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Excessive ovarian production of nerve growth factor elicits granulosa cell apoptosis by setting in motion a tumor necrosis factor α/stathmin-mediated death signaling pathway

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Abstract

Excessive nerve growth factor (NGF) production by the ovary, achieved via a transgenic approach, results in arrested antral follicle growth, reduced ovulatory capacity, and a predisposition to cyst formation in response to mildly elevated LH levels. Two salient features in these mutant mice (termed 17NF) are an elevated production of 17α-hydroxyprogesterone (17-OHP$_4$), testosterone, and estradiol (E$_2$) in response to gonadotropins, and an increased frequency of granulosa cell (GC) apoptosis. In this study, we show that the increase in steroidal response is associated with enhanced expression of *Cyp17a1*, *Hsd17b*, and *Cyp19a1*, which encode the enzymes catalyzing the synthesis of 17-OHP$_4$, testosterone, and E$_2$ respectively. Using a proteomic approach, we identified stathmin (STMN1), as a protein that is overproduced in 17NF ovaries. In its phosphorylated state, STMN1 mediates a cell death signal initiated by tumor necrosis factor α (TNF). STMN1 is expressed in GCs and excessive NGF increases its abundance as well as that of its forms phosphorylated at serine (Ser) 16, 25, and 38. TNF synthesis is also increased in 17NF ovaries, and this change is abolished by blocking neurotrophic tyrosine kinase receptors. Inhibiting TNF actions *in vivo* by administering a soluble TNF receptor prevented the increase in total and phosphorylated STMN1 production, as well as GC apoptosis in NGF-overproducing ovaries. These results indicate that an excess of NGF in the ovary promotes steroidogenesis by enhancing the expression of enzyme genes involved in 17-OHP$_4$, testosterone, and E$_2$ synthesis, and causes GC apoptosis by activating a TNF/ STMN1-mediated cell death pathway.
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Introduction

Regardless of the physiological role that nerve growth factor (NGF) may play in the regulation of normal tissue functions, its excess has been shown to initiate pathological changes in both endocrine and non-endocrine tissues (Davis *et al*. 1997, Hoyle *et al*. 1998, Edwards *et al*. 2005). The ovary is no exception as the development of follicular cysts in rats treated with estradiol (E$_2$) valerate is associated with overproduction of NGF in the gland (Lara *et al*. 2000, Stener-Victorin *et al*. 2000). This excess and that of the low-affinity NGF receptor (NGFR; also commonly known as the p75 neurotrophin receptor) are responsible, to a significant extent, for some of the ovarian abnormalities observed in these rats (Lara *et al*. 2000). Consistent with these findings, a selective increase in intraovarian NGF content via grafting of cells genetically engineered to produce NGF initiated several of the structural and functional alterations associated with the development of follicular cysts in the rat ovary, including appearance of precystic structures, an increase in the number of apoptotic follicles, and hyperandrogenemia (Dissen *et al*. 2000*a*). Thus, ovarian NGF may not only contribute to regulating normal follicle growth, but if produced at persistently elevated levels, it may also initiate ovarian pathology.

To more precisely define the mechanisms underlying this pathology, we generated transgenic mice carrying the NGF gene under the control of the 17α-hydroxylase/C$_{17–20}$ lyase (17α-OH) promoter (Dissen *et al*. 2009). Because this promoter is specifically expressed in

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androgen-producing cells (Gore-Langton & Armstrong 1994), these animals (termed 17NF) show selective overexpression of NGF in thecal/interstitial cells of the ovary (Dissen *et al*. 2009), the normal site of NGF production. Reproductive function is compromised; the age at vaginal opening was delayed by 1 week, and the age of the first fertile estrous cycle (determined by measuring the interval from exposure to a male and production of a litter of pups) was delayed by almost 2 months. This reduced reproductive capacity carries over into a lengthening of the interval between subsequent litters. Both the number of litters per dam and the number of pups per litter were reduced by 50%. Resembling the effect of local NGF overproduction by genetically engineered cells (Dissen *et al*. 2000*a*), the ovaries of NGF-overexpressing mice show accumulation of antral follicles, which are arrested at a medium–intermediate stage (Dissen *et al*. 2009). This developmental arrest is accompanied by a selective increase in 17α-hydroxyprogesterone (17-OHP_4), testosterone, and E_2 production in response to pregnant mare serum gonadotropin (PMSG), and an enhanced incidence of granulosa cell (GC) apoptosis.

We undertook this study to gain insights into the intraovarian mechanisms that may contribute to this dual ovarian phenotype in 17NF mice. We first determined if the enhanced 17-OHP_4, testosterone, and E_2 response to gonadotropins observed in 17NF mice is related to an increased expression of the genes encoding steroidogenic enzymes involved in the synthesis of these steroids. We then used a proteomic approach to identify proteins that may contribute to increase GC apoptosis in 17NF ovaries, and obtained results implicating stathmin (STMN1), a critical intermediate of the signaling pathway used by tumor necrosis factor (TNF) to promote cell death (Vancompernolle *et al*. 2000), as a major component of NGF-dependent GC apoptosis. A preliminary report of these findings has been published (Garcia-Rudaz *et al*. 2008).

Results

Excessive ovarian production of NGF results in selective changes in the expression of genes encoding steroidogenic enzymes

We previously observed that the ovaries from 17NF mice produced a slight, but significant increase in basal serum P_4 levels and release more 17-OHP_4, testosterone, and E_2 than wild-type (WT) mice in response to PMSG (Dissen *et al*. 2009; Fig. 1A). These increases are accompanied by a decrease in the release of P_4 following PMSG (Dissen *et al*. 2009; Fig. 1A). It was, therefore, of interest to determine whether the expression of genes encoding enzymes involved in the synthesis of these steroids is altered by the overproduction of NGF. No differences in the content of *Cyp11a1* mRNA were observed between WT and 17NF ovaries, although in both cases the mRNA levels increased in response to PMSG (Fig. 1B). *Cyp11a1* mRNA encodes the enzyme cytochrome P450, family 11, subfamily A, polypeptide 1 (also known as cytochrome P450 cholesterol side-chain cleavage enzyme), which catalyzes the conversion of cholesterol to pregnenolone. The abundance of *Star* mRNA was increased in untreated 17NF mice (Fig. 1B), suggesting that an augmented expression of STAR contributes to the elevated serum P_4 observed in mutant mice not exposed to PMSG (Dissen *et al*. 2009). This was the only change observed in the genes encoding the steroidogenic enzymes under basal conditions (Fig. 1B). Following PMSG treatment there was less *Star* mRNA in ovaries from 17NF mice than WT mice (Fig. 1B), coinciding with the decline in P_4 observed in these mice (Dissen *et al*. 2009). No differences in the abundance of *Hsd3b1* mRNA were found between WT and 17NF mice (Fig. 2B). This mRNA encodes hydroxy-δ-5-steroid dehydrogenase, 3β-, and steroid δ-isomerase 1 (also known as 3β-hydroxysteroid dehydrogenase (HSD3B1)), the enzyme that catalyzes the conversion of pregnenolone to P_4. The content of the mRNA encoding cytochrome P450, family 17, subfamily A, polypeptide 1 (more commonly known as 17α-hydroxylase/$C_{17–20}$ lyase; *Cyp17a1*), the enzyme that catalyzes the formation of 17-OHP_4 from P_4 (Fig. 1A) was increased in 17NF ovaries in response to PMSG (Fig. 1B). The levels of the mRNA encoding 17β-hydroxysteroid (17β) dehydrogenase 1 also known as 17β-hydroxysteroid dehydrogenase type 1 (*Hsd17b1*), which catalyzes the conversion of androstenedione to testosterone and estrone to E_2 (Fig. 1A) were also elevated in 17NF mice treated with PMSG (Fig. 1B). The increase in *Hsd17b1* mRNA content was specific to isoform 1 as the expression of isoform 4 (*Hsd17b4*) was not altered in the transgenic mice, even after PMSG treatment (data not shown). Finally, the mRNA abundance of *Cyp19a1*, which encodes cytochrome P450, family 19, subfamily A, polypeptide 1, the P450 aromatase (CYP19A1) enzyme that catalyzes the formation of E_2 and estrone from testosterone and androstenedione (respectively), rose more in 17NF ovaries in response to PMSG (Fig. 1B).

