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WNT7A/β-catenin signaling induces FGF1 and influences sensitivity to niclosamide in ovarian cancer

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Abstract

We previously characterized the link between WNT7A and the progression of ovarian cancer. Other groups have identified FGF1 as a relevant risk factor in ovarian cancer. Here, we show a linkage between these two signaling pathways that may be exploited to improve treatment and prognosis of patients with ovarian cancer. High expression of WNT7A and FGF1 are correlated in ovarian carcinomas and poor overall patient survival. A chromatin immunoprecipitation assay demonstrated that WNT7A/β-catenin signaling directly regulates *FGF1* expression via TCF binding elements in the *FGF1-1C* promoter locus. *In vitro* gene manipulation studies revealed that FGF1 is sufficient to drive the tumor promoting effects of WNT7A. *In vivo* xenograft studies confirmed that the stable overexpression of WNT7A or FGF1 induced a significant increase in tumor incidence, while FGF1 knockdown in WNT7A overexpressing cells caused a significant reduction in tumor size. Niclosamide most efficiently abrogated WNT7A/β-catenin signaling in our model, inhibited β-catenin transcriptional activity and cell viability, and increased cell death. Furthermore, niclosamide decreased cell migration following an increase in E-cadherin subsequent to decreased levels of SLUG. The effects of niclosamide on cell functions were more potent in WNT7A overexpressing cells. Oral niclosamide inhibited tumor growth and progression in an intraperitoneal xenograft mouse model representative of human ovarian cancer. Collectively, these results indicate that FGF1 is a direct downstream target of WNT7A/β-catenin signaling and this pathway has potential as a therapeutic target in ovarian cancer. Moreover, niclosamide is a promising inhibitor of this pathway and may have clinical relevance.

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No potential conflicts of interest were declared.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Keywords

FGF1; WNT7A; WNT/β-catenin pathway; ovarian cancer; therapeutic target

INTRODUCTION

Ovarian cancer (OvCa) remains the most common cause of death from gynecological malignancies and is the fifth leading overall cause of death from cancer in women. In 2014, approximately 22,000 new OvCa cases and 14,500 deaths are estimated from OvCa in the United States.[1] The dearth of specific signs, symptoms, or efficient early detection markers for this disease contributes to its diagnosis at advanced stages, resulting in low overall survival. Indeed, more than 75% of OvCa cases are diagnosed when there is widely metastatic disease in the peritoneal cavity.[2] Therefore, it is important to identify therapeutic targets and efficient drugs that can improve current OvCa treatment by preventing its dissemination.

WNT genes encode secreted glycoproteins, acting through frizzled receptor (FZD), that control cell fate, mortality, proliferation, differentiation and tissue growth.[3, 4] Gene mutations and changes in the expression of extracellular inhibitors and intranuclear transcription cofactors within the WNT pathway promote tumor progression and metastasis.[5, 6] The canonical pathway of WNT signaling results in the nuclear accumulation of β-catenin and transcriptional activation of target genes. WNT/β-catenin signaling plays a role in ovarian tumorigenesis,[7] as well as chemoresistance in cancer stem cells of all OvCa subtypes.[8] Our recent findings also suggest that the expression of WNT7A during the malignant transformation of OvCa plays a critical role in tumor progression mediated by the WNT/β-catenin signaling pathway.[9]

FGF1 is one of 23 members of the highly conserved polypeptide fibroblast growth factor family. FGF1 has strong mitogenic effects on a variety of different cell types in various stages of development, morphogenesis and angiogenesis in neoplastic or non-neoplastic tissues.[10, 11] FGF1 has been identified as a potential prognostic marker for OvCa.[12] Genetic variation of *FGF1* has the most significant association with increased OvCa risk within the FGF family.[13] Furthermore, *FGF1* expression is also a significant determinant of survival and response to platinum-based chemotherapy.[14] Thus, modulation of FGF1 by distinct mechanisms in OvCa may be important in ovarian tumor progression.

Niclosamide is an efficacious and minimally toxic, FDA-approved drug for the treatment of helminth parasites, specifically tapeworms, in humans. Several groups have reported that niclosamide is active against cancer cells and targets WNT signaling.[15–19] Niclosamide inhibits solid tumor growth in a colon cancer model by promoting FZD endocytosis, leading to the downregulation of DVL, β-catenin stabilization and TCF/LEF activity.[17, 20] Niclosamide inhibits tumor growth by targeting S100A4, which is a transcriptional target of WNT signaling,[18] and by suppressing LRP6 in prostate and breast cancer cells.[15] Given its proven safety record and mechanisms that target WNT signaling raises the possibility of repurposing niclosamide for OvCa treatment.

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In the present study, we show that *WNT7A* and *FGF1* expression are highly correlated in ovarian carcinomas, and FGF1 is a direct transcriptional target of WNT7A/β-catenin signaling. Niclosamide was an effective inhibitor of WNT7A/β-catenin signaling, including *FGF1* expression, and should be further explored as treatment for OvCa.

RESULTS

Analysis of FGF1 in OvCa

While analysis of *FGF1* gene expression or variation has indicated an association with OvCa risk,[12–14] the expression pattern of FGF1 in ovarian cancer has not been characterized. Therefore, immunoreactive FGF1 was examined using human OvCa tissues. FGF1 was low in normal (Figure 1a) and benign (Figure 1b) ovary, as well as benign fallopian tube epithelium (Figure 1c). FGF1 was highly detected in high grade invasive serous epithelial carcinomas (Figure 1d, black arrows), and surface epithelial cells of low grade serous carcinomas (Figure 1e, black arrows). Heterogeneous FGF1 was seen in the tumor microenvironment, with lymphocytes positive in serous (Figure 1f), but not in endometrioid carcinomas (Figure 1g). FGF1 was observed in fibroblasts of several histotypes (Figure 1d, h, open white arrows). FGF1 was positive in clear cell carcinomas (Figure 1h, black arrows). FGF1 was specifically detected in non-invasive cells lining the basal membrane (Figure 1i, black arrows) and in the surface epithelial cells of mucinous carcinomas (Figure 1j). Abundant FGF1 was also detected in epithelial fallopian tube carcinomas (Figure 1k, black arrows). FGF1 was significantly higher in serous, clear cell, mucinous and fallopian tube primary carcinomas compared to normal/benign ovarian and fallopian tube tissues. Specifically, serous and fallopian tube carcinomas showed higher levels of FGF1 than endometrioid carcinomas (Figure 1q). FGF1 was observed in metastases from serous (Figure 1l, q), clear cell (Figure 1n, q), mucinous (Figure 1o, q) and fallopian tube (Figure 1p, q) carcinomas, but not from endometrioid carcinomas (Figure 1m, q).

