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Targeting Wnt signaling with Signalomics in primary breast cancer and gynecological pathologies

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Summary

Breast and gynecological pathologies, such as endometriosis and cancers, rely on aberrant activation of pro-proliferative pathways like Wnt signaling. In addition to multiple omics techniques utilized to identify drivers and targetable vulnerabilities in these diseases, Signalomics has recently emerged as a new tool to rapidly assess the levels of activation of oncogenic signaling pathways in primary cells. Here we demonstrate the power of Signalomics when applied to patients with breast, ovarian, and endometrial cancers, as well as endometriosis. Assessing basal and stimulated levels of Wnt signaling in patients' surgical samples, we find these levels to correlate with the disease progression. We further show that pharmacological inhibition of the Wnt pathway in many of these primary samples suppresses cell proliferation, offering means for patient stratification prior to eventual targeted treatment. Altogether, our study is the first example of Signalomics applications in daily clinical settings, offering insights into disease mechanisms and personalized treatment.

Introduction

Gynecological diseases comprise a heterogeneous spectrum ranging from chronic inflammatory disorders such as endometriosis to malignant tumors. Although genomic profiling and targeted therapies have transformed clinical decision-making in oncology, a persistent challenge remains: disease phenotypes are ultimately driven by aberrations in signaling networks, which cannot always be inferred from DNA alterations alone. This gap is particularly pronounced in pathologies where aberrant tissue growth and invasion arise from context-dependent pathway activation, shaped by epigenetic remodeling and microenvironmental ligand-receptor cues rather than recurrent driver mutations[1].

In precision oncology, next-generation sequencing has become the dominant framework for patient stratification and therapeutic selection. Yet across tumor types, genotype-phenotype correlation is incomplete: signaling pathways may be strongly activated in the absence of canonical mutations, while apparently “actionable” alterations do not necessarily translate into pathway dependence or drug sensitivity[1]. These limitations motivate approaches that directly measure pathway output as a functional readout in patient-derived material.

To address this unmet need, we recently proposed Signalomics, a functional platform designed to quantitatively profile signaling pathway activities in freshly isolated patient-derived cells using reporter-based assays[2]. By capturing pathway activation states rather than static molecular features, Signalomics provides a complementary layer of information to genomics and transcriptomics and offers a practical framework for mapping inter-patient heterogeneity in clinically relevant signaling programs.

Among the signaling cascades implicated in gynecological disorders, the Wnt/β-catenin pathway represents a particularly compelling candidate for functional profiling. The Wnt pathway (Fig. 1A) is one of the most profoundly important oncogenic signaling pathways, regulating such aspects as the initial oncogenic transformation[3], growth of primary tumors[4,5], metastases[6], cancer stem cells[7], acquired chemo- and radio-resistance[8], and tumor microenvironment[9]. Through mutations in Wnt signaling components or through epigenetic dysregulations in their expression levels, aberrant Wnt signaling contributes to colorectal cancer, pancreatic cancer, hepatocellular carcinoma, melanoma, breast and gynecological cancers[4,10,11]. Aberrant Wnt signaling also emerges as a key player in endometriosis[12] – chronic inflammatory disorder characterized by ectopic endometrial-like tissue growth, fibrosis, and invasive behavior. It shares several hallmarks with cancer, including increased proliferative capacity, altered immune stromal interactions, and resistance to apoptosis[13].

Here, we apply the Signalomics framework to breast, ovarian, and endometrial cancers, as well as endometriosis, identifying Wnt signaling vulnerabilities in these malignancies and providing the patient stratification basis for eventual Wnt-targeting therapies. This work describes the first implementation of Signalomics in clinical applications, laying ground for inclusion of this technology in Molecular Tumor Boards and clinical practices.

Results

Wnt/β-catenin activation measurement in primary breast and gynecological cancer cells

Primary specimens were collected from adult patients with breast cancer (BC), gynecological malignancies (ovarian cancer (OC) and endometrial cancer (EC)), and endometriosis. Viable primary cells were isolated as described in the Methods section. Using the lentivirally-delivered Wnt reporter and the control reporter, we monitored basal Wnt activation levels in the samples (Fig. 1A), comparing them to the maximal possible Wnt activation levels obtained through the massive pathway activation using CHIR99021, a specific GSK3β inhibitor[14,15] (Fig. 1B). For the cancer samples analyzed in this manner, the relevant clinical data is provided in Supplementary Tables S1-S3. Analyses of the basal and stimulated Wnt signaling levels identified significant differences among the three cancer types, as well as noticeable heterogeneity within each cancer type. Thus, in BC and OC, basal Wnt activation was minimal, barely reaching 1-2% of the pathway activation achieved by GSK3β inhibition (Fig. 1C, D). In contrast, half of the analyzed EC samples exhibited the basal Wnt activation levels ≥50% of the maximal CHIR99021-stimulated Wnt activation levels (Fig. 1E).

Further, analysis across the cancer types permits identifying three gross groups of tumors in terms of their Wnt pathway levels. The first group is characterized by not only low levels of the basal pathway activation, but also by low levels of 'activatability' of the pathway achieved through GSK3β inhibition that reached <10% of the luciferase expression mediated by the constitutive CMV promoter. These are, e.g., BC11 and BC15, OC15 and OC39, or EC30 and EC31 (Fig. 1C-E). The second group is characterized by low basal, but relatively high stimulated Wnt pathway levels, represented by, e.g., BC14 and BC17, OC23 and OC27, or EC13 and EC17. And the third group, seen only in EC, are those where the basal levels of the pathway are high and comparable to the CHIR99021-induced levels (such as EC12, EC16, or EC19). These three groups can be metaphorically described as 'cold and non-ignitable' (group 1), 'cold and ignitable' (group 2), and 'hot and burning' (group 3) in terms of their levels of basal and inducible Wnt pathway levels, respectively. Given the key role of the Wnt pathway in multiple aspects of oncogenic transformation and progression, one could hypothesize that this 3-group distribution of the breast and gynecological tumors may reflect important clinical characteristics of the tumors / patients.

Gain-of-function mutations in *CTNNB1* – the gene encoding β-catenin – have been observed in EC with the frequency varying from 10% to 45% [16]. Sequencing revealed an activating T41A *CTNNB1* mutation in EC19 but not in other samples (Supplementary Fig. S1, Supplementary Table S3), making the *CTNNB1* mutation frequency in our cohort of 5%. Accordingly, EC19 displayed the highest basal levels of the Wnt pathway activation among our EC samples (Fig. 1E). However, several other EC samples also displayed high basal Wnt levels, indicating that the relatively high percentage of Wnt activation in EC has a non-mutational origin.

