Neurotrophins Acting Via TRKB Receptors Activate the JAGGED1-NOTCH2 Cell-Cell Communication Pathway to Facilitate Early Ovarian Development

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Tropomyosin-related kinase (TRK) receptor B (TRKB) mediates the supportive actions of neurotrophin 4/5 and brain-derived neurotrophic factor on early ovarian follicle development. Absence of TRKB receptors reduces granulosa cell (GC) proliferation and delays follicle growth. In the present study, we offer mechanistic insights into this phenomenon. DNA array and quantitative PCR analysis of ovaries from *TrkB*-null mice revealed that by the end of the first week of postnatal life, *Jagged1*, *Hes1*, and *Hey2* mRNA abundance is reduced in the absence of TRKB receptors. Although *Jagged1* encodes a NOTCH receptor ligand, *Hes1* and *Hey2* are downstream targets of the JAGGED1-NOTCH2 signaling system. *Jagged1* is predominantly expressed in oocytes, and the abundance of JAGGED1 is decreased in $TrkB^{-/-}$ oocytes. Lack of TRKB receptors also resulted in reduced expression of *c-Myc*, a NOTCH target gene that promotes entry into the cell cycle, but did not alter the expression of genes encoding core regulators of cell-cycle progression. Selective restoration of JAGGED1 synthesis in oocytes of $TrkB^{-/-}$ ovaries via lentiviral-mediated transfer of the *Jagged1* gene under the control of the growth differentiation factor 9 (*Gdf9*) promoter rescued *c-Myc* expression, GC proliferation, and follicle growth. These results suggest that neurotrophins acting via TRKB receptors facilitate early follicle growth by supporting a JAGGED1-NOTCH2 oocyte-to-GC communication pathway, which promotes GC proliferation via a c-MYC-dependent mechanism. **(*Endocrinology* 152: 5005–5016, 2011)**

Ovarian follicle development is tightly controlled by various endocrine, paracrine, and autocrine factors that act in a coordinated manner to regulate growth of the oocyte and its surrounding granulosa and theca cell layers (1–4). In rodents, initial follicle recruitment and the transition from primary to secondary follicles are mainly regulated by intraovarian factors, several of which have been identified (2). One of these regulatory systems uses neurotrophins (NT) as ligands and tropomyosin-related kinase (TRK) receptors, in addition to a common $p75^{NTR}$ receptor, for signaling (5–7). The NT were originally described as a family of polypeptide growth factors essential for the survival and differentiation of various neuronal populations in the central and peripheral nervous system (8, 9). It is now clear that they are also required for the development and function of organs as diverse as those comprising the cardiovascular, immune, endocrine, and reproductive systems (reviewed in Ref. 10). The four known mammalian NT include nerve growth factor, brain-derived neurotrophic factor (BDNF), NT3, and NT4/5. They are recognized by different TRK receptors: TRKA binds nerve growth factor, TRKB recognizes BDNF and NT4/5, and TRKC binds NT3 (6). The four NT are recognized by the pan-$p75^{NTR}$ (5). All of these molecules are expressed in feto-neonatal rodent ovaries and fetal human ovaries before the initiation of follicle assem-

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Abbreviations: BDNF, Brain-derived neurotrophic factor; CDK, cyclin-dependent kinase; CIP/KIP, cyclin and CDK inhibitors; CKI, CDK inhibitor; GC, granulosa cell; HA, hemagglutinin; INK4, inhibitors of CDK4; KO, knockout; LV, lentivirus; NICD, intracellular domain of NOTCH receptors; NT, neurotrophin; *Odc*1, ornithine decarboxylase1; PCNA, proliferating cell nuclear antigen; qPCR, quantitative PCR; TRK, tropomyosin-related kinase; WT, wild type.

bly (11–18). BDNF and NT4/5 have been shown to promote oocyte maturation (19) and *in vitro* follicular assembly (20), strongly suggesting a role for TRKB signaling in these processes.

Using *TrkB*-null mice, we (21) and others (12) demonstrated that TRKB signaling is required for oocyte survival and preantral follicular development. Because the ovary expresses both full-length, kinase domain-containing TRKB receptors and a truncated TRKB isoform lacking the tyrosine kinase domain of the receptor, we studied mice lacking both TRKB isoforms. Follicle assembly is reduced in these mutants (22), which in addition suffer a stage-selective deficiency in early follicular development that compromises the ability of follicles to grow beyond the primary stage. Proliferation of granulosa cells (GC), required for this transition, and expression of FSH receptors, which reflects the degree of biochemical differentiation of growing follicles, are also reduced. Although these observations and those of Spears *et al.* (12) demonstrate the importance of TRKB receptors in early ovarian development, they also raise the question as to the downstream molecules and cellular mechanisms underlying these novel functions of TRKB signaling in the ovary.

Earlier studies demonstrated that the JAGGED1-NOTCH2 complex contributes to maintaining oocyte-GC communication (23–27), with oocytes expressing JAGGED1 (the ligand) and GC expressing NOTCH2 (the receptor) (28). More recent studies have shown that NOTCH2 signaling is required for both follicle formation (29) and GC cell proliferation during early follicle development (27). Using DNA arrays as a high throughput strategy for gene discovery and a combination of molecular, morphologic, and gene transfer approaches, we now report that deficits in follicle development and GC proliferation observed in *TrkB*-null mice are due, to a significant extent, to perturbation of the JAGGED1-NOTCH2 cell-cell communication pathway. Our results also indicate that this perturbation results in reduced expression of *c-Myc*, a direct target of NOTCH signaling (30, 31) that drives cell cycle progression by promoting entry into the S (DNA synthesis) phase of the cell cycle (32, 33). Finally, our study indicates that oocytes play a critical role in sustaining TRKB-dependent early follicle growth, as evidenced by the effectiveness of oocyte-specific restoration of JAGGED1 synthesis to rescue the defects in *c-Myc* expression, follicle growth, and GC proliferation caused by the absence of TRKB receptors. A preliminary report of these findings has been presented (34).

Materials and Methods

TrkB-null mice

The *TrkB*-null mice used in this study, as well as additional details concerning their postnatal phenotype, were previously described (21, 35). $TrkB^{-/+}$ mice were bred to wild-type (WT) animals of the same genetic background, and the F1 progeny was used to produce *TrkB*-null and WT controls. The animals were maintained on a 12-h light, 12-h dark cycle (lights off at 1900 h), with food and water available *ad libitum*.

The breeders were fed with LabDiet 5001 (PMI Nutrition International Brentwood, St. Louis, MO). Animal usage was duly approved by the Institutional Animal Care and Use Committee of the Oregon National Primate Research Center, in accordance to the guidelines provided by the National Institutes of Health Guide and Use of Laboratory Animals.

