Regulation of Wilms' tumor gene expression by nerve growth factor and follicle-stimulating hormone in the immature mouse ovary

This study investigated the regulation of Wilms' tumor gene (WT1) in the ovary by nerve growth factor and FSH to better understand signals that initiate early follicular growth. Nerve growth factor showed a direct stimulatory effect on endogenous expression of WT1, whereas FSH attenuated basal and nerve growth factor-stimulated WT1 protein expression, which most likely depended on FSH responsiveness according to the follicle growth stage. (Fertil Steril® 2009;91:1451–4. ©2009 by American Society for Reproductive Medicine.)

The mechanisms that regulate the gradual exit of ovarian follicles from the large pool of nongrowing primordial follicles are poorly understood. To better understand the cellular signals that initiate follicular growth in mammals and the conditions necessary for sustained growth of early preantral follicles in vitro, we identified candidate genes expressed in early-stage follicles and examined their regulation of key transition genes or gene products.

Wilms' tumor gene (WT1) encodes a transcription factor that is highly expressed in the early stages of follicle development and is reduced as granulosa cells (GCs) differentiate into steroid-producing cells (1). In addition, WT1 overexpression regulates gonadal-specific gene promoters including aromatase, inhibin-α, and Müllerian-inhibiting substance (MIS) (2–6). This suggests that an inhibitory effect of WT1 could prevent premature differentiation of GCs during early follicular development. However, the regulatory factor of WT1 in the developing ovary is not clear. Nerve growth factor (NGF) appears to have a major role in the regulation of early follicle formation (7) and has been shown to regulate WT1 expression in nongonadal cells (8). We hypothesize that NGF may influence early follicle development by regulating WT1 expression in gonadal cells. In addition, FSH is one of the most important hormones for the growth and differentiation of secondary, antral, and preovulatory follicles. As follicles reach the two-layer stage, GCs undergo a third differentiation process that confers responsiveness to pituitary gonadotropins (9). The decline in WT1 mRNA expression in antral follicles (10) and the inverse relationship of FSH receptor and WT1 mRNA levels in the developing rat ovary (2) suggest that gonadotropin may also regulate WT1 expression or activity in the ovary. To better understand the factors that contribute to follicle recruitment, we studied the regulatory relationships between NGF, WT1, and FSH in mouse ovaries and GCs.

To investigate whether NGF or FSH regulates WT1, GRMO2 (mouse GC-derived line) cells were used for transient transfection by a WT1-promoter-Luc reporter construct (WT1-Luc). WT1-Luc consisted of -513 to +254 bp of the 5′ flanking sequence of the mouse WT1-promoter ligated into a luciferase reporter vector (pGL2-Luc; Promega Corp., Madison, WI). When GRMO2 cells reached 80% confluence, WT1-Luc was transiently transfected into cells using 0.2 μg of plasmid, and the p-Rous sarcoma virus–β-galactosidase (gal) vector (10 ng) was co-transfected as an internal control for transfection efficiency (11) in the presence of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Sixteen hours after transfection, cells were treated with NGF (100, 300 ng/mL), FSH (0.3, 1 IU/mL), or control media. In this study, treatment doses of NGF or FSH were determined based on previous reports (8, 9) that are known to be effective doses. After incubation for 16–24 hours, cells were then harvested, lysed, and assayed for luciferase activity using an AutoLumat (Berthold Technologies Co., Oak Ridge, TN). Luciferase activity is presented as relative light units and is normalized for β-gal activity.

