jun dose dependently at 24 h with maximal levels (~7-fold) attained at 5 ng of TNF/ml (Fig. 4A). Granulosa cells from TNFR1 null mice did not respond to TNF as evidenced by a lack of c-Jun protein expression (Fig. 4B). TNF also increased Ser63-phospho and Ser73-phospho c-Jun in parallel to c-Jun at 24 h.

Time course of c-Jun protein expression in TNF-treated granulosa cells: biphasic expression and MAPK cascades (Fig. 5)

The expression of c-Jun protein after TNF revealed a biphasic pattern with short-term induction (1–3 h) and long-term induction (12–24 h) (Fig. 5A). The time course of its phospho-forms (p63 and p73) was similar with that of c-Jun. Phospho-SAPK/JNK protein was quickly activated by TNF (1–3 h) and thereafter was not detectable (6–24 h). On the other hand, SAPK/JNK, phospho-p38MAPK, p38MAPK, phospho-ERK1/2 and ERK1/2 were not increased by TNF during the 24 h of culture (Fig. 5B). In fact, phospho-p38MAPK and phospho-ERK1/2 were not detectable.

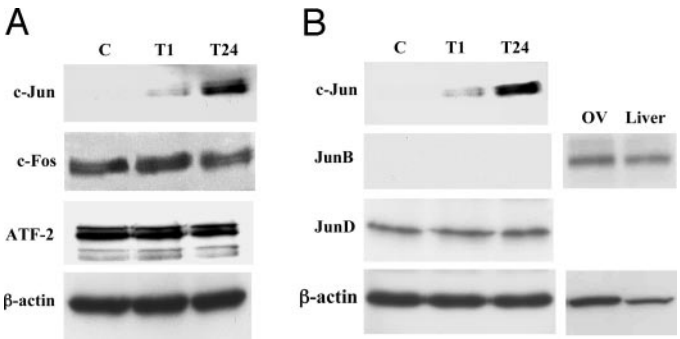

FIG. 2. *In vitro* effects of TNF on expression of c-Jun, c-Fos, ATF-2 (A), and Jun family members including c-Jun, JunB, and JunD (B) in mouse granulosa cells at 1 h (T1) and 24 h (T24) after TNF treatment (10 ng/ml). Each gel lane was loaded with 20 μg of total protein using whole cell lysates. β-Actin was used as a loading control and was reprobed from c-Jun (A) and JunB membranes (B). The whole ovary (OV) and the liver (Liver) from C57BL6 mice were used as positive controls for JunB. C, Control; T, TNF.

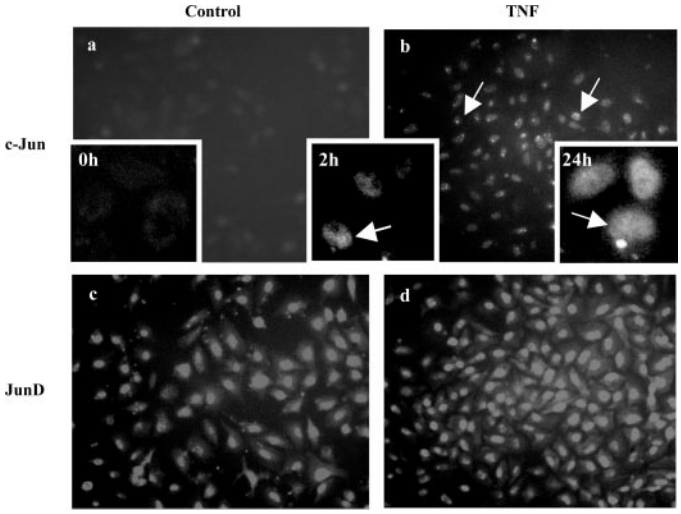

FIG. 3. *In vitro* effects of TNF (10 ng/ml) on cellular immunofluorescence of c-Jun and JunD. Magnification is ×100 except for *insets* at ×400. *White arrows* point to intense nuclear fluorescence. Cells were incubated with TNF (10 ng/ml) for 0, 2, or 24 h. A, c-Jun in control with no treatment after 24 h; B, c-Jun 24 h after TNF treatment; C, JunD in control with no treatment after 24 h; D, JunD 24 h after TNF treatment. Notice the larger number of cells in TNF treatment group stained for JunD (C *vs.* D).

Short-term induction of c-Jun protein by TNF and its MAPK cascades (Fig. 6)

Further analysis of the early increase in c-Jun protein revealed that it reached a maximum at 2 h after TNF (Fig. 6A). JunB was not expressed, and JunD was constitutively expressed and neither were affected by TNF. Phospho-SAPK/JNK protein was quickly increased by TNF within 15 min and thereafter remained quite low (Fig. 6B). Phospho-p38MAPK and phospho-ERK1/2 were not detectable with and without TNF treatment. Total SAPK/JNK, p38MARK, and ERK1/2 were constitutively expressed and unaffected by TNF.

Effect of MAPK inhibitors on TNF-induced c-Jun protein (Fig. 7)

A JNK inhibitor, SP600125 (10 μM) blocked the TNF-increase in c-Jun observed at 2 h (Fig. 7A). In contrast, SB203580 (10 μM), a p38MAPK inhibitor, and PD98059 (10 μM), an ERK inhibitor, did not block the increase in c-Jun by TNF at 2 h (Fig. 7A).

In long-term studies (24 h) (Fig. 7B), 5 and 10 μM SP600125 blocked the expression of c-Jun induced by TNF, but this was not observed at 1 μM. However, SP600125 at 10 μM was cytotoxic after 24 h incubation, as determined microscopically with cell rounding, floating, and detachment (data not shown). SB203580 and PD98059, each at 10 μM, did not block the TNF-increase in c-Jun protein.