Collection of ovarian tissue and genotyping

Ovaries from entire litters were collected at 0 (day of birth), 2, 3, 4, 6, 7, and 12 d after birth and used for different procedures (organ and cell culture, RNA extraction, immunohistochemistry, *in vitro* hybridization, and morphometric analysis) before establishing each genotype. Once the genotype was known, the ovaries were assigned to either a WT ($TrkB^{+/+}$) or a knockout (KO) ($TrkB^{-/-}$) group. No heterozygotes were included in the analyses. For identification of *TrkB* alleles, we used DNA isolated from 2-mm tail biopsies. Tail samples were lysed in a DirectPCR buffer containing proteinase K (Viagen Biotech, Inc., Los Angeles, CA), and 1 μl of the crude lysates was used for PCR. The *TrkB* WT allele and a DNA segment comprising both the targeting vector and a gene-specific sequence were detected using a set of three primers: *TrkB-C8* (5′-ACTGACATCCGTAAGCCAGT-3′), *TrkB-N2* (5′-ATGTCGCCCTGGCTGAAGTG-3′), and *PGK-3.1* (5′-GGTTCTAAGTACTGTGGTTTCC-3′). The size of the PCR product was 400 bp for the WT allele and 200 bp for the deleted allele (21, 22).

DNA microarrays

To identify mRNA that are differentially expressed in ovaries from $TrkB^{-/-}$ mice as compared with WT animals during the initiation of early follicular growth, we employed 7-d-old mice and a two-dye spotted cDNA microarray containing 8400 gene probes generated from a mouse NIA 15K gene set, printed in duplicate on glass slides by the Gene Microarray Shared Resource Facility of the Oregon Health and Science University (http://www.ohsu.edu/xd/research/research-cores/gmsr/). The procedure employed for RNA extraction, synthesis of cDNA from total RNA, cDNA labeling, and hybridization to DNA microarrays has been previously described (36). The signal intensities were analyzed using print tip group lowess, as recommended by Yang *et al.* (37), and implemented by Sandrine Dutoit in BioConductor (http://www.bioconductor.org/). The array results have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through Gene Expression Omnibus Series accession no. GSE8528.

Culture of ovaries with BDNF

To determine the effect of TRKB activation on *Jagged1*, *c-Myc*, and ornithine decarboxylase 1 (*Odc*1) mRNA levels, ovaries from 4-d-old WT mice were dissected under a stereomicroscope using aseptic conditions, placed on sterile lens paper, and cultured on metal grids in a 24-well plate at the interface of air/culture medium, under an atmosphere of 60% O_2-35% N_2-5% CO_2, as described (21, 38). One ovary from each animal

was cultured in the presence of BDNF (100 ng/ml); the contralateral ovary served as an untreated control. After 8 h of incubation, the tissues were frozen in dry ice and stored at −85 C until RNA extraction.

Culture of ovaries for lentivirus (LV)-mediated gene transfer

To increase *Jagged1* expression in oocytes of $TrkB^{-/-}$ mice, a cDNA containing the rat *Jagged1*-coding region fused to a sequence encoding a human influenza hemagglutinin (HA) epitope (kindly provided by Gerry Weinmaster; Department of Biological Chemistry, University of California, Los Angeles, CA) was inserted into the multiple cloning site of a LV vector (39). To restrict *Jagged1* expression to oocytes, the cytomegalovirus promoter of this vector was replaced by the *Gdf9* promoter (kindly provided by Austin Cooney; Baylor College of Medicine, Houston, TX). Infectious viral particles were produced and quantified as reported (40). The ovaries from 3-d-old $TrkB^{-/-}$ mice were dissected and incubated as described above. One ovary from each animal was treated for 4 d with this LV construct (termed LV-*Jagged1-HA*) and the contralateral ovary with a vector lacking *Jagged1* (LV-no *Jagged1*), each at 7×10^6 transducer units per milliliter per well. At the end of the culture period, the ovaries were either fixed for immunohistochemistry and morphometric analysis or frozen on dry ice and stored at −85 C until RNA extraction (see below).

RNA extraction, semiquantitative PCR (qPCR), and real-time PCR

A Micro RNeasy kit (QIAGEN, Germantown, MD) was used to isolate total RNA, and 250 ng of total RNA were reverse transcribed using Omniscript reverse transcriptase, as previously described (36, 40). In one set of experiments, in which we treated ovaries in culture with BDNF or NT4, we used the semi-qPCR procedure of Ambion (Austin, TX) to detect *Jagged1*, *c-Myc*, and *Odc1* mRNA. Before carrying out the amplification procedure, the optimal gene-specific primer concentrations, linear range of the PCR, and optimal primer concentration for the amplification of *Ppia* mRNA (used as an internal standard) were determined. *Ppia* mRNA encodes peptidylprolyl isomerase A, also known as cyclophilin A. The PCR were carried out in a 25-μl volume containing 0.5 μl of RT reaction and 25 pmol (*Jagged1*, *c-Myc*, or *Odc*) and 25 pmol (*Ppia*) of primers at a primer/competimer ratio of 1:1. The PCR amplification protocol consisted of 30 cycles of denaturing at 94 C (30 sec), annealing at 55 C (30 sec), and extension at 72 C (1 min). Equal volumes of the PCR were electrophoresed on 2% agarose gels stained with ethidium bromide, the gels were imaged in a Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA), and the images were quantitated using the image analysis Quantity One software (Bio-Rad Laboratories).

For all other studies, we measured mRNA levels by real-time PCR using SYBR Green PCR technology and reagents purchased from Applied Biosystems (Foster City, CA). Standard curves (threshold cycle number *vs.* log [RNA]) were constructed by using serial dilutions (1:10) of cDNA, assuming that the amount of cDNA is equal to the initial amount of mRNA. The threshold cycle number from each sample was referred to this curve to estimate the corresponding mRNA content, and each mRNA value was then normalized for procedural losses using the *18s* rRNA values estimated from the relative standard curve.

All PCR primers were designed using the software Primer Select 6.0 (DNASTAR, Inc., Madison, WI) (Table 1).

In situ hybridization

The cellular localization of *Jagged1* mRNA was assessed using the *in situ* hybridization procedure described by Simmons *et al.* (41) with minor modifications (42). A 445-bp mouse *Jagged1* cDNA complementary to nucleotides 3226–3670 in mouse *Jagged1* mRNA (NM_013822.4) was generated by PCR amplification of mouse ovary total RNA and cloned into the pGEM-T vector (Promega, Madison, WI). After linearization with *Nco*I, 500 ng of *Jagged1* cDNA template were transcribed with 250 μCi of ^{35}S-uridine triphosphate (PerkinElmer, Boston, MA). Hybridization reactions using this cRNA were performed on 14-μm