Immunohistochemical (IHC) analysis of β-catenin translocation from cytoplasm to the nucleus is a standard method for assessing Wnt pathway activation in cells and tissues[5,17]. We thus performed the IHC analysis in our EC, BC, and OC samples (Fig. 2A, B shows examples of predominantly

membranous vs. predominantly nuclear β-catenin-staining tumor samples), followed by calculation of the percentage of cells with nuclear β-catenin in each analyzed sample (Fig. 2C-E). At least three serial sections of the tumor from each patient were photographed. In BC and OC, the percentage of cells with the nuclear localization of β-catenin ranged from 0 to 50%. For EC, this level ranged from 20 to 80%. Importantly, a significant correlation between these IHC data and the direct and quantitative measurement of the basal TopFlash signal was found across the tumor samples (Fig. 2F). Thus, measuring Wnt activation in primary samples using a lentiviral reporter may serve as a quantitative alternative to IHC analysis.

Wnt signaling levels correlate with cancer progression

As Wnt signaling is widely considered as a driver in many cancers[18], we next investigated whether Wnt signaling levels in breast and gynecological cancers may correlate with the disease progression. Indeed, for EC – the only cancer consistently displaying high basal signaling levels, we found a significant correlation between the tumor grade and the basal Wnt signaling levels (Fig. 3A). Similarly, the stimulated Wnt levels also positively correlated with the tumor grade in EC (Fig. 3B).

While the low levels of basal Wnt signaling in BC did not correlate with the BC tumor grade (Supplementary Fig. S2A), a significant and positive correlation was found between the stimulated Wnt levels and the BC tumor grade (Fig. 3C).

As for OC, whose basal Wnt levels did not significantly correlate with the tumor grade (Supplementary Fig. S2B), a significant yet negative correlation was observed between the stimulated Wnt levels and the tumor grade (Fig. 3D).

These findings indicate different roles Wnt signaling may play in BC and gynecological cancers, as addressed in Discussion.

Wnt/β-catenin activation measurement in endometriosis

Similarly to the investigation in primary gynecological tumor samples, we assessed samples from 22 endometriosis patients. Three different endometriosis sample subtypes were collected, including rectovaginal endometriosis, colorectal endometriosis, and ovarian endometriomas (Supplementary Table S4). While all represented advanced endometriosis stages, the latter two represent more invasive forms[12,13]. Analysis of basal and stimulated Wnt signaling levels in the endometriosis samples demonstrated significant differences across and even within different endometriosis subtypes (Fig. 4A). However, analysis of the ratio of stimulated *vs.* basal Wnt signaling levels identified an interesting trend, with the samples of endometriosis infiltrated to the gut reaching significantly higher levels than those originating from ovarian cysts (Fig. 4B). This may reflect a growing importance of the Wnt pathway upon invasion of the endometriosis masses towards neighboring tissues.

Pharmacological inhibition of Wnt signaling reduces the proliferative capacity of patient-derived cells

Having established heterogeneity of Wnt/β-catenin pathway activity across patient-derived samples, we next examined whether pharmacological inhibition of Wnt signaling translates into functional suppression of tumor cell growth. To this end, primary cells were treated with two Wnt pathway inhibitors, ICG-001 and clofazimine, acting at different pathway components[5,19], and monitored by real-time impedance analysis. Integrated proliferative output was quantified as the area under the Cell Index curve (AUC; Cell Index × h).

We found that both inhibitors reduced AUC compared with untreated controls, indicating suppression of Wnt-dependent proliferative activity (Fig. 4C). Analysis with Holm-Sidac method demonstrated a significant reduction of AUC in the ICG-treated group versus control ($p<0.001$), as well as clofazimine-treated group versus control ($p<0.001$). However, treatment with ICG-001 was found efficient in higher number of patient-derived samples than clofazimine (75% versus 25% of responded patients' samples, respectively). Although the clofazimine responders represented a smaller group compared to ICG-001 responders, clofazimine is an approved drug prescribed for the treatment of leprosy and tuberculosis[20], ready for off-label applications in cancer[5,11], while ICG-001 remains a laboratory drug.

These findings support the functional relevance of Wnt signaling activity in primary tumor cells and demonstrate that pharmacological inhibition of the pathway reduces their proliferative capacity, laying ground for patient stratification for targeted treatment.

Discussion

In this study, we establish a functional platform for quantitative assessment of Wnt signaling activity in patient-derived, primary gynecological disease cells. By measuring both basal pathway output and inducible signaling capacity, we demonstrate that functional pathway activity can reveal clinically relevant heterogeneity that is not captured by static genomic or immunohistochemical analyses.

Wnt signaling is known to play important roles in breast and gynecological cancers[21-30]. Interestingly, while the presence of nuclear β-catenin (as a marker of overly activated canonical Wnt signaling) has been observed to correlate with poor survival of BC patients[22,31,32], we believe it is for the first time that a correlation between the quantified level of the Wnt pathway and the histological tumor grade of tumors is found for BC. Even more so, although the Wnt pathway is well implicated in EC, no correlations between the levels of the pathway activation, measured by nuclear β-catenin or other means, and the tumor grade have been previously observed[16,25]. Since the IHC data are inherently poorly quantitative, our method of robust Wnt level measurements in primary tumor cells provides a strong advantage to this previously standard IHC-based approach to monitor Wnt signaling in cancer. Interestingly, there appears to be a negative correlation between OC progression and the levels of Wnt pathway activation. Although Wnt signaling has been clearly implicated in OC[33], and we find sensitivity of primary OC cell proliferation to pathway inhibition by ICG-001, these findings may indicate differential role played by the canonical Wnt signaling in different stages of OC.

As expected, we find that the quantitative basal Wnt levels correlate well with the proportion of tumor cells with nuclear β-catenin. Remarkably, we further find that the stimulated (better than basal) Wnt signaling levels correlate with the tumor grade in BC and EC, discovering a new quantitative biomarker of breast and endometrial tumor progression. Noteworthy, IHC of β-catenin could not rival this novel biomarker, due to its inherent poorly quantitate nature, but also because it reflects the basal, rather than stimulated Wnt levels.

