Fibroblast Growth Factor 9 Has Oncogenic Activity and Is a Downstream Target of Wnt Signaling in Ovarian Endometrioid Adenocarcinomas

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Abstract

Wnt signaling plays a key role in development and adult tissues via effects on cell proliferation, motility, and differentiation. The cellular response to Wnt ligands largely depends on their ability to stabilize β-catenin and the ability of β-catenin to bind and activate T-cell factor (TCF) transcription factors. Roughly 40% of ovarian endometrioid adenocarcinomas (OEA) have constitutive activation of Wnt signaling as a result of oncogenic mutations in the β-catenin protein or inactivating mutations in key negative regulators of β-catenin, such as the adenomatous polyposis coli and Axin tumor suppressor proteins. We used oligonucleotide microarrays to identify genes of which expression was activated in OEAs with β-catenin dysregulation compared with OEs lacking Wnt/β-catenin pathway defects. Using microarray and quantitative PCR-based approaches, we found that fibroblast growth factor (FGF9) expression was increased >6-fold in primary OEs with Wnt/β-catenin pathway defects compared with OEs lacking such defects. Evidence that β-catenin and TCFs regulate FGF9 expression in several epithelial cell lines was obtained. We found FGF9 was mitogenic for epithelial cells and fibroblasts and FGF9 could stimulate invasion of epithelial and endothelial cells through Matrigel in transwell assays. Furthermore, FGF9 could promote neoplastic transformation of the E1A-immortalized RK3E epithelial cell line, and short hairpin RNA-mediated inhibition of endogenous FGF9 expression in the OEA cell line TOV112D, which carries a β-catenin mutation, inhibited neoplastic growth properties of the cells. Our findings support the notion that FGF9 is a key factor contributing to the cancer phenotype of OEs carrying Wnt/β-catenin pathway defects. (Cancer Res 2006; 66(3): 1354-62)

Introduction

Ovarian cancer is the most lethal gynecologic malignancy and is the fourth most frequent cause of cancer-related deaths in women in the United States (1). Malignant epithelial tumors compose the majority of ovarian cancers and there are several histologic subtypes, including serous, endometrioid, clear cell, and mucinous adenocarcinomas. A number of molecular studies, including comprehensive gene expression profiling, have shown that each of these histologic subtypes has a characteristic molecular signature (2, 3). As such, the different histologic types of ovarian carcinoma likely represent distinct, albeit partially overlapping, entities. Ovarian endometrioid adenocarcinomas (OEA) are the second most common type of ovarian cancer after serous carcinomas. Upwards of 40% of OEA harboring mutations of CTNNB1, the gene encoding β-catenin (β-cat; refs. 4–6). Mutations in β-cat are rare in serous, clear cell, and mucinous ovarian carcinomas. β-Cat is a critical component of the highly conserved canonical Wnt signaling pathway that plays a role in both vertebrate and invertebrate cell proliferation and differentiation (7, 8). Wnt signaling is activated by secreted Wnt ligands binding to a coreceptor complex composed of a transmembrane frizzled receptor and a low-density lipoprotein-related protein such as lipoprotein-related protein 5 or lipoprotein-related protein 6. Receptor activation likely leads to several consequences, most prominently inhibition of Axin and GSK3β, which play key roles in negative regulation of the free pool of β-cat. Stabilized β-cat is then able to enter the nucleus where it binds to T-cell factor (TCF)/lymphoid enhancer family DNA binding proteins and forms a transcription factor complex leading to transcriptional activation of specific downstream target genes, such as c-MYC, CCND1 (cyclin D1), and MMP-7 (9–12). Aberrant Wnt signaling is commonly seen in certain cancer types and a number of studies have been devoted to identifying the critical downstream effectors of this pathway that play a role in cancer progression and development. Recently, we used Affymetrix oligonucleotide (HuGeneFL) microarrays to compare the gene expression profiles of well-characterized OEA with intact Wnt signaling to those with known Wnt pathway defects (usually mutant β-cat) as a strategy to identify novel Wnt pathway target genes (4, 13, 14). Unsupervised principal component analysis of these data showed that Wnt pathway status is a major determinant of gene expression in OEA. The notable influence of deregulated Wnt signaling on global gene expression profile, as well as confirmation of several previously implicated pathway target genes (e.g., EPHB3, CCND1, MSX2, and MMP7) up-regulated in tumors with pathway defects, further validated this approach for identifying heretofore unrecognized Wnt pathway target genes (14). Clearly, a major challenge is identifying those genes on the list of candidates that likely play critical functional roles in OEA pathogenesis. In collaborative studies with our laboratory, Chamorro et al. (15) recently characterized FGF20 as a downstream target of the Wnt pathway that likely plays an important role in oncogenesis. Another member of the fibroblast growth factor (FGF) family, FGF9, also showed significantly increased expression in pathway-deregulated versus pathway-intact OES. Here, we report data implicating FGF9 as a downstream target of Wnt signaling and show that FGF9 has oncogenic activity, supporting its role in the molecular pathogenesis of OEs with Wnt pathway deregulation.
Materials and Methods