A proteomics approach revealed preferential expression of a protein involved in growth arrest in the ovaries from 17NF mice

To identify differentially expressed proteins in 17NF mice, we subjected ovarian lysates from WT and 17NF mice to two-dimensional gel electrophoresis-mass spectrometric (MS) analysis. Several spots were differentially expressed in the two-dimensional gel (Fig. 2). Spot quantification and statistical analysis (Phoretix 2D Evolution Software, PerkinElmer, Inc., Boston, MA, USA) of the gel identified four spots (2, 4, 5, and 6) as

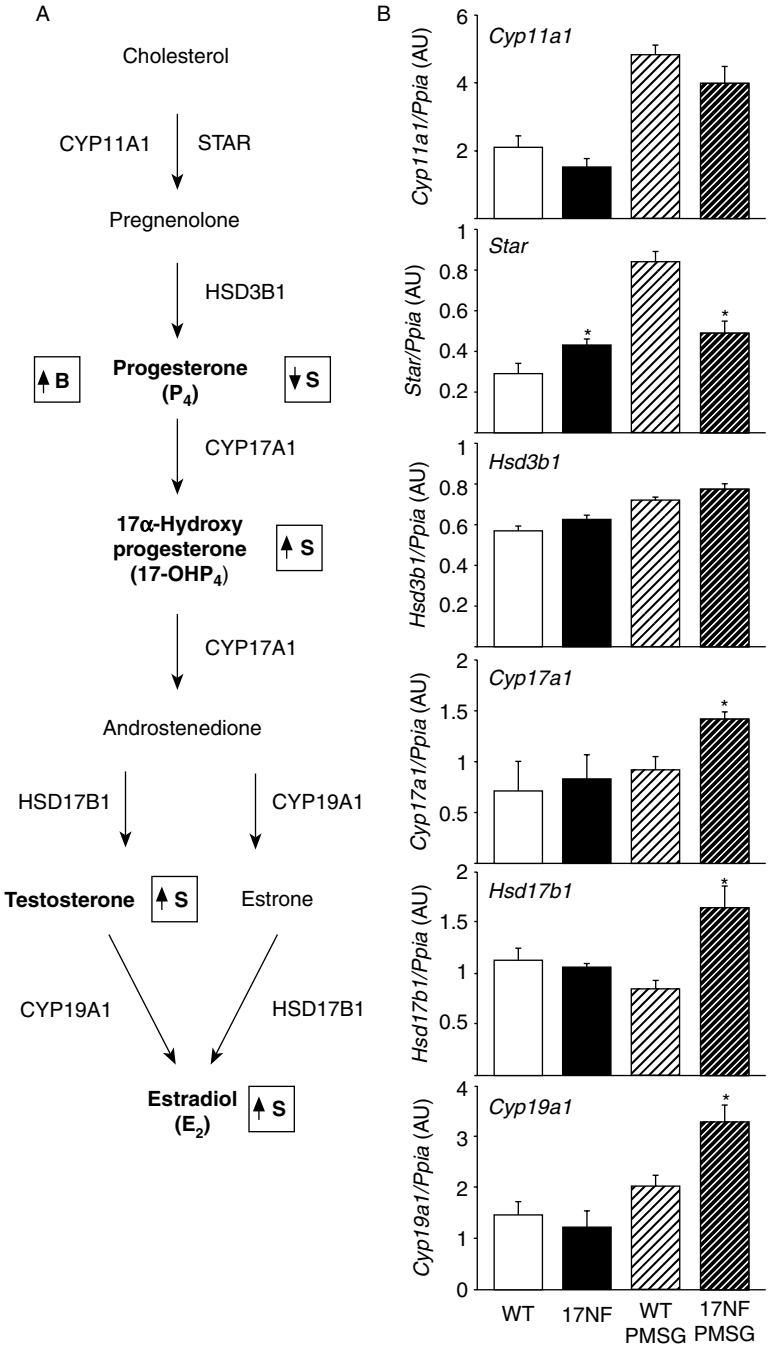

Figure 1 Expression of mRNAs encoding steroidogenic enzymes and STAR in the ovarian steroid hormone pathway. (A) Diagram of the ovarian steroidogenic pathway and the enzymes involved from cholesterol to E_2. Steroids whose levels in serum were previously shown to be altered in 17NF transgenic mice (Dissen *et al.* 2009) are shown in bold; down arrow, suppression; up arrow, increase; B, basal conditions; S, PMSG-stimulated conditions (see text). CYP11A1, cytochrome P450 cholesterol side-chain cleavage enzyme; STAR, steroidogenic acute regulatory protein; HSD3B1, 3β-hydroxysteroid dehydrogenase; CYP17A1, 17α-hydroxylase/$C_{17–20}$ lyase; HSD17B1, 17β-hydroxysteroid dehydrogenase type 1; CYP19A1, P450 aromatase. (B) Semi-quantitative PCR measurement of the mRNAs encoding six steroidogenic enzymes, and STAR, in the ovary of WT and NGF-overexpressing transgenic mice (17NF). *Ppia*, peptidylprolyl isomerase A (cyclophilin A); AU, arbitrary units. The ovaries were collected from 28- to 30-day-old animals. Each bar is the mean of 5 mice; vertical lines are S.E.M.; *$P<0.05$ versus respective WT groups.

having the highest degree of statistical confidence (100%, see Materials and Methods). Spots 4, 5, and 6 correspond to translationally modified forms of apolipoprotein AI (ApoAI), the major apoprotein of high-density lipoprotein (HDL). While the more basic spot (no. 6) – predominantly expressed in 17NF ovaries – represents proApoAI, the more acidic spots (4 and 5) represent biologically active, mature ApoAI isoforms, resulting from covalent phosphorylation of the pro-isoform (Beg *et al.* 1989). On the other hand, spot 2 corresponds to the phosphorylated form of STMN1/phosphoprotein p19 (STMN1), a developmentally regulated phosphoprotein (Doye *et al.* 1989) that becomes rapidly phosphorylated in response to signals leading to cell growth arrest (Braverman *et al.* 1986, Zhu *et al.* 1989).

To determine if STMN1 abundance is increased in 17NF ovaries, we assessed the content of the protein by both immunohistochemistry and western blot analysis using 30-day-old WT and 17NF mice. The immunohistochemical analysis revealed that STMN1 is mostly expressed in GCs, and that the level of expression is greater in follicles from 17NF mice than WT controls

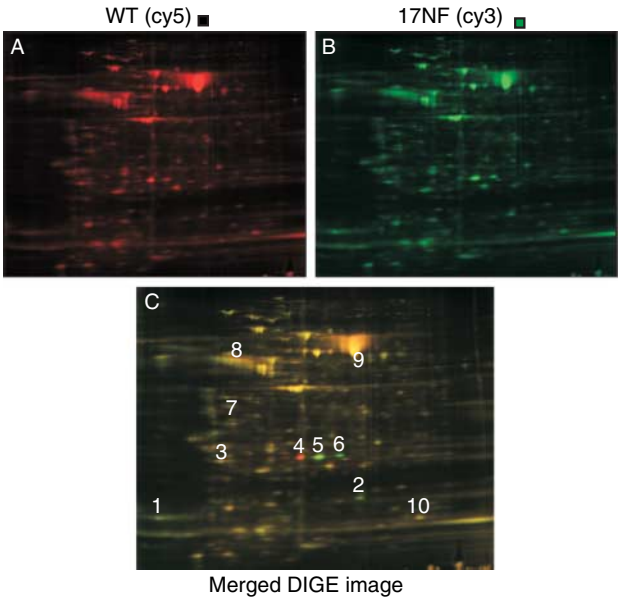

Figure 2 Protein expression profiles in 17NF and WT (B6D2) ovaries revealed by fluorescence two-dimensional differential gel electrophoresis (2-DIGE). Lysates (100 μg protein) were labeled with (A) Cy5 (WT, red color) and (B) Cy3 (17NF, green color) respectively. (C) Images from A and B were merged; numbers (1–10) point to differentially expressed proteins. Among these spots, four of them, spots 2, 4, 5, and 6 were identified with 100% statistical confidence, based on the sequence of more than two diagnostic peptides per protein (see Materials and Methods). Spots 4, 5, and 6 correspond to translationally modified forms of apolipoprotein AI (ApoAI), the major apoprotein of HDL. Spot 2 corresponds to the phosphorylated form of STMN1. For additional details see text.

