FGF16 Promotes Invasive Behavior of SKOV-3 Ovarian Cancer Cells through Activation of Mitogen-activated Protein Kinase (MAPK) Signaling Pathway*

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Background: The mechanism of FGF16-mediated invasions in ovarian cancer cells was not reported.

Results: Through the activation of FGFR-MAPK-pathway, FGF16 regulates SNAI1/CDH1/MMP2/-MMP9, promoting invasion of ovarian cancer cells. Wnt-signaling and PITX2 synergistically regulates FGF16 expression.

Conclusion: The oncogenic potential of FGF16 in ovarian cancer was established.

Significance: Growth factor/signaling pathways leading to increased proliferation/invasion of ovarian cancer were identified.

Uncontrolled cell growth and tissue invasion define the characteristic features of cancer. Several growth factors regulate these processes by inducing specific signaling pathways. We show that FGF16, a novel factor, is expressed in human ovary, and its expression is markedly increased in ovarian tumors. This finding indicated possible involvement of FGF16 in ovarian cancer progression. We observed that FGF16 stimulates the proliferation of human ovarian adenocarcinoma cells, SKOV-3 and OAW-42. Furthermore, through the activation of FGFR receptor-mediated intracellular MAPK pathway, FGF16 regulates the expression of MMP2, MMP9, SNAI1, and CDH1 and thus facilitates cellular invasion. Inhibition of FGFR as well as MAPK pathway reduces the proliferative and invasive behavior of ovarian cancer cells. Moreover, ovarian tumors with up-regulated PITX2 expression also showed activation of Wnt/β-catenin pathway that prompted us to investigate possible interaction among FGF16, PITX2, and Wnt pathway. We identified that PITX2 homeodomain transcription factor interacts with and regulates FGF16 expression. Furthermore, activation of the Wnt/β-catenin pathway induces FGF16 expression. Moreover, FGF16 promoter possesses the binding elements of PITX2 as well as T-cell factor (Wnt-responsive), in close proximity, where PITX2 and β-catenin binds to and synergistically activates the same. A detail study showed that both PITX2 and T-cell factor elements and the interaction with their binding partners are necessary for target gene expression. Taken together, our findings indicate that FGF16 in conjunction with Wnt pathway contributes to the cancer phenotype of ovarian cells and suggests that modulation of its expression in ovarian cells might be a promising therapeutic strategy for the treatment of invasive ovarian cancers.

Ovarian cancer is the most lethal gynecological malignancy and is the fourth most frequent cause of cancer-related deaths in women. The disease often manifests with little or no specific symptoms at the early stage, resulting in higher mortality (1). Malignant epithelial tumors represent the majority of ovarian cancers, and the histologic subtypes include serous, endometrioid, clear cell, and mucinous adenocarcinomas.

Growth factors are the major stimulators of cell proliferation upon binding to their specific high affinity cell surface receptors. Fibroblast growth factors (FGFs), originally identified in fibroblasts, constitute a large family of polypeptide growth factors in a variety of multicellular organisms, including invertebrates (2). Apart from normal development and wound healing, FGFs have been implicated in diverse cellular functions including chemotaxis, apoptosis, cell survival, migration, and differentiation (3, 4). In addition, substantial evidence supports the association of FGF and aberrant FGF signaling with the pathogenesis of cancers of different tissues (5–8). Phylogenetically 23 members of the human FGF family are clustered in several subfamilies; for example, FGF9, FGF20, and FGF16 constitute the FGF9 subfamily (9). FGF20 has been characterized to be the downstream target of the Wnt pathways and plays a major role in ovarian cancer (10). The expression of FGF9 is regulated by canonical Wnt signaling pathway in ovarian endometrioid adenocarcinoma and has oncogenic activity as well (11). Given the significant role of FGFs, particularly FGF9 subfamily in human ovarian cancer progression, we were interested to find out the role of FGF16 in this regard, which has not been highlighted so far.

FGF16, a member of FGF family, is a 207-amino acid protein containing a core region of 120 amino acids that binds to heparin and the FGF receptor (FGFR3; Ref. 12). Although originally characterized in the rat, FGF16 was shown to be expressed preferentially in brown adipose tissue in the embryo (13) and

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3 The abbreviations used are: FGFR, FGF receptor; PCNA, proliferating cell nuclear antigen; TCF, T-cell factor; CCND1, cyclin D1; LEF, lymphoid enhancer-binding factor; MMP, matrix metalloproteinase; rhFGF16, human recombinant FGF16; PD, PD173074; Q-PCR, quantitative PCR.
involved in the proliferation of brown adipocytes (14) and limb bud development in zebrafish (15). FGF16 induces migration of the primary human endothelial cells in culture (16). Previous reports suggest a significant role of FGF16 in cardiomyocyte proliferation (17) and development of coronary vasculature (18) in the embryonic mouse. However, its association with cellular proliferation and cancer in adult tissue has not been reported so far.

The involvement of homeobox genes in the progression of human cancer has been widely implicated. Pituitary homebox 2 (PITX2), a member of the bicoid/paired-like homeobox gene family, has extensive involvement in the development of multiple organs in vertebrates (19, 20). Three different isoforms of PITX2 (PITX2A/B/C) differ only in their amino terminus and regulate the transcription of their target genes differentially (21). Recently the association of PITX2 with different types of cancers (22, 23) has been highlighted as well. Evidence showing that the deregulated Wnt signaling pathway is frequently found in ovarian adenocarcinoma cells (24) and is strongly associated with ovarian tumorigenesis (25). Our earlier report also suggested the induction of the Wnt pathway by PITX2 in human ovarian carcinoma cells (26), which led us to determine whether this mechanism also exists regarding their involvement in the regulation of FGF16 expression. In addition, we identified for the first time the expression of FGF16 in human ovary that prompted us to investigate its possible involvement in growth, proliferation, and migration of human ovarian carcinoma cells.