Correlation between WNT7A and FGF1 levels and overall survival

Since FGF family members have previously been identified as downstream targets of WNT signaling,[21] and a role for the specific ligand WNT7A was elucidated in tumor growth and progression,[9] we next examined whether WNT7A is associated with FGF1 in OvCa. A total of 41 fresh-frozen ovarian samples including 5 normal/benign, 25 serous, 8 endometrioid, 3 clear cell and 0 mucinous were examined for *WNT7A* and *FGF1* mRNA levels (Figure 2a). The expression of *WNT7A* in serous carcinomas was correlated with high levels of *FGF1* expression. Elevated *WNT7A* was correlated with moderately increased *FGF1* levels in clear cell carcinomas. Significantly higher *WNT7A* was observed in serous (1506-fold) and clear cell (231-fold), but not in endometrioid carcinomas compared to normal/benign ovaries. *FGF1* transcripts were significantly higher (48-fold) in serous carcinomas compared to normal/benign ovaries and slightly increased in clear cell carcinomas (6.26-fold), but unchanged in endometrioid carcinomas.

Using previously generated cell lines,[9] a correlation between *FGF1* and *WNT7A* was observed in cells with knockdown or overexpression of WNT7A, and in orthotopic tumors that developed from these cells (Figure 2b). However, no other *FGF* family members, nor

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FGF receptors, appeared to consistently follow *WNT7A* expression (Supplementary Figures 1 and 2). To further explore the correlation between *WNT7A* and *FGF1* expression in OvCa, we evaluated the prognostic and predictive impact of *WNT7A* and *FGF1* using Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) datasets. A total of 961 primary ovarian tumors with completed data sets were selected for survival analysis. High expression of both *WNT7A* and *FGF1* was significantly correlated with poor survival as determined by log-rank test (P=0.0304, Figure 2c). Furthermore, the 5-year (60 months) survival rate was 34.2% in women with high *WNT7A* and *FGF1* expressing tumors as compared to 49.4% in women with low *WNT7A* and *FGF1* expressing tumors. These findings were similar to previously reported survival rates of advanced OvCa patients 5 years after initial diagnosis.[22, 23]

FGF1 is a direct transcriptional target of WNT7A/β-catenin signaling

The *FGF1* gene is composed of a single protein isoform and has four alternative tissue-specific promoters subject to alternative splicing (Figure 3a).[24, 25] Each promoter is coupled with its 5′ untranslated exon, giving rise to four mRNAs with distinct UTRs, containing exons *1A*, *1B*, *1C* and *1D*, respectively. Therefore, we examined specific *FGF1* transcript(s) in OvCa using 4 different forward primers designed in each untranslated exon and one reverse primer designed in exon 1. SKOV3.ip1 cells, which express high endogenous *WNT7A* and *FGF1* (Supplementary Figure 3), show low or no expression of transcripts *1A*, *1B* or *1D*, and high expression of the *1C* transcript (12-fold higher than that of *1A* and *1B* (Figure 3b)). The *1C* transcript was reduced in WNT7A knockdown SKOV3.ip1 cells, suggesting that *1C* is specific to OvCa and possibly regulated by WNT7A. Similarly, *FGF1-1C* was the most abundant transcript in human malignant ovarian tissues (Figure 3c).

To determine whether *FGF1* is likely to be a direct rather than indirect β-catenin/TCF target gene, we searched the *FGF1* genomic locus and flanking DNA sequences for consensus TCF/LEF-binding elements (WWCAAWG, W=A/T). We found several putative binding sites at the *1A*, *1B* and *1C* locus, but not at the *1D* locus (Figure 3a). Therefore, we performed chromatin immunoprecipitation (ChIP) assays using a TCF4 antibody to analyze chromatin isolated from SKOV3.ip1, parental A2780 (lacking endogenous WNT7A), and A2780 cells overexpressing WNT7A (Figure 3d). DNA from the anti-TCF4 ChIP of SKOV3.ip1 was approximately 8-fold enriched in site 8, which contains the consensus TCF4 binding site located in the *1C* locus, when compared to IgG control. Interestingly, site 8 enrichment (5.5-fold) was observed in WNT7A overexpressing A2780 cells, but not in control A2780 cells. TCF/LEF binding sites in the promoters of *AXIN2* and *SP5*, well-known direct β-catenin/TCF target genes, were used as positive controls. Irrelevant sites ~4 kb and ~500 kb downstream of the *FGF1* locus (Non1 and Non2) and the *RPL19* locus on a different chromosome served as negative controls. Dominant negative (DN)-TCF4 expression in SKOV3.ip1 cells decreased *FGF1*, but not *WNT7A* levels (Supplementary Figure 4a). Specifically, *FGF-1C* transcript expression was decreased in DN-TCF4 expressing cells (Supplementary Figure 4b), confirming the ChIP results in Figure 3d. Collectively, these results suggest that at least one site within the *FGF1* promoter is directly regulated by WNT7A/β-catenin/TCF in OvCa.

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WNT7A-FGF1 signaling promotes neoplastic transformation in OvCa

To understand whether FGF1 is necessary and/or sufficient for the effects of WNT7A in OvCa tumor progression, we generated stable WNT7A or FGF1 overexpressing or knockdown human OvCa cell lines (Supplementary Figure 5). Relative *WNT7A* and *FGF1* expression in a panel of OvCa cell lines was determined (Supplementary Figure 3) and A2780 cells were chosen for further analysis, as these cells have been widely used for gene manipulation and xenograft study, and showed lack of or low endogenous *WNT7A* and *FGF1*. The expression of FGF1 correlated with the upregulation of WNT7A in a genetically manipulated A2780 line, whereas WNT7A expression was not induced by FGF1 overexpression. Therefore, stable FGF1 knockdown in WNT7A overexpressing cells and their control cells were also generated.

Using these stable cell lines, we examined the *in vitro* effects of WNT7A and/or FGF1 on cell proliferation and adhesion (Supplementary Figure 6), using these assays as indicators of the roles of WNT7A and/or FGF1 in tumor growth *in vivo*. Cell doubling time was significantly shorter and ability to adhere to plastic was significantly greater in WNT7A overexpressing cells as compared to vector control cells. FGF1 overexpressing cells also exhibited a significant increase in cell adhesion, but no obvious decrease in cell doubling time was seen. However, FGF1 knockdown in WNT7A overexpressing cells showed that the effect of WNT7A on cell doubling time and adhesion was attenuated in the absence of FGF1.

Next, we examined the effects of WNT7A and/or FGF1 on tumor growth *in vivo*. Nude mice were intraperitoneally inoculated with control, WNT7A overexpressing, or FGF1 overexpressing cells (n=6 each group, Figure 4a). After 5 weeks, a significant increase in tumor burden, but not tumor number, was observed in mice injected with WNT7A or FGF1 overexpressing cells (1776.1 ± 555.3 mg or 1275.8 ± 396.2 mg, respectively compared to pcDNA5, 151.8 ± 55.2 mg), suggesting that both WNT7A and FGF1 have potential roles in tumor growth. The increased tumor growth by WNT7A overexpression was abrogated by inhibiting FGF1 (n=6 each group, Figure 4b), confirming that FGF1 is downstream of WNT7A signaling in OvCa cells. FGF1 knockdown in WNT7A overexpressing cells resulted in reduced tumor burden (265.3 ± 106.5 mg) compared to mice injected with cells overexpressing WNT7A and control shRNA (1372.8 ± 474.4 mg). We confirmed that expression of *WNT7A* and *FGF1* was maintained in orthotopic tumors.