(Fig. 3A and B). This difference is evident in both preantral (Fig. 3C and D) and antral follicles (Fig. 3E and F). Sections incubated without primary antibody exhibited no detectable immunostaining (data not shown). Consistent with these immunohistochemical observations and those of the two-dimensional gel analysis, STMN1 abundance, quantified by western blots, was significantly ($P<0.05$) increased in the ovaries from 17NF mice compared with WT controls (Fig. 3G).

STMN1 phosphorylation is increased in 17NF ovaries

STMN1 is a cytoplasmic phosphoprotein highly expressed in rapidly proliferating tissues (Braverman *et al.* 1986, Rubin & Atweh 2004). It regulates microtubule assembly by promoting microtubule depolymerization (Rubin & Atweh 2004), an event required for the formation of the mitotic spindle, a structure critical for cell division. The actions of STMN1 are terminated by phosphorylation; for instance, activation of the apoptosis signal-regulating kinase 1 (ASK1)/p38 MAP kinase complex, results in STMN1 phosphorylation so that the microtubule destabilizing activity of STMN1 is turned off (Mizumura *et al.* 2006). Cell death then ensues via a mitochondrial-dependent pathway not yet well characterized. STMN1 phosphorylation at serine (Ser) 16, 25, 38, and 63 (henceforth referred to as 16P, 25P, 38P, and 63P) accounts for all the major functional STMN1 phosphor-forms *in vivo* (Beretta *et al.* 1993). To determine the pattern of STMN1 phosphorylation in the ovaries of 17NF mice, we used antibodies that specifically recognize 16P, 25P, and 38P (Gavet *et al.* 1998). The antibodies also recognize a reduced electrophoretic mobility form of phosphorylated STMN1, known as spot '17', which migrates as a 23-kDa species (Gavet *et al.* 1998). The ovaries of 17NF mice showed a marked increase in the 19-kDa STMN1 species phosphorylated at 16P, 25P, and 38P compared with WT mice (Fig. 4A–D). In addition to the 19-kDa species, the lower mobility 23 kDa 25P and 38P forms were also highly expressed in the ovaries of 17NF mice compared with those of WT mice (Fig. 4A, C, and D) respectively. Interestingly, neither 17NF nor WT ovaries showed a 23 kDa 16P form (Fig. 4A and B),

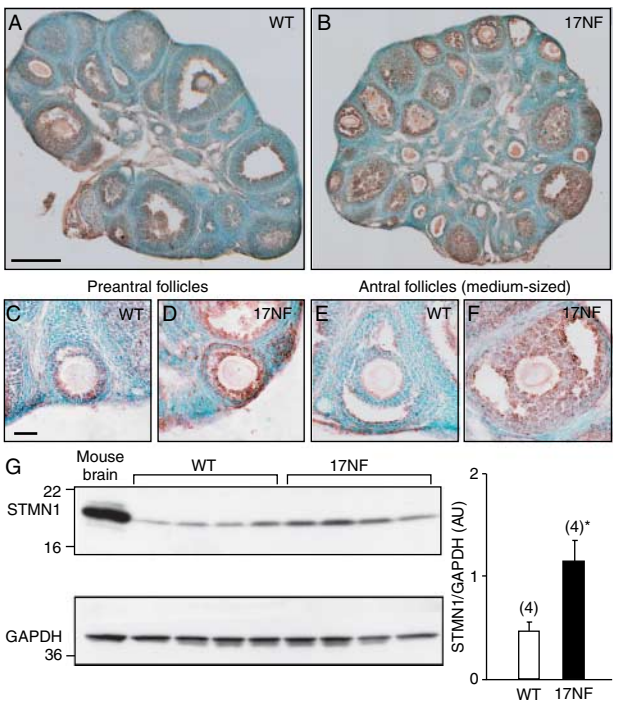

Figure 3 STMN1 is predominantly expressed in GCs of antral follicles, and is more abundant in the ovaries from 17NF mice than in those of WT animals. (A) Immunoreactive STMN1 in WT ovaries. (B) Increased abundance of STMN1 in GCs of 17NF ovaries; bar = 200 μm. Notice the increased number of small antral follicles, and the absence of large, preovulatory follicles in the 17NF ovary. (C–F) Immunoreactive STMN1 is more abundant in preantral (C and D) and medium-sized antral follicles (E and F) of 17NF animals than WT controls. Sections incubated without primary antibody exhibited no discernible immunostaining (not shown). Ten sections from two animals of each genotype were examined; representative sections are presented; bar = 50 μm. (G) Ovarian STMN1 levels quantified by western blot analysis are greater (*$P=0.02$) in 17NF ovaries compared with WT ovaries. AU, arbitrary units; vertical lines represent S.E.M. and numbers at top of bars are number of mice per group.

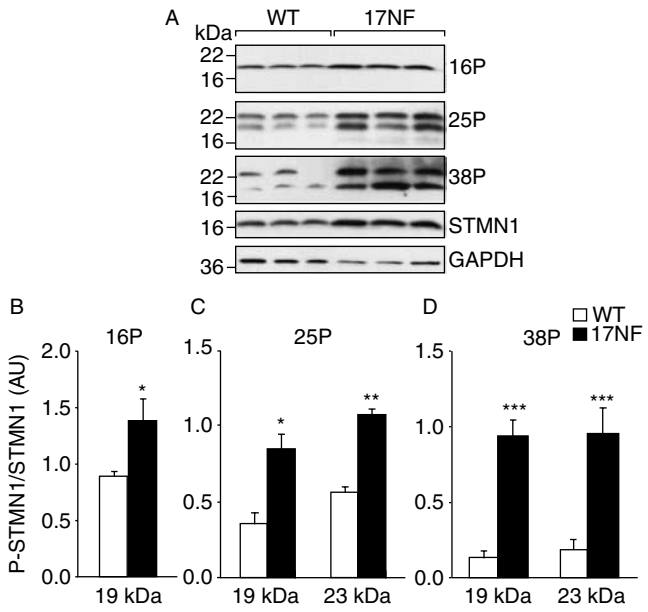

Figure 4 The abundance of three major phosphorylated forms of STMN1 (phosphorylated on 16P, 25P, and 38P respectively) is increased in the ovaries from 17NF mice compared with WT ovaries. (A) The phospho-forms were identified by western blot analysis using ovaries from prepubertal, 30-day-old mice, and antibodies specific for each form (Gavet *et al*. 1998). Quantitative results for each phospho-isoform (16P, 25P, and 38P) are shown in panels B–D respectively. The three antibodies recognize both the 19 kDa phosphorylated STMN1 form and a reduced electrophoretic mobility species known as stathmin '17', which migrates as a ~23 kDa species (Gavet *et al*. 1998). Notice that neither WT nor 17NF ovaries express STMN1 '17' phosphorylated on 16P. AU, arbitrary units; each bar is the mean of four mice; vertical lines represent S.E.M. $*P<0.05$, $**P<0.01$, and $***P<0.001$ versus WT groups.

previously reported in HeLa cells (Gavet *et al*. 1998). The increases in total and phosphorylated STMN1 abundance were discerned despite the fact (revealed by GAPDH blotting) that the lanes containing 17NF ovary samples were underloaded in comparison to the lanes containing WT ovary samples.