Immature CD1 mice were used to investigate whether the NGF-stimulatory effect on WT1 expression differs by developmental age. The Northwestern University ACUC committee approved all procedures involving animals. Ovaries collected from 7-day-old mice were cultured on 0.4-μm pore millicell CM filters (Millipore, Bedford, MA) in a 24-well tissue culture plate (Costar, High Wycombe, United Kingdom). Each well contained 500 μL of α-minimal essential medium (MEM) (GIBO, Santa Clara, CA) supplemented with glucose (4.5 g/L), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were cultured in the

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presence or absence of NGF (R&D Systems, Minneapolis, MN) or FSH (NV Organon, Oss, the Netherlands) under 5% CO_2–balanced nitrogen for 2 days. The GCs were from small antral follicles of 21-day-old mice and were cultured in α-MEM in the presence or absence of NGF or FSH under 5% CO_2–balanced nitrogen for 24 hours. At the end of culture, ovaries or GCs were frozen and stored at -80°C for RNA or protein extraction.

Total RNA was isolated using the RNeasy extraction kit (Qiagen Inc., Valencia, CA). After quantification of RNA and verification of its integrity, 1 μg of total RNA was annealed (5 minutes at 70°C) to oligo$(dT)_{18}$ primers and was reverse transcribed with the Advantage RT for PCR kit (BD Biosciences; Clontech, Palo Alto, CA). The primers were designed with the Primers Express program (PE Applied Biosystems, Foster City, CA): forward, 5′-AGGTT TTCTCGCTCAGACCA-3′ and reverse, 5′-CTGGTGTGG GTCTTCAGAT-3′ (GenBank accession no. AAA40573). Amplification of 18S ribosomal RNA (rRNA) (forward, 5′-CATGGCCGT TCTTAGTTGGT-3′ and reverse, 5′-ATGCCAGAGTCTC GTTCGTT-3′) (GenBank accession no. emb|X00686) was used to normalize each reaction (amplification product size 123 and 67 bp for WT1 and 18S rRNA, respectively). Real-time polymerase chain reaction (PCR) reactions were carried out in a total volume of 25 μL, using SYBR Green supermix (BioRad, Hercules, CA) using an iCycler Thermal Cycler (BioRad). The PCR reaction was as follows: 10 minutes at 95°C, 35 cycles of 95°C for 15 seconds, 60°C for 45 seconds, and 72°C for 1 minute. Samples were run in triplicate in 96-well optical plates (BioRad), and the mean values were compared with the nontreated control value to calculate the relative amount of transcript.

For immunobloting analysis, GCs or cultured ovaries were washed with cold phosphate-buffered saline (PBS) before lysis in Laemmli buffer containing β-mercaptoethanol (Bio-Rad). Cell lysates or ovarian extracts were boiled for 3 minutes. Samples were loaded 50 μg per lane and resolved using 4%–12% NuPAGE gel electrophoresis (Invitrogen). Proteins were transferred onto Hybond-P membranes (Amersham Pharmacia Biotech, Arlington Heights, IL). Membranes were blocked for 2 hours at room temperature in TBS-0.1% Tween containing 5% fat-free dry milk and were incubated with an anti-WT1 antibody (C19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted at 1:500 for 3 hours at room temperature. Membranes were washed and blotted with peroxidase-conjugated donkey antirabbit second antibody (1:8,000) (Boehringer Mannheim, Indianapolis, IN). Immunolabeled proteins were detected using the enhanced chemiluminescence kit (Amersham Pharmacia Biotech). The 52-kDa WT1 protein is indicated in Figure 1C. To ensure that lysates were loaded equally, the blots were stripped and incubated with an anti-β-actin antibody (1:1,000 dilution; Sigma, St. Louis, MO). The relative WT1 protein level was estimated by analyzing the Western blot signals using the ImageQuant (Molecular Dynamics, Piscataway, NJ) program, normalized to the β-actin band. Values were calculated as fold changes to the control.

All data were analyzed by a Student's t-test, with $P<.05$ considered significant. Data were expressed as mean $\pm$ SD of duplicate or triplicate measurements of triplicate cultures for all experiments.