Niclosamide can inhibit WNT7A/β-catenin signaling in OvCa

Several WNT inhibitors have been found to effectively block WNT signaling.[26–29] To determine whether WNT7A/β-catenin signaling could be a therapeutic target in the context of OvCa treatment, we searched the LOPAC library containing 1280 pharmacological active compounds (Sigma-Aldrich, St. Louis, MO, USA) to find compounds inhibiting WNT signaling. A total of 14 small molecules were selected to determine WNT7A/β-catenin inhibitory activity in OvCa cell lines (Supplementary Figure 7a). Half of the small molecules significantly inhibited the activity of the TCF/LEF luciferase reporter in A2780 cells stimulated by WNT7A. Niclosamide exhibited the most significant inhibitory effect on WNT7A/β-catenin signaling. Thus, we chose to focus on niclosamide for further study.

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Next, we assessed the molecular targets of niclosamide within the WNT/β-catenin signaling pathway in OvCa cells (Supplementary Figure 7bcd). Our results indicated potential regulation of WNT7A production, as niclosamide dose-dependently inhibited *WNT7A* levels. TCF/LEF reporter activity in cells stimulated by exogenous (A2780) or endogenous (SKOV3.ip1) WNT7A was suppressed by niclosamide. We also confirmed that niclosamide inhibited TCF/LEF activity stimulated by a constitutively active β-catenin (S33Y).

Niclosamide regulates OvCa cell functions and inhibits tumor growth and progression

To determine whether niclosamide has therapeutic potential in OvCa, control and WNT7A knockdown SKOV3.ip1 cells [9] were used to assess cell proliferation, apoptosis, and migration (Figure 5). Niclosamide dose-dependently decreased cell number in SKOV3.ip1 cells, whereas cells depleted of WNT7A were less sensitive to the anti-proliferative effects of niclosamide (IC50=1.2 or 9.8 μM in SKOV3.ip1 or WNT7A knockdown cells, respectively, Figure 5a). Similarly, cleaved-caspase 3 was increased up to 2.7-fold by treatment with 10 μM niclosamide in SKOV3.ip1 cells, while WNT7A knockdown cells were less sensitive to this effect (Figure 5b). This result was confirmed by immunoblot analysis, showing that niclosamide increased cleaved caspase-3 and PARP, and decreased total caspase-3 and PARP. Niclosamide inhibited invasive features in SKOV3.ip1 cells (Figure 5c). It is well known that the SKOV3.ip1 cells primarily contain CDH1 (E-cadherin) negative cells. Our results showed that niclosamide dose-dependently increased CDH1 and decreased its transcriptional repressor SLUG in SKOV3.ip1, while niclosamide did not affect CDH1 and SLUG in WNT7A knockdown cells. Consistent with these results, migration was inhibited in WNT7A knockdown cells, and was dose-dependently decreased by niclosamide in parental SKOV3.ip1 cells.

We next determined the effect of niclosamide on ovarian tumor growth and progression (Figure 6). Five weeks after injection with SKOV3.ip1 cells, mice that received vehicle treatment (n=6) developed disseminated abdominal disease mimicking OvCa in patients. Niclosamide treated mice (n=6) had significantly fewer implants on the mesentery and intraperitoneal region (13.8 ± 2.0 vs 78.5 ± 8.7 implants) and their total tumor weight was 172 ± 30 mg vs 936 ± 121 mg. Decreased tumor cell proliferation, as determined by Ki67 immunohistochemistry, was correlated with tumor growth and quantitatively significant. TUNEL analysis revealed a significant number of apoptotic cells in the tumors of mice treated with niclosamide.

While niclosamide reduced *FGF1* expression in SKOV3.ip1 cells, FGF1 itself did not influence the efficacy of niclosamide (Supplementary Figure 8). When FGF1 only was overexpressed in A2780 cells, no differences in cell viability or death were observed compared to control following niclosamide exposure, whereas cells overexpressing WNT7A were more sensitive to the effects of niclosamide. Moreover, WNT7A overexpressing cells with and without FGF1 knockdown were equally sensitive to the effects of niclosamide, suggesting that niclosamide targets WNT7A/β-catenin rather than FGF1 directly. However, because FGF1 is a downstream target of aberrant WNT7A/β-catenin signaling and abundant WNT7A and FGF1 are observed in ovarian cancer, indirect downregulation of FGF1 by niclosamide remains clinically relevant.

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DISCUSSION

FGF1 has been identified as an adverse prognostic factor in OvCa risk.[12–14] In the present study, we show that *FGF1* is a downstream target of WNT7A signaling via β-catenin activation. First, FGF1 is abundant in OvCa, correlates with high *WNT7A*, especially in serous carcinomas. High expression of both *WNT7A* and *FGF1* is associated with poor survival (Figures 1 and 2). Second, the *FGF1* gene is directly regulated by WNT7A/β-catenin signaling (Figure 3). Third, FGF1 is sufficient to drive the tumor promoting effects of WNT7A (Figure 4).

Our previous study demonstrated that WNT7A is the sole ligand activating β-catenin/TCF signaling in OvCa.[9] We found that the *FGF1-1C* transcript is specifically regulated in a WNT7A and β-catenin/TCF-dependent manner in OvCa. The ChIP analysis indicates that a single consensus TCF/LEF binding site at the *1C* promoter locus is critical for WNT7A-mediated TCF binding, while binding sites within the *1A* and *1B* loci are not occupied in the context of WNT7A. It has been reported that *1C* and *1D* transcripts are potential markers for cell proliferation, while *1A* and *1B* are specific for the maintenance and survival of cells.[24] Although *FGF1-1C* specific functions in OvCa pathogenesis have yet to be determined, *FGF1-1C* is directly transcribed from activation of β-catenin/TCF signaling stimulated by WNT7A.

FGF1 binds receptors of FGFR1, 2, 3 and 4 with high-affinity.[11] Two receptors, FGFR2IIIb and FGFR4 have been implicated in OvCa progression,[30, 31] and FGFR4 has been identified as a potential therapeutic target in OvCa.[31] Several studies have reported that other members of the FGF family are WNT/β-catenin target genes. *FGF9* and *FGF20* are indirect and/or direct transcriptional targets of β-catenin/TCF signaling in endometrioid OvCa, in which the WNT pathway is often constitutively activated, usually via missense mutation of *CTNNB1*.[21, 32] However, no members of the FGF family or the FGF receptors, other than FGF1, have been correlated with WNT7A expression in OvCa and none were directly regulated by WNT7A in our model. These results suggest that FGF1 is an important mediator of the tumor promoting events of WNT7A-dependent pathogenesis.