Wnt Signaling and PITX2 Protein Synergistically Regulate FGF16

| Gene name | Forward primer (5′-3′) | Reverse primer (5′-3′) | Amplicon size | Tm |
|-----------|------------------------|------------------------|---------------|----|
| FGF16     | GGGTACCAACAGCAAGCCCACAACCT | GGAACCTCCAGCTATTTGTCCCCACACT | 1.4 kb | 59 |

MATERIALS AND METHODS

Primary Tumor Samples—Surgical sections of tumor tissues obtained from primary ovarian cancer patients were used for quantitative PCR assay and immunohistochemical staining. Ovarian tissues obtained from individuals undergoing oophorectomies for indications other than ovarian cancer were used as controls. Written informed consent was obtained from all patients in their vernacular. The study was approved by the Institutional Independent Ethics and Research Oversight Committees.

Cell Culture, Treatment of Growth Factor, and Inhibitors—Human ovarian adenocarcinoma cells SKOV-3 (ATCC, Manassas, VA) and OAW-42 (Sigma) were maintained in McCoy’s 5A (Sigma) and DMEM (Invitrogen), respectively; both were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin (all from Invitrogen). Chinese hamster ovary (CHO) cells were cultured in Ham’s/F-12 medium (Invitrogen) supplemented with 10% FBS and penicillin/streptomycin. Human recombinant FGF16 (rhFGF16; R&D systems, Minneapolis, MN) was used at 100 ng/ml. The FGFR inhibitor (PD173074, Calbiochem) and the MEK inhibitor (U0126, Promega, Madison, WI) were used at 50 ng/ml for 1 h. Treatment of 20 mM lithium chloride (LiCl) or sodium chloride (NaCl) was applied for 24 h. Before each treatment, the cells were serum-starved for 16 h, and the control cells were treated with vehicles (0.1% BSA in 1× PBS or DMSO). Recombinant human DKK1 (30 ng/ml; R&D Systems) was added to 10^5 cells/well in 6-well plate, and after 30 min, 1 µg of PITX2 expression vectors was transfection into the cells in serum-free medium. After 6 h of incubation, the medium was replaced with fresh and complete medium. 24 h post-transfection, the cells were harvested for RNA isolation.

Expression and Reporter Constructs—Expression plasmids containing the cytomegalovirus (CMV) promoter linked to full-length cDNAs of three isoforms of PITX2 (PITX2-A/B/C), β-catenin, LEF-1, and FGF16, were constructed in pcDNA 3.1 MycHisC (Invitrogen). The 1.4-kb upstream promoter region of FGF16 gene was PCR-amplified using human genomic DNA as template and then cloned into pGL3 basic vector (Promega) at HindIII/Kpn1 site. The primer sequences used to clone the promoter are given in Table 1, where the restriction enzyme sites are underlined. All constructs were sequenced by ABI Prism Automated DNA Sequencer (PerkinElmer Life Sciences). Sequence alignment and data analysis were performed through BLAST search (NCBI GenBank™).

Site-directed Mutagenesis—The PITX2-specific bicoid and bicoxid-like elements and Wnt-response elements present in the upstream region of FGF16 promoter were either deleted or substituted by PCR-based method. The wild-type clone of FGF16 promoter in pGL3 vector was used as template. Pfu DNA polymerase-based enzyme cocktails were used for PCR-based mutation introduction to minimize undesirable mutations following the PCR conditions 95 °C for 30 s, 55 °C for 30 s, and extension at 72 °C for 30 s or 1 min for 35 cycles. The mutations were confirmed by sequencing followed by BLAST alignment. The information of the primers is shown in Table 2.

Transient Transfection and Luciferase Assay—For the reporter assay, 5 × 10^4 cells seeded on 12-well culture plates were transiently transfected individually or in different combinations with the vectors like FGF16—luciferase (0.4 µg), PITX2A/B/C, β-catenin, and LEF1 expression vectors (0.4 µg). Here pRL-CMV vector (Promega, 0.04 µg) was used for normalization of luciferase activity, and the Lipofectamine 2000 (Invitrogen) was used as the transfection reagent. On the following day the cells were harvested, and the firefly as well as renilla luciferase activity was determined in cell lysates using Glomax 96-microplate luminometer (Promega) and dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized with that of renilla luciferase for the correction of transfection efficiency, and the reporter gene expression was represented as relative luciferase units. Each transfection was performed in triplicate, and the experiments were repeated three times. To overexpress the cDNAs of β-catenin, LEF-1, FGF16, and PITX2 isoforms, the respective
expression constructs were transfected at 1 μg/10^5 cells/well in a 6-well plate using Lipofectamine 2000 (Invitrogen) and 24 h post-transfection, and the cells were harvested for RNA isolation. In these experiments, pcDNA3.1-transfected cells were treated as control.

**Chromatin Immunoprecipitation (ChIP) and DNA Microarrays (chip)—**ChIP-on-chip (with SKOV-3 cells) and ChIP assays were performed as described previously (26). A parallel set of ChIP assay was performed using PITX2 isoform-specific antibodies (Innovenagen) with SKOV-3 cells transfected with PITX2A/B/C cDNAs. ChIP with β-catenin antibody (Chemi- conc, Temecula, CA) was performed with NaCl/LiCl-treated cells. For ChIP-PCR, equal amounts of the immunoprecipitated (IP) and total input DNA was used with following conditions: 95 °C for 30 s, annealing at a specific temperature of 30 s, and 72 °C for 20 s for 30 cycles. The information of the primers is shown in Table 3.

**siRNA and Transfection—**The RNA interference was carried out by the ON-TARGET plus SMART pool siRNAs against PITX2, FGF16, and non-targeting siRNA at 20 nm/well using 2 μl of Dharmafect-1 transfection reagent (Dharmacon) in cells seeded in 6-well culture plates. After 48 h of incubation, the cells were harvested, and the RNAs were isolated to perform Q-PCR assay. The siRNA against β-catenin (Santa Cruz Biotechnology) was used at 20 nm/well in cells seeded in 6-well plate.

**Quantitative Real-time PCR (Q-PCR)—**Total RNA was isolated from human ovarian tissues and cell-lines using TRI reagent (Sigma) following standard protocol (27). First-strand cDNA synthesis was carried out using iScript kit (Bio-Rad) from the 500 ng of isolated RNA. Relative expression levels of the specific genes were quantified by Q-PCR using fluorescent power SYBR Green-I kit on the ABI 7500 Real Time PCR system (Applied Biosystems) after normalization with the expression of 18 S rRNA (all from Cell Signaling Technology, Beverly, MA; dilution 1:1000), anti-LEF-1, anti-FGF16, and anti-MMP9 (all Santa Cruz; dilution 1:1000), and anti-β-catenin (Chemicon), anti-PITX2A, anti-PITX2B, and anti-PITX2C (Invogen).