Primary tumor samples. Oligonucleotide microarray-based gene expression profiling of primary OEsAs with and without Wnt signaling pathway deregulation has been reported previously in detail (14). All tumors were manually microdissected before RNA extraction to ensure each sample contained at least 70% tumor cells. Analysis of tissues from human subjects was approved by the University of Michigan Institutional Review Board (IRB-MED #2001-0568).

Quantitative reverse transcription-PCR analysis of FGF9 mRNA expression. RNA was extracted from primary tumor tissues or cultured cells harvested in Trizol (Invitrogen, Carlsbad, CA), then further purified using RNeasy columns (Qiagen, Valencia, CA) and treated with DNase I. First-strand cDNA was synthesized from DNase I–treated mRNA samples using random hexamer primers (Amersham Biosciences, Piscataway, NJ) and Superscript II (Invitrogen). Microarray data for FGF9 were verified using TaqMan reverse transcription-PCR (RT-PCR; see http://dot.ped.med.umich.edu:2000/pub/Ovary/FGF9/index.html for primer sequences). Reactions were carried out on an Applied Biosystems 7700 system (Foster City, CA) with the following conditions: 50°C for 2 minutes and 95°C for 10 minutes (initial denaturation) followed by 40 cycles of 95°C for 15 seconds (denaturing) and 60°C for 1 minute (annealing and extension). The threshold cycle ($C_t$; i.e., the cycle number at which the amount of amplified gene of interest reached a fixed threshold) was determined. Quantitation of gene expression was calculated by the comparative $C_t$ method using HPRT1 as the internal control gene. Selected RT-PCR experiments for evaluation of FGF9 expression were done using the SYBR Green method. 2 $\times$ SYBR Green PCR master mix (Applied Biosystems) was used in 25-$\mu$L reactions set up in duplicate with the previously described primer sequences. Reactions were run at 50°C for 2 minutes and 95°C for 10 minutes (initial denaturation) followed by 40 cycles of 95°C for 15 seconds (denaturing) and 60°C for 1 minute (annealing and extension). This was followed by dissociation curve analysis to ensure the fluorescence signal was not derived from primer-dimer formation. The following conditions were used: 95°C for 15 seconds, 60°C for 20 seconds, and 95°C for 15 seconds with a ramp time of 19 minutes 59 seconds between the annealing and denaturation steps. Experiments were done in duplicate and the SE expression values were determined.

Protein isolation and Western blotting. Primary tumor samples were homogenized in 250 $\mu$L of radioimmunoprecipitation assay lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS] containing complete protease inhibitor tablets (Roche, Indianapolis, IN) per instructions of the manufacturer. Samples were centrifuged twice at 12,000 rpm for 30 minutes at 4°C. The protein concentration in each sample was determined by bicinchoninic acid assay (Pierce Chemicals, Rockford, IL). Fifty micrograms of protein were separated on SDS-12% polyacrylamide gels and then transferred to Immobilon P membranes (Millipore, Bedford, MA) by semidry electroblotting. Western blots were done according to standard methods using a murine monoclonal anti-FGF9 primary antibody (R&D Systems, Minneapolis, MN) at 1:125 dilution or a murine monoclonal anti-c-myc antibody (9E10, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:1,000, and horseradish peroxidase–conjugated goat anti-mouse antibody (Pierce) at 1:4,000. Membranes were probed with monoclonal anti-FGF9 antibody (9E10, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:20,000 dilution. The antigen-antibody complexes were detected by enhanced chemiluminescence (Amersham) followed by exposure to X-Omat film (Kodak, Rochester, NY).

Comparison of FGF amino acid sequence homology. The amino acid sequences of FGF family members were obtained at www.nlhm.ncbi.org. The sequences were imported into MegaAlign and a phylogenetic tree was generated using the Clustal W algorithm. The sequences were then aligned in the CLUSTAL W program and the alignment was imported into MEGA 2.0. The consensus tree was generated using the Clustal W algorithm.