Assessment of other TNF signaling cascades on c-Jun protein expression (Fig. 8)

After transient transfection of granulosa cells with p50/p65 vectors of NF-κB, the luciferase activity indicating activation of NF-κB, one of the main signaling cascades of TNF,

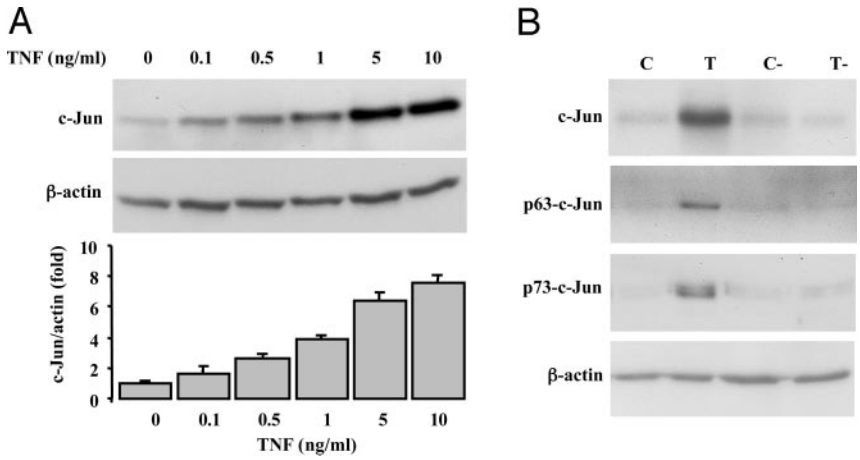

FIG. 4. Dose-dependent *in vitro* effects of TNF on c-Jun in cultured mouse granulosa cells from intact C57BL6 mice (A). Cells were incubated with 0, 0.1, 0.5, 1, 5, and 10 ng of TNF/ml of medium for 24 h. Effects of TNF on expression of c-Jun, Ser p63-c-Jun, and Ser p73-c-Jun in granulosa cells from intact (C, T) and TNFR1 knockout mice (C−, T−). Each lane was loaded with 20 μg total protein from whole cell lysates. β-Actin was used as a loading control and was reprobed from the p73-c-Jun membrane (B). A, Data represent mean and range relative to OD normalized by β-actin from two experiments, and therefore no statistics are included. C, Control-treated cells from C57BL6 mice; T, TNF treatment of cells from C57BL6 mice; C−, control-treated cells from TNFR1 knockout mice; T−, TNF treatment of cells from TNFR1 knockout mice; p63-c-Jun, serine 63-phospho-c-Jun; p73-c-Jun, serine 73-phospho-c-Jun.

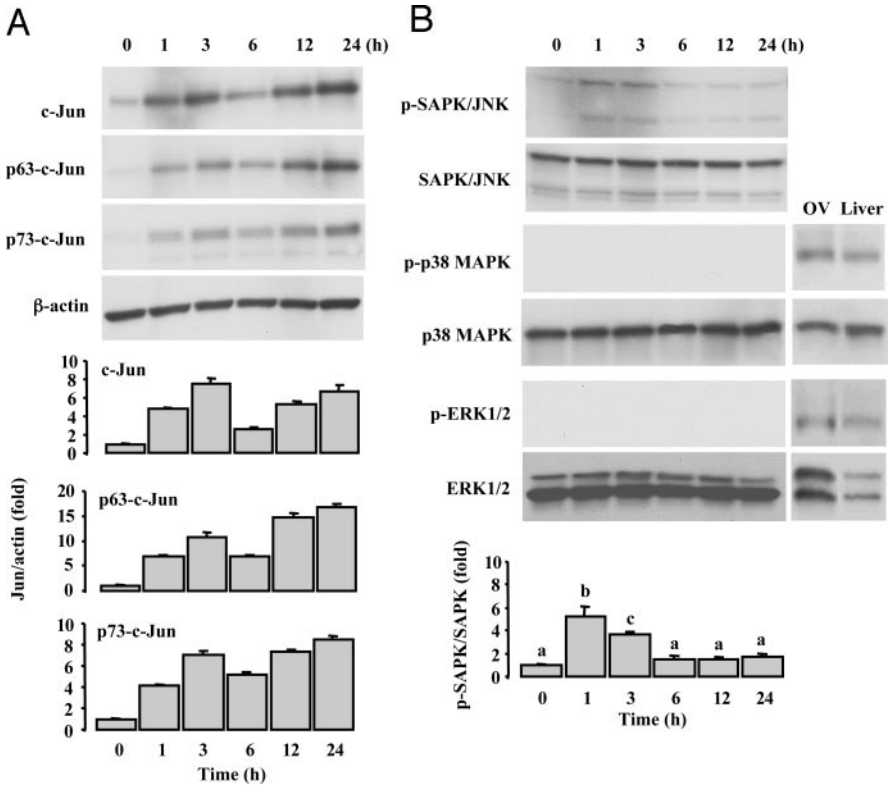

FIG. 5. *In vitro* effects of TNF (10 ng/ml) on the time course of Jun proteins (A) and MAPK induction (B) in mouse granulosa cells. Cells were incubated with TNF (10 ng/ml) for 0, 1, 3, 6, 12, and 24 h. Each lane was loaded with 20 μg total protein using whole cell lysates. β-Actin was used as a loading control for c-Jun and its phospho-forms and was reprobed from the p73-c-Jun membrane (A). A, Data represent mean and range relative to OD normalized by β-actin from two experiments with no statistical analyses. B, Data represent the mean and SEM from three experiments where *different superscripts* indicate significant difference ($P \leq 0.05$) between fold within a group compared with 0. The whole ovary (OV) and the liver (Liver) from C57BL6 mice were used as positive controls for MAPK phosphoproteins. Each total MAPK protein was used as loading controls for its MAPK phosphoproteins.

was increased approximately 17-fold (Fig. 8A, *right panel*). But activation of NF-κB did not affect the expression of c-Jun by 24 h (Fig. 8A, *left panel)*. TNF did not affect total NF-κB protein as determined by Western blots (data not shown). In addition, BAY 11-7082 and DEX, both well-known NF-κB inhibitors, did not alter the short-term induction of c-Jun by TNF at 2 h (Fig. 8B). Because BAY 11-7082 was very cytotoxic even at 1 μM concentration (data not shown), it could not be used for a 24-h exposure. C2-ceramide (1 μM) and cAMP (500 μM), each alone, did not increase c-Jun (Fig. 8C).