The importance of inappropriate WNT signaling for the development and progression of many cancers, including OvCa, has been well documented.[7–9] Therefore, it is plausible to search for therapeutic drugs that target the WNT7A/β-catenin-FGF1 pathway. While several small molecules that target this pathway have been identified[33] and, for a few of them, a precise mechanism of inhibition has been determined,[27, 33, 34] niclosamide was selected as the most efficient inhibitor of WNT7A-dependent TCF/LEF reporter activity. Niclosamide has recently been identified to target stem-like OvCa-initiating cells by a drug screening method, and gene expression array indicated involvement of WNT hyperactivity.[35] In the present study, we found that niclosamide could directly target β-catenin-TCF/LEF transcriptional activity, specifically; the effects of niclosamide on cell functions are more robust in WNT7A overexpressing cells. Our results also revealed that niclosamide reduced tumor growth and progression. We show that WNT7A is highest in serous carcinomas, the most common OvCa subtype,[9] and recent studies suggest niclosamide is effective in OvCa stem cells.[35] While the importance of WNT7A in OvCa stem cells is not known, WNT7A is

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one of the critical factors determining cell fate in female reproductive development.[36, 37] The regulation of *WNT7A* in OvCa may lead us to understand the etiology and/or function of stem cells in aggressive serous carcinomas.

Although our results demonstrated that niclosamide efficacy in OvCa depended on WNT7A, but not FGF1 function, other signaling pathways could be additional targets. Niclosamide is reported to target not only WNT signaling but also mTORC1, STAT3 and NFκB pathways in several cancers.[38–42] Furthermore, we showed that niclosamide increased E-cadherin and decreased SLUG proteins. E-cadherin establishes cell polarity, mediates inhibition of proliferation and inhibits tumor cell growth.[43, 44] E-cadherin is often downregulated during tumor progression, leading to increased tumor invasiveness and metastasis.[45, 46] Therefore, it is likely that niclosamide reduces invasive and aggressive features in OvCa. While the potential interaction between these cascades and WNT signaling is an intriguing area for future study, overall niclosamide has broad potential as an effective inhibitor to treat OvCa patients.

In the present study, we determined that WNT7A-FGF1 signaling is capable of inducing tumor growth, indicating a critical role in the aggressive progression of OvCa. Furthermore, we found that niclosamide is an effective therapeutic inhibitor of WNT7A/β-catenin signaling. New drugs are desperately needed to treat OvCa, and niclosamide is FDA-approved with a favorable safety profile. If niclosamide continues to hold promise in further pre-clinical studies, repurposing may ultimately prove to have a tremendous impact on the lives of OvCa patients in the clinical setting.

MATERIALS AND METHODS

Reagents and plasmids

Short hairpin RNAs (shRNA), cDNAs encoding human *WNT7A* and *FGF1*, and pcDNA5/FRT/V5-His, pcDNA6/TR and pcDNA5/TO plasmids were purchased from Sigma-Aldrich, Thermo Scientific (Rockford, IL, USA) and Life Technologies (Life Technologies, Grand Island, NY, USA), respectively. Niclosamide, iCRT3, iCRT14, Pyrvinium, Bafilomycin, Quercetin, NSC668036 and LiCl were purchased from Sigma-Aldrich. XAV939 and IWR were purchased from Cayman Chemical (Ann Arbor, MI, USA). IWP and Box5 were purchased from Thermo Scientific. ICG001, CCT031374 and iCRT5 were obtained from R&D Systems (Minneapolis, MN USA).

Tissue samples and cell lines

Tissue microarray paraffin-embedded and fresh-frozen ovarian specimens were obtained from The University of Chicago and Southern Illinois University. Clinical and histopathologic information was collected and verified for each sample by a gynecologic pathologist following International Federation of Gynecology and Obstetrics (FIGO) classifications for stages I–IV.

OVCAR3, OVCAR5, SKOV3, TOV-112D, TOV-21G, OV-90, MDAH 2774 and ES2cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). A2780 and OVCAR4 cells were purchased from Sigma-Aldrich and NCI, respectively.

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KURAMOCHI, OVKATE and OVSAHO were purchased form JCRB cell bank (Osaka, Japan). HEY, HEYA8, OVCAR8, IGROV-1, OVCA420, OVCA429, OVCA432, OVCA433 and SKOV3.ip1 cells were purchased from the cell bank of The University of Texas MD Anderson Cancer Center. All cells were authenticated by short tandem repeat (STR) analysis and passaged within 6 months of receipt. All cells were tested routinely for cell proliferation and BrdU incorporation as well as mycoplasma contamination, and showed similar growth rates and negative mycoplasma throughout all experiments. TOV-112D, TOV-21G, and OV-90 cells were grown in 1:1 MCDB 105:M199 with 15% FBS and penicillin/streptomycin, other cells were cultured in DMEM with 10% FBS, 200mM glutamine and penicillin/streptomycin. All cell lines were grown at 37 °C in a humidified 5% CO_2 incubator.

DN-TCF4 expressing SKOV3.ip1 cells were generated using the T-REx system with pcDNA6/TR and pcDNA5/TO vectors (Life Technologies). A2780 cells were transfected with empty vector (pcDNA5/FRT), or plasmids encoding WNT7A tagged with V5 or FGF1 using the Flp-In system (Life Technologies). Hygromycin resistant stable clones were used for further analysis based on WNT7A and FGF1 expression detected by qPCR and western blot. Furthermore, stable FGF1 knockdown in WNT7A overexpressing cells and their control cells were generated using shRNA gene knockdown methods as described previously.[9] Stable clones doubly resistant for hygromycin and puromycin were selected and WNT7A and FGF1 expression were confirmed by qPCR and western blot.

QPCR, western blot, TUNEL and cleaved-caspase assays

Total RNA was isolated from tissues and cells, and cDNA was synthesized from total RNA. Relative gene expression was determined by SYBR green incorporation using a Bio-Rad myCycler as described previously.[47] A table of oligonucleotides used for each gene is presented in Supplementary Table 1. Ten micrograms of total protein from whole cell lysates were separated on SDS-PAGE gels and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked and incubated overnight at 4°C with primary antibodies. Bound antibody was visualized with IRDye 700 or 800 conjugated affinity-purified secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA, USA) using the Odyssey infrared imaging system (LI-COR, Lincoln, NE, USA). A list of antibodies is shown in Supplementary Table 2. The TUNEL assay was performed according to manufacturer's instructions using ApopTag Fluorescein In Situ Apoptosis Detection Kit (Thermo Scientific). To assess apoptosis, cleaved-caspase 3 was quantitated using a PathScan Cleaved Caspase 3 Sandwich ELISA Kit according to manufacturer's instructions (Cell Signaling).