PCR products were initially cloned into pCR-BluntII-TOPO (Invitrogen). Following screening and sequence verification, the FGF9 cDNA was excised and subcloned into pcDNA3.1/myc-His–B (Invitrogen). The myc-tagged FGF9 cDNA was further subcloned into pPGS.

Cell lines and transfections. TOV112D (OEA), HEC-1A (endometrial adenocarcinoma), and RK3E (EA-1 immortalized rat kidney epithelial cells) were obtained from the American Type Culture Collection (Manassas, VA). Human umbilical vein endothelial cells (HUVEC) were procured from Cambrex (Walkersville, MD). Photographing Phoenix (AP) cells were acquired from G. Nolan (Stanford University, Palo Alto, CA). OVCA420 cells, derived from an ovarian carcinoma, were obtained from D. Fishman (Northwestern University, Chicago, IL). Mouse mammary epithelial cell lines (Rac311) with stable expression of either Wnt-1 or empty vector control were provided by L. Howe (Weill Medical College of Cornell University, New York, NY). Ovarian surface epithelial cells immortalized with SV40 IgTag (IOSE-80) were a gift from N. Auersperg (University of British Columbia, Vancouver, Canada). AP, OVCA420, TOV112D, IOSE-80, Rac311, and RK3E cells were cultured in DMEM (Life Technologies, Inc., Gaithersburg, MD) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. HUVECs were maintained in endothelial growth medium from Cambrex. Generation of TOV112D/pPGS, TOV112D/dnTCF, and RK3E/S33Y-ER has been previously described (14, 18); the cell lines derived from TOV112D were maintained in medium with 0.5 $\mu$g/mL G418 (Life Technologies) and RK3E/S33Y-ER cells were cultured in medium with 0.5 mg/mL puromycin. HEC-1A/S33Y-ER cells were generated using previously described methods (18) and grown in McCoy’s 5A modified medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.5 mg/mL puromycin. RK3E/dnTCF and control cells were generated by infecting RK3E cells with viral supernatant from AP cells transfected with 15 $\mu$g of pPGS/dnTCF or pPGS. Cells were selected in 1 mg/mL G418 for 2 weeks; after which, they were grown in 0.5 mg/mL G418. RK3E and HEC-1A cells stably expressing the inducible S33Y-ER construct were either mock treated with ethanol or treated with 0.5 $\mu$mol/L 4-OH-tamoxifen (Sigma, St. Louis, MO) to activate the S33Y-ER fusion protein. In selected experiments, new protein synthesis was inhibited by addition of 0.5 $\mu$mol/L cycloheximide (Sigma). All drugs were added simultaneously to the cells in complete medium and cells were harvested at the indicated time points. To assess the effects of inhibiting GSK3β on FGF9 gene expression, OVCA420 cells were treated with 0 to 30 mmol/L LiCl. Cells were harvested at 24 hours in Trizol according to the instructions of the manufacturer. OVCA420 cells were also treated with 30 mmol/L LiCl, with and without cycloheximide, over a 0 to 24 hour time course to investigate whether FGF9 transcript induction may be dependent on short-lived cofactors that are exhausted over the time course necessary for induction of the S33Y-ER construct.

Cell proliferation assays. Cells were plated at 1.3 × 10^4/cm^2 in 24-well plates. For studies comparing the growth kinetics of RK3E parental cells to those stably expressing FGF9, cells were grown in complete medium for the duration of the assay. Cells were trypsinized and counted using a hemocytometer at the indicated time points. In studies using recombinant FGF9 protein (R&D Systems), cells were plated on day 1 and were changed to serum-free medium the following day. After 16 to 24 hours in serum-free conditions, the cells were treated with recombinant FGF9 in medium containing 1% FBS. The medium was refreshed after 72 to 96 hours and cells were trypsinized and counted at the indicated time points. Three independent experiments were done and error was calculated as the SE.

Focus formation assays. RK3E cells were infected in 100-mm dishes at 70 to 80% confluency with viral supernatant (pPGS, pPGS/S33Y (-cat)) diluted 1:1 with complete medium supplemented with 4 $\mu$g/mL Polybrene (Sigma). Medium was changed every 3 to 4 days over a total of 26 days. Cells were stained with methylene blue and colonies were photographed and counted. Four independent experiments were done in duplicate and error was calculated as the SE.

Soft agar assays. Underlayers (1-1.5 mL) of 0.6% agar medium were prepared in 35-mm dishes by combining equal volumes of 1.2% Noble agar (Difco, Detroit, MI) and 2 $\times$ DMEM with 40% FBS (Life Technologies). Cells (5 × 10^5) were then plated in 0.3% agar medium. The surface was kept wet.
by periodic addition of a small amount of growth medium. After 3 to 4 weeks, dishes were stained with methylene blue and colonies were photographed and counted. Four independent experiments were done in triplicate and error was calculated as SE.