Involvement of c-Jun in cell proliferation (Fig. 9)

Antisense oligonucleotides for c-*jun* blocked completely the relative increase in granulosa cells induced by TNF, whereas its scrambled oligonucleotides did not block the increase (Fig. 9A). Also, antisense oligonucleotides for c-*jun* reduced the TNF increase in granulosa c-Jun protein, whereas its scrambled oligonucleotides had no effect (Fig. 9B). c-Fos protein was not altered by antisense c-Jun or its scrambled oligonucleotides (Fig. 9B).

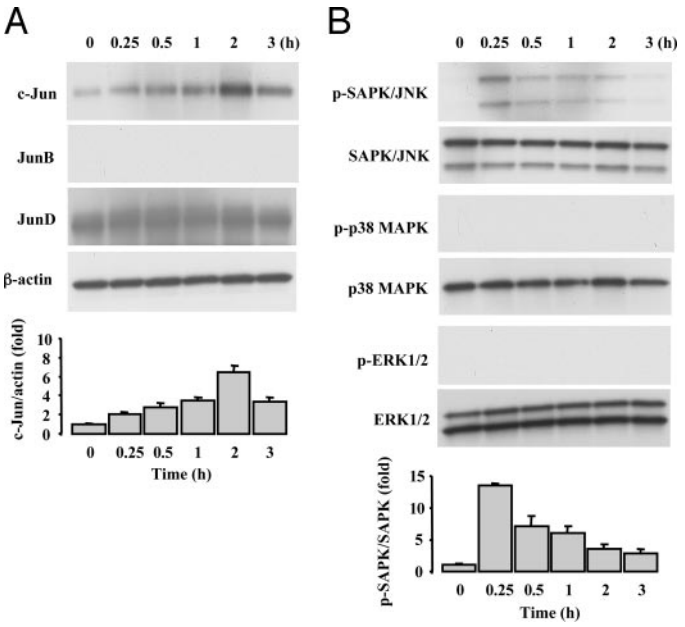

FIG. 6. Effects of TNF on short-term (0–3 h) induction of Jun proteins (A) and MAPKs (B). Cells were incubated with TNF (10 ng/ml) for 0, 0.25, 0.5, 1, 2, and 3 h. Each lane was loaded with 20 μg total protein from whole cell lysates. β-Actin was used as a loading control for Jun family and was reprobed from the JunB membrane (A). Data in panels A and B represent the mean and range relative to OD normalized by β-actin from two experiments and thus no statistical analyses were performed. Each total MAPK protein was used as loading controls for its MAPK phosphoproteins.

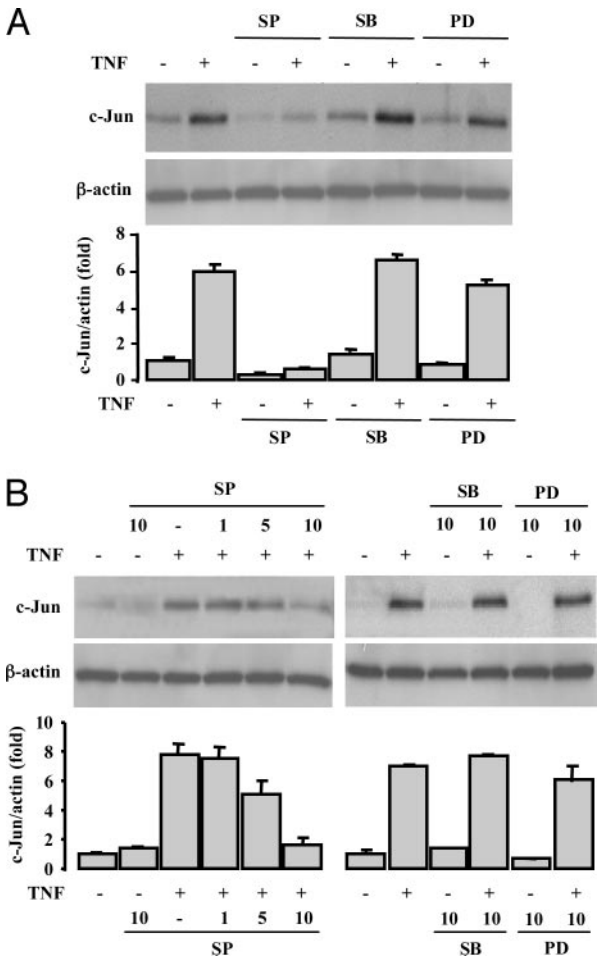

FIG. 7. Effects of SAPK/JNK, p38MAPK and ERK inhibitors on c-Jun expression in mouse granulosa cells after 2 h (A) and 24 h (B) of incubation with TNF (10 ng/ml). SP600125 (SP), a JNK inhibitor, was used at either 10 μM (A) or 1–10 μM (B). SB203580 (SB), a p38MAPK inhibitor, and PD98059 (PD), an ERK inhibitor, were used at 10 μM each. All MAPK inhibitors were incubated with the cells 1 h before addition of TNF. Each lane was loaded with 20 μg total protein from whole cell lysates. β-Actin was used as a loading control for c-Jun. Data represent mean and range relative to OD normalized by β-actin from two experiments.