Immunohistochemistry

Immunolocalization of FGF1 in a total of 537 human specimens including 10 normal/benign (8 ovaries and 2 fallopian tubes), 147 serous, 20 endometrioid, 22 clear cell, 7 mucinous and 32 fallopian tube primary tumors, and 299 metastases from 199 serous, 8 endometrioid, 15 clear cell, 4 mucinous and 73 fallopian tube tumors; and Ki67 in xenografts from nude mice were examined in cross-sections (5 μm) of paraffin-embedded tissue sections using specific primary antibodies and a Vectastain Elite ABC Kit (Vector laboratories, Burlingame, CA,

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USA). Immunostaining was semiquantitatively scored by Image J, which is a public domain Java image processing program developed by NIH.

Cell proliferation, adhesion and migration

Cell proliferation, adhesion and migration assays were performed following our previously described methods.[9, 48] To assess cell proliferation, cells (2×10^4/well) were seeded in 24-well plates. Doubling time of cells was calculated from the growth rate during the exponential growth phase (0–72 h) using the formula, $Td = 0.693t/\ln(N_t/N_0)$, where t is time in days, N_t is cell number at time t, and N_0 is cell number at the initial time.[49, 50] To determine the effect of niclosamide on cell viability, cells were treated with vehicle (0.1% DMSO) or niclosamide (0–10 μM) for 48 hours before being harvested and counted by trypan blue exclusion. To assess cell adhesion, cells (1×10^5/well) were seeded in 24-well plates and harvested after 1 h incubation. Cell migration assays were performed using a modified Boyden Chamber method with 8-μm pore size polycarbonate membrane 24-well transwells. Cells were treated with vehicle or niclosamide for 24 hours before being harvested, re-plated (75×10^3 cells per well in 100 μl of serum-free medium) into upper wells, and placed in lower wells containing 500 μl serum-free medium in order to determine unstimulated migratory ability.

Chromatin immunoprecipitation assay

ChIP assays were performed using the Magna ChIP G-chromatin immunoprecipitation Kit (Thermo Scientific) as described previously.[51] Sheared chromatin from 1×10^6 cells was immunoprecipitated with mouse monoclonal TCF4 antibody or control mouse IgG (Thermo Scientific). Primers for the qPCR are listed in Supplementary Table 3.

Animal experiments

For *in vivo* animal studies, 7–8 week-old female nude mice (*nu/nu* BALB/*c*, Jackson Laboratories) were injected with 1×10^6 cells i.p. in a total volume of 400 μl. Body weights were monitored every 7 days thereafter. Mice were euthanized and necropsied 5 weeks after injection. Niclosamide (200 mg/kg B.W.) or vehicle control (PEG400) was given daily by oral gavage for 5 weeks. Harvested tumors were either frozen or fixed with 4% paraformaldehyde in PBS for further analysis.

Statistical analyses

Quantitative data were subjected to least-squares ANOVA and differences between individual means were tested by a Tukey multiple-range test using Prism 4.0 (Graphpad, San Diego, CA, USA). QPCR data were corrected for differences in sample loading using the *RPL19* data as a covariate. Tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. A p-value of 0.05 or less was considered significant. Data are presented as least-square means (LSM) with standard error of the means (SEM). The Kaplan-Meier method was used to calculate the survival rates and was evaluated by the log-rank test using GEO and TCGA datasets: GSE9891,[52] GSE14764,[53] GSE26712[54] and TCGA-OV, that contained 285, 80, 195 and 570 samples of normal and cancerous ovary, peripheral tissues and fallopian tubes, respectively. A total of

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961 primary ovarian tumors with completed data sets (151, 68, 185 and 557 from GSE9891, GSE14764, GSE26712 and TCGA-OV, respectively) were selected for survival analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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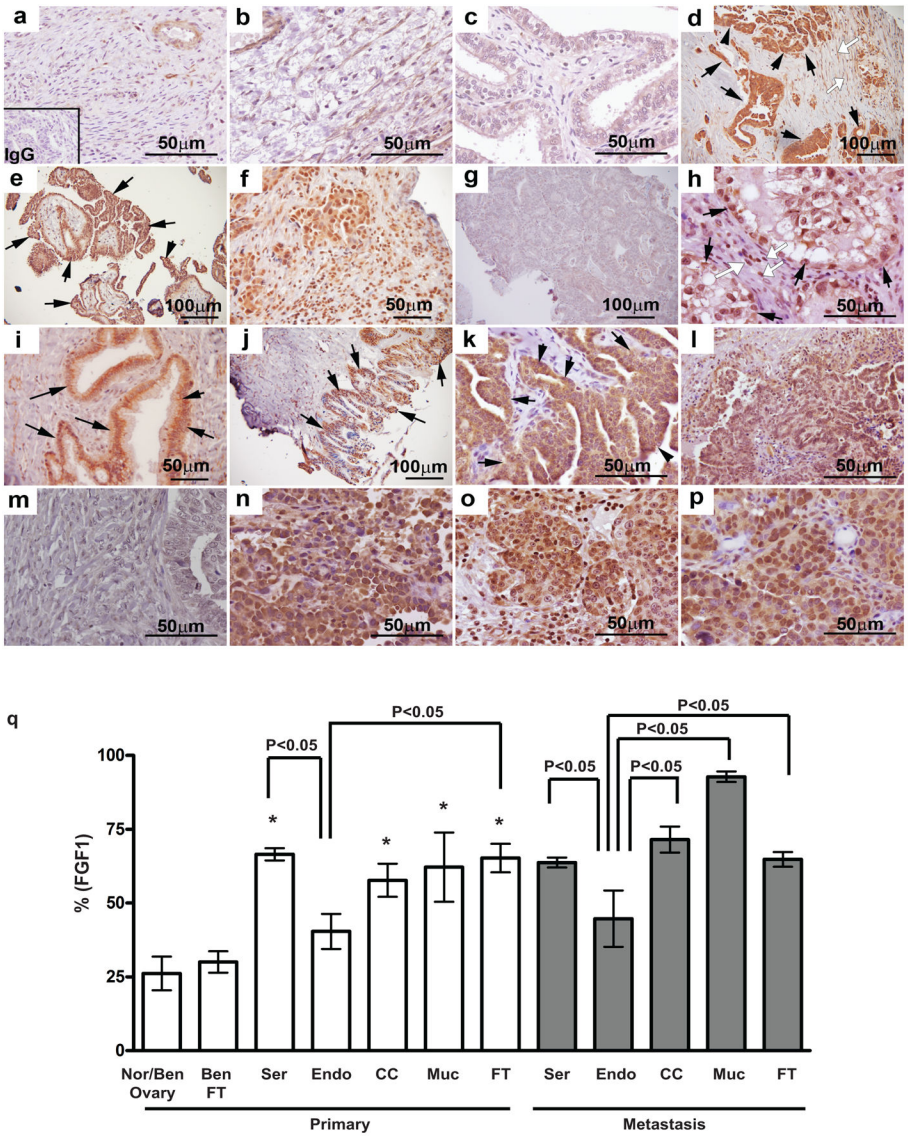

Figure 1.