**In vitro invasion assays.** Transwell membranes coated with Matrigel (BD Biosciences, San Jose, CA) were used to assay invasion in vitro. RK3E cells were trypsinized and plated at $5 \times 10^4$ per well (6.25 $\times 10^4$ per well for HUVECs) in the upper chamber in serum-free medium. FBS or recombinant FGF9 was added to the medium in the lower chamber. After 16 to 24 hours of incubation, medium was aspirated from the top and bottom wells and noninvasing cells were removed from the top well with a cotton swab. The remaining cells were fixed with 3% paraformaldehyde and stained with 4,6-diamidino-2-phenylindole. Stained membranes were excised, mounted on slides, and photographed. Three independent 20$\times$ fields for each well were counted for quantitation. Three independent experiments were done and error was calculated as the SE.

**Tumorigenicity assays.** Polyclonal populations of RK3E stably transduced by recombinant retroviruses with and without FGF9 cDNA were evaluated for tumorigenicity in nude mice. Five-week-old female athymic nude (nu/nu) mice (Charles River Laboratories, Wilmington, MA) were s.c. injected in the left and right flanks with $5 \times 10^6$ cells in 0.2 mL of HBSS without phenol red (Life Technologies). Groups of five mice were bilaterally injected with FGF9-expressing or control RK3E cells. Mice were examined two to three times per week for tumor formation at the injection sites. Tumor measurements were taken once per week when applicable and three for tumor growth at the injection sites and for tumor dissemination. Tumors were measured with linear calipers and stored at $-80^\circ$C for further analysis. Volume reported was determined by the standard ellipsoid formula $[\text{length} \times \text{width} \times \text{depth} \times (\pi/6)]$. All animal studies were conducted in accordance with a study protocol approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA approval #8082).

**Short hairpin RNA–mediated knockdown of FGF9 expression in TOV112D.** Four short hairpin RNA (shRNA) sequences, one scrambled and three for FGF9, were cloned into pSuper.retro.neo.gfp (Oligoengine, Seattle, WA) using BglII and HindIII restriction sites. See http://dot.ped.med.umich.edu/2000/pub/Ovary/FGF9/index.html for specific sequences. Following sequence verification of the constructs, 15 $\mu$g of purified plasmid DNA was transfected into AP cells. Viral supernatant was collected 48 hours after transfection. TOV112D cells were infected using a 1:1 virus-media ratio plus 4 $\mu$g/mL of Polybrene. Transduced cells were selected in 100 $\mu$g/mL G418. Following selection, FGF9 transcript levels relative to HPRT1 were determined using quantitative RT-PCR.

**Results**

**Selected FGFs are candidate Wnt pathway target genes.** We have previously shown that comparison of gene expression in primary OEAs with intact versus deregulated $\beta$-cat/TCF signaling is a powerful strategy for identifying novel Wnt pathway target genes (14). As proof of principle, and as previously noted, our list of candidate Wnt pathway genes included several genes that have been reported independently as likely $\beta$-cat/TCF transcriptional targets in other systems. We have reanalyzed our original and two additional OEAs (one with wild-type and one with mutant $\beta$-catenin) using higher-density oligonucleotide microarrays (Affymetrix U133A; full data set to be published separately). Two members of the FGF family, FGF20 ($P = 1.2e^{-07}$) and FGF9 ($P = 6.1e^{-06}$), were up-regulated >6-fold in OEAs with Wnt signaling pathway defects ($n = 12$) compared with those with intact Wnt signaling ($n = 18$). These two

![Figure 1](https://example.com/figure1.png)

**Figure 1.** FGF9 mRNA and protein levels are up-regulated in OEAs with Wnt pathway defects. A, FGF9 expression in OEAs with Wnt pathway defects (shaded columns) was compared with OEAs with intact signaling (open columns). Relative expression based on the Affymetrix U133A microarray data is shown in arbitrary units. B, quantitative RT-PCR analysis confirms FGF9 up-regulation in Wnt pathway–defective OEAs. Columns, mean fold expression of FGF9 (normalized to HPRT) in OEAs with (shaded) and without (open) Wnt pathway deregulation; bars, SE. C, immunoblot analysis of lysates from selected primary OEAs shows readily detectable expression of FGF9 protein. Normal proliferative endometrium [NE-18(PCI)] was used as a positive control.
FGFs are among those genes showing the most significant and largest fold differential expression of all genes measured on the U133A array. In collaborative studies with Chamorro et al. (15), we recently reported FGF20 as a transcriptional target of β-catenin that is implicated in tumorigenesis and maintenance of the transformed phenotype. We compared the amino acid sequence homology of the 23 known FGF family members to examine amino acid homology of those FGFs that emerged as candidate Wnt pathway target genes. Notably, the amino acid sequences of both FGF9 and 20 are closely related, sharing >70% sequence identity.