Discussion

The main findings in the present study were that TNF increased c-Jun protein in a time- and dose-dependent manner in mouse granulosa cells through the TNFR1 and that blockage of c-Jun using antisense oligonucleotides decreased TNF-induced cell proliferation. The TNF-induced c-Jun protein was closely related with increases in the phosphorylated forms of SAPK/JNK. SAPK/JNK is one of three subfamilies of MAPK including also ERK1 and 2, and p38MAPK. In the mouse granulosa cell, TNF increased only the phosphorylated forms of SAPK/JNK (Figs. 5B and 6B). However, in other cell types TNF can increase all three subfamilies of MAPK (29, 30). The TNF-induced increase in phospho-SAPK/JNK in mouse granulosa cells appeared to be specific and related to c-Jun based on the following observations: 1) TNF activated only the phosphorylated form of SAPK/JNK within 15 min (not the nonphosphorylated form, Fig. 6B); 2) SP600125, a JNK inhibitor, blocked the increase in TNF-induced c-Jun protein (Fig. 7A); 3) phosphorylated and nonphosphorylated forms of ERK1 and 2 and p38MAPK did not change after TNF treatment; 4) SB203580 (a p38MAPK inhibitor) and PD98059 (an ERK inhibitor) had no effect on the increase in c-Jun protein induced by TNF (Fig. 7); and 5) lastly, there were no changes in immunoactive ATF-2 and c-*fos* (Fig. 2A). Previous studies using different cell types have shown that SAPK/JNK is involved in the regulation of c-Jun (19, 31), that ERK1 and 2 contribute to c-*fos* induction (31), and that p38MAPK contributes to induction of ATF-2 (19). The latter two did not change in response to TNF in the mouse granulosa cell (Fig. 2A). In addition, unpublished observations from our laboratory reveal that TNF did not affect phosphorylation of ATF-2 (data not shown).

Ser63 and Ser73 sites within c-Jun were phosphorylated in concert with the phosphoryation of SAPK/JNK at 1 and 3 h after TNF treatment but not at 12 and 24 h (Fig. 5, A and B). Interestingly, TNF induced the phosphorylation of c-Jun (Figs. 4 and 5), and this coincided with an increase in total c-Jun protein as revealed by the similar biphasic time courses between c-Jun and its phospho-forms (Fig. 5A). SAPK/JNK can phosphorylate the c-Jun *trans*-activating domain at Ser63 and Ser73 (19); thus, the present data are consistent with that observation.

The mechanism for the increase in c-Jun protein after TNF treatment in the mouse granulosa cell is currently unknown. However, most genes encoding AP-1 sites such as c-Jun behave as immediate-early genes and are induced rapidly without *de novo* protein synthesis (31). The increase in c-Jun

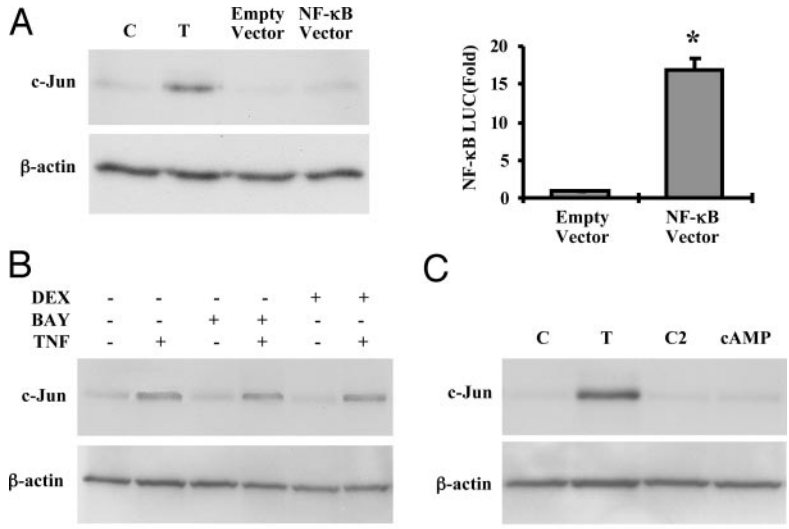

FIG. 8. Effects of NF-κB activation on the expression of c-Jun protein (A). After transient transfection for 3 h with p65-pRc/RSV and p50-pcDNAI vectors or the corresponding empty vector, cells were incubated for 24 h. A, *Right panel*, NF-κB activation using NF-κB luciferase vector. *Asterisk* indicates significant ($P \leq 0.05$) difference between groups as determined by the Student's *t* test. Data are mean ± SEM from three determinations for the luciferase activity. Effects of NF-κB inhibitors, DEX (1 μM), and BAY 11-7082 (BAY, 1 μM) on the short-term induction (2 h) of c-Jun by TNF (10 ng/ml) (B). Effects of C2-ceramide (C2, 1 μM) and cAMP (500 μM), each alone, on the expression of c-Jun protein at 24 h (C). BAY and DEX were added 1 h before addition of TNF (10 ng/ml). Each lane was loaded with 20 μg total protein from whole cell lysates. β-Actin was used as a loading control for c-Jun. C, Control; T, TNF.