TABLE 1. RT-PCR primer list

Gene	Forward primer	Reverse primer	GenBank
Jagged1[a]	GCACGCCGACAAAACACCCGAACT	ATTAGGACCGCTGGCAGATGTGGA	NM_013822
Jagged1	CCGAGGACTATGAGGGCAAGAA	GGGGACCACAGACGTTAGAAGAG	NM_013822
Notch2	GTGGACGGCATCAATCGCTACA	GGGGCATATACACCGGAAACCAT	NM_010928
Hes1	GCCGCCGCCGCTTGTGC	GGGATGACCGGGCCGCTGTGAG	NM_008235
Hey2	ATTTTGAAGATGCTCCAGGCTACAG	CACTCTCGGAATCCAATGCTCA	NM_013904
Ppia[a]	GGCAAATGCTGGACCAAACACAA	GGTAAAATGCCCGCAAGTCAAAAG	NM_008907
Cdk2	GGGGGATGACCGCAGTGT	GGGTCCCCAGAGTCCGAAAGAT	NM_183417
Cdk4	TGTACGGCTGATGGATGTCTGTGC	GCCCGGTGGAGGTGCTTTGTC	NM_009870
CyclinD2	GCCGCAGTCACCCCTCACGA	TGCTCCCACGCTTCCAGTTGC	NM_009829
CyclinE1	TGTCCTCGCTGCTTCTGCTTTGTA	CGGATAACCATGGCGAACGGAACC	NM_007633
$p15^{INK4b}$	AGGGGCGCGGCTGGATGT	CCTAGATGGGGCTGGGGAGAAAGA	NM_007670
$p16^{INK4a}$	CAAGAGCGGGGACATCAAGACATC	ACGTTCCCAGCGGTACACAAAGAC	NM_009877
$p18^{INK4c}$	GGGGCATCGGAACCATAAG	AACCCCATTTGCCTCCATCA	NM_007671
$p19^{INK4d}$	GAAGAAGGGAGTGGGAGGAGCAGT	CCAAAAGGGGTGAGAAAAACAAAT	NM_009878
$p21^{Cip1}$	TGGGCCCGGAACATCTCAGG	CGTGGGCACTTCAGGGTTTTCTCT	NM_007669
$p27^{Kip1}$	GGTGGACCAAATGCCTGACTCGT	TCTGTTCTGTTGGCCCTTTTGTTT	NM_009875
$p57^{Kip2}$	GGGTGCTGAGCCGGGTGATGA	CTCCGGCTCCTCGTCCTTCTCCTC	NM_009876
c-Myc	GCCCACCACCAGCAGCGACTCT	GGGGTTTGCCTCTTCTCCACAG	NM_001177352
Odc1[a]	GCCCGGCTCTGACGATGAA	CCGCTCTCCTGGGCACAAG	NM_013614

[a] Semiquantitative RT-PCR.

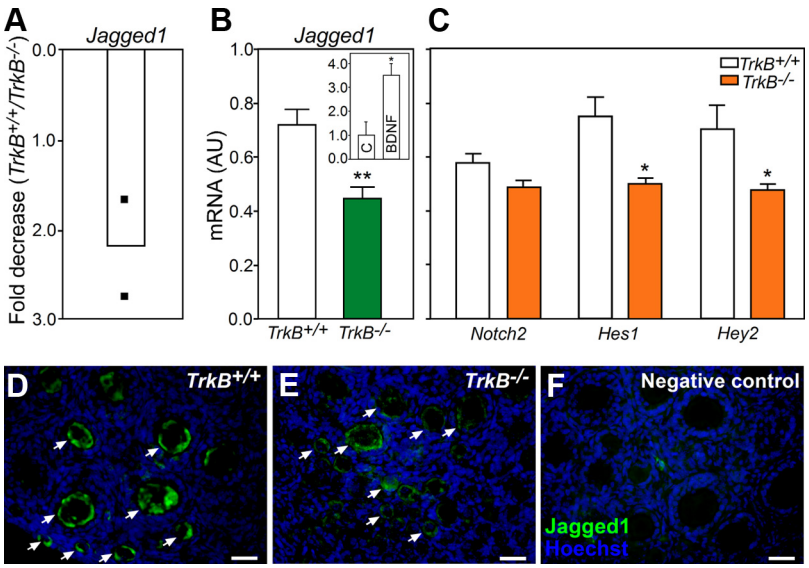

FIG. 1. Absence of TRKB receptors result in reduced expression of *Jagged1* and NOTCH target genes in the mouse ovary. Panel A, Decrease in *Jagged1* mRNA content in the ovary of 7-d-old $TrkB^{-/-}$ mice detected using cDNA microarrays. Changes in mRNA content are expressed as fold-decrease with respect to mRNA values in $TrkB^{+/+}$ mice of the same age. *Filled squares* represent the values detected in independent microarray determinations. Panel B, *Jagged1* mRNA content was reduced in the ovary of 7-d-old $TrkB^{-/-}$ mice, as assessed by real-time PCR. *Inset*, *Jagged1* mRNA content increased in 7-d-old WT ovaries treated *in vitro* with BDNF (100 ng/ml, 8 h) compared to control (C) ovaries incubated with vehicle. Relative mRNA values are expressed as arbitrary units (AU), normalized using *18s* RNA or *Ppia* mRNA values as the normalizing unit. Panel C, *Hes1* and *Hey2*, but not *Notch*, mRNA abundance, was also reduced in *TrkB*-null ovaries. Panel D, JAGGED1 immunoreactive material (*green color*) mostly localizes to oocytes in the ovary from 7-d-old $TrkB^{+/+}$ mice. Panel E, JAGGED1 immunoreactivity was noticeably decreased in oocytes of $TrkB^{-/-}$ mice. Panel F, Section incubated without JAGGED1 antibodies. Cell nuclei stained with the DNA-binding dye Hoechst are shown in *blue*. *White arrows* point to examples of oocytes showing JAGGED1 staining. *Columns* in B and C represent means from four to five animals per group, and *vertical lines* are SEM. *, $P < 0.05$; **, $P < 0.01$ *vs.* WT controls. *Scale bars*, 50 μm.

cryostat sections derived from ovaries collected at postnatal d 4, 6, and 12 and fixed by immersion in 4% paraformaldehyde and 0.1 M sodium borate buffer (pH 9.5) (overnight at 4 C). After an overnight hybridization at 55 C, the sections were washed (36, 42) and exposed to an autoradiography nuclear emulsion (Kodak, Rochester, NY) for 3 wk. After developing the reaction, the sections were counterstained with hematoxylin (Sigma, St. Louis, MO), dehydrated in ascending alcohols, and coverslipped for microscopic examination. Control sections were incubated with a ^{35}S-labeled sense *Jagged1* RNA probe transcribed from the same cDNA template used to prepare the antisense probe but in the opposite direction.

Immunohistochemistry

After collection, the ovaries were immersed in Zamboni's fixative overnight at 4 C and processed as described (21, 40), before preparing 14-μm cryostat sections. JAGGED1 was detected with a rabbit polyclonal antibody (SC-6011, diluted 1:400; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The HA.11 epitope tag used in the LV-*Jagged1* construct was detected with a mouse monoclonal antibody (MMS-101R, diluted 1:1000; Covance, Berkeley, CA). After an overnight incubation at 4 C, the immunoreactions were developed by incubating the sections for 1 h at room temperature with Alexa Fluor 488 donkey antirabbit (1:500) and Alexa Fluor 568 donkey antimouse (1:500), respectively. Cell nuclei were stained with the vital dye Hoechst (Molecular Probes, Eugene, OR) as reported (21, 40).

Assessment of cell proliferation

The ovaries from 3-d-old $TrkB^{-/-}$ mice, incubated with LV-*Jagged1* or LV-no *Jagged1* for 4 d, were fixed in Zamboni's fixative, embedded in paraffin, sectioned at 14 μm, and subjected to immunohistochemistry for proliferating cell nuclear antigen (PCNA), as reported (21, 43), using a monoclonal antibody to PCNA (Mab PC-10, 1:100; Santa Cruz Biotechnology, Inc.) and developing the immunoreaction with a diaminobenzidine, H_2O_2, and nickel chloride solution, followed by counterstaining with Nuclear Fast Red (undiluted, 10 min at room temperature; Vector Laboratories, Burlingame, CA). Positive cells were counted using the public domain software ImageJ 1.42q (National Institutes of Health, Bethesda, MD; http://rsbweb.nih.gov/ij/index.html).