Both FGF9 mRNA and protein are up-regulated in OEs with Wnt pathway defects. Our microarray-based gene expression analysis of 30 primary OEs showed FGF9 transcripts to be significantly and consistently up-regulated in OEs with Wnt pathway defects compared with those with intact Wnt signaling (Fig. 1A). The microarray data were validated through studies of a partially overlapping set of OEs using TaqMan quantitative RT-PCR analysis with FGF9 specific primers (Fig. 1B). Protein lysates were generated from selected OEs with varying levels of FGF9 transcripts. Normal proliferative phase endometrium (NE-18) was used as a positive control for FGF9 protein expression (19). FGF9 protein was readily detectable in the primary tumor samples, particularly those with increased mRNA based on microarray analysis such as OE-18T (Fig. 1C). Increased expression of FGF9 was also observed in two OE-derived cell lines, TOV112D and MDAH2774 (t test, P = 0.02 and P = 0.08, respectively) compared with lines derived from other histologic types of ovarian carcinoma (data not shown). Previous studies from our laboratory have shown Wnt pathway defects in TOV112D and MDAH2774 and both cell lines showed increased TCF-dependent transcriptional activity (4).

FGF9 is a downstream target of Wnt pathway activation. Based on quantitative RT-PCR assays, FGF9 transcripts are 2-fold up-regulated in a polyclonal RK3E cell population stably expressing mutant β-cat compared with cells transfected with vector alone (data not shown). In addition, mouse mammary epithelial cells (Rac311) stably expressing Wnt1 show markedly increased FGF9 expression compared with control cells (Fig. 2A). To assess the effects on FGF9 gene expression in human ovarian cancer cells following transient stabilization of wild-type β-cat, OVCA420 cells were treated with LiCl, a GSK3β inhibitor. OVCA420 cells treated with 0 to 30 mmol/L LiCl showed a dose-dependent increase in FGF9 transcripts (Fig. 2B). To further investigate whether the FGF9 gene is a downstream transcriptional target of β-cat/TCF, we evaluated FGF9 expression in TOV112D cells stably expressing a dominant-negative version of TCF4 (dnTCF-4), which is known to diminish transcriptional activity of TCF-responsive promoters (17, 18). TOV112D cells have an activating mutation of β-cat (S37A) and high endogenous levels of FGF9 transcripts. Consistent with expected results for a Wnt pathway target gene, FGF9 transcripts were reduced in cells expressing dnTCF (open columns) versus cells expressing empty vector (shaded columns) (Fig. 2C). Finally, to determine the effects of acute

**Figure 2.** FGF9 transcript levels increase following Wnt pathway activation. A, quantitative RT-PCR analysis shows increased FGF9 expression (relative to HPRT) in polyclonal and clonal Rac311 cells stably expressing Wnt1 compared with cells transfected with vector alone. B, LiCl treatment of OVCA420 cells leads to increased FGF9 transcripts in a dose-dependent fashion. Cells were treated with the indicated doses of LiCl and, at 24 hours, RNA was extracted from cells harvested in Trizol. cDNA was synthesized, then analyzed by quantitative RT-PCR. Relative FGF9 expression (normalized to HPRT). C, FGF9 transcript levels are reduced in TOV112D cells stably expressing dnTCF (open columns) versus cells expressing empty vector (shaded columns). RNA was analyzed for FGF9 expression by quantitative RT-PCR as above. D, FGF9 transcripts are increased following 4-OH-tamoxifen treatment of HEC-1A cells expressing β-cat-S33Y-ER. Cells were treated with 0.5 μmol/L 4-OH-tamoxifen for 0 to 48 hours; RNA was analyzed for FGF9 expression by quantitative RT-PCR as above.
activation of mutant β-catenin, HEC-1A endometrial carcinoma cells were transfected with a construct allowing inducible expression of mutant β-catenin. In this construct, mutant β-catenin is fused to the mouse estrogen receptor. A point mutation in the estrogen receptor ligand binding domain renders the fusion protein insensitive to estrogen but sensitive to stimulation with 4-OH-tamoxifen. FGF9 transcripts were significantly increased in these cells 48 hours after treatment with 4-OH-tamoxifen, further supporting FGF9 as a downstream target of β-catenin/TCF (Fig. 2D). The relatively late induction of these transcripts compared with previously reported direct target genes, such as AXIN 2 (18), suggests that FGF9 may be an indirect, rather than direct, transcriptional target of Wnt signaling.