Nerve growth factor dose-dependently stimulated WT1 promoter activity in transiently transfected GROM2 cells with WT1-Luc (Fig. 1A), similar to NGF regulating the expression of WT1 in neuronal cells (8). However, FSH did not have an effect on WT1 promoter activity. As expected, NGF had a stimulatory effect on endogenous expression of WT1 mRNA (Fig. 1B) and protein (Fig. 1C). This effect was profound in 7-day-old mice (Fig. 1B, left) and was less in the GCs of 21-day-old mice ovaries (Fig. 1B, right).

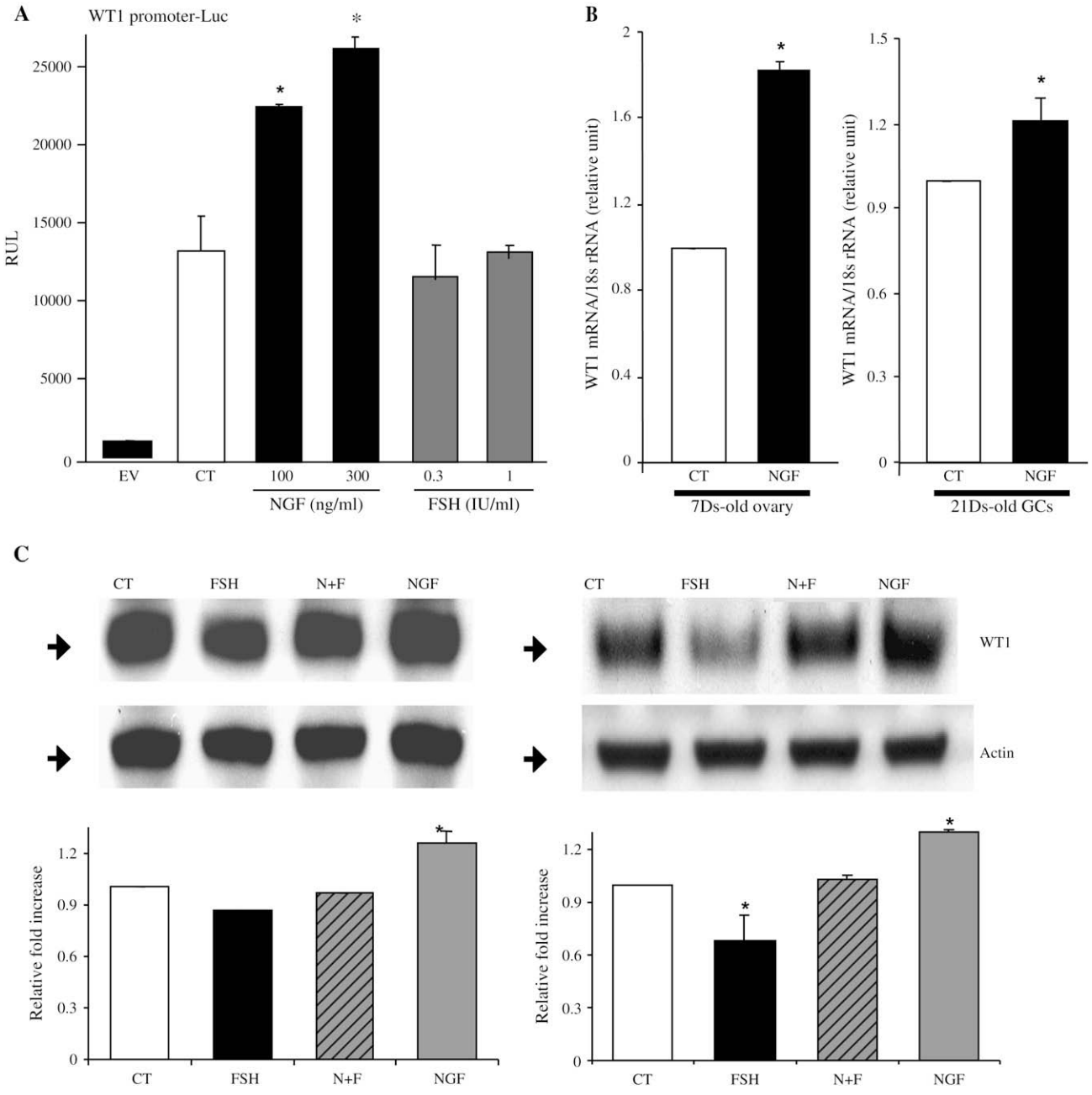
Although WT1 promoter activity was not changed (Fig. 1A), FSH treatment attenuated basal and NGF-stimulated WT1 protein expressions (Fig. 1C). This was not profound in 7-day-old mice (Fig. 1C, left). It is possible that the basal expression of FSH receptor at this age is too low to respond to FSH. These data suggest that FSH down-regulates WT1 expression, and this likely depends on FSH responsiveness to GCs differentiation (amount of FSH receptor) according to the follicle growth stage.