Production of TNF, an activator of the ASK1/p38 MAP kinase/ STMN1 pathway is elevated in 17NF ovaries

One of the mechanisms by which TNF promotes cell death is by inducing STMN1 phosphorylation (Vancompernolle *et al*. 2000). NGF has been shown to be a potent stimulus for TNF release in other cell systems (Bullock & Johnson 1996, Barouch *et al*. 2001). These findings and the earlier observations that TNF is an apoptotic signal for GCs (Kaipia *et al*. 1996) and also suppresses gonadotropin-induced steroidogenesis in these cells (Adashi *et al*. 1989), raise the possibility that the increase in apoptosis and reduced follicle growth observed in 17NF ovaries may involve TNF. Our results show that *Tnf* mRNA levels were increased ($P<0.05$) in 17NF ovaries compared with WT controls (Fig. 5A). The ovaries from 17NF mice also contain more ($P=0.02$) TNF protein than WT ovaries (Fig. 5B), indicating that TNF synthesis is increased in the presence of excessive amounts of NGF. *In vitro* treatment of the ovaries with the neurotrophic tyrosine kinase, receptor (NTRK) tyrosine kinase inhibitor K252a significantly ($P=0.02$) decreased TNF protein levels in 17NF ovaries, suggesting that the stimulatory effect of NGF on TNF production is mediated by high affinity NTRK1 tyrosine kinase NGFRs.

Blockade of TNF actions prevents the increase in STMN1 and phosphorylated STMN1 abundance, in addition to GC apoptosis in 17NF ovaries

To directly examine the notion that the increase in STMN1 and STMN1 phosphorylation levels, as well as the enhanced level of apoptosis observed in 17NF ovaries, are caused by TNF, we treated 27-day-old 17NF mice for 4 days with Etanercept (Enbrel), at a dose shown by others to inhibit TNF actions (Peppel *et al*. 1991, 1993, Kolls *et al*. 1994). We selected 16P for analysis, because the apoptotic effects of TNF have been shown to require STMN1 phosphorylation at 16P (Vancompernolle *et al*. 2000). We also studied 38P, because TNF uses, but does not require, this phosphorylated form (in conjunction with 25P) to inhibit the microtubule destabilizing activity of STMN1, and induce cell death (Vancompernolle *et al*. 2000). The 17NF ovaries had increased levels of total STMN1 (Fig. 6A), as well as 16P and 38P (Fig. 6B and C respectively). These increases were all blunted by Enbrel treatment to values near the levels detected in WT controls (Fig. 6A–C). This indicates that inhibition of TNF signaling prevents the overall increase in STMN1 levels observed in NGF overexpressing ovaries. Enbrel treatment resulted in a specific

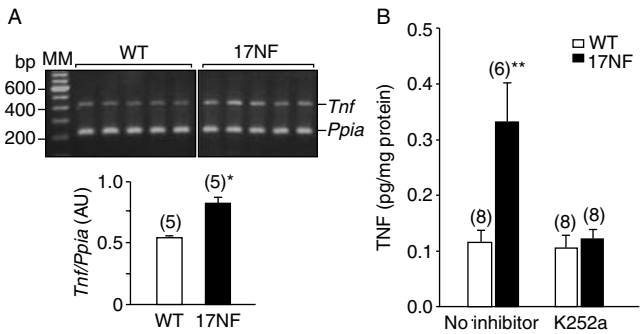

Figure 5 *Tnf* mRNA and TNF protein abundance are increased in 17NF ovaries compared with WT controls, and this increase in TNF production is abolished by blocking high-affinity NTRK receptors. (A) *Tnf* mRNA content in 30-day-old WT and 17NF ovaries measured by semi-quantitative PCR. *Ppia*, peptidylprolyl isomerase A (cyclophilin A). (B) TNF protein measured by ELISA in protein extracts from 28- to 30-day-old ovaries incubated for 3 h in KRB buffer at 37 °C. Some ovaries were treated during this time with 100 ng/ml of K252a, a blocker of NTRK tyrosine kinase activity. MM, molecular marker; $*P<0.05$, and $**P<0.02$ versus WT group; AU, arbitrary units; vertical lines represent S.E.M. and numbers of top of bars are number of ovaries per group.

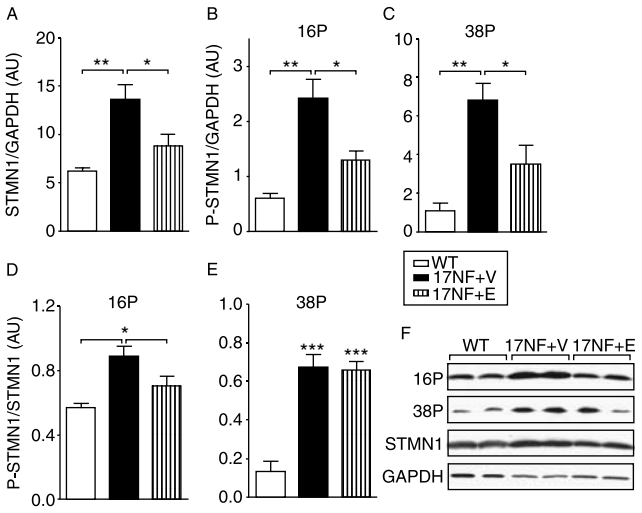

Figure 6 Blocking TNF actions via *in vivo* administration of a soluble TNF receptor 2 form coupled to the Fc portion of human IgG1 (Enbrel) prevents the increase in abundance of total STMN1 and phosphorylated STMN1 forms observed in ovaries overexpressing NGF. (A) Total STMN1 expressed as a ratio of the GAPDH signal; (B) 16P content normalized using GAPDH as the reference unit; (C) 38P normalized similarly; (D) 16P expressed as a fraction of non-phosphorylated stathmin; (E) 38P similarly expressed; (F) representative western blots. WT, wild-type ovaries; 17NF+V, ovaries from NGF-overexpressing mice treated with vehicle; 17NF+E, ovaries from NGF-overexpressing mice treated with Enbrel. AU, arbitrary units; columns are mean±S.E.M; each group is the mean of 4–8 animals. *$P<0.05$ versus 17NF group not treated with Enbrel; **$P<0.01$ and ***$P<0.001$ versus WT group.

decrease in 16P, but not 38P, abundance in relation to total STMN1 levels (Fig. 6D and E), a finding consistent with the notion that phosphorylation of 16P is a primary link in the signaling pathway used by TNF to induce cell death (Vancompernolle *et al.* 2000). A representative western blot illustrating these changes is shown in Fig. 6F.

A previous study showed that small-to-medium-size (101–300 μm) follicles have increased GC apoptosis in 17NF ovaries (Dissen *et al.* 2009). The ovaries from 17NF mice treated with Enbrel have a lower incidence of apoptotic antral follicles than the ovaries from untreated 17NF animals (Fig. 7A). Importantly, this reduction occurred specifically in small-to-medium-size follicles (101–300 μm; Fig. 7B). Examples of this difference are shown in Fig. 7C and D, which show that the ovary of a 17NF mouse treated with Enbrel (Fig. 7D) has a reduced number of apoptotic medium-size follicles (arrows) compared with the ovary of a 17NF mouse treated with vehicle (Fig. 7C). These results indicate that GC death in 17NF mice is to a significant extent mediated by an increased production of TNF.