Morphometric analysis

Ovaries from 3-d-old mice, maintained for 4 d in organ culture, were fixed in Kahle's fixative, embedded in paraffin, serially sectioned at 6 μm, stained with Weigert's iron hematoxylin, and counterstained with picric acid-methyl blue, as reported (21, 38, 43). Every section was imaged as described (21), and the degree of follicle development was subjected to morphometric analysis counting only follicles in which the nucleus of the oocyte was visible (21, 43). Follicles were classified according to well-established criteria (44) that we have previously used (21, 38, 43).

Statistical analysis

Quantitative data were analyzed using SigmaStat 3.1 software (Systat Software, Inc., San Jose, CA). The data were first subjected to a normality test and an equal variance test. Data that passed these two tests were then analyzed as follows: comparison of two groups was performed with the Student's *t* test, data sets containing more than two groups were analyzed with one-way ANOVA followed by Student-Newman-Keuls multiple test for individual means.

Results

Absence of TRKB receptors results in reduced expression of JAGGED1 and NOTCH2 target genes in postnatal mouse ovaries

To identify genes that may be controlled by TRKB receptor-dependent signaling in the infantile mouse ovary,

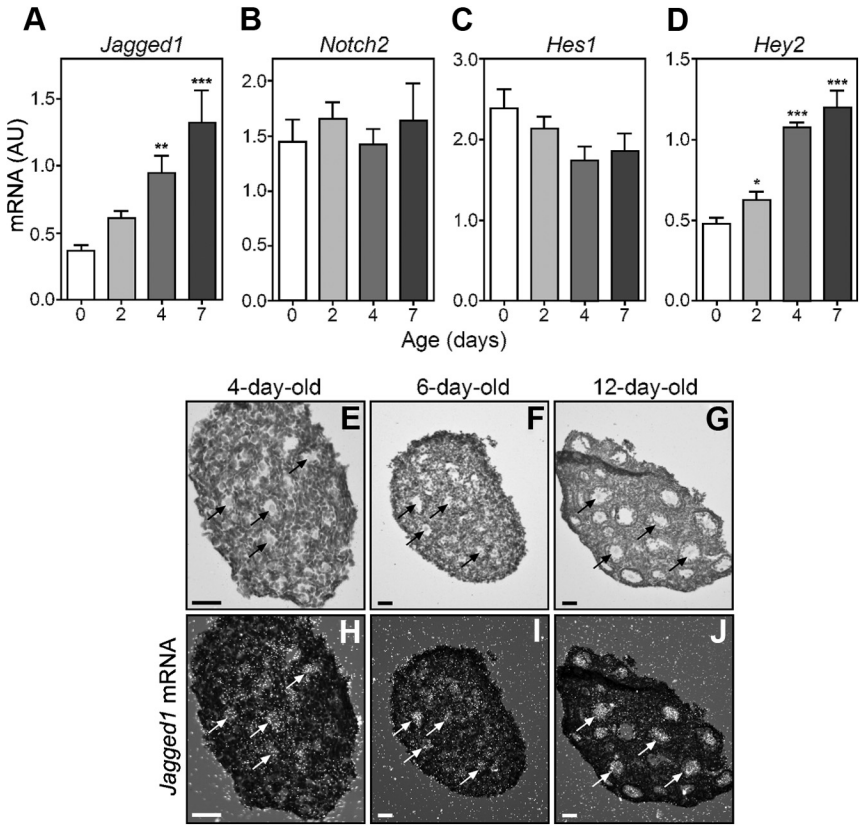

FIG. 2. Changes in ovarian content of *Jagged1*, *Notch2*, *Hes1*, and *Hey2* mRNA during the first postnatal week of life of the mouse, as assessed by real-time PCR. A, *Jagged1* mRNA. B, *Notch2* mRNA. C, *Hes1* mRNA. D, *Hey2* mRNA. Relative mRNA values are expressed as arbitrary units (AU), normalized using *18s* RNA values as the normalizing unit. E–J, *In situ* hybridization, using a mouse-specific ^{35}S-uridine triphosphate-labeled *Jagged1* cRNA probe, shows that *Jagged1* mRNA is exclusively expressed in oocytes and that the abundance of *Jagged1* mRNA increases during the first 12 d of postnatal life. Bright field images are shown in E–G and dark field images in H–J. *Black and white arrows* point to examples of *Jagged1* mRNA-containing oocytes. *Bars* represent the mean of four to five mice per group, and *vertical lines* are SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* 0-d-old group. *Scale bar*, 50 μm.

we interrogated ovaries from 7-d-old $TrkB^{+/+}$ and $TrkB^{-/-}$ mice employing DNA microarrays and real-time PCR. Among the genes whose expression decreased in the absence of TRKB signaling, we identified *Jagged1* (Fig. 1A), one of the Notch ligands. Using real-time PCR, we confirmed the array result (Fig. 1B) and found that the expression of *Hes1* and *Hey2*, two of the well-characterized NOTCH target genes (45, 46), was also decreased (Fig. 1C). In contrast, there were no differences in *Notch2* mRNA expression between WT and KO ovaries (Fig. 1C). Incubation of WT ovaries from 7-d-old mice with the TRKB ligand BDNF (100 ng/ml, 8 h) resulted in a 3-fold increase in *Jagged1* mRNA content (Fig. 1B, *inset*), suggesting that *Jagged1* expression decreases in $TrkB^{-/-}$ ovaries due to the absence of TRKB-mediated signaling. Next, we performed immunohistochemical experiments to verify the cellular sites of JAGGED1 expression and to determine whether the content of JAGGED1 protein is also decreased in $TrkB^{-/-}$ ovaries. JAGGED1 was seen exclusively in oocytes, and the intensity of the fluorescence signal was distinctly lower in *TrkB*-null than in WT ovaries (Fig. 1, D and E). No specific staining was observed in sections incubated without the primary antibody (Fig. 1F).

Ontogeny of *Jagged1*, *Notch2*, *Hes1*, and *Hey2* mRNA expression in the postnatal mouse ovary

We next performed real-time PCR measurements to determine whether the expression of *Jagged1*, *Notch2*, and *Notch2* target genes changes during the first 7 d of life, *i.e.* the period of time when the primordial, primary, and secondary follicles form. In agreement with an earlier report (29), we observed that *Jagged1* and *Hey2* mRNA abundance increase about 3-fold between the day of birth and the end of the first postnatal week of life (Fig. 2, A and D). In contrast, there were no significant changes in *Notch2* and *Hes1* mRNA expression (Fig. 2, B and C). Hybridization histochemistry demonstrated negligible *Jagged1* expression on the day of birth (data not shown), before follicular assembly, and a gradual increase in *Jagged1* mRNA abundance restricted to oocytes between 4 and 12 d of age (Fig. 2, E–G, bright field images, and H–J, dark field images), a time during which primary follicles (Fig. 2, E and H) reach the large preantral stage (Fig. 2, G and J).