Expression of WT1 mRNA has been described in primordial, primary, and secondary rat follicles, and mRNA levels decrease with follicle maturation (10). Nerve growth factor and its two membrane-anchored receptors, trkA and p75NTR, are also expressed in the developing rat ovary (12), and the data from NGF null mice indicate that NGF is necessary for early follicular development (7). Nerve growth factor has been shown to regulate the expression levels of WT1 in a nongonadal cell line (8) and a gonadal cell line (Fig. 1A). The stimulatory effect of NGF was demonstrated in the mouse ovary (Fig. 1B,C). The up-regulation of WT1 by NGF was greater in younger mice. As NGF increases FSH receptor mRNA in ovaries of younger rats (13), NGF may determine early follicle differentiation by regulating FSH receptor (13) and WT1 expression.

The stage-specific expression pattern of WT1 (10) and the inverse expression of FSH receptor mRNA (2) suggests that it may be a factor in the maintenance of follicles within early stages of development. Because FSH decreased WT1 protein expression in 21-day-old mice GCs (Fig. 1C, right), but not in 7-day-old mice ovaries (Fig. 1C, left), the repressive effects by FSH might depend on FSH responsiveness of GCs by follicle stage and serial changes likely induced by FSH (Fig. 1A). In addition, the NGF-stimulated WT1 level was attenuated by FSH both in 21-day-old mice GCs and 7-day-old mice ovaries. Thus, increasing FSH may down-regulate WT1 expression in certain follicles, relieving the repressive effects of WT1 and selecting them for further follicle development.

FIGURE 1

Regulation of Wilms' tumor gene (WT1) expression by nerve growth factor (NGF) and FSH. (**A**) The WT1-promoter reporter activity in transiently transfected GRMO2 cells with WT1-promoter-Luc by treatment with FSH (0.3, 1 IU/mL) or NGF (100, 300 ng/mL). Control (CT); empty vector (EV); relative light units (RLU) (**B**) Analysis of WT1 mRNA levels by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in cultured ovaries of 7-day-old mice (*left*) or granulosa cells (GCs) of 21-day-old mice (*right*) in the presence or absence of NGF (100 ng/mL).(**C**) Immunoblot analysis of WT1 protein in cultured ovaries of 7-day-old mice (*left upper panel*) or GCs of 21-day-old mice (*right upper panel*) and quantitative analysis of WT1 expression (*lower panels*) after treatment with FSH (1 IU/mL), NGF (100 ng/mL), or both (N+F). Data are represented as the mean ± SD. **P*<.05 compared to control. *Arrowheads* indicate specific bands of WT1 (52 kDa) and β-actin (42 kDa).



Roh. WT1 regulated by NGF and FSH in the mice ovary. Fertil Steril 2009.

We suggest that NGF acts as an ovarian paracrine factor that may direct some follicles to further grow through the up-regulation of FSH receptor (13) and WT1. With cell survival-promoting hormones such as FSH, some follicles may be relieved from WT1 growth suppression. Investigation of WT1 and its regulation by NGF in the ovary gives a better understanding of the interplay between the endocrine and paracrine factors that govern the release of small preantral follicles.

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