5α-Androstane-3β,17β-diol does not contribute to promote GC apoptosis in 17NF ovaries

Evidence has emerged showing that 5α-androstane-3β,17β-diol (3β-diol) can also result in GC apoptosis via binding to estrogen receptor β (ESR2; Weihua *et al.* 2002, Omoto *et al.* 2005). To determine if this signaling system also contributes to promoting GC apoptosis in 17NF ovaries, we performed three experiments. In the first experiment, we measured the content of *Hsd3b1* mRNA. Although HSD3B1, encoded by this mRNA, converts pregnenolone into P_4 (Fig. 1A), it also catalyzes the conversion of dihydrotestosterone into 3β-diol (Fig. 8A). As shown in Fig. 1, the abundance of *Hsd3b1* mRNA content was similar in 17NF ovaries and WT controls, either in the presence or absence of PMSG stimulation (Fig. 1B). In a second experiment, we measured the content of *Cyp7b1* mRNA, which encodes cytochrome P450, family 7, subfamily B, polypeptide 1 also known as cytochrome P450 7b1, an enzyme that catalyzes the metabolism of 3β-diol into inactive products (Fig. 8A). *Cyp7b1* mRNA levels were substantially greater in 17NF ovaries than WT controls under both basal conditions and after PMSG stimulation (Fig. 8B). These results indicate that the intraovarian

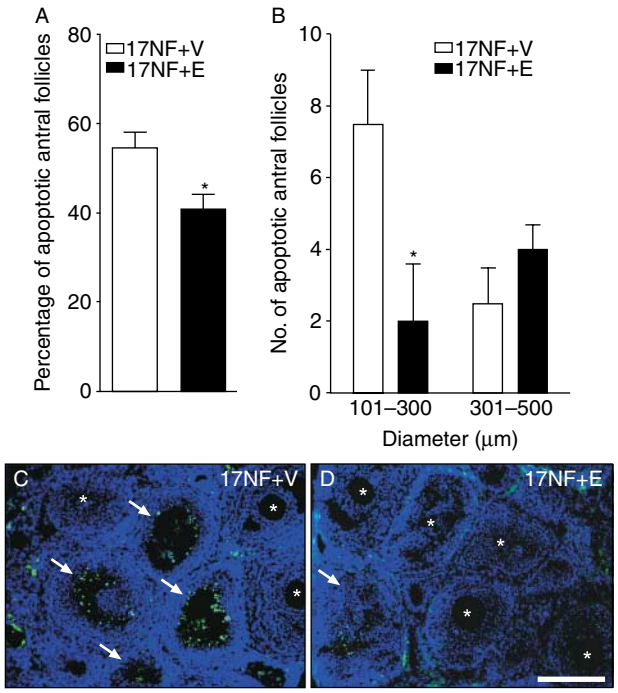

Figure 7 Blocking TNF actions via *in vivo* administration of Enbrel (once daily for 4 days) prevents the increase in GC apoptosis observed in NGF overexpressing ovaries, as assessed by quantitative evaluation of TUNEL reactions. (A) The ovaries of 17NF mice treated with Enbrel (E) show a lower incidence of total apoptotic antral follicles compared with vehicle (V)-treated 17NF mice. (B) This difference is due to a lower number of apoptotic small-to-medium-size (101–300 μm) follicles in the ovaries of Enbrel-treated 17NF mice than in vehicle-treated 17NF animals; (C) representative image of an ovary from a 17NF mouse treated with vehicle; (D) image of an ovary from a 17NF mouse treated with Enbrel. Arrows point to apoptotic medium-size follicles; and asterisks identify healthy medium-size follicles. Bar=200 μm; columns are mean±S.E.M. Each group is the mean of four animals. *$P<0.05$ versus 17NF group treated with vehicle.

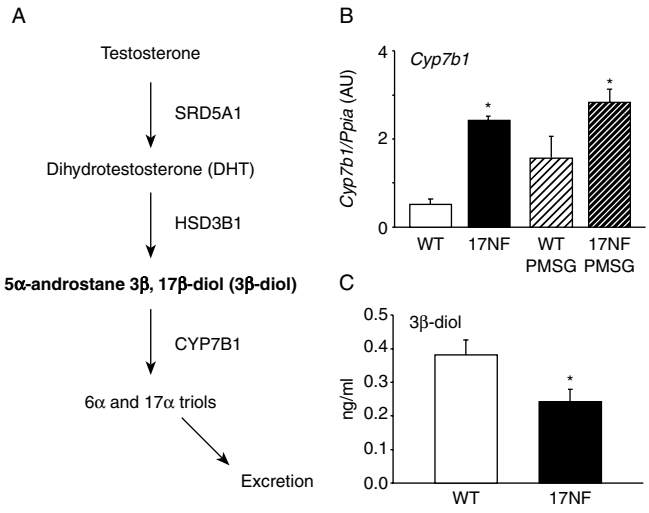

Figure 8 The content of *Cyp7b1* mRNA, which encodes CYP7B1 (the enzyme that catalyzes the metabolic degradation of 3β-diol) is increased in the ovaries of 17NF mice as assessed by semi-quantitative PCR, and plasma levels of 3β-diol are reduced in these animals compared with WT animals as assessed by RIA. (A) Diagram showing the 3β-diol biosynthetic/metabolic pathway; 3β-diol is in bold. (B) Content of *Cyp7b1* mRNA in ovaries of 17NF and WT mice; the ovaries were collected from 28- to 30-day-old animals. AU, arbitrary units; each bar is the mean of five mice. (C) Serum concentration of 5α-androstane-3β,17β-diol (3β-diol); each bar is the mean of 12 mice. Bars are mean±S.E.M.; *$P<0.05$ versus respective WT groups.

metabolism of 3β-diol is accelerated, instead of reduced, in 17NF ovaries. Consistent with this interpretation, serum 3β-diol levels were significantly lower in 17NF than WT mice (Fig. 8C).

In a third experiment, we used *Esr2*-null mice to determine if apoptosis still occurs in GCs of 17NF mice in the absence of ESR2. GCs are the predominant intraovarian site of ESR2 expression in rodents (Byers *et al*. 1997, Fitzpatrick *et al*. 1999, Sar & Welsch 1999, Sharma *et al*. 1999). The results showed that ovaries from 17NF/*Esr2*$^{-/-}$ animals had the same fraction of apoptotic follicles than 17NF ovaries (30.3±5 and 38.7±4% respectively). These results indicate that neither an increased production of 3β-diol nor increased ESR2-mediated signaling contribute to promote GC apoptosis in 17NF ovaries.

Discussion

This report provides insights into the cellular mechanisms underlying some of the deleterious effects that an excess of NGF has on ovarian function. We previously reported that 17NF mice release more 17-OHP$_4$, testosterone, and E$_2$ than WT mice in response to PMSG, and that the incidence of GC apoptosis was increased in the mutant ovaries (Dissen *et al*. 2009). The present results indicate that the increased response of these steroids to gonadotropins is likely related to an enhanced expression of the genes encoding HSD3B1, HSD17B1, and CYP19A1 respectively, and that the elevated incidence of GC apoptosis involves a TNF/STMN1-mediated pathway, not previously known to operate in the ovary.

In all likelihood, the elevated steroidogenic enzyme gene expression observed in 17NF ovaries is related to the increased number of medium-sized follicles observed in NGF overexpressing ovaries. Of interest in this context is the striking similarity that exists between the increased steroid output of the NGF overproducing ovary in response to gonadotropins and the abnormal steroidal output observed in patients in which follicle growth – like in 17NF ovaries – fails to progress efficiently to the periovulatory stage. For instance, patients with polycystic ovarian morphology (PCOM) exhibit an enhanced 17-OHP$_4$ response to GNRH (Mortensen *et al*. 2006), adult subjects with PCOM respond to hCG with a greater increase in testosterone (Adams *et al*. 2004), and adolescents with PCOS, release more E$_2$ when challenged with gonadotropins (Adams *et al*. 2004, Mortensen *et al*. 2006).