Why would CHIR99021 (GSK3β inhibitor[14] as a specific receptor-independent Wnt signaling pathway stimulator)-induced Wnt pathway levels in tumor cells be a better biomarker of the tumor progression than the basal levels? In other words, why would the maximal potentially achievable levels of the pathway be more informative in this context than the actual levels of activation captured at a given time point? We suspect that the inherent heterogeneity / variability of the cancer cells within a tumor, both in their spatial and their temporal sense, offers the answer. Indeed, a snapshot taken at any given moment of tumor progression reveals the number of cells currently active in their Wnt signaling, but what probably drives the tumor progression more is the potential of this cancer cell population to engage the Wnt pathway at a critical time point during the tumor history. This potential is less effectively captured by the measurement of the basal Wnt signaling levels (which turn out to be rather low in our BC samples) but is revealed by the measurement of the CHIR99021-induced levels of the pathway.

Taken together, these observations support a unifying model in which inducible pathway capacity represents a functionally meaningful dimension of disease biology that is not captured by static genomic, transcriptomic, or immunohistochemical measurements. Signalomics directly quantifies both basal pathway output and inducible signaling reserve in primary patient-derived cells, thereby capturing

clinically relevant functional heterogeneity. In oncology, this provides a rational basis for incorporating functional pathway profiling into molecular tumor board decision-making to complement genomic data and refine therapeutic stratification. In the context of the patients analyzed in this study, such an approach would identify breast and gynecological cancer patients with high Wnt signaling capacity who may benefit from Wnt-targeting interventions, including off-label clofazimine[5,11,17,20,34], potentially in combination with chemo- or immune-therapies[5,35,36].

Beyond oncology, the same functional framework is applicable to personalized diagnostics and treatment stratification in proliferative and inflammation-associated diseases such as endometriosis, where pathway hyperactivation may influence lesion progression and therapeutic responsiveness. Indeed, our dataset in endometriosis provides a complementary demonstration of the value of functional Wnt pathway readouts in gynecological disorders. In endometriosis lesions, both basal and stimulated levels of the Wnt pathway are heterogeneous. However, a trend emerging from our studies shows that the stimulated-to-basal ratio increases upon progression from ovarian endometriomas to rectovaginal lesions, and even more so – colorectal lesions. This subtype dependence is consistent with the view that Wnt/β-catenin signaling is involved in key endometriosis-associated processes including invasion and fibrogenesis[12,37], and represent first-ever direct measurements of the Wnt pathway levels in primary cells of this disease. Prior work has shown that pharmacological suppression of Wnt signaling can reduce lesion-associated fibrotic programs and improve disease features in preclinical models[38]. Thus, our endometriosis data support extending Signalomics beyond its oncology use. By quantifying both basal pathway output and inducible signaling capacity in primary patient-derived cells, Signalomics captures functional heterogeneity that is poorly reflected by static readouts. This principle is directly applicable to personalized functional diagnostics and treatment stratification not only in cancer, but also in proliferative and inflammation-associated diseases such as endometriosis.

Taken together, the present study aims at generating a system for detecting the activity of the Wnt signaling pathway in breast and gynecological diseases as the first step for a larger study of the entire panel of oncogenic signaling pathways activities. We believe that such a system for detecting the activity of signaling pathways will become a working routine of Molecular Tumor Boards in the future, complementing the existing molecular cell analysis methods for personalized cancer treatment. In addition to the translational significance of our study, it also offers a large fundamental opportunity, as it assesses how widespread the Wnt (and other pathways') activation is in individual tumors, as well as the level of this activation and its responsiveness to specific inhibitors and activators. Furthermore, Signalomics measures the importance of the given pathway for cell proliferative properties in each specific case of the studied cancer, creating the basis for patient stratification for targeted therapies.

By harnessing the power of Signalomics, clinicians can gain deeper insights into the intricate molecular signaling patterns of tumors and leverage this knowledge to inform personalized treatment decisions. By embracing the potential of Signalomics, we can move closer to the goal of providing patients with the most effective and targeted therapies tailored to their specific cancer/ disease biology.

Methods

Primary tumor samples

Human tumor samples were obtained from patients who were diagnosed with ovarian cancer (OC), endometrial cancer (EC), or breast cancer (BC) and underwent surgical resection at the Oncogynecology and Mammology Institute of the Kulakov National Medical Research Center of Obstetrics, Gynecology and Perinatology (Moscow). Informed consent was obtained from the patients, and all procedures were approved by the Ethics Committee of the Kulakov National Medical Research Center. Primary tumor specimens were collected from adult patients with BC (n=30), OC (n=30), and EC (n=30), taken 30-60min after surgery. Collected tumor samples were placed in sterile 50ml centrifuge tubes containing DMEM/F12 (Biolot, Russia) with 500U/ml of penicillin and 500µg/ml of streptomycin (PanEco, Russia) when transporting from the operating room to the research laboratory. Next, the tissues were mechanically chopped with a scalpel and placed in a culture medium DMEM/F12 with 10% FBS (Gibco, USA). The tissue pieces were then subjected to enzymatic digestion using collagenase-1 (Gibco, USA) at 1000U/ml. The digestion process occurred at 37°C for a duration ranging from 2h for EC and OC to 15h for BC. Subsequently, the resulting suspensions were washed in 0.9% NaCl and briefly spinned down, and the supernatants were discarded. The pellets were resuspended in fresh 0.9% NaCl and strained via 100µm pore cell strainer. To remove any residual erythrocytes from the tumor samples, cells were incubated in a Red Blood Cell Lysis Buffer (Miltenyi Biotec, USA). Isolation of the epithelial cell population from the samples was performed by MACS using commercial kits for negative selection with magnetic particles attached to antibodies against non-epithelial cell markers (Tumor Cell Isolation Kit, human, Miltenyi Biotec, USA). Cancer cells were then resuspended in DMEM/F12 with 10% FBS and seeded in white 96-well culture plates at the density of 80,000-100,000 per well. Cells were used for the subsequent procedures as early as DIV (day *in vitro*) 1-2 to avoid artificial effects of lengthy cell culturing on the signaling pathways. Isolation of viable cancer cells was successful for 16 samples of BC (53% success rate), 14 samples of OC (47%), and 19 samples of EC (63%). The roughly 50% of cases of the unsuccessful isolation of viable cancer cells likely occurred for the tumor samples on the longer side of the 30-60min waiting to be treated by our protocol after surgery, stressing the need of access to the freshly obtained surgical isolates.