**Confocal Microscopy—**SKOV-3 cells were seeded (1 × 10^5 cells/well) on coverslips in 6-well plate and grown to 70% confluency. After the treatment with rhFGF16, U0126, or PD173074, the cells were fixed with 4% paraformaldehyde for 15 min followed by permeabilization with 0.1% Triton X-100. Cells were kept in blocking solution (5% goat serum, 0.3% Triton X-100 in PBS) for 1 h and then incubated with rabbit anti-phospho-ERK1/2 antibody (Thr-202/Tyr-204; dilution 1:200) for 2 h followed by Alexa Fluor-488-conjugated secondary antibody (Invitrogen; dilution 1:400) for 1 h. The cells were then stained with 1 mg/ml DAPI for 5 min followed by washing with PBS. The stained cells were observed under an Andor spinning disk confocal microscope (Andor Technology plc, Belfast, Ireland) using Andor iXON3 ultra EMCCD camera. The average fluorescence intensity was calculated using Andor IQ2 software and shown as a histogram.

**Cell Proliferation Assays—**Cells (750 cells/well) were plated in 96-well culture plates and incubated in culture medium for 6 h. After treatment with 100 ng/ml rhFGF16 for 6 h, cell growth was determined using BrdU incorporation assay kit (Calbiochem) as described previously (26). Each experiment was run in triplicate. For the growth curve assay, 8 × 10^3 cells/well were seeded in 6-well plates. The cells were treated with 100 ng/ml rhFGF16 in medium with 1% FBS. Cell counting was performed in triplicate using an automated cell counter (TC-10, Bio-Rad) at days 3, 5, 7, and 9, where the first day after seeding represents day 0.

**In Vitro Invasion Assay—**Transwell membranes coated with Matrigel (BD Biosciences) were used to assay in vitro invasion as mentioned previously (28). In brief, 2.5 × 10^5 cells were seeded in the upper chamber in serum-free medium, and FBS or rhFGF16 was added in the lower chamber. After incubating for 22 h at 37 °C in 5% CO_2_ atmosphere, the medium was aspirated from the top and bottom wells, and the non-invading cells were removed from the top wells with a cotton swab. The remaining cells were fixed and stained with toluidine blue. The stained membranes were then excised, mounted on slides, observed under a microscope, and photographed. Three independent fields at 20× magnification for each well were counted, and three independent experiments were performed followed by statistical analysis.

**Immunohistochemistry followed by Immunofluorescence-based Detection—**Isolated cancerous and normal ovaries were used for formalin fixation, processing, and paraffin block preparation using standardized protocol (29). The paraffin sections of 5-μm thickness were prepared by a microtome (Leica...
**TABLE 3**
The sequence, respective amplicon sizes and Tm of the oligonucleotide primers used in ChIP-PCR

| Gene Name | Forward primer (5’-3’) | Reverse primer (5’-3’) | Amplicon size | Tm °C |
|-----------|------------------------|------------------------|---------------|-------|
| FGF16     | GGTTGACCTGACCCAGAGAG   | ACCAAGTCATTTGTCCAC    | 150 bp        | 59    |
| FGF16     | GTTCTCCTCCTCCGTTAAT    | GTTGCGGCAGATGTTGG     | 250 bp        | 58    |

**TABLE 4**
The sequence, respective amplicon size and Tm of the oligonucleotide primers used in Q-PCR

| Gene Name | Forward primer (5’-3’) | Reverse primer (5’-3’) | Amplicon size | Tm °C |
|-----------|------------------------|------------------------|---------------|-------|
| PCNA      | GAAGGCTCTCTGAGGAAAAG   | AGGCTGCTCCACAAAGAG    | 70 bp         | 59    |
| FGF16     | CACCGGCTCTCCTCTGCTCT  | AGGCGGCTCTCCAGGAAA    | 68 bp         | 58    |
| CCND1     | TATGCTGCTTGACTCTGTGA  | ACCAAGCTGACCAACATGAAAA| 90 bp         | 60    |
| 18S rRNA  | GATTCTGCTGTGGTGTTGTC  | AAAGGAATCTGGGACACCA   | 134 bp        | 60    |
| POLR2A    | TGAGCCGGCCGGGACCTTTCT | GGCCTGCTGCTTCACACT    | 141 bp        | 60    |
| C-MYC     | TCAAGGAGGGGACACACAAC  | GGCCTTTTTCATGTTGTCCA  | 110 bp        | 60    |
| PITX2     | CGGAGGAAATGGTCTGCTGAC| GCAGGCTTCTGGACACAAAACC| 78 bp         | 58    |
| AXIN2     | CCGAGGACACACTCTTCTCT  | TGAGACCTGCTGACACATTT  | 80 bp         | 60    |
| CTNNB1    | CTTCTGTCCTCCGCAAGATCT| CTTGCCGCTCTGCTGATTC   | 100 bp        | 60    |
| PITX2A    | GGCTGCTGCTGCTTGAGAGAG| GCCAGGCTTCTGGAGCCAC   | 80 bp         | 60    |
| PITX2B    | GGGGTCTGAGTGGTCTCCTTCT| CCTCTGCAGCATGCTGAGG   | 80 bp         | 60    |
| PITX2C    | GCCTGCTCTCATCTGCTGTCTT| GTAGGGTCTGAGGACACCA   | 80 bp         | 60    |
| RC2       | CATGCTGCTCTGCTGCTGCTG| GCCTGCTGCTGCACTTACTGA| 83 bp         | 60    |
| CDH1      | GCTAGCTGACACCAACGATCT| TGGAGTGTGCTGACTGACTGA| 100 bp        | 60    |
| MMP2      | TGATGTGACGACAGAAAC    | GCTGCTGAGGAGCAAGTT    | 90 bp         | 60    |
| MMP9      | ACCCTGAGATTCAGACTGAGAC| GAGAAATTGACCTAGCCAGC  | 113 bp        | 60    |
| SNAI1     | TGCAAGAATCTAATACAGGCSA| AGAGAGCAGTATGGAGCCAG | 140 bp        | 60    |