Analysis of FGF1 in ovarian cancer. (a–p) Representative photomicrographs of immunoreactive FGF1 in normal/benign, primary and metastasized ovarian and fallopian tube tumors (A total of 537 specimens including 10 normal/benign (8 ovaries and 2 fallopian tubes), 147 serous, 20 endometrioid, 22 clear cell, 7 mucinous and 32 fallopian tube primary tumors, and 299 metastasized tumors from 199 serous, 8 endometrioid, 15 clear cell, 4 mucinous and 73 fallopian tubes). (a) Normal ovary. (b) Benign ovary. (c) Benign fallopian tube. (d) Black arrows point to high grade invasive serous epithelial cells. Open white arrows point to fibroblasts. (e) Black arrows point to epithelial cells of low grade serous carcinoma. (f) Serous carcinoma lymphocytes. (g) Endometrioid carcinoma. (h) Black arrows point to epithelial cells of clear cell carcinoma. Open white arrows point to fibroblasts. (i) Black arrows point to non-invasive cells lining the basal membrane of mucinous carcinoma. (j) Black arrows point to surface epithelium of mucinous carcinoma. (k) Black arrows point to epithelial fallopian tube carcinoma. (l) Serous carcinoma

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metastasis in peritoneum. (m) Endometrioid carcinoma metastasis in omentum. (n) Clear cell carcinoma metastasis in peritoneum. (o) Mucinous carcinoma metastasis in omentum. (p) Fallopian tube carcinoma metastasis in ovary. (q) Immunoreactive FGF1 levels were quantitatively scored by Image J analysis. Nor/Ben, normal/benign; Ser, serous; Endo, endometrioid; CC, clear cell; Muc, mucinous; FT, fallopian tube. *P<0.05 vs. normal/benign ovaries and fallopian tubes.

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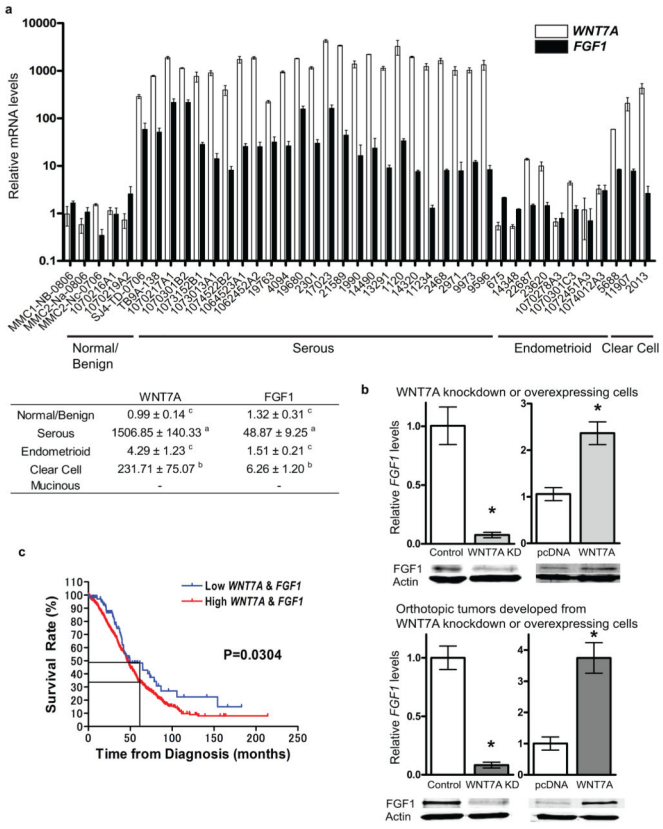

Figure 2.

Expression of FGF1 correlates with WNT7A in ovarian cancer. (a) Relative *WNT7A* and *FGF1* expression. A total of 41 fresh-frozen ovarian samples including 5 normal/benign, 25 serous, 8 endometrioid, 3 clear cell and 0 mucinous were examined for *WNT7A* and *FGF1* levels. Duplicates of each sample were analyzed by qPCR. Values were normalized against *RPL19* and are expressed as fold above normal/benign (± SEM), which was arbitrarily given a value of 1. Mean vales from each subtype are shown in table. Different letters denote transcripts that have statistically significant (P<0.01) differences in mean expression levels. (b) Relative FGF1 mRNA and protein expression in WNT7A knockdown SKOV3.ip1 cells and WNT7A overexpressing SKOV3 cells. *P<0.05 vs. control or pcDNA. Relative FGF1 mRNA and protein expression in orthotopic tumors developed from WNT7A knockdown SKOV3.ip1 cells or WNT7A overexpressing SKOV3 cells. *P<0.05 vs. control or pcDNA. (c) WNT7A and FGF1 expression correlates with survival. Overall survival rate was calculated in 151, 68, 185, 557 patients from GSE9891, GSE14764, GSE26712 and TCGA-OV, respectively, in relation to the expression of *WNT7A* and *FGF1* by Kaplan-Meier method using Prism 4.0. The P-value was determined by the log rank test.

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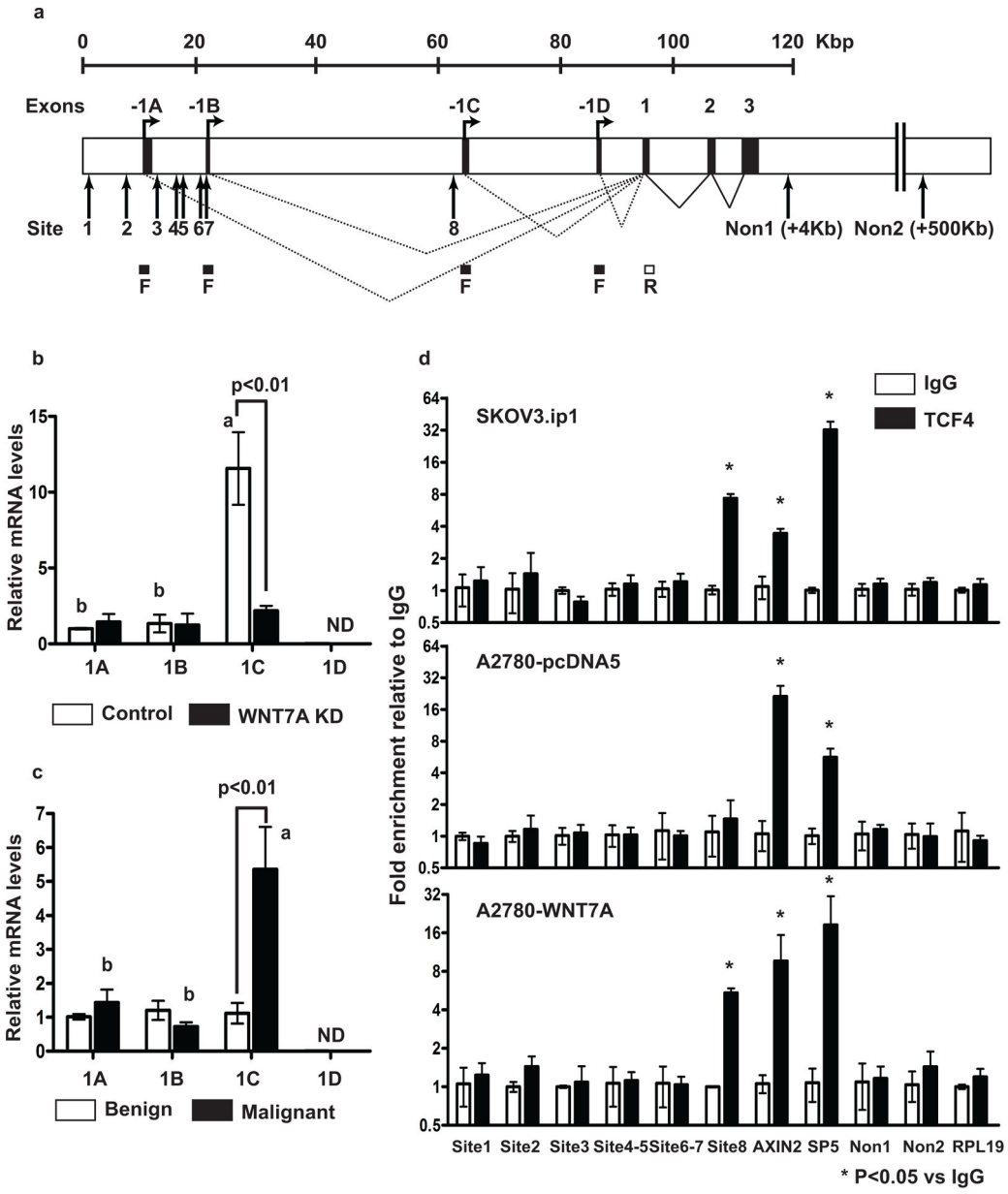