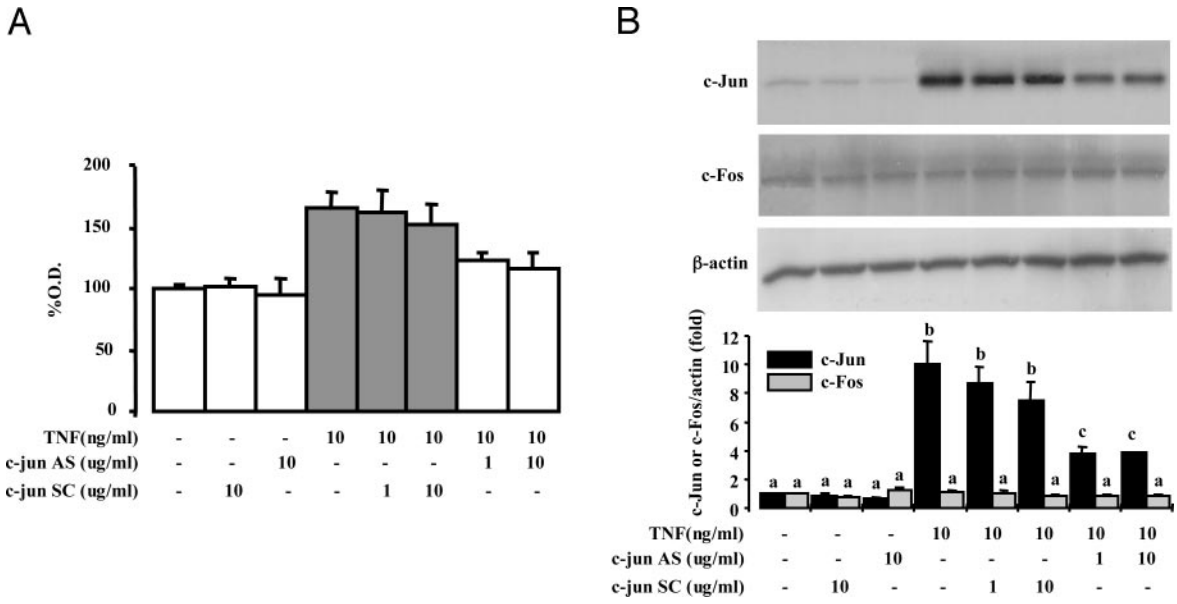

FIG. 9. Effects of antisense (AS) and scrambled (SC) oligonucleotides for c-*jun* on TNF-induced cell proliferation at 24 h (A). Effects of AS and SC oligonucleotides for c-*jun* on c-Jun and c-Fos protein expression in the absence or presence of TNF at 24 h (B). Cells were incubated with TNF for 24 h in the absence or presence of AS and SC oligonucleotides. Data represent mean ± SEM from eight determinations in at least four experiments (A). *Different color bars* indicate significance ($P \leq 0.05$) between groups using the Student-Newman-Keuls method (A). Data represent mean ± SEM relative to OD normalized by β-actin from three experiments (B). *Different superscripts* indicate significant difference ($P \leq 0.05$) between groups.

might be related to a decrease in protein degradation because phosphorylation of c-Jun has been shown to reduce ubiquitin-dependent degradation (32). Thus, the increases in c-Jun at 1–3 h and at 12–24 h after TNF treatment in mouse granulosa cells may be due to decreased degradation of c-Jun as observed in a different cell type (32). Whether the promoter of c-Jun is activated and leads to increased transcription after TNF treatment remains unknown. Possibly both reduced degradation and increased transcription may eventually account for the increase in c-Jun protein after TNF treatment.

The physiological roles of TNF in ovarian granulosa cell function still remain elusive. A combination of *in vitro* and *in vivo* studies shed some light on these roles. First of all, TNF has been identified in whole ovaries, oocytes, follicular fluid, corpora lutea, and granulosa cells of many species including rodents, cows, pigs, and humans and numerous potential roles of TNF in follicular development, steroidogenesis, and luteal function have been reported (33). *In vitro* TNF is well known to inhibit gonadotropin-induced granulosa cell and theca cell steroidogenesis (33), and the inhibition of steroidogenesis by TNF does not occur in TNFR1 knockout mice, indicating the necessity of the TNFR1 (15). TNFR1 knockout mice exhibit early puberty and undergo normal estrous cycles but enter periods of acyclicity associated with aging sooner than mice with intact TNFR1 receptors; whether this

early decline in cyclicity is due to hypothalamic or ovarian aging is unknown. Bioactive TNF has been identified in the immature rat ovary and TNF decreased in response to exogenous gonadotropins as follicular development proceeds (34). The present observation raises the question as to whether *in vivo* TNF has any role in granulosa cell proliferation. The granulosa cells used in the current study were from preantral and early antral follicles of nonovulatory 28-d-old immature mice. One role of TNF in the ovary may be to stimulate proliferation of granulosa in a cohort of small follicles that may be independent or only slightly dependent on gonadotropins for their growth. Previous *in vivo* studies using mice have shown maintenance and growth of small preantral follicles in the absence of the pituitary even though the absolute number of follicles was reduced when compared with control nonhypophysectomized mice (35). The effects of TNF on proliferation of granulosa cells from larger follicles including early antral and preovulatory follicles are unknown. There have been divergent reports even in the same species regarding effects of TNF on proliferation of granulosa cells. In granulosa cells, TNF has been reported to induce apoptosis (22, 23), have no effect (5, 16, 36–38), and induce proliferation (10, 25, 26). The reasons for these differences are unknown but may likely be related to species differences, culture conditions, and differentiation status of the granulosal cells. One report has indicated that TNF influences the balance between follicular growth (proliferation) and atresia (apoptosis) (13) with still unknown mechanisms. It is known from *in vitro* and *in vivo* studies that FSH stimulates granulosa cell proliferation in antral follicles. Thus, after administration of equine chorionic gonadotropin (eCG) (a FSH-like acting hormone) to immature rats, the decline in bioactive TNF in the ovary (34) could potentially slow growth of small preantral follicles while allowing synchronous growth of antral follicles in response to the exogenous gonadotropin. Another consideration is that TNF may increase proliferation (10, 13) as well as initial apoptosis in the granulosa of follicles (13). In healthy pig follicles, immunoreactive TNF was intense in the outer layer of granulosa and this correlated with increased TNF receptor-associated factor 2, the mRNAs for each and increased proliferating cells (10). In atretic follicles, TNF receptor-associated factor 2 was reduced and many apoptotic granulosa cells were observed.