Endometriosis samples

Primary endometriosis samples were obtained from 22 adult female patients aged 20-45 years diagnosed with endometriosis at the Surgery Department of the Kulakov National Medical Research Center of Obstetrics, Gynecology and Perinatology. The main subtypes included rectovaginal endometriosis, endometriotic cysts, and colorectal endometriosis. Women did not receive hormonal therapy such as gonadotropin-releasing hormone agonists or sex steroids and did not use intrauterine contraception for at least 6m prior to surgery. All included patients had regular menstrual cycles (26-32 days) with a confirmed menstrual history. Informed consent was obtained from all patients, and all study procedures were approved by the Ethics Committee of the Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology (Moscow). Primary tissue samples were handled with the

same protocol as for cancer specimens with a slight distinction: the epithelial and stromal cell fractions were not separated and were seeded together.

Generation of lentiviral particles

To detect Wnt/β-catenin activity in tumor cells, the TopFlash plasmid was used (#12456, Addgene, USA). This reporter cassette containing 8 repeats of the TCF/LEF binding sites, a minimal promoter and a sequence encoding firefly luciferase was cloned into the lentiviral backbone (Lenti-TopFlash). Cloning was verified by restriction enzyme digestion and Sanger sequencing. A control lentiviral plasmid encoded the same lentiviral backbone and firefly luciferase downstream the CMV promoter. Assembly of lentiviral particles followed standard procedures and was performed in the HEK-LTV cell line. HEK-LTV cells were grown in 75cm^2 or 175cm^2 flasks and were taken at 70% confluence for transfection. The transfer plasmid, psPAX2 (Addgene #12260) and pMD2.G (Addgene #12259) were mixed in the ratio of 3:2:1 (by weight, with 12μg/ml of total DNA final concentration). X-tremeGENE HP DNA (Roche, Switzerland, 40μl/ml of final concentration) was added to DNA and mixed by tapping. After 30min incubation, the transfection solution was mixed with the DMEM+10% FBS medium; 10μl of the transfection mixture with 100μl of DMEM+10% FBS was added per 1cm^2 of surface with cultured cells. After 24h, the transfection medium was removed and replaced with the equal volume of fresh DMEM+10% FBS. After 72h, the medium was collected and replaced with a fresh medium. The collected medium (batch I) was stored at 4˚C. After 120h, batch II was also gathered; batch I and batch II were centrifuged for 10min 1000*g* and then passed through the 0.45μm polyethersulfone membrane filter. To obtain pure lentiviruses, the medium was then centrifuged at 70000*g* for 1.5h, the pellet was resuspended in cold sterile PBS (500μl of PBS per lentivirus pellet from one 75cm^2 flask), aliquoted and stored at -80˚C. Successful transduction was observed for 14 BC samples (87% success rate), 6 OC samples out of 8 tested (75%), and 14 EC samples out of 18 tested (78%). Successful transduction was observed in 100% cases of endometriosis.

Cell treatments

On 1-2 DIV after seeding, cancer and endometriosis cells were treated with the lentiviruses. Lentiviral Wnt reporter particles were added at 1:5 dilution to cells while control lentiviruses were used at the 1:25 dilution ratio. After 72h incubation with lentiviruses, one group of cells transduced by Wnt reporter particles was also treated by 10μM CHIR99021 (Sigma-Aldrich, USA), a specific inhibitor of GSK-3β and a potent inductor of Wnt/β-catenin pathway, and has been incubated with it for additional 24h. Then cells were loaded with calcein AM (C1430, ThermoFisher Scientific, USA) for 1h and the fluorescence was measured at the 530nm wavelength.

Luciferase assay

Prior to luciferase activity measurement, cells were lysed in a 50μl per well luciferase assay buffer (100μM luciferin, 1500μM ATP, 4mM EGTA, 4mM DTT, 5mM $MgSO_4$, 25mM Gly-Gly, 0.5% Triton X-100). Luciferase activity was determined by a spectrophotometer Victor Nivo (Perkin Elmer, USA) and normalized with respect to calcein fluorescence measurement. The relative Wnt activity was determined

then by normalizing this ratio against that of control lentivirus luciferase activity and was expressed as a percent of control lentivirus signal which was set as a 100%.

Data availability

All data generated or analyzed in the study are included in this article and its supplementary information files.

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Author contributions

Conceptualization: V.L.K.; Methodology: T.V.D., A.E.I., A.K., and D.N.S.; Formal Analysis: T.V.D., A.E.I., A.K., and D.N.S.; Investigation: T.V.D., A.E.I., A.K., and D.N.S.; Resources: V.V.K., M.R.D., A.V.A., and G.I.T.; Writing – Original Draft: T.V.D., A.E.I., A.K., D.N.S., and V.L.K.; Writing – Review & Editing: T.V.D., A.E.I., A.K., D.N.S., and V.L.K.; Supervision: A.A.S., V.V.R., L.A.A., G.T.S., and V.L.K.; Funding Acquisition: T.V.D., G.T.S., and V.L.K.

Competing interests

V.L.K. is a co-founder and holds equity shares in Wynveris SA, a company based in Geneva, Switzerland. V.L.K. is a co-inventor on a patent application "New Frizzled receptors inhibitors and uses thereof", which was filed by the University of Geneva.

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Figure legends

Figure 1. Basal and activated Wnt signaling levels in primary breast and gynecological tumors. (**A, B**) Scheme of the Wnt pathway and of the measurement of its activity using the TopFlash reporter under basal conditions (A) and upon artificial pathway activation with the GSK3β inhibitor CHIR99021 (B). The gain-of-function mutation in *CTNNB1* leads to a similar overactivation of the pathway through stabilization of β-catenin. (**C-E**) Levels of Wnt activation in breast (C), ovarian (D), and endometrial cancer (E) specimens measured by the TopFlash assay. The basal levels of Wnt/β-catenin response (blue) were compared to the peak levels of the Wnt/β-catenin response after 24h treatment with CHIR99021 (red). Relative luminescence units (RLU) of TopFlash were normalized to RLU of the control lentivirus (encoding CMV-firefly luciferase) and to cell number (calculated by calcein staining). Data are shown as a mean ± SD (n≥3). Statistical significance by multiple t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Black boxed bars in (E) highlight the EC19 specimen harboring the heterozygous T41A mutation in *CTNNB1*.