Figure 3.

FGF1 is a direct β-catenin/TCF target. (a) Human *FGF1* gene and transcripts. Alternative splicing of untranslated exons (*1A*, *1B*, *1C* and *1D*) to exon 1 will generate mRNAs *1A*, *1B*, *1C* and *1D*. A total of 8 consensus TCF/LEF-binding elements (WWCAAWG, W=A/T) at the *FGF1* genomic locus are shown. F; forward primers designed in *1A*, *1B*, *1C* or *1D* and R; reverse primer designed in exon 1. Relative *FGF1* alternative splicing mRNA levels in (b) control and WNT7A knockdown SKOV3.ip1 cells, and (c) human ovarian normal/benign (n=5) and malignant (n=36) tumors. Different letters denote transcripts that have statistically significant (P<0.01) differences in mean expression levels. ND: non detectable. (d) ChIP assay of DNA isolated from SKOV3.ip1, A2780-pcDNA5 and WNT7A overexpressing cells immunoprecipitated with TCF4 antibody. Immunoprecipitated DNA

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was analyzed by qPCR and normalized to input. TCF4 binding sites at AXIN2 and SP5 loci were used as positive controls. Three irrelevant non TCF4 binding sites on the same chromosome of *FGF1* and different chromosome at the *RPL19* locus were used as negative controls.

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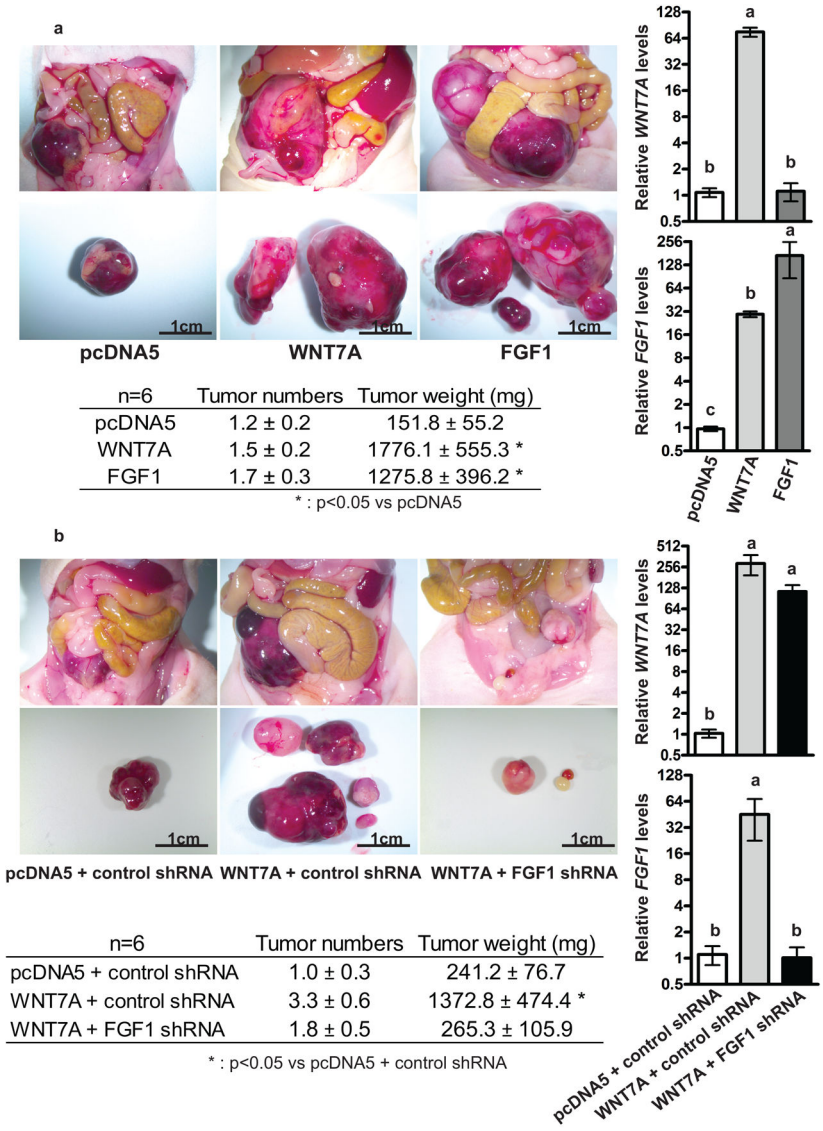

n=6	Tumor numbers	Tumor weight (mg)
pcDNA5	1.2 ± 0.2	151.8 ± 55.2
WNT7A	1.5 ± 0.2	1776.1 ± 555.3 *
FGF1	1.7 ± 0.3	1275.8 ± 396.2 *

* : p<0.05 vs pcDNA5

n=6	Tumor numbers	Tumor weight (mg)
pcDNA5 + control shRNA	1.0 ± 0.3	241.2 ± 76.7
WNT7A + control shRNA	3.3 ± 0.6	1372.8 ± 474.4 *
WNT7A + FGF1 shRNA	1.8 ± 0.5	265.3 ± 105.9

* : p<0.05 vs pcDNA5 + control shRNA

Figure 4.