Gonadotropins such as FSH and LH increase c-Jun in rat granulosa cells (39–41). The gonadotropins are known to act in synergy and promote the proliferation of granulosa cells that coincides with the growth of follicles. Whether c-Jun is required for gonadotropin-induced granulosa cell proliferation is unknown. In addition, physiological levels of estradiol *in vitro* have been shown to increase c-Jun in rat granulosa cells, whereas progesterone had no effect (42). It is well known that exogenous estradiol administered *in vivo* to immature mice and rats increases granulosa cell proliferation, although there are species-specific variations in the responses (43–45). A common intracellular signaling pathway for stimulation of granulosa cell proliferation may be c-Jun because gonadotropins, estradiol, and TNF activate this important bZIP transcription factor.

In contrast, luteinization of rat and pig granulosa cells coincided with a decrease in c-Jun and an increase of JunD (41, 46) and interestingly, luteinization is associated with a decrease in granulosal-luteal cell proliferation (47, 48). In fact, granulosa cell proliferation is significantly curtailed in the preovulatory follicles and corpora lutea after the LH surge (49). In addition, antiluteinization effects of *in vitro* TNF have been reported including inhibition of FSH-stimulated LH receptor in rat granulosa cells (12) and down-regulation of progesterone receptor in pig granulosa cells (13). Whether the latter are related to changes in c-Jun is unknown.

c-Jun has been detected in granulosa cells of atretic rat follicles implicating it in programmed granulosa cell death (50). This result seems to contrast with the present observations. However, increased tritiated thymidine has been detected in granulosa cells of atretic mouse follicles indicating that some cell division and/or DNA repair is ongoing during follicular demise (35).

It is well recognized that TNF activates NF-κB in many of its actions on cells (51). In fact, TNF activates NF-κB in rat granulosa cells (52). Addition of TNF to rat granulosa cells *in vitro* induces apoptosis only in the presence of cycloheximide (38, 52), indicating that normally in the absence of cycloheximide TNF is protective (38, 52). Two factors, X-linked inhibitor of apoptosis and Flice-like inhibitory protein, were identified after TNF stimulation of granulosa cells *in vitro* that protected the cells from apoptosis (38, 52). In the present study, transient transfection with p50 and p65 vectors representing active components of NF-κB had no effect on TNF-induced c-Jun (Fig. 8A). In addition, NF-κB inhibitors such as BAY 11-7082 and DEX did not alter the short-term induction of c-Jun by TNF (Fig. 8B). These observations indicate that TNF-induced c-Jun was likely independent of NF-κB. Collectively, the observations indicate the possibility that protection from apoptosis and induction of cell proliferation through c-Jun may be through different intracellular signaling mechanisms because activation of NF-κB did not induce cell proliferation (data not shown).

TNF also activates ceramide as a second messenger and ceramide *in vitro* inhibits gonadotropin-induced aromatase activity for estradiol secretion (53, 54). In the present study, C2-ceramide (1 μM) did not affect c-Jun in mouse granulosa cells (Fig. 8C), nor did it increase cell proliferation *in vitro* (data not shown). Thus, the inhibitory effects of TNF and ceramide on granulosa cell estradiol secretion may likely be independent of cell proliferation.

Because gonadotropins use cAMP as a second messenger and increase proliferation of granulosa cells *in vivo*, the effects of cAMP on c-Jun were investigated. cAMP did not induce c-Jun (Fig. 8C). Some studies have shown that TNF can reduce gonadotropin-stimulated cAMP in rat granulosa cells (5, 12), and others have reported that TNF did not alter cAMP levels in human granulosa cells (7, 55, 56); however, *in vitro* TNF can inhibit cAMP-induced steroidogenesis in humans (7) and mice (15). Although the present data indicate that cAMP signaling was not involved in TNF-induced c-Jun, whether cAMP, an inducer of luteinization and steroidogenesis, modifies the ability of TNF, an antiluteinization factor, to induce c-Jun and granulosa cell proliferation is unknown.

In summary, TNF induced c-Jun specifically and dose dependently in mouse granulosa cells, and this was medi-

ated by TNFR1 with a biphasic pattern of short-term (1–3 h) and long-term induction (12–24 h). Among the MAPK cascades, SAPK/JNK signaling appeared to be involved in TNF-induced c-Jun and blockage of c-Jun with antisense oligonucleotides reduced TNF-induced granulosa cell proliferation.

Acknowledgments

We thank Dr. Tom Maniatis (Harvard University, Cambridge, MA) for p65-pRc/RSV and p50-pcDNAI vectors.

Received July 11, 2003. Accepted November 5, 2003.

Address all correspondence and requests for reprints to: Dr. Paul F. Terranova, Center of Reproductive Sciences, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66160-7417. E-mail: pterrano@kumc.edu.

This research was supported by grants from the National Cancer Institute (CA50616, to P.F.T.) and National Institute of Child Health and Human Development (Specialized Cooperative Center Program in Reproduction Research, U54HD33994).