Oocyte-specific increase in JAGGED1 expression rescues deficits in secondary follicle development and GC proliferation in *TrkB*-null mice

A previous study showed that 7-d-old ovaries lacking all isoforms of the TRKB receptor have a deficiency in secondary follicle development and GC proliferation (21). To determine whether this deficit is related to decreased *Jagged1* expression, we exposed ovaries from 3-d-old $TrkB^{-/-}$ mice for 4 d to a lentiviral construct expressing *Jagged1*, tagged with the human influenza HA epitope tag, under the control of the *Gdf9* promoter (Fig. 3A). Immunohistofluorescence analysis of the ovaries 4 d later using antibodies to HA revealed that the JAGGED1-HA protein was exclusively expressed in oocytes, where it was cor-

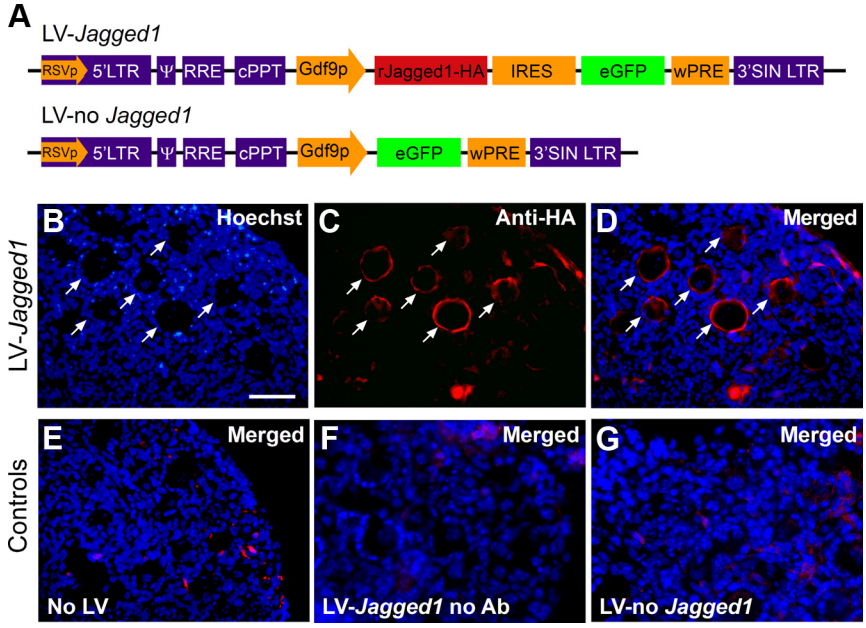

FIG. 3. Lentiviral-mediated delivery of *Jagged1*, using the *Gdf9* promoter to target expression of a JAGGED1-HA fusion protein to oocytes, correctly targets JAGGED1 to the cell membrane of oocytes. A, Map of the lentiviral delivery construct (LV-*Jagged1*) used in this study. The lentiviral vector employed has been previously described (79). The 3′LTR of this vector contains a 400-bp deletion that results in the self-inactivation (SIN) of the vector. The other components include the packaging signal (ψ), the Rev response element binding site (RRE), the central polypurine tract (cPPT), and the woodchuck-hepatitis-virus posttranslational regulatory element (wPRE). The LV-*Jagged1* construct contains a bicistronic transgene cassette in which expression of a *Jagged1-HA* cDNA is driven by the rat *Gdf9* promoter (*Gdf9p*). The *Jagged1*-HA cDNA is linked to an enhanced green fluorescent protein (eGFP) cDNA via an internal ribosome entry site (IRES). A construct lacking *Jagged1-HA* (LV-no *Jagged1*) was used as a negative control. B–D, Immunohistofluorescent images of sections from 3-d-old mouse ovaries cultured for 4 d in the presence of LV-*Jagged1* and stained with monoclonal antibodies against the HA epitope. E, Section from an ovary not infected with LV. F, Section from an ovary infected with LV-*Jagged1* and incubated without HA antibodies. G, Section from an ovary infected with LV-no *Jagged1*. JAGGED1 immunoreactive cells are seen in *red*, and cell nuclei stained with the DNA-binding dye Hoechst are shown in *blue*. *Scale bar*, 50 μm.

rectly targeted to the cell membrane (Fig. 3, B–D). We verified the specificity of this localization using three different negative controls: 1) ovaries incubated without LV (Fig. 3E), 2) ovaries incubated in presence of LV-*Jagged1-HA* but without adding the first antibody to the immunohistochemical reaction (Fig. 3F), and 3) ovaries incubated with the LV vector alone (Fig. 3G). In no case was HA oocyte staining observed.

Next, we performed a morphometric analysis of cultured $TrkB^{-/-}$ ovaries to compare the degree of follicular growth achieved after infection with LV-*Jagged1* in comparison with ovaries infected with LV-no *Jagged1*. We observed that the number of secondary follicles per ovary was significantly ($P < 0.05$) increased in the mutant ovaries infected with LV-*Jagged1* as compared with ovaries infected with LV-no *Jagged1* (Fig. 4A). Representative images showing this difference are shown in Fig. 4, B and C. No overt changes in other ovarian structures were observed between the two groups.

To determine whether this increase in follicle number is accompanied by changes in GC proliferation, we estimated the number of GC immunopositive for the proliferation marker PCNA 4 d after infecting $TrkB^{-/-}$ ovaries with LV-*Jagged1* or LV-no *Jagged1*. We found a 2.5-fold increase in the number of follicles containing PCNA-positive cells in $TrkB^{-/-}$ ovaries infected with LV-*Jagged1* compared with controls (Fig. 5A) and a similar increase in the number of PCNA-immunopositive cells per follicle (Fig. 5B). The microphotographs depicted in Fig. 5, C–E, illustrate these changes (examples denoted by *arrows*).

Loss of TRKB signaling results in decreased expression of *c-Myc*

Follicular growth requires proliferation of GC (47–49), a process that involves the coordinated participation of cyclins, cyclin-dependent kinases (CDK), and CDK inhibitors (CKI) (50, 51). In addition, early follicular growth appears to involve the participation of the oncoprotein c-MYC (27, 52, 53). It was, therefore, important to determine whether the reduction in GC proliferation observed in $TrkB^{-/-}$ ovaries (21) is related to changes in expression of these genes. In the absence of TRKB receptors, *c-Myc* mRNA abundance was significantly reduced, as assessed by qPCR on postnatal d 7 (Fig. 6A). This decrease is, to a significant extent, due to the absence of TRKB-mediated signaling, because WT ovaries responded to NT4/5, a TRKB ligand, with increased *c-Myc* expression after 8 h in organ culture (Fig. 6A, *inset*).