Activation of FGF9 as an indirect, rather than direct, transcriptional target of the Wnt signaling pathway. Human FGF9 contains at least two consensus TCF/lymphoid enhancer family binding sites (5′-WWCAAWGG-3′; ref. 20) in the sequences within 5 kb upstream of the transcriptional start site. Attempts to show binding of β-catenin/TCF to these sites using chromatin immunoprecipitation assays were unsuccessful. To determine whether activation of FGF9 transcription by β-catenin/TCF is dependent on new protein synthesis (more in keeping with FGF9 as an indirect, rather than direct, transcriptional target of Wnt signaling), HEC-1A/S33Y-ER cells were treated with 4-OH-tamoxifen in the presence of cycloheximide, an inhibitor of new protein synthesis. As expected, increased levels of FGF9 transcripts were noted within 24 hours after activation of the β-catenin-S33Y-ER fusion protein with 4-OH-tamoxifen. This effect was completely abrogated by treatment with cycloheximide (Fig. 3A). Similar results were obtained with polyclonal RK3E cells expressing the S33Y-ER fusion construct (data not shown). In contrast, the direct β-catenin/TCF target gene AXIN2 showed increased expression following treatment with 4-OH-tamoxifen that was not blocked by cycloheximide (Fig. 3B). We also considered the possibility that FGF9 is a direct transcriptional target of β-catenin/TCF but dependent on short lived cofactors that might be exhausted in the roughly 12-hour interval required to fully activate the S33Y-ER construct. In such a scenario,
these short-lived cofactors could not be regenerated in the presence of cycloheximide. To explore this possibility, OVCA420 cells were treated with LiCl, which allows for acute stabilization of the wild-type β-catenin protein in the presence of cycloheximide over time periods as short as 2 hours. Again, FGF9 transcript levels diminished when cells were treated with cycloheximide (Fig. 3C). Taken together, the findings support FGF9 as an indirect, rather than direct, transcriptional target of β-catenin/TCF.

**FGF9 is mitogenic for some cell types, including epithelial cells.** The effect of FGF9 on the growth kinetics of epithelial cells was tested using polyclonal RK3E cells overexpressing FGF9. Expression of exogenous myc-tagged FGF9 was verified by RT-PCR and Western blotting (Fig. 4A). Increased growth of RK3E/FGF9 cells compared with parental RK3E cells was apparent at the 2-day time point and was even more dramatic by day 4 (Fig. 4B). Similar results were obtained when clonal RK3E cells expressing FGF9 were compared with parental cells (data not shown) and when RK3E parental cells were treated with 10 ng/mL recombinant FGF9 (Fig. 4C). We also tested the effect of recombinant FGF9 on the growth rate kinetics of 10SE-80, NIH 3T3 fibroblasts, and HUVECs. We found that FGF9 strongly stimulated the proliferation of NIH 3T3 cells and was weakly mitogenic for IOSE-80 cells. In keeping with studies by others (21), we found that FGF9 did not stimulate proliferation of HUVECs. This observation stands in stark contrast to comparable analyses of FGF family members FGF1 and FGF2, which have been shown to be strong mitogens for endothelial cells and potent angiogenic agents (22, 23). Our findings indicate that FGF9 is mitogenic for only some cell types, including epithelial cells.

**FGF9 promotes invasion of epithelial and endothelial cells.** Given that FGF9 is mitogenic for at least some types of epithelial cells, we wished to determine whether FGF9 might affect other cellular processes involved in cancer development or progression. To the best of our knowledge, the effect of FGF9 on cell motility/invasion in a cancer context has not previously been examined. We investigated whether FGF9 could promote invasion of endothelial and/or epithelial cells using transwells coated with Matrigel. Noninvasive cells remain in the upper chamber whereas invasive cells digest the Matrigel and migrate through the pores in the membrane. FGF9 stimulated invasion of HUVECs at 1 ng/mL (2-fold) and 10 ng/mL (8-fold; Fig. 5A). We also evaluated the ability of FGF9 to stimulate invasion of epithelial cells. The invasiveness of clonal RK3E cells expressing FGF9 was compared with RK3E cells transfected with vector alone. Overexpression of FGF9 led to a 20-fold increase in the number of invasive cells (Fig. 5B). To ensure increased invasion was not simply a reflection of clonal variation, the assays were repeated with RK3E parental cells treated with 1 and 10 ng/mL recombinant FGF9. Recombinant FGF9 stimulated invasion of RK3E cells in these experiments as well (data not shown).