References

1. **Zolti M, Meirom R, Shemesh M, Wollach D, Mashiach S, Shore L, Rafael ZB** 1990 Granulosa cells as a source and target organ for tumor necrosis factor-α. FEBS Lett 261:253–255
2. **Chen HL, Marcinkiewicz JL, Sancho-Tello M, Hunt JS, Terranova PF** 1993 Tumor necrosis factor-α gene expression in mouse oocytes and follicular cells. Biol Reprod 48:707–714
3. **Marcinkiewicz JL, Krishna A, Cheung CMY, Terranova PF** 1994 Oocytic tumor necrosis factor α: localization in the neonatal ovary and throughout follicular development in the adult rat. Biol Reprod 50:1251–1260
4. **Kondo H, Maruo T, Mochizuki M** 1995 Immunohistochemical evidence for the presence of tumor necrosis factor-α in the infant and adult human ovary. Endocr J 42:771–780
5. **Adashi EY, Resnick CE, Croft CS, Payne DW** 1989 Tumor necrosis factor α inhibits gonadotropin hormonal action in nontransformed ovarian granulosa cells. A modulatory noncytotoxic property. J Biol Chem 264:11591–11597
6. **Spicer LJ, Alpizar E** 1994 Effects of cytokines on FSH-induced estradiol production by bovine granulosa cells in vitro: dependence on size of follicle. Domest Anim Endocrinol 11:25–34
7. **Montgomery Rice V, Limback SD, Roby KF, Terranova PF** 1999 Tumor necrosis factor α inhibition of follicle-stimulating hormone-induced granulosa cell estradiol secretion in the human does not involve reduction of cAMP secretion but inhibition at post-cAMP site(s). Endocrine 10:19–23
8. **Ghersevich S, Isomaa V, Vihko P** 2001 Cytokine regulation of the expression of estrogenic biosynthetic enzymes in cultured rat granulosa cells. Mol Cell Endocrinol 172:21–30
9. **Lanuza GM, Saragueta PE, Bussmann UA, Baranao JL** 2002 Paradoxical effects of tumour necrosis factor-α on rat granulosa cell DNA synthesis. Reprod Fertil Dev 14:133–139
10. **Nakayama M, Manabe N, Inoue N, Matsui T, Miyamoto H** 2003 Changes in the expression of tumor necrosis factor (TNF) α, TNFα receptor (TNFR) 2, and TNFR-associated factor 2 in granulosa cells during atresia in pig ovaries. Biol Reprod 68:530–535
11. **Brannstrom M, Bonello N, Wang LJ, Norman RJ** 1995 Effects of tumour necrosis factor α (TNF α) on ovulation in the rat ovary. Reprod Fertil Dev 7:67–73
12. **Darbon JM, Oury F, Laredo J, Bayard F** 1989 Tumor necrosis factor-α inhibits follicle-stimulating hormone-induced differentiation in cultured rat granulosa cells. Biochem Biophys Res Commun 163:1038–1046
13. **Prange-Kiel J, Kreutzkamm C, Wehrenberg U, Rune GM** 2001 Role of tumor necrosis factor in preovulatory follicles of swine. Biol Reprod 65:928–935
14. **Cianci A, Calogero AE, Palumbo MA, Burrello N, Ciotta L, Palumbo G, Bernardini R** 1996 Relationship between tumour necrosis factor α and sex steroid concentrations in the follicular fluid of women with immunological infertility. Hum Reprod 11:265–268
15. **Roby KF, Son DS, Terranova PF** 1999 Alterations of events related to ovarian function in tumor necrosis factor receptor type 1 knockout mice. Biol Reprod 61:1616–1621
16. **Spicer LJ** 1998 Tumor necrosis factor-α (TNF-α) inhibits steroidogenesis of bovine ovarian granulosa and thecal cells in vitro. Involvement of TNF-α receptors. Endocrine 8:109–115
17. **Spicer LJ** 2001 Receptors for insulin-like growth factor-I and tumor necrosis factor-α are hormonally regulated in bovine granulosa and thecal cells. Anim Reprod Sci 67:45–58
18. **Zhang M, Tracey KJ** 1998 Tumor necrosis factor. In: Thamson A, ed. The cytokine handbook. San Diego: Academic Press; 517–548
19. **Kyriakis JM** 1999 Activation of the AP-1 transcription factor by inflammatory cytokines of the TNF family. Gene Expr 7:217–231
20. **Leppä S, Bohmann D** 1999 Diverse functions of JNK signaling and c-Jun in stress response and apoptosis. Oncogene 18:6158–6162
21. **Mechta-Grigoriou F, Gerald D, Yaniv M** 2001 The mammalian Jun proteins: redundancy and specificity. Oncogene 20:2378–2389
22. **Basini G, Mainardi GL, Bussolati S, Tamanini C** 2002 Steroidogenesis, proliferation and apoptosis in bovine granulosa cells: role of tumour necrosis factor-α and its possible signalling mechanisms. Reprod Fertil Dev 14:141–150
23. **Sasson R, Winder N, Kees S, Amsterdam A** 2002 Induction of apoptosis in granulosa cells by TNF α and its attenuation by glucocorticoids involve modulation of Bcl-2. Biochem Biophys Res Commun 294:51–59
24. **Wada S, Manabe N, Nakayama M, Inou N, Matsui T, Miyamoto H** 2002 TRAIL-decoy receptor 1 plays inhibitory role in apoptosis of granulosa cells from pig ovarian follicles. J Vet Med Sci 64:435–439
25. **Wang LJ, Brannstrom M, Robertson SA, Norman RJ** 1992 Tumor necrosis factor α in the human ovary: presence in follicular fluid and effects on cell proliferation and prostaglandin production. Fertil Steril 58:934–940
26. **Yan Z, Hunter V, Weed J, Hutchison S, Lyles R, Terranova P** 1993 Tumor necrosis factor α alters steroidogenesis and stimulates proliferation of human granulosal cells in vitro. Fertil Steril 59:332–338
27. **Peluso JJ, Luciano AM, Pappalardo A, White BA** 1995 Cellular and molecular mechanisms that mediate insulin-dependent rat granulosa cell mitosis. Biol Reprod 52:124–130
28. **Sambrook J, Fritsch EF, Maniatis T** 1989 Detection and analysis of proteins expressed from cloned genes. Molecular cloning: a laboratory manual. 2nd ed. Vol 3. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 18: 18.47–18.75
29. **Clerk A, Harrison JG, Long CS, Sugden PH** 1999 Pro-inflammatory cytokines stimulate mitogen-activated protein kinase subfamilies, increase phosphorylation of c-Jun and ATF2 and upregulate c-Jun protein in neonatal rat ventricular myocytes. J Mol Cell Cardiol 31:2087–2099
30. **Chen CC, Sun YT, Chen JJ, Chang YJ** 2001 Tumor necrosis factor-α-induced cyclooxygenase-2 expression via sequential activation of ceramide-dependent mitogen-activated protein kinases, and IκB kinase 1/2 in human alveolar epithelial cells. Mol Pharmacol 59:493–500
31. **Karin M** 1995 The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 270:16483–16486
32. **Musti AM, Treier M, Bohmann D** 1997 Reduced ubiquitin-dependent degradation of c-Jun after phosphorylation by MAP kinases. Science 275:400–402
33. **Terranova PF, Rice VM** 1997 Review: cytokine involvement in ovarian processes. Am J Reprod Immunol 37:50–63
34. **Rice VM, Limback SD, Roby KF, Terranova PF** 1998 Changes in circulating and ovarian concentrations of bioactive tumour necrosis factor α during the first ovulation at puberty in rats and in gonadotrophin-treated immature rats. J Reprod Fertil 113:337–341
35. **Wang XN, Greenwald GS** 1993 Hypophysectomy of the cyclic mouse. I. Effects of folliculogenesis, oocyte growth and follicle-stimulating hormone and human chorionic gonadotropin receptors. Biol Reprod 48:585–594
36. **Veldhuis JD, Garmey JC, Urban RJ, Demers LM, Aggarwal BB** 1991 Ovarian actions of tumor necrosis factor-α (TNF α): pleiotropic effects of TNF α on differentiated functions of untransformed swine granulosa cells. Endocrinology 129:641–648
37. **Best CL, Pudney J, Anderson DJ, Hill JA** 1994 Modulation of human granulosa cell steroid production in vitro by tumor necrosis factor α: implication of white blood cells in culture. Obstet Gynecol 84:121–127
38. **Xiao CW, Asselin E, Tsang BK** 2002 Nuclear factor κB-mediated induction of Flice-like inhibitory protein prevents tumor necrosis factor α-induced apoptosis in rat granulosa cells. Biol Reprod 67:436–441
39. **Delidow BC, Lynch JP, White BA, Peluso JJ** 1992 Regulation of proto-oncogene expression and deoxyribonucleic acid synthesis in granulosa cells of perifused immature rat ovaries. Biol Reprod 47:428–435
40. **Ness JM, Kasson BG** 1992 Gonadotropin regulation of c-fos and c-jun messenger ribonucleic acids in cultured rat granulosa cells. Mol Cell Endocrinol 90:17–25
41. **Sharma SC, Richards JS** 2000 Regulation of AP1 (Jun/Fos) factor expression and activation in ovarian granulosa cells. Relation of JunD and Fra2 to terminal differentiation. J Biol Chem 275:33718–33728
42. **Shelley ME, Hossain A, McDonough PG, Khan I** 1994 Differential c-jun gene expression with tonically administered steroids in rat ovary and uterus. Am J Obstet Gynecol 170:1410–1415
43. **Kim I, Greenwald GS** 1987 Effect of estrogens on follicular development and ovarian and uterine estrogen receptors in the immature rabbit, guinea pig and mouse. Endocrinol Jpn 34:871–878
44. **Kim I, Greenwald GS** 1989 Effects of progesterone or luteinizing hormone on folliculogenesis in intact or hypophysectomized immature hamsters. Biol Reprod 41:74–78
45. **Wang XN, Greenwald GS** 1993 Synergistic effects of steroids with FSH on folliculogenesis, steroidogenesis and FSH- and hCG-receptors in hypophysectomized mice. J Reprod Fertil 99:403–413
46. **Rusovici R, LaVoie HA** 2003 Expression and distribution of AP-1 transcription factors in the porcine ovary. Biol Reprod 69:64–74