**TABLE 5**
Histotypes of human ovarian tissue samples used to check the expression of FGF16 and PITX2 are mentioned in the following table

| Histotype                         | No. of samples | Intensity of staining |
|-----------------------------------|----------------|----------------------|
|                                   |                | Strong | Weak | Absent |
| Normal                            | 20             | 0      | 8    | 12     |
| Highly differentiated             | 20             | 18     | 2    | 0      |
| Poorly differentiated             | 5              | 5      | 0    | 0      |

RM2235, Wetzlar, Germany) and were adhered onto the poly-L-lysine (Sigma)-coated glass slides. The slides were deparafﬁnized and rehydrated, then blocked in 5% BSA in 1 × TBS-Tween for 30 min and incubated for 2 h with the primary antibody diluted in 1 × TBS containing 0.1% BSA. The slides were then washed and incubated for 1 h with the secondary antibody (Alexa Fluor-488) followed by DAPI. The stained slides were observed under a confocal microscope. The anti-PITX2 antibody (1:500, Chemicon) and anti-FGF16 (1:100, Santa Cruz) were used as the primary antibodies. The immunofluorescence staining was performed in all collected samples, and the images obtained from the representative slides have been shown.

**Statistical Analysis**—All data were expressed as the mean ± S.E., and statistical significance was calculated by two-tailed Student’s t test. p < 0.05 was considered to be signiﬁcant. The experiments were repeated at least three times in duplicate unless otherwise stated.

**RESULTS**

Up-regulation of FGF16/PITX2 and Activation of the Wnt Signaling Pathway in Human Ovarian Cancer—The abberant activation of FGF-signaling pathway is frequently associated with human cancer (6). To understand the involvement of FGF16-associated signaling pathway in ovarian cancer initiation and progression, the expression pattern of the same was investigated in human ovarian cancer samples (Table 5). The Q-PCR assay conﬁrmed that the expression of FGF16 is ~4.6-fold (p < 0.001) higher in ovarian tumors (Fig. 1a) compared with non-cancerous normal ones. β-Catenin (CTNNB1), the central molecule of canonical Wnt pathway, was up-regulated by ~3-fold (p < 0.01) in ovarian cancer tissues, and the universal target gene of this pathway, AXIN2, was also found ~5-fold higher (p < 0.01; Fig. 1b) in cancerous tissue. In a parallel experiment, the expression of three isoforms of PITX2 homeodo-

main transcription factor (PITX2A/B/C) was found up-regulated by ~5-fold (p < 0.01; Fig. 1c) compared with normal ovarian tissue. Confocal imaging of FGF16 (Fig. 1d) and PITX2 (Fig. 1e) in tissue sections of normal (A) and human ovarian cancer (B) also supported the increased expression of both in cancer samples. Staining of tissue sections with only secondary antibody and DAPI (negative control) showed the speciﬁcity of staining (Fig. 1e). As both PITX2 protein and Wnt signaling pathway are shown to be associated with ovarian cancer along with the up-regulation of FGF16, we hypothesized that there might be a positive correlation among Wnt/β-catenin signaling and PITX2 in regulating the expression of FGF16 in ovarian cancer cells.

**PITX2 Binds to FGF16 Promoter and Isoform-specifically Regulates Its Expression in Ovarian Cancer Cells**—To identify the PITX2-mediated regulation of genes/signaling pathways involved in increased cell proliferation/invasion, we had earlier performed a ChIP-on-chip assay with PITX2 antibody in human ovarian carcinoma cells, SKOV-3 (26). We found that the promoter of FGF16 was enriched by PITX2. FGF16 is highly conserved within species, and it is also expressed in SKOV-3 and OAW-42 cells as we conﬁrmed by Q-PCR assay with gene-speciﬁc primers. To re-validate the microarray result, we performed ChIP-PCR with PITX2-IP DNA with a primer pair designed within the probe-hybridization region (ChIP-on-chip) in the proximal FGF16 promoter. Ampliﬁcation from this
DNA (Fig. 2a) indicated that PITX2 physically interacts with FGF16 promoter, which was observed in OAW-42 cells as well (Fig. 2b). The primers of an unrelated gene (GAPDH promoter) did not show any PCR amplification from the PITX2-IP DNA (Fig. 2c). Furthermore, binding of all three isoforms of PITX2 was observed in FGF16 promoter by performing a ChIP assay with isoform-specific antibodies of PITX2 (Fig. 2d). The binding of PITX2B/C with FGF16 promoter was stronger compared with PITX2A (Fig. 2e). In addition, transfection of SKOV-3 cells with PITX2A or -B or -C was used for ChIP with specific antibodies followed by PCR assay (Fig. 2e), which confirmed the binding of all PITX2 isoforms with FGF16 promoter. All PCR products were sequenced to confirm their identities.

The ectopic overexpression of PITX2 in transfected cells was confirmed by Western blot with isoform-specific antibodies (Fig. 2f) and Q-PCR assay (Fig. 2, g and i). Subsequently, the expression of FGF16 was found to be up-regulated (p < 0.01) isoform-specifically in both SKOV-3 (Fig. 2h) and OAW-42 (Fig. 2i) cells. Furthermore, down-regulation of PITX2 mRNA (~80%; p < 0.005) using specific siRNA reduced FGF16 gene expression by ~70% (p < 0.001; Fig. 2k), confirming regulation of FGF16 by PITX2. As the sequence analysis revealed the presence of PITX2-specific bicoid and bicoid-like elements within the proximal promoter (Fig. 2) of human FGF16, the PITX2-mediated transactivation of FGF16 was investigated. For that, the sequence containing those elements was cloned in pGL3 reporter vector and checked the activation of the FGF16 promoter upon overexpression of three PITX2 isoforms in CHO cells that do not have endogenous PITX2 expression. Here, PITX2C activated FGF16 promoter by ~7-fold, whereas PITX2B and PITX2A activated it by ~5- and ~3-fold, respectively (Fig. 2m), suggesting the interaction of PITX2 with the FGF16 promoter.