A well-established target of c-MYC is *Odc1* (54, 55), which encodes ODC, the rate-limiting step in polyamine biosynthesis. ODC plays an essential role in cell proliferation (56). DNA microarrays revealed that *Odc1* expression was reduced (2-fold decrease) in the ovaries from 7-d-old $TrkB^{-/-}$ mice compared with WT ovaries (data not shown). Using real-time PCR, we confirmed the array result (Fig. 6B). WT ovaries treated *in vitro* with NT4/5 (100 ng/ml, 8 h) showed an increase in *Odc1* mRNA abundance (Fig. 6B, *inset*), suggesting that, as is the case of *c-Myc*, *Odc1* expression is also enhanced by TRKB-mediated signaling. In contrast, no changes were observed

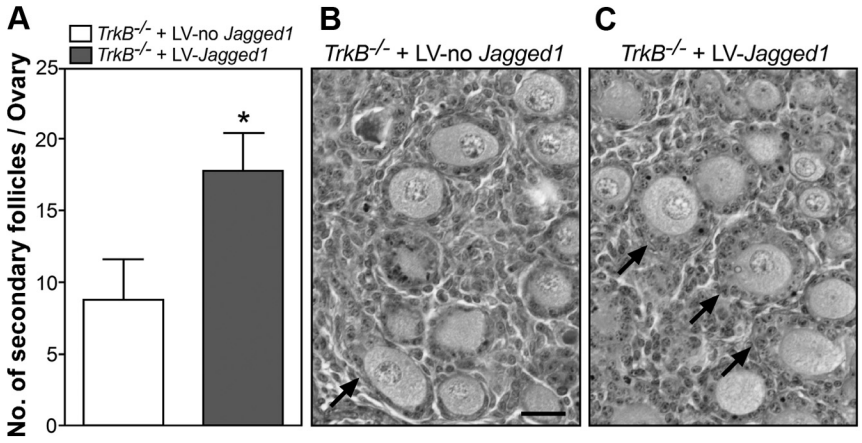

FIG. 4. Oocyte-specific restoration of JAGGED1 expression, via lentiviral-mediated gene transfer, rescues the deficit in follicle growth of $TrkB^{-/-}$ ovaries. A, Increased number of secondary follicles in $TrkB^{-/-}$ ovaries incubated for 4 d with LV-*Jagged1* in comparison with $TrkB^{-/-}$ ovaries infected with LV-no *Jagged1*. B, Section from a $TrkB^{-/-}$ ovary infected with LV-no *Jagged1*. C, Section from a $TrkB^{-/-}$ ovary infected with LV-*Jagged1*. *Arrows* point to secondary follicles, which contain an oocyte surrounded by two layers of GC. *Scale bar*, 50 μm. *Columns* represent the mean of four mice per group, and *vertical lines* are SEM. One ovary from each animal was infected with LV-*Jagged1* and the contralateral ovary from the same animal with LV-no *Jagged1*. *, $P < 0.05$.

in the gene expression of several core regulators of the cell cycle, including two mRNA encoding cyclins (*CycD2* and *CycE1*), two mRNA encoding CDK (*Cdk2* and *Cdk4*), and mRNA encoding CKI of either the inhibitors of CDK4 (INK4) gene family ($p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$) or the cyclin and CDK inhibitors (CIP/KIP) family ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) (Fig. 6, C and D).

Restoring JAGGED1 expression in oocytes of *TrkB*-null ovaries rescues *c-Myc* expression

$TrkB^{-/-}$ ovaries infected with LV-*Jagged1-HA* showed increased levels of *c-Myc* mRNA after 4 d of treatment in organ culture (Fig. 6E). This change is not due to a general effect of JAGGED1 on cell cycle regulators, because neither $p19^{INK4D}$ nor $p27^{Kip1}$ mRNA levels, selected as examples of each class of genes, changed in LV-*Jagged1-HA*-infected ovaries (Fig. 6F). These results suggest that NOTCH signaling supports GC proliferation by activating a c-MYC-dependent pathway and not by affecting the expression of core regulators of cell-cycle progression.

Discussion

Studies in several species, including rodents, humans, cattle, and pigs, have established the concept that NT are physiological components of the intraovarian machinery controlling both the assembly of primordial follicles and the growth of newly formed follicles (reviewed in Ref. 57). The mechanisms underlying the supportive actions of NT on these two developmental events have not been yet elucidated. The present results identify the NOTCH signaling pathway as a mediator of the process by which NT acting via TRKB receptors facilitate early follicle development and stimulate GC proliferation in primary follicles. Our results indicate that activation of this pathway involves a NOTCH ligand (JAGGED1) produced in oocytes and NOTCH receptors, presumably located in GC. Although *Jagged1* expression is reduced in the absence of TRKB receptors, oocyte-specific restoration of JAGGED1 synthesis in *TrkB*-null ovaries reinvigorates follicle growth and GC proliferation, suggesting that JAGGED1 produced in oocytes is crucial for NT4/5-BDNF (the TRKB ligands) to promote early follicle development. We also observed loss of *c-Myc* expression in the ovaries of *TrkB*-null mice and rescue of this deficit by the oocyte-specific restoration of JAGGED1 synthesis. Considering that c-MYC, which drives cell proliferation by promoting entry into the cell cycle (32), is a direct target of the NOTCH signaling system (30, 31), our results implicate c-MYC as a downstream mediator of TRKB-dependent stimulation of GC proliferation. None of the aforementioned deficits can be attributed to a general effect related to the absence of TRKB receptors, because all of these deficits were rescued by specifically recovering JAGGED1 synthesis in oocytes of the mutant mice.

NOTCH signaling is an evolutionarily conserved mechanism that regulates cell fate, differentiation, and growth in a vast array of tissues (45, 58, 59). There are at least four NOTCH receptors (NOTCH1–NOTCH4) and at least five ligands, including JAGGED1, JAGGED2, Δ-like 1, Δ-like 2, and Δ-like 3 (59, 60). A major feature of NOTCH signaling is that it mediates communication between adjacent cells. Upon ligand binding, the intracellular domain of NOTCH receptors (NICD) is released by proteolytic cleavage (61) and translocates to the nucleus, where it binds to a repressor of the C-promoter binding factor 1/suppressor of hairless/Lag-1 family to convert it into a trans-activating complex, which then promotes the transcription of target genes, including *Hes1*, *Hey2*, *c-Myc*, and others (45, 46, 59).

Several components of the NOTCH signaling pathway have been previously described in the ovary. Although JAGGED1 is expressed exclusively in germ cells, and oocytes of primordial, primary, and secondary follicles,