FGF1 promotes the tumorigenic functions of WNT7A. (a) WNT7A or FGF1 overexpression increased tumor growth. Nude mice were injected i.p. with vector control cells, WNT7A overexpressing, or FGF1 overexpressing cells. The images provide a direct view of the abdominopelvic cavity and tumors isolated from mice. Total tumor numbers and weight 5 weeks after i.p. injection are shown (n=6 each group). Relative *WNT7A* or *FGF1* expression in orthotopic tumors that developed from vector control, WNT7A overexpressing, or FGF1 overexpressing cells. Different letters denote groups that have statistically significant (P<0.05) differences in mean expression levels. (b) FGF1 knockdown inhibits WNT7A-dependent tumor growth. FGF1 knockdown in WNT7A overexpressing cells inhibited tumor growth. Nude mice were injected i.p. with vector control, or control or FGF1 knockdown WNT7A overexpressing cells. The images provide a direct view of the abdominopelvic cavity and tumors isolated from the mice. Total tumor numbers and weight 5 weeks after i.p. injection are shown (n=6 each group). Relative *WNT7A* or *FGF1* expression in orthotopic

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tumors that developed from vector control, or control or FGF1 knockdown WNT7A overexpressing cells. Different letters denote groups that have statistically significant ($P<0.05$) differences in mean expression levels.

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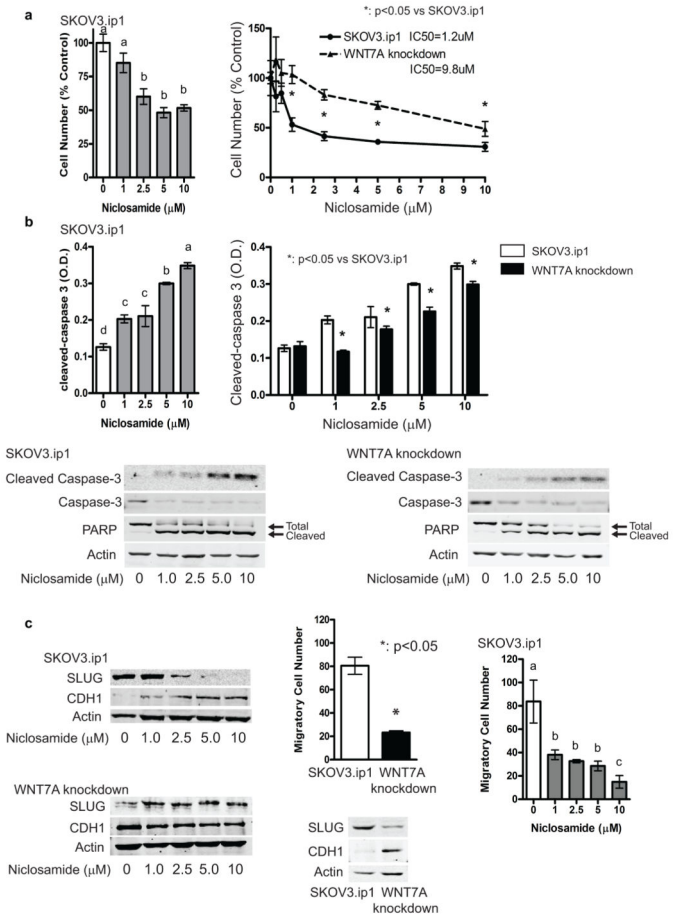

Figure 5.

Niclosamide effectively inhibits ovarian cancer cell functions in WNT7A expressing cells. (a) Effects of niclosamide on cell viability in SKOV3.ip1 control and WNT7A knockdown cells. Different letters denote groups that have statistically significant (P<0.05) differences in mean cell number. (b) Effects of niclosamide on cleavage of the apoptosis effector molecule caspase-3 and its target PARP in SKOV3.ip1 and WNT7A knockdown cells by ELISA and/or western blots. Different letters denote groups that have statistically significant (P<0.05) differences in mean O.D. (c) Effects of niclosamide on CDH1 and SLUG expression levels, and cell migration in SKOV3.ip1 control and WNT7A knockdown cells. Different letters denote groups that have statistically significant (P<0.05) differences in mean cell number.

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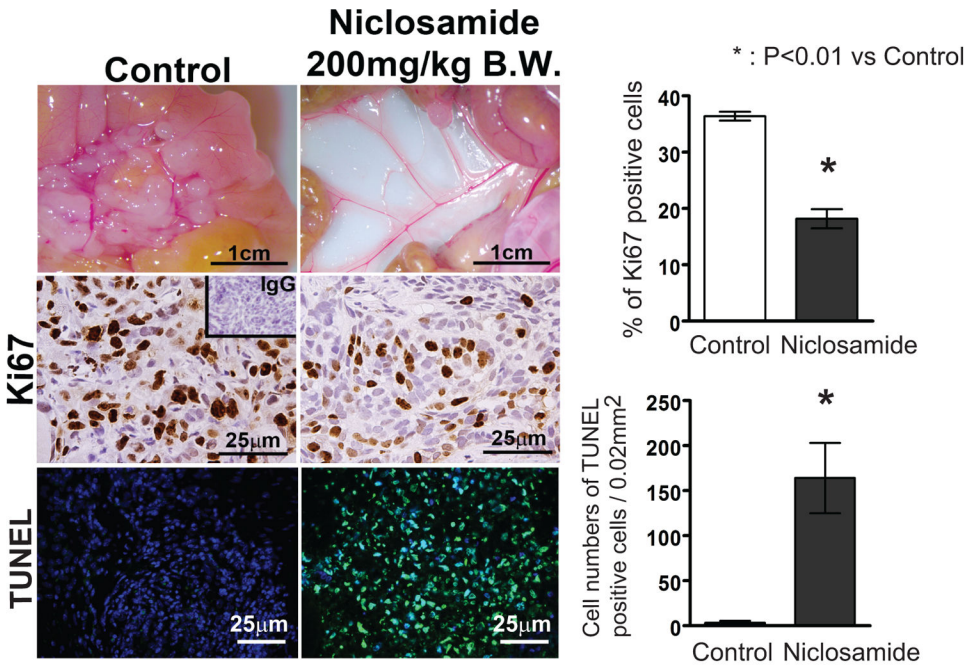

n=6	Tumor numbers	Tumor weight (mg)
Control	78.5 ± 8.7	936 ± 121
Niclosamide (200mg/kg B.W.)	13.8 ± 2.0 *	172 ± 30 *

* : p<0.01 vs control

Figure 6.

Effects of niclosamide on tumor growth in ovarian cancer. (a) Mice were injected i.p. with SKOV3.ip1 cells and treated with niclosamide (0 and 200 mg/kg/day B.W.) by oral gavage for 5 weeks starting 3 days after i.p. injection. Tumor implant number and total tumor weight 5 weeks after niclosamide treatment are shown (n=6 each group). Cell proliferation and apoptosis were determined by immunoreactive Ki67 staining and TUNEL assay.