# INVESTIGATING THE ANTI-TUMOR EFFECTS OF TWO NOVEL β-CATENIN INHIBITORS IN OVARIAN CANCER CELLS

VU HONG LOAN NGUYEN

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## Abstract

Epithelial ovarian cancer (EOC) is the deadliest female malignancy. The development of chemoresistance and relapse becomes a major challenge in treating ovarian cancer. The Wnt/β-catenin pathway, which plays a critical role in developmental and physiological processes, have been indicated to contribute to EOC development. Aberrant activation of this pathway was reported to promote cancer stem cell self-renewal, metastasis, and chemoresistance in all subtypes of EOC, suggesting the Wnt/β-catenin pathway is a strong candidate for the development of targeted therapies. In this study, we have identified and investigated the anti-tumor effects of two novel β-catenin inhibitors, referred to as compound 2 (C2) and C10. Results from functional assays showed that C2 and C10 inhibited EOC cell proliferation, anchorage-independent growth, and spheroid formation. In addition, C10 treatment inhibited the expression of stem cell markers of EOC cells, suggesting the small molecule inhibitors target ovarian cancer stem cells. Using TOPflash reporter assays, we found that C2 and C10 inhibited the transcriptional activity of β-catenin/TCF complex. Finally, preliminary results suggest that C2 and C10 bind directly to recombinant β-catenin. Taken together, this study identified two novel potent β-catenin inhibitors that have strong anti-tumor effects. These compounds could be potentially developed into targeted therapies for ovarian cancer patients who harbor abnormal activation of Wnt/β-catenin signaling.

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**Chapter 1**



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## List of Abbreviations

- ABCG2: ATP-binding cassette sub-family G member 2
- ALDH: Aldehyde dehydrogenase
- APC: Adenomatous polyposis coli
- ATP: Adenosine triphosphate
- BCL9: B-Cell Lymphoma 9 Protein
- bFGF: Basic fibroblast growth factors
- BMI1: Polycomb complex protein BMI-1
- BMP-10: Bone morphogenetic protein-10
- BRAF: B-Raf Proto-Oncogene
- BSA: Bovine serum albumin
- C2: Compound 2
- C10: Compound 10
- C-kit: Mast/stem cell growth factor receptor Kit
- CBP: Creb-binding protein
- CCNG2: Cyclin G2
- CCNY: Cyclin Y
- CD: cluster of differentiation
- CDK: cyclin-dependent kinase
- circRNA: circular RNA
- CK1: Casein kinase 1
- CSC: Cancer stem cell
- CTNNB1: β-catenin
- CUL4A: Cullin-4a
- DACT1: Dishevelled binding agonist of β-catenin 1 or Dapper 1
- DKK: Dickkopf-related protein
- DMSO: Dimethyl sulfoxide
- Dvl: Disheveled
- ECM: extracellular matrix
- EGF: Epidermal growth factor
- EMT: Epithelial-to-Mesenchymal Transition
- RNF43: RING-Type E3 Ubiquitin ligase
- EOC: Epithelial ovarian cancer
- EPCAM: Epithelial cell adhesion molecule
- ERBB2: Receptor tyrosine-protein kinase erbB-2
- FHL2: Four and half LIM domain protein 2
- FILIP1L: Filamin A interacting protein 1-like
- FN: Fibronectin
- FOXA2: Forkhead box protein A2
- FOXM1: Forkhead box protein M1
- FOXO3: Forkhead box protein O3
- FTE: Fallopian tube epithelium