Our study does not address the issue of the signaling mechanism mediating this effect of NGF on steroidogenic enzyme gene expression. Neurotrophins acting via high-affinity NTRK receptors can activate at least four intracellular signaling pathways, including those requiring RAS/extracellular signal-regulated kinase (ERK) protein kinase, phosphatidylinositol-3-OH kinase/AKT kinase, phospholipase C-γ1 and nuclear factor κB (Patapoutain & Reichardt 2001). Despite this diversity of signaling options, different cell types may not respond to NTRK stimulation with activation of the same pathway (reviewed in Patapoutain & Reichardt (2001)), indicating that signaling molecules are connected to NTRK receptors in a cell-specific manner. In many cellular systems, including the ovary (Julio-Pieper *et al*. 2009), NGF preferentially uses the same ERK pathway mediating EGF action (Chao 1992, Szeberényi & Erhardt 1994); because binding of EGF to its receptor and transactivation of the EGF receptor by LH results in increased steroidogenesis (Makarevich *et al*. 2002, Evaul & Hammes 2008), it would appear plausible that the effect of NGF on the expression of steroidogenic enzyme genes is similarly mediated, at least in thecal–interstitial cells, the site of NGF overexpression. However, the increased *Cyp19a1* gene expression cannot be due to a direct effect of NGF on GCs, because in rodents these cells lack both NTRK1 (Dissen *et al*. 1996) and NGFR (Dissen *et al*. 1991). Therefore, it is likely that this change is due to a secondary effect of NGF, which acting on thecal–interstitial cells, stimulates the release of diffusible factors that, upon recognition by GCs, set in motion a signaling pathway linked to *Cyp19a1* gene expression. One of these factors may be prostaglandin E$_2$, which is released by thecal cells in response to NGF (Dissen *et al*. 2000*b*) and has been shown to induce expression of several steroidogenic

genes including *Cyp19a1* (Brueggemeier *et al*. 2003, Attar *et al*. 2009).

A similar theca–GC interaction may be less relevant in the human ovary because human GCs express NTRK1 receptors (Abir *et al*. 2005, Salas *et al*. 2006). Considering that in both the developing central nervous systems and some pediatric tumors of neural origin, NTRK1 receptors mediate a cell death signal (Harel *et al*. 2009, Nikoletopoulou *et al*. 2010), it is formally possible that an excess of NGF in human GCs may induce cell death directly, without the intermediacy of TNF of thecal–interstitial origin. However, if NGF-induced GC apoptosis requires NGFR in addition to NTRK1 (an involvement not ruled out by our results), then the rodent and human ovary would behave similarly because in both cases GCs lack these receptors (Dissen *et al*. 1991, Anesetti *et al*. 2001).

A proteomics approach allowed us to unveil a potentially important pathway mediating the deleterious effects of NGF on GC survival and follicle growth. We identified phosphorylated STMN1 as a protein preferentially expressed in 17NF ovaries in comparison to WT controls. STMN1 is a cytoplasmic phosphoprotein highly expressed in proliferating cells (Rubin & Atweh 2004). In its unphosphorylated state, STMN1 promotes depolymerization of microtubules and prevents the polymerization of tubulin heterodimers. As a consequence of these actions, cell proliferation decreases and the cells accumulate in the G_2/M phases of the cell cycle (Gavet *et al*. 1998, Rubin & Atweh 2004). The actions of STMN1 are terminated by phosphorylation (Gavet *et al*. 1998), which occurs when the cells enter mitosis (Gavet *et al*. 1998). However, studies involving inhibition and overexpression of STMN1 expression have shown that STMN1 is not only important for the initiation and progression of mitosis, but also for the exit from mitosis (reviewed in Rubin & Atweh (2004)). As such, STMN1 is considered to be an essential component of the cell cycle.

This function notwithstanding, recent studies have shown that STMN1 plays a role in cell death. A pathway that causes STMN1 phosphorylation is the ASK1/p38-mediated cascade (Mizumura *et al*. 2006), which mediates both cytokine and cellular stress-mediated apoptotic cell death (Matsuzawa & Ichijo 2001). TNF and interleukin 1 stand out among the cytokines that use the ASK1/p38 pathway to induce apoptosis; osmotic shock, u.v. radiation, heat shock, and oxidative stress are cellular stresses that also use the ASK1/p38 pathway to elicit cell death (Matsuzawa & Ichijo 2001). TNF can also induce STMN1 phosphorylation and cell death by activating other kinases, such as protein kinase A (Zhang *et al*. 1988, Gradin *et al*. 1998), the MEK/ERK pathway (Lovric *et al*. 1998), and the Ca^{2+}/calmodulin-dependent kinase pathway (Lawler 1998).

Our results show that phosphorylated STMN1 is more abundant in 17NF ovaries than in WT controls, and that – consistent with its reported abundance in proliferating cells – STMN1 is predominantly expressed in GCs of antral follicles. To the best of our knowledge, the presence of STMN1 in the ovary has never been reported. However surprising this gap in current knowledge might be, our results also show that an even more distinct change in 17NF ovaries is an abundance of phosphorylated forms of STMN1. All forms of phosphorylated STMN1, we measured (16P, 25P, and 38P) are overexpressed in 17NF ovaries, suggesting that this posttranslational modification is strongly favored by an excess of NGF. Although NGF is able to induce STMN1 phosphorylation by itself (Doye *et al*. 1990), such an effect may not take place in rodent GCs, because as mentioned earlier rodent GCs do not contain NGFRs. However, as human GCs contain NTRK1 receptors it is possible that NGF may directly induce STMN1 phosphorylation in these cells.

An ovarian factor known to induce GC apoptosis (Kaipia *et al*. 1996), and more recently shown to promote cell death by hyperphosphorylating STMN1 (Vancompernolle *et al*. 2000), is TNF. The downstream cellular mechanisms underlying this effect are not well understood. Resembling the pattern of phosphorylation observed in 17NF ovaries, TNF has been shown to induce phosphorylation of all four major phosphorylation sites of the protein, including 16P, 25P, 38P, and 63P (Vancompernolle *et al*. 2000). However, only phosphorylation at 16P and 63P is required for TNF to promote cell death via microtubule stabilization (Vancompernolle *et al*. 2000). Phosphorylation at the other two sites appears to occur only after 16P and 63P are phosphorylated, and if prevented, the lack of phosphorylation blocks neither TNF-induced microtubule stabilization nor TNF-induced cell death (Vancompernolle *et al*. 2000). Our results show that TNF production is increased in 17NF ovaries, and that this change is likely due to activation of NTRK1 receptors. They also demonstrate that blocking TNF actions in 17NF mice *in vivo* not only diminishes the increased levels of STMN1 and its 16P and 38P forms, but also reduces the number of follicles with apoptotic GCs observed in these animals. The relevance that these findings might have to the understanding of the cell–cell mechanisms underlying NGF-induced GC atresia is considerable, because NGF has been shown to be a potent stimulus for TNF release in other cell systems (Bullock & Johnson 1996, Barouch *et al*. 2001), and TNF is a well-known apoptotic signal for GCs (Kaipia *et al*. 1996) that also suppresses gonadotropin-induced steroidogenesis in these cells (Adashi *et al*. 1989). A NGF–TNF relationship has never been examined in the ovary, but it is likely to be functional because interstitial–thecal cells, the site of NGF production, are also a site of TNF synthesis (Chen *et al*. 1993).

Although NGF/proNGF can promote cell death by activating NGFR (Dechant & Barde 2002, Barker 2004, Lu *et al*. 2005) and use this receptor to stimulate TNF

release (Lebrun-Julien *et al*. 2010), it is unlikely that this mechanism operates in GCs, because neither rodent nor human GCs express NGFR (Dissen *et al*. 1991, 1996, Anesetti *et al*. 2001). The possibility exists, however, that NGFR expressed in thecal–interstitial cells of both species contribute (in conjunction with NTRK1 receptors) to mediating the effect of NGF on TNF production, and hence, the TNF-dependent increase in GC apoptosis. Further studies are required to resolve this issue.