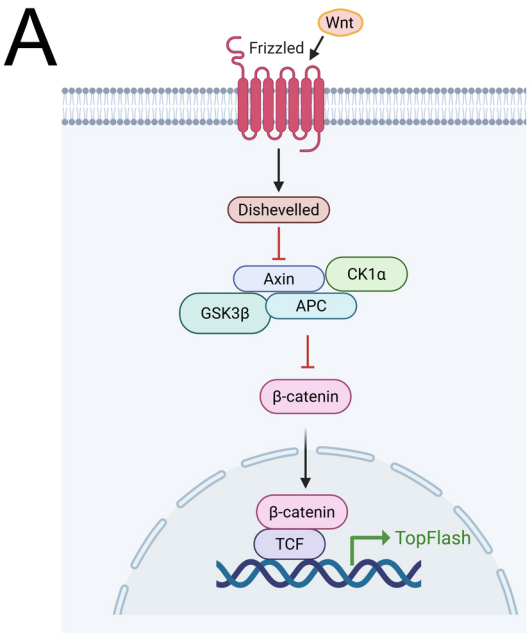
Figure 2. Nuclear β-catenin immunostaining correlates with basal Wnt signaling levels in gynecological tumors. (**A**, **B**) Examples of predominantly membranous (A, breast cancer sample BC31) and predominantly nuclear (B, endometrial cancer sample EC19) β-catenin staining in tumors. DAPI stains nuclei. Scale bar 20µm. (**C**-**E**) Quantification of the percentage of nuclear β-catenin-positive cells in breast cancer (C), endometrial cancer (D), and ovarian cancer (E) immunohistochemistry sections. (**D**) Regression analysis reveals a strong correlation between basal Wnt activation levels and the percentage of nuclear β-catenin-positive cells in the samples. Data is demonstrated as a mean ± SD (n=3, where n is a distinct slide with a sliced tumor specimen). R^2 value and p-value are shown.

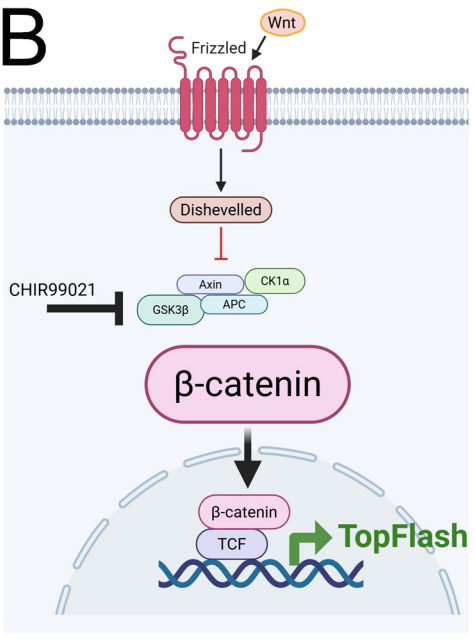
Figure 3. Wnt signaling levels correlate with tumor progression. (**A, B**) In endometrial cancer, both basal levels of the Wnt pathway activation (A) and CHIR99021-stimulated levels of the Wnt pathway activation (B) positively correlate with the tumor grade (see Supplementary Table S3). (**C**) CHIR99021-stimulated levels of the Wnt pathway activation in breast cancer positively correlate with the tumor grade (see Supplementary Table S1). (**D**) CHIR99021-stimulated levels of the Wnt pathway activation in ovarian cancer negatively correlate with the tumor grade (see Supplementary Table S2). Regression analysis with the resulting R^2 value and p-value are shown.

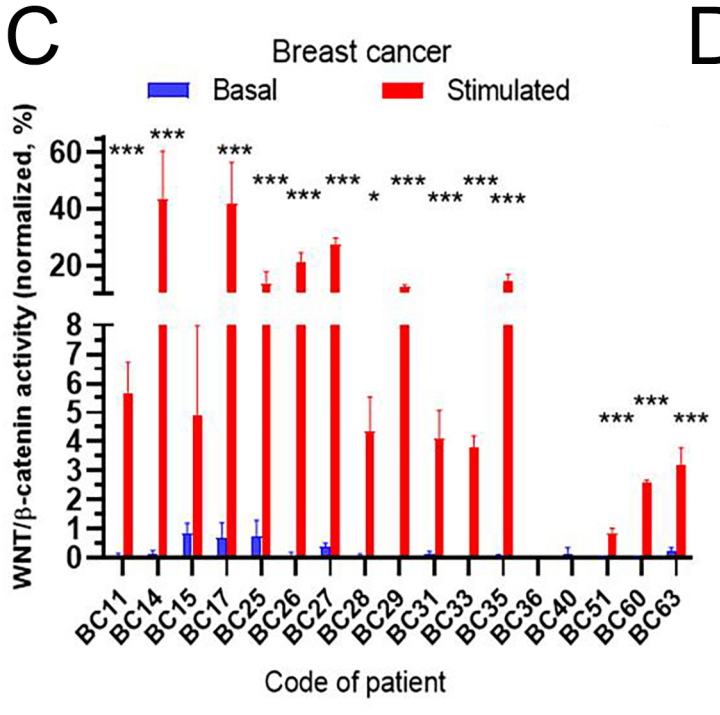
Figure 4. Wnt levels in endometriosis and antiproliferative effects of Wnt pathway inhibition. (**A**) Basal and stimulated Wnt pathway levels in primary endometriosis from different locations: gut, cysts, and rectovaginal, measured by the TopFlash assay. (B) Ratio of CHIR99021-stimulated to basal levels of Wnt signaling is higher in invasive lesions than in ovaries. Data are shown as a mean ± sem (n≥3). p-value by t-test is shown. (C) Proliferation of primary breast, ovarian, and endometrial cancer tumor samples is affected by Wnt pathway inhibitors ICG-001 and clofazimine (both at 10µM). Data in (A, C) are shown as a mean ± SD (n≥3). Statistical significance by multiple t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