Activation of Canonical Wnt Signaling Pathway Induces the Expression of FGF16—To assess the expression of FGF16 after transient stabilization of β-catenin, both SKOV-3 and OAW-42 cells were treated with 20 mM LiCl, which induced FGF16 mRNA by ~25-fold (p < 0.001; Fig. 3a) and ~4-fold (p < 0.001; Fig. 3b), respectively. Here, the activation of Wnt pathway by LiCl was confirmed by the increase in canonical Wnt target gene, CCND1 (p < 0.001; Fig. 3, a and b) in both cells. In addition, knockdown of β-catenin by 20 nM siRNA transfection significantly (p < 0.001) reduced its own expression as well as the FGF16 mRNA (~80%; p < 0.001) and CCND1 (p < 0.005) in SKOV-3 (Fig. 3c) and OAW-42 (Fig. 3d) cells. The bioinformatics analysis revealed the presence of two consensus T-cell factor (TCF) binding Wnt response elements (5’-WWCAAWWG-3’, where W represents A/T; Fig. 3e) in the human FGF16 promoter, where β-catenin is in complex with TCF, and LEF1 binds and transactivates the target gene expression. Additionally, a putative PITX2 binding bicoid-like element was also found at the vicinity of the TCF response element. This prompted us to look for the interaction between FGF16 promoter with β-catenin-TCF complex as well as with PITX2. For this, a ChIP-PCR assay was performed with anti-β-catenin antibody after LiCl treatment (of both SKOV-3 and
Expression proximity on the possibility of synergistic regulation of FGF16.

Temporary transfected into the cells followed by isolation of RNA. The expression of -B, and -C was checked by Western blot analysis in control (a), (b), and (c), respectively. (d) shows the ChIP of the FGF16 promoter from the chromatin input and from PITX2-IP DNA. (e) shows the amplification of the FGF16 promoter with the same primer sets (used in Fig. 3) from those cells using primers of an unrelated gene did not show amplification from PITX2-IP DNA. (f) shows the amplification of the FGF16 promoter and regulates its expression in human ovarian cancer cells.

**FIGURE 2.** PITX2 binds to FGF16 promoter and regulates its expression in human ovarian cancer cells. ChIP with SKOV-3 (a) and OAW-42 (b) cells followed by the PCR showed the amplification of the FGF16 promoter from the chromatin input and from PITX2-IP DNA as indicated in the lanes. The PITX2 antibody used in a and b recognizes all three isoforms of PITX2. c, primers of an unrelated gene did not show amplification from PITX2-IP DNA. d, ChIP with PITX2 isoform-specific antibodies was performed in SKOV-3 cells followed by PCR showed amplification of FGF16 promoter from the IP-DNA. e, upon transient transfection of either PITX2A or -B or -C, SKOV-3 cells were used for ChIP with isoform-specific antibodies followed by PCR with the primers used in a, f, the expression of PITX2A, -B, and -C was checked by Western blot analysis in control (UT) and transfected (T) cells with specific antibodies. g, three isoforms of PITX2 (PITX2A/B/C) were transiently transfected into the cells followed by isolation of RNA. The expression of PITX2 isoforms (g, for SKOV-3; i, for OAW-42) and FGF16 (h, for SKOV-3; j, for OAW-42) were checked in the PITX2-overexpressed cells (b) by Q-PCR using specific primers. k, SKOV-3 cells were transiently transfected with 20 nm non-targeting (NT)-siRNA and PITX2-siRNA followed by Western blot analysis of PITX2 and a-tubulin proteins (top). m, CHO cells were transiently co-transfected with the pGL3-FGF16 construct alone or in combination with PITX2 isoforms followed by luciferase assay. The FGF promoter activity was calculated in terms of -fold change in PITX2-overexpressed cells compared with empty vector-transfected cells, after normalization with renilla luciferase activity. The statistical analysis is done as described under "Materials and Methods."

OAW-42 cells) showing more intense amplified product of FGF16 promoter compared with NaCl-treated cells at the same number of PCR cycles (compare lane C and D; Fig. 3f). Immuno precipitation with PITX2 antibody in SKOV-3 and OAW-42 cells followed by PCR with same primer sets (used in Fig. 3f) also resulted in amplification of FGF16 promoter (Fig. 3g), suggesting the association of both PITX2 as well as -catenin in close proximity on FGF16 proximal promoter.

**PI TX2, -Cat enin, and LEF-1 Synergistically Induce FGF16 Expression**—Binding of both PITX2 and -catenin within close proximity on FGF16 promoter prompted us to determine the possibility of synergistic regulation of FGF16 expression by PITX2 and -catenin. The transfection of either -catenin or LEF1 could increase the FGF16 level in SKOV-3 cells by only ~2-fold, whereas co-transfection of both could increase it to ~2.6-fold (Fig. 4a) compared with empty vector-transfected cells. In contrast, co-transfection of either of the PITX2 isoforms along with -catenin and LEF1 remarkably enhanced the FGF16 mRNA level. However, co-transfection of 20 nm PITX2-siRNA and the -catenin and LEF1 expression vectors sharply decreased (p < 0.005; Fig. 4a) the FGF16 mRNA level. Thus, synergistic activation of FGF16 by PITX2, -catenin, and LEF1 was confirmed in SKOV-3 cells, which was also evidenced in OAW-42 cells as well (Fig. 4b). Next, we attempted to check whether the activated Wnt pathway and PITX2 could independently induce the FGF16 expression. For that, -catenin was first knocked down by specific siRNA followed by transient overexpression of PITX2 isoforms, and the Q-PCR assay showed reduction (p < 0.01; Fig. 4c) in FGF16 expression. Additionally, PITX2 overexpression in DKK1 pretreated cells
Wnt Signaling and PITX2 Protein Synergistically Regulate FGF16