47. **Monniaux D, Huet C, Besnard N, Clement F, Bosc M, Pisselet C, Monget P, Mariana JC** 1997 Follicular growth and ovarian dynamics in mammals. J Reprod Fertil Suppl 51:3–23
48. **Hillier SG** 2001 Gonadotropic control of ovarian follicular growth and development. Mol Cell Endocrinol 179:39–46
49. **Rao MC, Midgley Jr AR, Richards JS** 1978 Hormonal regulation of ovarian cellular proliferation. Cell 14:71–78
50. **Zhou J, Refuerzo J, Bondy C** 1995 Granulosa cell DNA synthesis is strictly correlated with the presence of insulin-like growth factor I and absence of c-fos/c-jun expression. Mol Endocrinol 9:924–931
51. **Leong KG, Karsan A** 2000 Signaling pathways mediated by tumor necrosis factor α. Histol Histopathol 15:1303–1325
52. **Xiao CW, Ash K, Tsang BK** 2001 Nuclear factor-κB-mediated X-linked inhibitor of apoptosis protein expression prevents rat granulosa cells from tumor necrosis factor α-induced apoptosis. Endocrinology 142:557–563
53. **Santana P, Llanes L, Hernandez I, Gallardo G, Quintana J, Gonzalez J, Estevez F, Ruiz de Galarreta C, Fanjul LF** 1995 Ceramide mediates tumor necrosis factor effects on P450-aromatase activity in cultured granulosa cells. Endocrinology 136:2345–2348
54. **Kaipia A, Chun SY, Eisenhauer K, Hsueh AJ** 1996 Tumor necrosis factor-α and its second messenger, ceramide, stimulate apoptosis in cultures ovarian follicles. Endocrinology 137:4864–4870
55. **Rice VM, Williams VR, Limback SD, Terranova PF** 1996 Tumour necrosis factor-α inhibits follicle-stimulating hormone-induced granulosa cell oestradiol secretion in the human: dependence on size of follicle. Hum Reprod 11:1256–1261
56. **Montgomery Rice V, Limback SD, Roby KF, Terranova PF** 1998 Differential responses of granulosa cells from small and large follicles to follicle stimulating hormone (FSH) during the menstrual cycle and acyclicity: effects of tumour necrosis factor-α. Hum Reprod 13:1285–1291

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**