**FGF9 promotes neoplastic transformation of epithelial cells.** The ability of FGF9 expression to promote a transformed phenotype was tested in a focus formation assay. Cancer-derived mutant forms of β-catenin, as well as several other known oncogenes, readily

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**Figure 5.** FGF9 stimulates invasion of HUVECs and RK3E cells in transwell invasion assays. A, HUVECs were plated on Matrigel-coated membranes in the upper chamber of transwells. Cells penetrating the membrane were fixed and 4',6-diamidino-2-phenylindole stained after 16 to 24 hours as described in Materials and Methods. B, invasiveness of control versus clonal FGF9-expressing RK3E cells was also assessed using transwell invasion assays. Migrating cells were stained 4',6-diamidino-2-phenylindole stained, photographed, and counted. C, summary of three independent experiments comparing invasiveness of RK3E control cells to RK3E cells expressing exogenous FGF9. Overexpression of FGF9 led to a 20-fold increase in the number of invasive cells (P = 0.01).
generate foci of morphologically transformed cells when introduced into E1A-immortalized rat kidney (RK3E) cells (16, 24). Expression of FGF9 was sufficient to allow RK3E cells to form foci, albeit with reduced efficiency compared with mutant (S33Y) β-catenin (Fig. 6A and B). FGF9-induced focus formation was not strongly reduced by expression of dnTCF, unlike transformation by mutant β-catenin. These findings are consistent with FGF9 activation being downstream of β-catenin/TCF. Individual foci of FGF9-transformed RK3E cells were picked and clonally expanded for soft agar assays. A subset (20%) of clonal RK3E/FGF9 (n = 13) lines were able to grow in soft agar, further supporting a role for FGF9 in transformation (Figs. 6C and D). Polyclonal RK3E cells stably expressing FGF9 and RK3E cells transfected with vector alone were also tested for tumorigenicity in nude mice. S.c. tumors formed at 5 of 10 sites injected with polyclonal FGF9-expressing RK3E cells (average tumor volume at eight weeks: 254 mm³). Tumors were visible 3 weeks postinjection. None of the 10 sites injected with RK3E control cells showed any tumor growth 8 weeks postinjection. OEA-derived TOV112D cells contain mutant β-catenin (S37A) and exhibit anchorage-independent growth in soft agar. We used stable expression of shRNAs to knock down FGF9 expression in TOV112D cells (Fig. 7A). Two independent shRNAs showed substantial reduction of FGF9 expression compared with a scrambled-sequence control shRNA. Although shRNA-mediated knockdown of FGF9 expression did not affect the growth kinetics of TOV112D cells (Fig. 7B), anchorage-independent growth of TOV112D cells was markedly reduced in cells treated with the FGF9 shRNAs (Fig. 7C).

Taken together, the findings show that FGF9 has oncogenic activity, inducing loss of contact inhibition in both monolayer and three-dimensional cultures.

### Discussion

FGF9, also known as glial activating factor, is one of 23 members of the highly conserved FGF family. FGF9 is a secreted, glycosylated 26-kDa protein that has mitogenic effects on a variety of different cell types. Interestingly, FGF9 has been shown to be involved in the cyclical proliferation of the uterine endometrium, the nonneoplastic tissue that OEs most closely mimic histologically (19). The high-affinity receptors for FGF9 are FGFR2 (IIIc isoform) and FGFR3 (IIIb and IIIc isoforms; refs. 25, 26). The receptor FGFR2IIb has been characterized in epithelial ovarian cancers and implicated in ovarian cancer development (27). Interestingly, our microarray-based gene expression data revealed that whereas expression of FGFR2 is readily detectable in most of our OEs, it is not differentially up-regulated in OEs with Wnt pathway defects (data not shown). Taken together with our data, the findings suggest that FGF9, and not its cognate receptor(s), may be the main mediator of tumor promoting events by FGF signaling in OEA pathogenesis.

Several studies have reported other members of the FGF family as Wnt pathway target genes. FGF4 was determined to be a downstream target of Wnt signaling using tooth rudiments as a model system (28). FGF18 and FGF-BP, a chaperone protein for FGFs, have been reported as target genes in colorectal carcinogenesis (29, 30).
In addition to FGF9, our comprehensive gene expression analysis implicated at least one other FGF family member, FGF20, as a putative Wnt pathway target gene in OEAs. Chamorro et al. (15) have recently shown that FGF20 is likely a direct β-catenin/TCF transcriptional target that is up-regulated in human OEAs with deregulated Wnt signaling and in adenomas from ApcMin/+ mice, compared with normal intestinal tissue. FGF20 seems to be required for maintenance of the anchorage-independent growth capability of RK3E cells transformed by β-catenin. Three other FGFs (FGF3, FGF13, and FGF14) showed >2-fold up-regulation in Wnt pathway–defective OEAs. Their role in ovarian cancer pathogenesis remains largely unstudied.