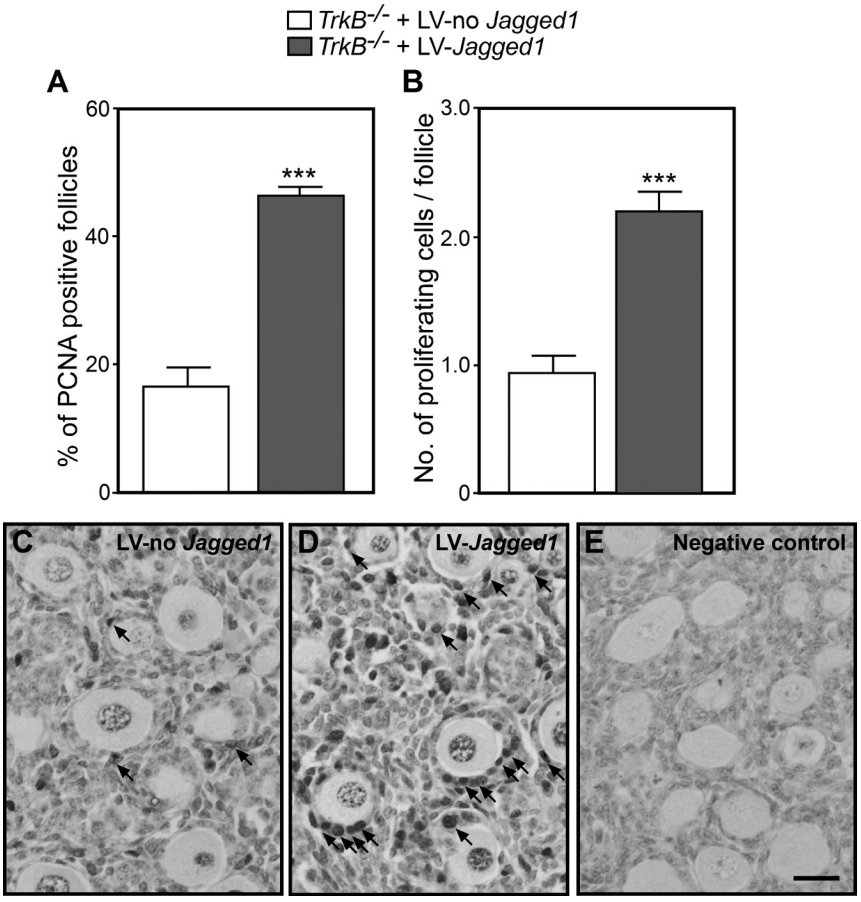

FIG. 5. Oocyte-specific restoration of JAGGED1 expression, via lentiviral-mediated gene transfer, rescues the deficit in GC proliferation of *TrkB*$^{-/-}$ ovaries. A, Percent of follicles showing at least one PCNA-positive GC. B, Number of PCNA-positive GC per follicle (primary and secondary). C, Image of a section from a *TrkB*$^{-/-}$ ovary incubated for 4 d with LV-no *Jagged1*. D, A section from a *TrkB*$^{-/-}$ ovary incubated for 4 d with a LV-*Jagged1*. E, Ovarian section immunostained in absence of primary antibodies. *Columns* represent the mean of four mice per group, and *vertical lines* are SEM. In each group, four sections per ovary were used for quantification. One ovary from each animal was infected with LV-*Jagged1* and the contralateral ovary from the same animal with LV-no *Jagged1*. *Scale bar*, 50 μm. ***, $P <$ 0.001 *vs.* LV-no *Jagged1*.

NOTCH2, HES1, and HEY2 are expressed mainly in GC (27–29). Although it has been known for some time that the NOTCH system plays a crucial role in the control of follicle cell and oocyte development in *Drosophila* (23–25), it is only recently that the importance of NOTCH signaling in primordial follicle formation and GC proliferation during early follicle development in mammals has been documented (27, 29). An essential requirement for NOTCH activity is the addition of *N*-acetylglucosamine to its extracellular domain by Fringe proteins (62). Lunatic Fringe (*Lfng*), one of these proteins, specifically facilitates JAGGED1/NOTCH2 signaling (63). The finding that mice lacking *lunatic fringe* are infertile and that the infertility is due to an ovarian defect (26) adds further credence to the notion that NOTCH signaling is required for normal ovarian development.

It is surprising that the ability of NT to activate *Hes1* transcription has been known for more than 15 yr (64), but only recently evidence has been provided demonstrating the existence of a functional relationship between NT and the NOTCH signaling pathway (65). Our results suggest that a mechanism by which activation of TRKB receptors enhances NOTCH signaling in the ovary is by stimulating the synthesis of JAGGED1 in oocytes. Whether this is a direct effect or involves the production of an intermediate molecule produced in GC is unclear. Because expression of full-length TRKB receptors is minimal in oocytes of neonatal-infantile animals (12, 21), a direct effect would imply a role for truncated TRKB receptors in inducing *Jagged1* expression. These receptors are the most abundant TRKB isoform expressed in infantile mouse ovaries (12, 21), but they lack canonical signaling motifs. They may, however, be able to initiate intracellular signaling via pathways other than those activated by full-length TrkB receptors (66, 67). A more detailed examination of this issue is warranted.

An involvement of the NOTCH system in stimulating GC proliferation of primary follicles was recently demonstrated by Zhang *et al.* (27), who exposed mouse primary ovarian follicles cultured *in vitro* to γ-secretase inhibitors to block the proteolytic release of NICD and observed arrest of follicle growth and inhibition of GC proliferation. These authors also showed that overexpression of NICD promotes GC proliferation (27). By showing that GC proliferation is diminished in the absence of TRKB-mediated signaling (21) and that selective restoration of JAGGED1 production in oocytes rescues the defect in follicle growth and GC proliferation seen in *TrkB*-null mutants, our results provide a functional link between the NT and NOTCH signaling systems.

NOTCH signaling promotes progression of the cell cycle in all species thus far examined (25, 68–71), suggesting that the loss of GC proliferation observed in *TrkB*-null ovaries may be related to loss-of-function of one or more regulatory components of the cell cycle. Cell-cycle progression is promoted by phase-specific kinase complexes