Finally, our results rule out the contribution of 3β-diol to NGF-dependent GC death. This androgen metabolite may act as a signal for the arrest of GC growth (Omoto *et al*. 2005) via activation of ESR2 receptors (Kuiper *et al*. 1997, Weihua *et al*. 2002), which are abundant in GCs of antral follicles (Krege *et al*. 1998). Our results make clear that 17NF ovaries do not produce more 3β-diol than WT ovaries, and that ESR2 receptors – which mediate 3β-diol growth inhibitory effects (Weihua *et al*. 2002, Omoto *et al*. 2005) – are neither responsible for the arrest of follicle growth nor the enhanced rate of GC apoptosis observed in 17NF ovaries.

Altogether, these observations suggest a novel mechanism by which an excess of NGF causes GC apoptosis. According to this concept, NGF stimulates TNF production, and this cytokine then acts on GCs to induce apoptosis using an STMN1-mediated pathway.

Materials and Methods

Animals, treatments, and tissue collection

Transgenic 17NF mice were generated at the OHSU Transgenic/Gene Targeting Core as described (Dissen *et al*. 2009). *Esr2*-null mice (Krege *et al*. 1998) were kindly provided by Dr Kenneth Korach (National Institute of Health, Research, Triangle Park, NC, USA). They were used to assess the contribution of ESR2 to the increase in GC apoptosis observed in 17NF mice; double mutant mice were generated by first breeding homozygote 17NF mice to $Esr2^{+/-}$ animals, and then the progeny of these animals were intrabred to generate 17NF/$Esr2^{-/-}$ mice. Another group of 17NF animals was treated with Etanercept (trade name Enbrel; Immunex Corp., Thousand Oaks, CA, USA) at a dose reported to inhibit TNF actions (Peppel *et al*. 1991, 1993, Kolls *et al*. 1994). The animals were giving daily i.p. injections of Enbrel (8 μg/g body weight (BW) in a volume of 5 μl/g BW) for 4 days starting on day 27, and were killed 5 h after the last injection. Control mice were injected with distilled water. Etanercept is a fusion protein consisting of the extracellular domain of the TNF receptor 2 fused to the Fc component of human IgG1. Animal usage was duly approved by the Institutional Animal Care and Use Committee of the Oregon National Primate Research Center.

RNA extraction and semi-quantitative PCR

Ovaries were collected from WT and 17NF prepubertal mice (29–31 days old). To induce follicular development half of the mice were given an i.p. injection of PMSG (5 IU) 48 h before removing the ovaries. Total RNA from both ovaries of individual mice was extracted using the RNeasy Mini Kit (Qiagen). RNA samples were treated with DNase (Promega) before 1 μg was reverse transcribed with the Omniscript reverse transcriptase kit (Qiagen). Semi-quantitative PCR was carried out as described previously (Romero *et al*. 2002) using the primers given in Table 1.

Fluorescence two-dimensional gel electrophoresis-MS

To identify downstream proteins selectively expressed in the ovaries of 17NF animals, we used the comparative proteomics technique of fluorescence two-dimensional differential gel electrophoresis followed by time-of-flight ion MS (Righetti *et al*. 2004). Lysates (100 μg) from WT and 17NF 30-day-old

Table 1 PCR primers used for semi-quantitative PCR.

mRNA	Primer sequence	Range	Product
Cyp11a1	Forward: GCGCCTGGAGCCATCAAGAACT	491–512	442
NM_019779	Reverse: CCCCCAGGAGGCTATAAAGGACAC	909–932	
Star	Forward: CCGGGTGGATGGGTCAAGTT	190–209	428
NM_011485	Reverse: GCGCACGCTCACGAAGTCTC	598–617	
Hsd3b1	Forward: TGCAGGGCCCAACTCGTA	514–531	313
NM_008293	Reverse: TGCCCAGGCCACATTTTC	807–826	
Cyp17a1	Forward: GAAGGCCAGGACCCAAGTGTG	992–1012	418
NM_007809	Reverse: CTAAGAAGCGCTCAGGCATAAACC	1384–1409	
Hsd17b1	Forward: AGGCCGCCAGGACTCAAG	174–191	273
NM_010475	Reverse: GCACACGCCCAGAGTGGCGCCTCT	421–446	
Cyp19a1	Forward: ACGGGCCCTGGTCTTAT[a]	498–764	404
NM_007810	Reverse: CTCTCAGCGAAAATCAAATCA	879–901	
Cyp7b1	Forward: TTACTGCTCTCGGCCCTGTTCCTC	156–179	502
NM_007825	Reverse: TCGCAAATGTGATCTCAAATACCA	632–657	
Tnf	Forward: CAGGGGCCACCACGCTCTTC	281–300	419
NM_013693	Reverse: CTTGGGGCAGGGGCTCTTGAC	677–699	
Ppia	Forward: GGCAAATGCTGGACCAAACACAA	341–363	223
NM_008907	Reverse: GGTAAAATGCCCGCAAGTCAAAAG	538–563	

[a]Primers designed for a separate rat project; some mismatches are present compared with mouse sequence, but they generate a single PCR product from the mouse ovary. *Ppia* mRNA (peptidylprolyl isomerase A) encodes cyclophilin A, and is constitutively expressed).

mouse ovaries were labeled using Cy5 and Cy3 fluorescent cyanine (Cy) dyes (GE Healthcare Biosciences, Piscataway, NJ, USA) at a concentration of 400 pmol dye/50 μg protein. Labeled proteins were dissolved in isoelectric focusing (IEF) buffer containing 0.5% ampholytes and rehydrated passively onto a 24 cm immobilized pH gradient (IPG) strip (pH 4–7) for 12 h at room temperature. After rehydration, the IPG strip was subjected to IEF for ∼10 h to attain a total of 65 KV-h. Focused proteins were reduced in the presence of 1% dithiothreitol for 15 min and then alkylated with 2.5% iodoacetamide. IPG strips were loaded onto an 8–16% gradient polyacrylamide gel (24×20 cm), and electrophoresed at 80–90 V for 18 h. Following electrophoresis, the gel was scanned in a Typhoon 9400 scanner (GE Healthcare Biosciences) using appropriate lasers and filters at a photomultiplier (PMT) voltage of 550. Gel images in both channels were overlaid and the differences were visualized using ImageQuant software, version 5.2 (GE Healthcare Biosciences).

Individual spots were excised from the gel and subjected to in-gel digestion with trypsin for 24 h at 37 °C. Following tryptic digestion, the peptide solution was filtered through a 0.22 μm Durapore filter (Millipore, Billerica, MA, USA), vacuum-dried and reconstituted in 5% formic acid and analyzed on a hybrid quadrupole time-of-flight mass spectrometer (Q-Tof-2) connected to a CapLC (Waters Corporation, Milford, MA, USA). An MS/MSMS survey method was used to acquire MS and MS/MS spectra. Masses from 400 to 1500 kDa were scanned for MS survey, and masses from 50 to 1900 kDa were scanned for MS/MS. Data analysis was performed by ProteinLynx Global Server v2.1 (Waters Corporation) and by *de novo* sequencing using a PEAKS algorithm, combined with the OpenSea alignment algorithm (v 1.3.1) (Searle *et al*. 2005). Peptides consisting of five or more amino acids were used and matched to either a non-redundant mouse International Protein Index (IPI) or the Swiss-Prot database to identify the corresponding proteins. Proteins with two or more peptides by both ProteinLynx (significance score >10.6) and OpenSea (significance score >100) scoring algorithms were chosen (Searle *et al*. 2005).