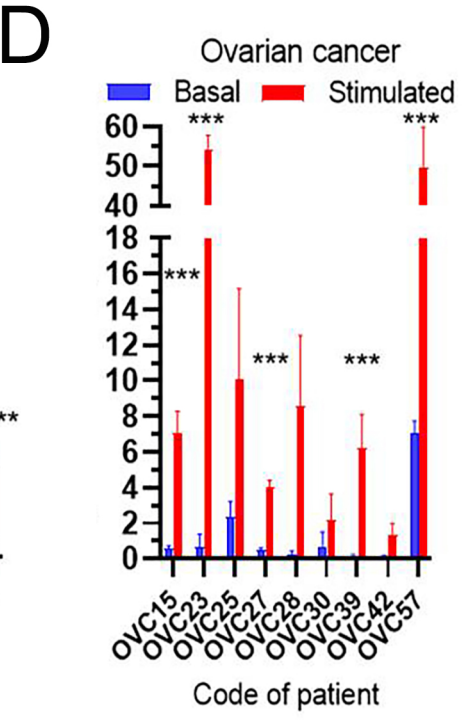
Supplementary Figure S1. Heterozygous T41A mutation is detected in *CTNNB1* exon 3 of EC19 tumor specimen DNA according to Sanger sequencing data. (**A**) Normal forward and reverse nucleotide and amino acid sequence around codons 37 to 43 of *CTNNB1*. (**B**) Sanger sequencing (forward and reverse) reveal the heterozygous c.121A>G (p.Thr41Ala) mutation. (**C**) Higher resolution Sanger sequencing (forward and reverse) data. Q – quality. Related to Fig. 1.

Supplementary Figure S2. Lack of significant correlation between basal Wnt signaling levels and tumor grades in breast (**A**) and ovarian (**B**) cancer. (see Supplementary Tables S1-S2). Regression analysis with the resulting R^2 value and p-value are shown. Related to Fig. 3.


A
Wnt
Frizzled
Dishevelled
Axin
CK1α
GSK3β
APC
β-catenin
β-catenin
TCF
TopFlash


B
Wnt
Frizzled
Dishevelled
CHIR99021
Axin
CK1α
GSK3β
APC
β-catenin
β-catenin
TCF
TopFlash


C
Breast cancer
Basal
Stimulated
WNT/β-catenin activity (normalized, %)
Code of patient
BC11
BC14
BC15
BC17
BC25
BC26
BC27
BC28
BC29
BC31
BC33
BC35
BC36
BC40
BC51
BC60
BC63


D
Ovarian cancer
Basal
Stimulated
Code of patient
OVC15
OVC23
OVC25
OVC27
OVC28
OVC30
OVC39
OVC42
OVC57

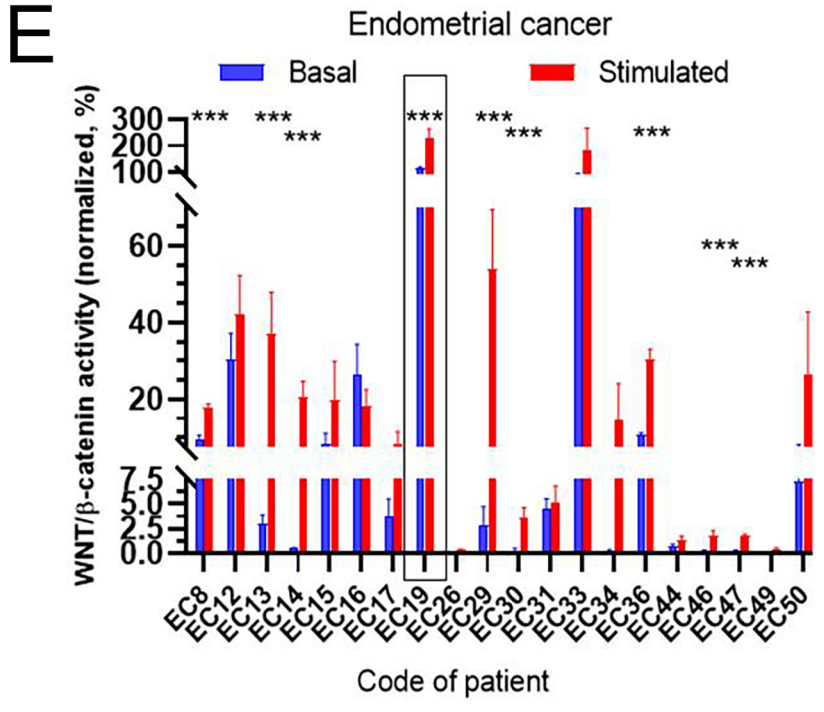
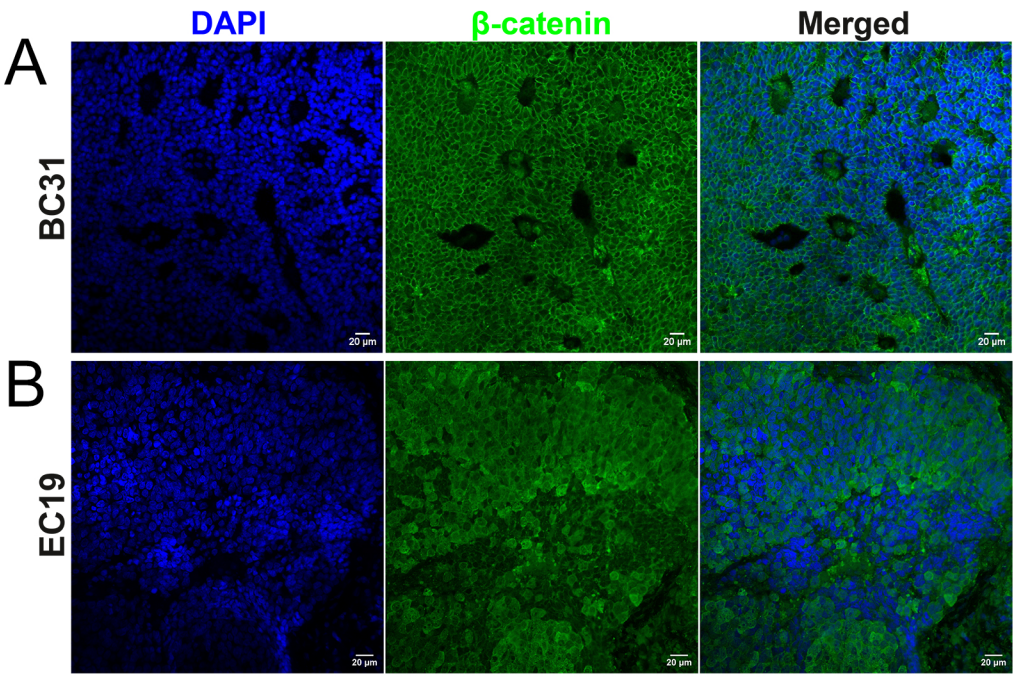
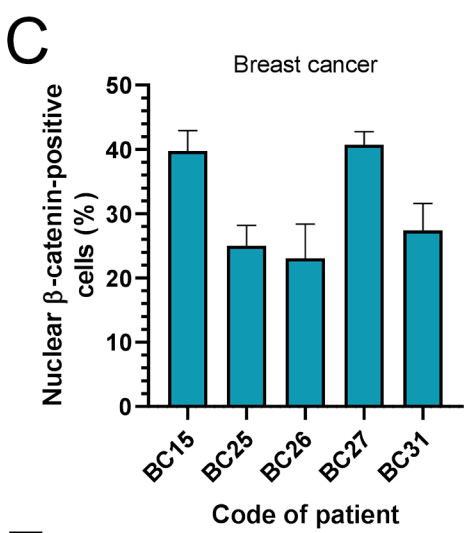
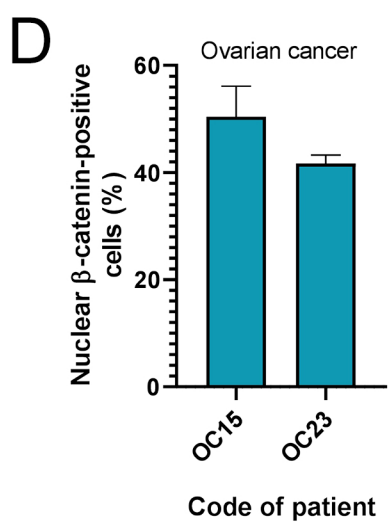
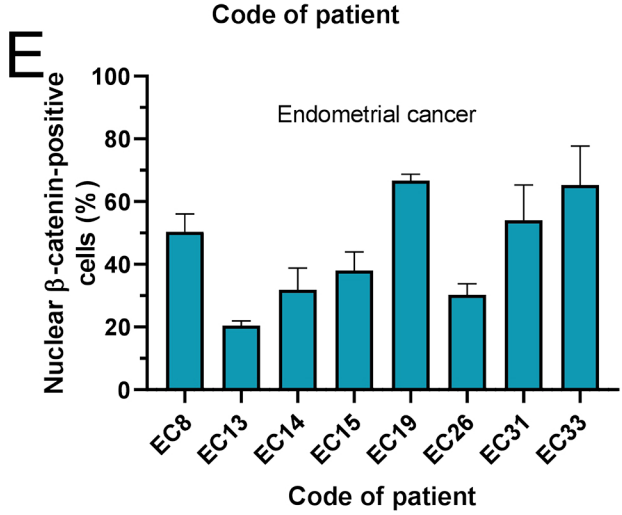