The expression of FGF16 is induced by activation of the Wnt pathway. 16-h serum-starved SKOV-3 (a) and OAW-42 (b) cells were treated with 20 mM NaCl or LiCl followed by isolation of RNA and Q-PCR analysis of FGF16 and CCND1. c and d, SKOV-3 and OAW-42 cells were transiently transfected with β-catenin-siRNA or non-targeting (NT)-siRNA as the control followed by Western blot analysis of β-catenin and α-tubulin. RNA isolation and Q-PCR assay was also performed with primers of β-catenin, FGF16 and CCND1 (c, i, and d). The statistical analysis was done as described under "Materials and Methods." * represents $p < 0.05$. a, a schematic representation of 1.4 kb upstream sequence of FGF16 promoter shows the canonical TCF elements (■), PITX2-specific bicoid and bicoid-like elements (not in scale). The solid arrows mark the positions of primers that were used to amplify the region during ChIP-PCR. The positions of respective cis elements were marked with respect to the TSS (plain arrow). f, SKOV-3 and OAW-42 cells were treated with or without 20 mM LiCl and then subjected to ChIP with β-catenin antibody. IP, immunoprecipitation. PCR with the primers of FGF16 shows the amplification from chromatin input (lane 8) and from the IP-DNA (lanes C and D). g, ChIP with PITX2 antibody followed by PCR with the primers of FGF16 used in f showed the amplification from the input and the immunoprecipitation-DNA.

also showed significant reduction ($p < 0.005$; Fig. 4d) in PITX2-induced FGF16 activation. The synergistic regulation of FGF16 expression was further demonstrated by luciferase reporter assay. For that, the 1.4-kb upstream region of FGF16 promoter (Fig. 3e) was cloned into pGL3-basic vector and transfectected alone or in combination with three isoforms of PITX2, LEF1, and β-catenin into CHO cells. Either β-catenin or LEF1 could activate the FGF16 reporter by only 2–3-fold, whereas the presence of PITX2A along with either of them increased the reporter activity by ~5-fold (Fig. 4e). Co-transfection of PITX2A and β-catenin or LEF1 sharply increased the reporter activity by ~8- and ~10-fold, respectively, and this activity was maximal when all three clones were co-transfected (~12-fold). This synergistic activation was also observed in case of PITX2B (Fig. 4f) and PITX2C (Fig. 4g). Thus, PITX2 in concert with β-catenin and LEF-1 showed a combinatorial effect in the activation of FGF16 promoter.

The sequences of PITX2 and TCF binding elements of FGF16 promoter (Fig. 3e) were mutated and cloned into pGL3-basic vector. The respective clones (Fig. 4h) were transfected alone or in combination with β-catenin/LEF1 into CHO cells to check the luciferase-reporter activity. The reporter activities of the clones with mutated PITX2 binding elements (Mut1 (Fig. 4i) and Mut2 (Fig. 4k)) and TCF binding element (Mut3; Fig. 4l) were drastically reduced compared with that of the wild-type (WT; Fig. 4i) clone. Although we observed the activities of another clone with mutated TCF binding element (Mut4; Fig. 4m), it was less compared with that of the WT.

FGF16 Enhances the Proliferation of Ovarian Cancer Cells—The biological effect of FGF16 on the growth kinetics of ovarian cancer cells was assessed. The transient transfection of FGF16 in SKOV-3 cells enhanced the mRNA levels of the proliferation marker, PCNA, and the pro-survival gene, BCL2, by ~3.5-fold ($p < 0.05$; Fig. 5a) and ~10-fold ($p < 0.01$; Fig. 5a), respectively. The induction of PCNA and BCL2 expression by FGF16 over-expression was observed (Fig. 5b) in OAW-42 cells as well. In contrast, the siRNA-mediated down-regulation of FGF16 significantly reduced the expression of PCNA and BCL2 by ~50% in both ovarian cells (Fig. 5, c and d). Furthermore, Western blot analysis revealed the up-regulation of the cell cycle-regulating proteins, phospho-retinoblastoma (p-Rb), cyclinD1, and cyclinD2 (Fig. 5e) upon treatment with rhFGF16 in SKOV-3 cells. In addition, BrdU incorporation assay clearly
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(a) SKOV.3
(b) OAW.42
(c) SKOV.3
(d) OAW.42

(e) Fold change in luciferase activity
(f) Fold change in luciferase activity
(g) Fold change in luciferase activity

(h) FGF16 promoter

△ of TAAT : Mut1
△ of AAT : Mut2
△ of AA and substitution of WRE : Mut3
△ of WRE : Mut4

Mut2-specific elements
Wnt-response element (ATCAAAG)

△ Indicates deletion.

(i) Fold change in luciferase activity

(j) Fold change in luciferase activity

(k) Fold change in luciferase activity

(l) Fold change in luciferase activity

(m) Fold change in luciferase activity
indicated a significant increase in cell proliferation (Fig. 5f) upon rhFGF16 treatment. On the contrary, knockdown of FGF16 by siRNA transfection significantly reduced cell growth by ~40% (p < 0.01; Fig. 5g). Moreover, a growth curve assay performed by cell counting for 7 days also revealed greater proliferative activity (Fig. 5, h and i) in cells stimulated with rhFGF16. All these experiments cumulatively supported the proliferative property of FGF16.

**FGF16 Induces MAPK Signaling Pathway through the Activation of FGFR**—We investigated the activation of ERK1/2 by rhFGF16 in both SKOV-3 and OAW-42 cells. Stimulation with rhFGF16 (100 ng/ml) significantly increased the p-ERK1/2 level.