Western blots

In one series of experiments, ovaries were collected from WT and 17NF mice (29–31 days old). Brain tissue, collected at the same time, served as a positive control. In a second series, we collected ovaries from 17NF mice treated with Enbrel (see above) and 17NF animals treated with the diluent (distilled water). The ovaries (4/tube) were homogenized in 500 μl of freshly prepared RIPA lysis buffer (10 mM Tris, pH 7.4, 0.1% SDS, 0.5% deoxycholic acid, 1% Triton X-100, NaCl 150 mM, 80 μM aprotinin, 2 μM leupeptin, 1.5 μM pepstatin, and 1 mM phenylmethylsulphonyl fluoride (PMSF)). After clearing the homogenates by centrifugation, protein concentrations were estimated by the Bradford method (Bio-Rad). Laemmli sample buffer (6×) was then added to each sample to a final concentration of 1×. The samples were boiled for 5 min before loading them (25 μg protein/sample) onto a 4–20% precast SDS–PAGE gel (Invitrogen; Prevot *et al*. 2003). After electrophoresis at 130 V for 2 h, the proteins were transferred for 1.5 h at 4 °C onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked in 5% non-fat milk for 1 h, and then incubated overnight at 4 °C with a rabbit polyclonal antibody against non-phosphorylated-STMN1 (1:20 000; Calbiochem, San Diego, CA, USA) followed by an anti-rabbit HRP antibody (1 h at room temperature, 1:50 000; Invitrogen). The signal was developed by enhanced chemiluminescence using the western lightning chemiluminescence substrate (PerkinElmer Life Sciences). To correct for procedural losses, the membrane was washed several times in Tris buffered saline Tween-20 (TBST; 10 mM Tris, 150 mM NaCl, pH 7.5 plus 0.2% Tween-20) before exposure (overnight at 4 °C) to a mouse MAB against GAPDH (AbCam, Inc., Cambridge, MA, USA; 1:20 000 dilution), followed by an anti-mouse HRP antibody (1 h at room temperature, 1:50 000; Invitrogen, previously Zymed, San Francisco, CA, USA). To detect the phosphorylated forms of STMN1, 80 μg protein were loaded onto 18% precast SDS–PAGE gels, subjected to electrophoresis for 2 h and then transferred to membranes as above. Before blocking with 5% non-fat milk, membranes were fixed with 0.25% glutaraldehyde for 20 min at room temperature (Gavet *et al*. 1998). Three different rabbit polyclonal antibodies that recognize STMN1 phosphorylated on 16P, 25P, or 38P, respectively (Gavet *et al*. 1998) were used. The antibody to STMN1 16P was used at a 1:200 000 dilution whereas the antibodies to STMN1 25P and 38P were used at 1:2000 dilution. The membranes were incubated with these antibodies overnight at 4 °C, followed in all cases by an anti-rabbit HRP antibody (1 h at room temperature, 1:25 000; Invitrogen). To avoid interference by the different P-STMN1 antibodies, membranes were stripped before applying a new antibody. Briefly, membranes were incubated at 65 °C under constant shaking with a stripping solution containing Tris–HCl 62.5 mM pH 6.7, 2% SDS and 0.7% β-mercaptoethanol, and then washed several times in TBST. STMN1-P antibodies were kindly provided by Dr Andre Sobel (Institut National de la Sante et de la Recherche Medicale Unite 153, Paris, France). For quantitation purposes, the membranes were extensively washed in TBST before exposing them to the antibodies that recognize non-phosphorylated STMN1, as outlined above.

Immunohistochemistry

The ovaries from 28-day-old WT and 17NF mice were fixed by immersion in Zamboni's fixative, cryostat-sectioned at 14 μm intervals, and processed for STMN1 immunohistochemistry (Dissen *et al*. 1995) utilizing the same rabbit polyclonal antibody (1:20 000 dilution, overnight at 4 °C) against non-phosphorylated STMN1 used for western blots. The immunoreaction was developed the next day using a biotinylated donkey anti-rabbit γ-globulin antibody (1:250, 1 h at room temperature; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), followed by diaminobenzidine, as reported (Dissen *et al*. 1991). Thereafter, the sections were counterstained with 0.25% methyl green.

Apoptosis

Apoptotic ovarian cells were detected using the *In Situ* Cell Death Detection Kit coupled with fluorescent detection

(TUNEL; Roche Diagnostics), following the manufacturer's instructions. The ovaries analyzed were from 30-day-old 17NF mice treated with Enbrel or diluent and from 29- to 31-day-old 17NF/*Esr2*$^{-/-}$ and 17NF/*Esr2*$^{+/+}$ mice. The ovaries were immersion-fixed overnight at 4 °C in 4% paraformaldehyde–PBS, and then cryoprotected in 20% sucrose–PBS 24 h at 4 °C before embedding them in OCT compound (Miles, Inc., Elkhart, IN, USA), and dry ice freezing. The whole ovary was then serially sectioned at 14 µm intervals. One series from each ovary, consisting of one every fourth section, was permeabilized for 30 min at 4 °C with a 0.5% citrate, 1% Triton X-100 permeabilization solution and then subjected to TUNEL reaction. The DNA strand breaks characteristic of apoptotic cells were identified by labeling the breaks with fluorescein-labeled dUTP, so that the nuclei emit a green fluorescence. For quantitation analysis, apoptotic GCs from antral follicles in which the oocyte was visible, were counted and the antral follicle diameter was measured with an eyepiece using a 10× objective. Follicles were considered apoptotic if they had more than six visible green cells at 10× magnification. The proportion of antral follicles showing apoptosis was then calculated.

Measurement of TNF by ELISA

Prepubertal female 26-day-old 17NF and WT mice were given an i.p. injection of PMSG (5 IU) 48 h before removing the ovaries for short-term incubation (Advis *et al.* 1979). The incubation was carried out in Krebs–Ringer bicarbonate solution (KRB; pH 7.4), containing 0.1 mg/ml BSA at 37 °C, continuously flushed with 95% of O_2 and 5% CO_2, saturated with water and with constant shaking (60 cycles/min). Briefly, the ovaries were halved and preincubated individually in small plastic flasks containing 250 µl/ovary of KRB supplemented with glucose (1 mg/ml) for 30 min. After this preincubation period, the medium was replaced by fresh KRB supplemented with 2.5 IU hCG per ovary. One ovary from each 17NF and WT mice was treated with 100 nM of the NTRK receptor inhibitor K252a (Tocris Bioscience, Ellisville, MO, USA). The contralateral ovary from the same animal received no treatment. After 3 h of incubation, the ovaries were collected for protein extraction. Individual ovaries were homogenized in 120 µl of homogenization buffer containing 25 mM Tris–HCl pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM PMSF, and 80 µM Aprotinin. The lysates were centrifuged at 10 000 ***g*** 10 min and supernatants (100 µl) were collected for TNF measurement. TNF was measured using a commercial ELISA kit (Mouse TNF; eBioscience, San Diego, CA, USA cat# 88-7324-22) following the manufacturer recommendations. The sensitivity for this assay was 8 pg/ml.

Measurement of 3β-diol levels

The levels of 3β-diol in serum from WT and 17NF mice were determined by RIA (Wahlgren *et al.* 2008) using a specific anti-3β-diol polyclonal antibody (BioSite, Stockholm, Sweden). The radioactive trace, 5α-[1α, 2α-^{3}H (N)] androstane-3β,17β-diol (specificity activity, 45 Ci/mmol), was obtained from NEN Life Science Products (Boston, MA, USA). For these particular assays, the inter- and intra-assay variations were 12 and 8% respectively.

Statistical analysis

The results were analyzed using SigmaStat 3.1 software (Systat Software, Inc., San Jose, CA, USA). The data were first subjected to a normality test and an equal variance test. Data that passed these two tests were then analyzed with the Student's *t*-test. Data that failed either the normality or equal variance test were analyzed by the non-parametric Mann–Whitney Rank Sum Test method.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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