E
Endometrial cancer
Basal
Stimulated
WNT/β-catenin activity (normalized, %)
Code of patient
EC8
EC12
EC13
EC14
EC15
EC16
EC17
EC19
EC26
EC29
EC30
EC31
EC33
EC34
EC36
EC44
EC46
EC47
EC49
EC50

Figure 1


DAPI
β-catenin
Merged
A
BC31
B
EC19
20 μm


C
Breast cancer
Nuclear β-catenin-positive cells (%)
50
40
30
20
10
0
BC15
BC25
BC26
BC27
BC31
Code of patient


D
Ovarian cancer
Nuclear β-catenin-positive cells (%)
60
40
20
0
OC15
OC23
Code of patient


E
Endometrial cancer
Nuclear β-catenin-positive cells (%)
100
80
60
40
20
0
EC8
EC13
EC14
EC15
EC19
EC26
EC31
EC33
Code of patient

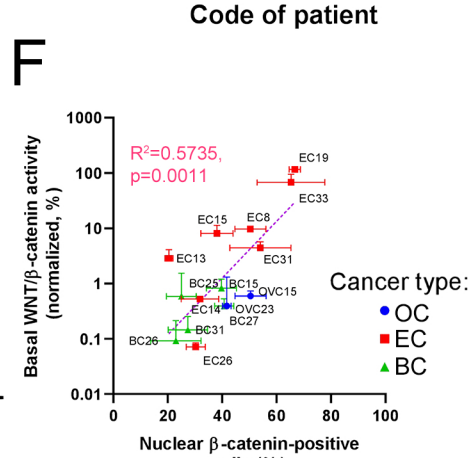

F
R²=0.5735,
p=0.0011
Basal WNT/β-catenin activity (normalized, %)
1000
100
10
1
0.1
0.01
EC19
EC33
EC15
EC8
EC13
EC31
BC25
BC15
QVC15
EC14
OVC23
BC27
BC31
BC26
EC26
0
20
40
60
80
100
Nuclear β-catenin-positive cells (%)
Cancer type:
OC
EC
BC