**FIGURE 4.** The expression of FGF16 is synergistically up-regulated by PITX2, LEF-1, and β-catenin. a and b, 1 μg each of LEF-1, β-catenin, and PITX2A/B/C expression clones were transiently transfected alone or in different combinations in SKOV-3 (a) and OAW-42 (b) cells, and Q-PCR performed with the RNAs isolated from those cells showed the expression level of FGF16 mRNA. The expression of LEF1 and β-catenin was checked by Western blot analysis in control (UT) and transfected (T) cells (c). SKOV-3 and OAW-42 cells were co-transfected with β-catenin siRNA alone or in combination with LEF-1 and PITX2A, -B, and -C expression clones followed by isolation of RNA and Q-PCR with the primer of FGF16. d, cells pretreated with DKK1 were transiently transfected with PITX2A/B/C individually, and Q-PCR performed with the RNAs isolated from those cells showed the expression level of FGF16 mRNA. Relative gene expression is indicated as fold-change in the y axis (mean ± S.E.). e–g, a 1.4-kb sequence of FGF16 promoter was cloned (with primers marked with hollow arrows in Fig. 3e) in pGL3-basic vector. The CHO cells were co-transfected with this construct and LEF-1, β-catenin, and PITX2A/B/C expression clones in different combinations, and luciferase reporter assay was performed. The relative luciferase activities are shown as the mean fold-activation compared with the pGL3-basic vector after normalization with renilla luciferase activity. h, the WT and mutated sequences of PITX2 and TCF binding elements of the FGF16 promoter were shown, and the respective deletion/substitution mutations has been schematically presented for each mutated clone, Mut1, Mut2, Mut3, and Mut4. WRE, Wnt response element. i–m, the fold-change in luciferase reporter activities for each WT and Mut1–4 clone is shown upon transient transfections in given combinations in CHO cells. The -fold change in overexpressed cells was compared with empty vector-transfected cells after normalization with renilla luciferase activity. The statistical analysis was done as described previously. * represents p < 0.05.
by ~2-fold (p < 0.001; Fig. 6a). The FGF16-induced active ERK level was significantly reduced (p < 0.01) in U0126 pretreated cells (Fig. 6a). The active-MAPK level was also induced by rhFGF16 treatment in OAW-42 cells, which was severely reduced upon U0126 pretreatment (Fig. 6b). As FGFs signal upon binding to their cell-surface FGFRs, we investigated the effect of FGFR-inhibitor, PD173074 (PD), on the activation of the downstream MAPK signaling cascade. Treatment of cells with PD reduced the p-ERK1/2 level, which was induced significantly (p < 0.01) when rhFGF16 was added in PD-pretreated SKOV-3 (Fig. 6c) and OAW-42 (Fig. 6d) cells.

Immunostaining of active ERK1/2 followed by confocal imaging supported the induced level of ERK1/2 in rhFGF16-treated SKOV-3 cells (p < 0.0001; Fig. 6e, B), which was remarkably reduced upon pretreatment with U0126 (p < 0.001; Fig. 6e, D) as well as with PD (p < 0.01; Fig. 6e, F). The signal intensity of confocal images was calculated and graphically represented with significance (Fig. 6f). Next, we examined the effect of blocking FGFR activity on the proliferation of SKOV-3 and OAW-42 cells by BrdU incorporation assay (Fig. 6g), which was found to be severely reduced (p < 0.01) compared with DMSO-treated cells. The long term growth assay for 7 days also supported the reduced growth rate by PD treatment (p < 0.01) in SKOV-3 (Fig. 6h) and OAW-42 (Fig. 6i) cells. Overall, the data suggested that FGF16 activates MAPK signaling pathway through FGFR.
rhFGF16 induced the invasion of cells by >2-fold (p < 0.01; Fig. 7c), and the inhibition of either the MAPK signaling pathway or FGFR severely reduced (p < 0.01; Fig. 7c) the FGF16-induced invasion. This suggests that FGF16 induced the cellular invasion through the activation of FGFR-mediated MAPK-signaling pathway. To gain insight into the molecular mechanism of FGF16-induced cellular invasion, the status of the relevant genes involved in this process was assessed. Treatment with rhFGF16 reduced CDH1 expression by ~2-fold (Fig. 7d) but up-regulated SNAI1 (Fig. 7e), MMP2 (Fig. 7f) and MMP9 (Fig. 7g) expression in SKOV-3 cells. This regulation of gene expression was served through the FGF16-mediated activation of the MAPK pathway as pretreatment of U0126 significantly blocked the FGF16-induced effects. Western immunoblot with specific antibodies also supported the regulation at the protein level (Fig. 7h).

**DISCUSSION**

Induced proliferation and invasion are defining features of cancer cells, particularly for ovarian cancer. The mechanism behind these physiological events as well as the factors involved in these processes needs to be unraveled for proper understanding of cancer progression. Different growth factors, including VEGF (28) and FGFs, are strongly associated with cancer. Our report is the first of its kind to show that FGF16, a novel growth factor, is expressed in human ovary, and its expression is significantly increased in ovarian cancer (Fig. 1). Furthermore, we found its expression in human ovarian adenocarcinoma cells, SKOV-3 and OAW-42. Up-regulated expression of FGF16 in human ovarian cancer led us to explore its possible oncogenic potential. We observed a significant increase in proliferation of SKOV-3 and OAW-42 cells treated with exogenous FGF16, as indicated by induction in proliferating marker PCNA and pro-survival gene BCL2. The proteins involved in cell cycle progression were induced by FGF16 treatment. The BrdU incorporation assay and growth curve assay further supported the mitogenic potential of FGF16 (Fig. 5).

FGF proteins exert their biological functions by activating intracellular signaling pathways, including MAPK, PI3K, and PKC (30–33). We demonstrated the activation of the MAPK signaling pathway upon exogenous FGF16 treatment (Fig. 6) in both SKOV-3 and OAW-42 cells. FGFs, being diffusible in nature, induce their biological responses by binding to and activating specific FGFRs, a subfamily of cell surface receptor-tyro-sine kinases. The mammalian FGFR family consists of four closely related receptors, FGFR1–4 (34), and all of them are expressed in SKOV-3 and OAW-42 cells. A specific isofrom of FGFR2 is expressed only in epithelial ovarian carcinomas (36) but not in its normal counterpart. PD173074 inhibits the receptor-tyro-sine kinase activity of FGFR (37), and our results also showed that PD significantly reduced the FGF16-mediated activation of the MAPK signaling pathway in SKOV-3 cells and decreased cell proliferation as well (Fig. 6). Previously, it has been demonstrated that reduction in FGFR by RNAi significantly inhibited the proliferation of SKOV-3 cells (35) and signaling through.