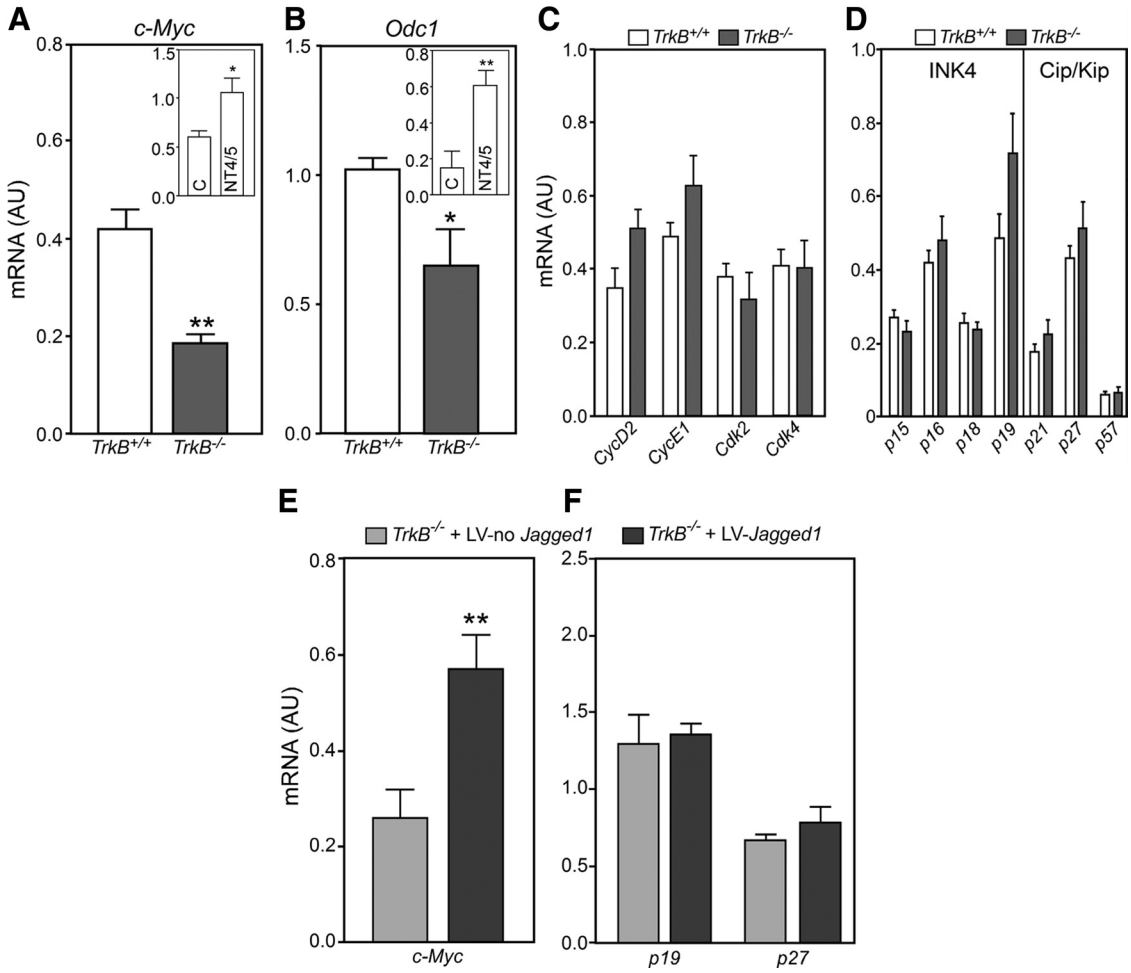

FIG. 6. TrkB signaling sustains *c-Myc* and *Odc1* expression but not the expression of core regulatory components of the cell cycle. Panel A, *c-Myc* mRNA content was reduced in 7-d-old $TrkB^{-/-}$ ovaries as compared with WT animals. *Inset*, *In vitro* exposure of WT ovaries to NT4/5 (100 ng/ml, 8 h) increased c-*Myc* mRNA abundance as compared to control (C) ovaries incubated with vehicle. Panel B, *Odc1* mRNA abundance was also decreased in $TrkB^{-/-}$ ovaries. *Inset*, NT4/5 increased *Odc1* mRNA abundance in WT ovaries. Panels C and D, The content of mRNA encoding cyclins (*CycD2* and *CycE1*), CDK (*Cdk2* and *Cdk4*), and the CKI of the INK4 family ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$) and CIP/KIP family ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) remain unaltered in $TrkB^{-/-}$ ovaries as compared with $TrkB^{+/+}$ mice. Panel E, *c-Myc* mRNA levels were increased in $TrkB^{-/-}$ ovaries after oocyte-specific restoration of JAGGED1 synthesis. The ovaries from 3-d-old mice were incubated for 4 d with a lentiviral construct carrying the *Jagged1*-coding region under the control of the *Gdf9* promoter. Panel F, Neither $p19^{INK4D}$ nor $p27^{Kip1}$ mRNA levels changed after lentiviral-mediated restoration of JAGGED1 synthesis. Control ovaries were infected with a LV lacking *Jagged1* cDNA (LV-no *Jagged1*). *Each column* represents the mean of four to five mice per group, and *vertical lines* are SEM. One ovary from each animal was infected with LV-*Jagged1* and the contralateral ovary with LV-no *Jagged1*. *, $P < 0.05$; **, $P < 0.01$ *vs.* their respective controls. AU, Arbitrary units.

composed of cyclin and CDK. Although cyclin D-CDK4/6 complexes promote G_1 progression, cyclin E-CDK2 complexes facilitate completion of the G_1 phase (72–74). CDK activity is, in turn, regulated by CKI, which induce cell-cycle arrest by blocking the activity of cyclin-CDK complexes (75–78). Two CKI families have been described: members of the INK4 family ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$) bind to and inhibit cyclin D-CDK4/6 complexes; members of the CIP/KIP family ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) bind to and inhibit the cyclin E-CDK2 complex, as well as other cyclin-CDK complexes operating throughout the cell cycle (77, 78).

We measured two mRNA encoding cyclins (*CycD2* and *CycE1*), two encoding CDK (*Cdk2* and *Cdk4*), four encoding CKI of the INK4 family ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$), and three encoding members of the CIP/KIP family ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) in ovaries from *TrkB*-null mice and found that their abundance was similar to that of WT ovaries. In addition, we measured two mRNA encoding CKI ($p19^{INK4d}$ and $p27^{Kip1}$) after restoring *Jagged1* expression in oocytes and again found the abundance of these mRNA to be unaltered. These results suggest that the deficit in GC proliferation seen in *TrkB*-null ovaries is not due to decreased expression of core regulatory components of the cell cycle.

In contrast to these results, expression of the proto-oncogene *c-Myc*, a *bona fide* NOTCH target (30, 31), decreased in the absence of TRKB signaling and increased

after oocyte-specific restoration of JAGGED1 synthesis. These findings suggest that the proliferative and growth-inducing actions of TRKB-activated NOTCH signaling are mediated by c-MYC. Earlier studies support this conclusion. For instance, several years ago, it was reported that expression of both *c-Myc* mRNA and c-MYC is restricted to preantral follicles (52, 53). A very recent report showed that inhibition of NOTCH2 signaling in primary follicles decreased GC proliferation and reduced *c-Myc* expression and that overexpression of the NOTCH2 intracellular domain increases GC proliferation and induces *c-Myc* expression (27). Further supporting an involvement of *c-Myc* in mediating TRKB-activated Notch-dependent GC proliferation is provided by the reduction in *Odc1* mRNA abundance observed in *TrkB* KO ovaries and the increase in *Odc1* expression elicited in WT ovaries by the ligand-dependent activation of TRKB receptors. *Odc1* encodes ODC, an enzyme that plays an essential role in cell proliferation (56) and whose transcription is activated by c-MYC (54, 55).

It has been shown that c-MYC activates the *Cdc25A* gene, which encodes a phosphatase controlling CDK2 activity, in addition to the genes encoding cyclin D2 and CDK4. It has also been reported that c-MYC represses $p21^{Cip1}$, suggesting that c-MYC drives the cell cycle by prolonging activation of cyclin E/CDK2 complexes (32). The lack of changes in *CycD2*, *Cdk4*, and $p21^{Cip1}$ expression that we observed in our studies may be interpreted as indicating a lack of c-MYC effect on the expression of these genes in the infantile ovary. However, in several instances, c-MYC appears to act via a hit-and-run mechanism (33), raising the possibility that changes in expression of these cell cycle components in our model are evanescent and no longer detected after several days in culture.

In sum, our results suggest that NOTCH signaling is one of the cell-cell communication pathways used by NT to control early follicular growth and that the coordinated activation of TRKB and NOTCH signaling represents one of the mechanisms of reciprocal oocyte-GC communication underlying the initiation of follicular growth. Our results are consistent with a model in which BDNF/NT4/5 produced by GC would activate (directly or indirectly) *Jagged1* expression. In turn, JAGGED1 would activate NOTCH2 receptors in GC, which would promote GC proliferation and follicle growth by inducing expression of the cell cycle regulator factor c-MYC. Although the early stages of follicle development may be the most sensitive to NOTCH signaling, it is possible that NT-initiated cell-cell communication mediated through JAGGED1-NOTCH also plays a role in later stages of follicle development. Studies using mice in which TRKB receptors are conditionally deleted from the ovary in a cell-specific manner will be useful to address this question.

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