FGF9 is part of a gene cluster previously implicated as a downstream target of Wnt signaling in Ciona savignyi development (31). In developmental systems, Wnts have been shown to confer competence for FGF signaling in specific settings [reviewed by Dailey et al. (32)]. Our study offers convincing support for FGF9 as a downstream target of the Wnt signaling pathway in a human disease context. In contrast to the other FGFs and FGF-related proteins previously reported as direct targets of Wnt signaling, our data suggest that FGF9 is more likely an indirect downstream target of β-catenin. Further studies are required to determine the molecular mechanisms by which FGF9 is up-regulated in OEAs with Wnt pathway defects. Nevertheless, whether or not FGF9 is a direct or an indirect target of β-catenin/TCF transcriptional activation, the findings presented here substantiate the relevance of FGF9 to OEA pathogenesis.

Our data suggest that FGF9 may be at least one downstream target of the Wnt signaling pathway that mediates increased proliferation and invasiveness of cells with aberrant pathway activation, although this effect may well be context dependent. Activation of Wnt signaling has been associated with increased invasion and metastasis in other studies. For example, inhibition of Wnt signaling by overexpression of Dikkopf 3 reduces invasion and motility of osteosarcoma cells (33). Similarly, Yang et al. (34) have recently reported that activation of Wnt/β-catenin signaling inhibits death receptor–mediated apoptosis and promotes motility and invasion of head and neck squamous cell carcinoma cells. Interestingly, FGF9 has been reported to stimulate production of matrix metalloproteinases, such as MMP1, in human gliomas (35). FGF9 has oncogenic properties that support a role for its up-regulation in OEA pathogenesis. Previous studies have shown FGF9 can transform murine fibroblasts and promote growth of these cells in nude mice (36). Matsumoto-Yoshitomi and colleagues found that transfection of human FGF9 cDNA into BALB/c 3T3 clone A31 cells led to their morphologic transformation, focus formation, growth in soft agar, and tumorigenicity in nude mice. Treatment with a neutralizing antihuman FGF9 monoclonal antibody inhibited all of these properties. Moreover, injection of the neutralizing antibody caused existing tumors to regress. These investigators concluded that the cellular transformation mediated by FGF9 is likely produced by autocrine stimulation. In addition,
studies by Tsai et al. (19) showed that FGF9 promotes endometrial proliferation through autocrine stimulation. Our study is the first to show that FGF9 can promote neoplastic transformation of epithelial cells. This finding is relevant because the ovarian surface epithelium is generally regarded to be the cell of origin for most ovarian adenocarcinomas. Given the expression of the cognate receptor(s) in most ovarian carcinoma samples tested, it is likely that FGF9 mediates at least some of its key effects via autocrine mechanisms in OEA pathogenesis as well.

Few, if any, prior studies have addressed the effects of FGF9 on cellular properties that affect cancer progression, such as motility, invasiveness, or angiogenesis. Such studies may have been deterred by early data showing that FGF9 does not affect the growth kinetics of endothelial cells, unlike FGF1 and FGF2, which are strong mitogens for this cell type. The potent angiogenic properties of FGF1 and FGF2 have also been extensively characterized (22, 37).

We confirmed that FGF9 is not mitogenic for endothelial cells, making it unlikely that FGF9 is an initiator of angiogenesis. This does not, however, exclude a possible role for FGF9 in potentiating or modulating angiogenesis. Studies exploring the role of FGF9 in angiogenesis and other processes, such as extracellular matrix degradation, cell motility, cell adhesion, and tubule formation, should provide additional insights into the role of FGF9 in tumor development and progression.

In conclusion, we have shown that FGF9 is a downstream target of Wnt signaling in the context of a particular type of human ovarian cancers (i.e., OEAs). In addition, we have shown that FGF9 promotes invasion of endothelial and epithelial cells, suggesting that it may play a role in angiogenesis and metastasis. Further, our studies show FGF9 not only promotes growth of epithelial cells in monolayer culture but also promotes transformation of these cells in focus formation and soft agar assays. Taken together, the findings implicate FGF9 as an important mediator of development and/or progression of OEAs, and perhaps other tumors with deregulated Wnt signaling.

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