**FIGURE 7.** FGF16 stimulates invasion of ovarian cancer cells and regulates the expression of relevant genes through MAPK pathway. Cells were plated in Matrigel-coated membranes in the upper chamber of transwells. rhFGF16 (rh; 100 ng/ml), U0126 (U; 50 ng/ml), PD (50 ng/ml), or in combination of rhFGF16 and inhibitors were added to the medium in the lower chamber. SKOV-3 (a) and OAW-42 (b) cells penetrating the membrane were fixed, stained, and photographed. c, the respective percent values of migrated cells treated as above with respect to control is shown as a histogram. d–g, cells were treated as earlier for 6 h followed by RNA isolation and Q-PCR with the primers of SNAI1, CDH1, MMP2, and MMP9. h, lysates of the SKOV-3 cells treated as indicated were immunoblotted with respective antibodies, and the representative gel image is shown. The statistical analysis was done as described under “Materials and Methods.” * represents p < 0.05.
FGFR is involved in proliferation of breast cancer cells (38). Therefore, the present report is the first of its kind to show that FGF16, in particular, activates FGFR signaling in ovarian epithelial carcinoma cells.

Invasion/metastasis is the characteristic feature of cancer cells, and the growth factors, including FGFs are actively involved in regulating this process. We observed a significant increase in invasive behavior of SKOV-3 cells by FGF16 (Fig. 7). The transition of primary tumor cells to invasive metastatic cancer is characterized by the ability to overcome cell-cell adhesion to invade surrounding tissue. E-cadherin, located at the junctions of epithelial cells, is reduced in late-stage ovarian cancers as well as in ascites-derived tumor cells (39). Low E-cadherin expression in ovarian cancer cells enhances invasion (40) leading to poor patient survival (41). Snail, a transcription factor, represses E-cadherin and thus contributes to the cancer progression (42). In addition, the interaction of Snail-MMP and cancer progression has been evident in pancreas, liver, and breast tissues (43–45). We demonstrated that FGF16 up-regulates Snail as well as reduces the E-cadherin expression and thus promotes invasion and metastasis of ovarian cancer cells. On the other hand, the MMP family enzymes, particularly MMP-2 (gelatinase A) and MMP-9 (gelatinase B), degrade extracellular matrix components, especially basement membrane collagens, gelatin, and laminin 5 (46, 47), and thus contributes to the cellular invasion. Through the degradation of extracellular matrix (ECM), MMPs release the ECM-sequestered growth factors, which in turn facilitate the survival, proliferation, and angiogenesis of tumor cells (46). In this report we demonstrated that FGF16-mediated up-regulation of MMP2 and MMP9 facilitates cancer progression (Fig. 7). Moreover, we have shown that the MAPK-signaling pathway is involved in FGF16-regulated expression of Snail, E-cadherin, and MMPs as well as invasion of SKOV-3 cells.

The expression of growth factor genes is regulated intricately by different proteins to maintain their proper homeostasis. Our earlier finding demonstrated the regulation of the canonical Wnt signaling pathway by PITX2 as well as the existence of both the positive and negative feedback loop by PITX2 in SKOV-3 cells (26). Along with it, PITX2 shows a distinct pattern of expression during developmental stages of gonads (29). A lot of emphasis has been given demonstrating the active involvement of PITX2 in organ development (19–20) and cellular proliferation (48) as well. Being such a significant transcription factor, we verified the possible role of PITX2 in human ovarian cancer, and interestingly, we found tumor sections with up-regulated expression of FGF16 showed a significant increase in PITX2 expression as well (Fig. 1). Earlier reports evidenced the association of PITX2 in cancer of other tissues (22, 23). In the present work we found that all three isoforms of PITX2 bind to the proximal promoter of FGF16, although their binding intensities differ. ChIP-PCR assay (Fig. 2d) suggests the binding of PITX2A is weakest compared with other two isoforms. Subsequently, we found that PITX2 isoforms differentially activated FGF16 expression (Fig. 2) in both cell types.

The presence of two canonical TCF binding elements (49) within 600 bp upstream of FGF16 TSS raises the possibility of Wnt/β-catenin pathway-mediated regulation of FGF16, and it is corroborated by the activation of the canonical Wnt signaling pathway in human ovarian tumors (Fig. 1). Subsequently, LiCl treatment and β-catenin siRNA transfection (Fig. 3) confirmed that FGF16 is indeed induced by canonical Wnt pathway. FGF16 shares 75 and 62% amino acid sequence homology with its closely related members, FGF9 and FGF20, respectively (50), and both were found to be regulated by canonical Wnt pathway. In addition, FGF4 and FGF18 have recently been reported as direct targets of β-catenin (51, 52). Interestingly, FGF16 promoter possesses both PITX2 and TCF binding elements in close proximity to which both PITX2 and β-catenin bind and synergistically activate its expression (Fig. 4) in ovarian cancer cells. The significance of these binding elements for the activation of FGF16 expression by PITX2 and β-catenin was elucidated by mutating their sequences. The luciferase reporter activities of those mutated clones upon transfection into cells suggest the presence of PITX2-specific (5′-TAATAA-3′ and 5′-TAATCC-3′) elements is highly essential for transactivation of FGF16. Additionally, the TCF elements also contribute to this activation (Fig. 4). The induction of FGF16 expression by PITX2 and activated Wnt signaling pathway is served independent of each other. Knockdown of PITX2 by siRNA transfection (Fig. 4a) significantly reduced β-catenin-mediated induction of FGF16. On the other hand, knockout of β-catenin (Fig. 4c) or inhibition of the Wnt pathway by the canonical Wnt inhibitor, DKK1, showed the same effect on FGF16 expression. Earlier reports established the physical interaction between β-catenin and PITX2 (48, 53). Therefore, it can be hypothesized that through the regulation of growth factor gene expression, PITX2 may contribute to ovarian cancer progression. Therefore, the FGF16 expression in ovarian cancer cells is tightly regulated by PITX2 as well as Wnt signaling pathway, and the maintenance of its homeostasis in dynamic situation within a cell is finely tuned by all the associated factors.

The present study describes the first comprehensive investigation on the regulation of FGF16 in ovarian carcinoma cells, where PITX2 and β-catenin individually as well as synergistically activate its expression. The functional investigation demonstrates that through the activation of FGFR signaling, FGF16 induces the endogenous MAPK level and ultimately enhances cell proliferation as well as invasion. Our study underscores the importance of FGF16 in the proliferation and invasion during cancer progression. Novel therapeutic agents considering both Wnt and FGFR signaling pathways as targets may be effective for the treatment of invasive ovarian cancers in future.

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