Figure 2

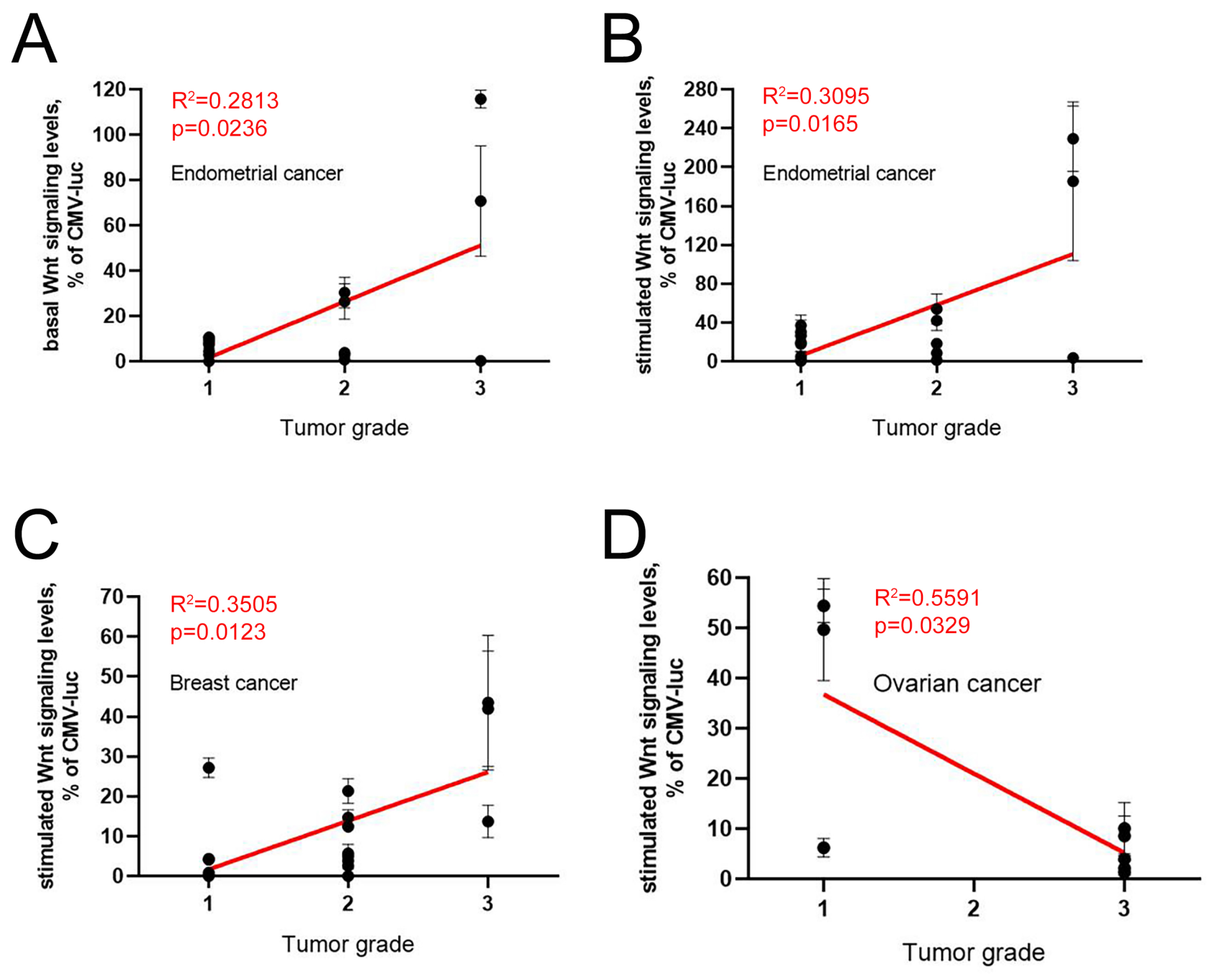

A
basal Wnt signaling levels, % of CMV-luc
120
100
80
60
40
20
0
R²=0.2813
p=0.0236
Endometrial cancer
1
2
3
Tumor grade
B
stimulated Wnt signaling levels, % of CMV-luc
280
240
200
160
120
80
40
0
R²=0.3095
p=0.0165
Endometrial cancer
1
2
3
Tumor grade
C
stimulated Wnt signaling levels, % of CMV-luc
70
60
50
40
30
20
10
0
R²=0.3505
p=0.0123
Breast cancer
1
2
3
Tumor grade
D
stimulated Wnt signaling levels, % of CMV-luc
60
50
40
30
20
10
0
R²=0.5591
p=0.0329
Ovarian cancer
1
2
3
Tumor grade

Figure 3

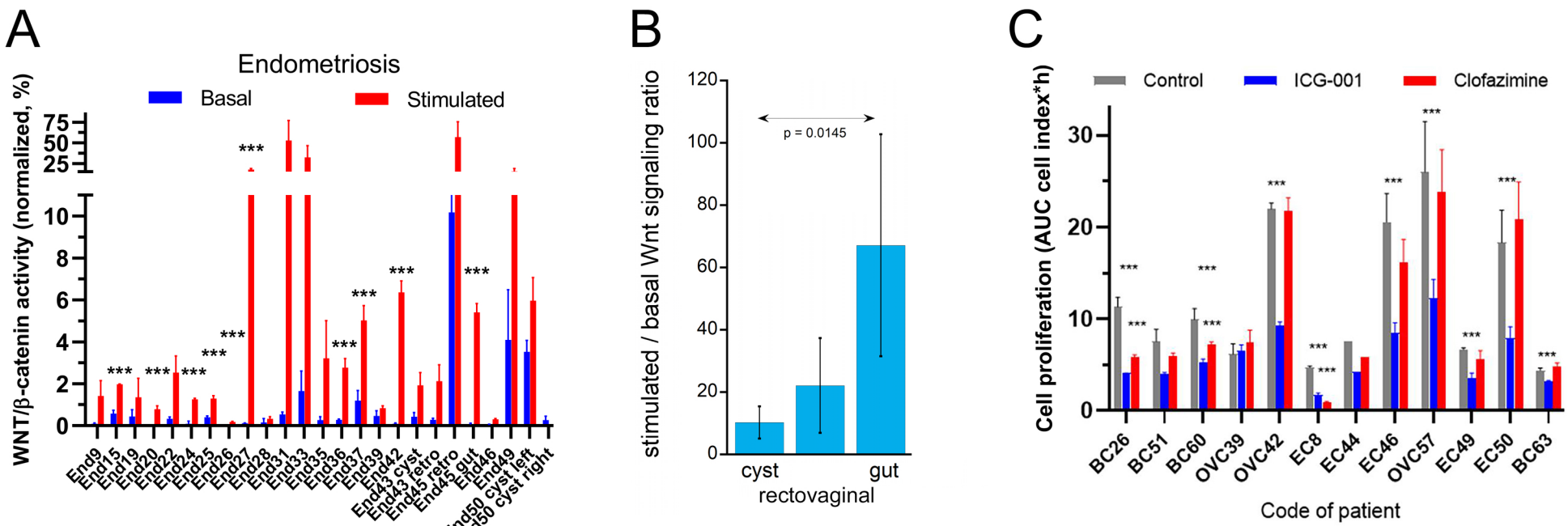

A
Endometriosis
Basal
Stimulated
WNT/β-catenin activity (normalized, %)
End9
End15
End19
End20
End22
End24
End25
End26
End27
End28
End31
End33
End35
End36
End37
End39
End42
End43 cyst
End43 retro
End45 retro
End45 gut
End46
End49
End50 cyst left
End50 cyst right
B
stimulated / basal Wnt signaling ratio
p = 0.0145
cyst
gut
rectovaginal
C
Control
ICG-001
Clofazimine
Cell proliferation (AUC cell index*h)
BC26
BC51
BC60
OVC39
OVC42
EC8
EC44
EC46
OVC57
EC49
EC50
BC63
Code of patient

Figure 4

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFig.S1.pdf
- SupplementaryFig.S2May11.pdf
- SupplementaryTableS1.docx
- SupplementaryTableS2.docx
- SupplementaryTableS3.docx
- SupplementaryTableS4Sept2.docx