- Fzd: Frizzled receptor
- GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
- GSK3β: Glycogen synthase kinase 3β
- hCTR1: human copper transporter 1
- ICAT: β-catenin interacting protein 1
- IDO: indoleamine 2, 3-dioxygenase
- IL: interleukin
- Jak: Janus kinase
- JRK: Jerky protein homolog
- KIAA0101: PCNA-associated factor
- LGR: Leucine-rich repeat-containing G protein-coupled receptor
- LPA: lysophosphatidic acid
- LPAR: lysophosphatidic acid receptors
- LRP: low-density lipoprotein receptor-related protein miRNA: micro-RNA
- MARCH7: Membrane-associated ring finger protein 7
- MMP: Matrix metalloproteinase
- NANOG: Homeobox protein NANOG
- OC: Ovarian cancer
- OCT: Octamer-binding protein
- OSE: Ovarian surface epithelium
- PARP: Poly (ADP-ribose) polymerase
- PC: Pyruvate carboxylase
- PD-L1: Programmed cell-death ligand
- PI3K: phosphoinositide 3-kinase
- PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
- PP2A: Protein phosphatase 2
- PPP2R1A: Protein Phosphatase 2 Scaffold Subunit Alpha
- PORCN: Porcupine O-Acyltransferase
- PTEN: Phosphatase and tensin homolog
- PVDF: Polyvinylidene difluoride
- Pygo: Pygopus
- Rab14: Ras-related protein Rab-14
- RIPA: Radioimmunoprecipitation assay
- SCF-βTrCP: multiprotein E3 ubiquitin ligase complex
- sE-cadherin: Soluble E-cadherin
- SFRP: Secreted Frizzled-Related Protein
- siRNA: small interfering RNA
- SP1: Specificity protein 1
- STICs: serous tubal intraepithelial carcinomas
- TCF/LEF: T cell factor/lymphoid enhancer factor
- TCGA: The Cancer Genome Atlas
- TET: Ten-eleven translocation methylcytosine dioxygenase
- TG2: Tissue transglutaminase 2
- TGFβ: Transforming growth factor beta

- Th17: T helper 17 cell
- TNKS: Tankyrase
- Treg: regulatory T cells
- USP25: Ubiquitin carboxyl-terminal hydrolase 25
- VEGF: vascular endothelial growth factor

# CHAPTER 1

# LITERATURE REVIEW

## 1. Ovarian cancer

Ovarian cancer is ranked as the fifth leading cause of death due to cancer in females (1). The term “ovarian cancer” is generally used to describe any cancer relating to the ovary (2). Based on the origin where ovarian tumors arise, ovarian cancer is classified into three categories: epithelial, germ cells, and stromal ovarian cancer. Among them, epithelial ovarian cancer (EOC) is accounted for more than 85% of ovarian cancer cases, and responsible for most ovarian-related deaths (2). The lack of specific disease-related symptoms and effective prognosis markers together with the lack of effective screening methods at the early stages contribute to the high morbidity and mortality of EOC.

### 1.1. Classification of ovarian cancer

#### 1.1.1. Histological subtypes

EOC is heterogeneous cancer which is further subcategorized into numerous subtypes based on the morphology and molecular pathology. This includes high-grade serous carcinomas (HGSC) which accounts for 70%-74% of EOC cases, endometrioid carcinomas (EC) (7-24%), clear cell carcinomas (CCC) (10%- 26%), low-grade serous carcinomas (LGSC) (3%-5%), and mucinous carcinomas (CC) (2%-6%) (2). Accumulating evidence suggests that ovarian carcinomas might not strictly arise from inside the ovaries (Fig. 1) (2). It could start from sites outside and spread to the ovaries. However, the origin of the subtypes of ovarian carcinomas is still under investigation. For many years, EOC tumors were believed to originated from ovarian surface epithelium (OSE) due to its dominant mass in the ovaries (2). However, the assumption is still controversial. Gene expression profiling revealed that HGSCs are closely related to fallopian tube epithelium (FTE) (3, 4). In addition, serous tubal intraepithelial carcinomas (STICs) were suggested to be the precursor

lesion of HGSCs. STICs were detected in the fimbria region at the end of the fallopian tube which is close to the ovary (5) and was found in 50-70% of women with HGSC (6-8). HGSC is not the only EOC subtype which was suggested to originate from tissues outside of the ovary. The high correlation between endometriosis and ECs and CCCs was indicated in several studies (9-13). Molecular genetic studies have shown that endometriotic cysts could transition into ECs or CCCs (14-16). The origin of LGSCs and MCs remains unclear. They were found to be frequently associated with borderline tumors (2).

Genetic instability is one of the characteristics of cancer which contribute to its pathology. Genetic alterations and molecular defects of different subtypes of EOC have been studied extensively (2). Some tumors were developed from germline mutations, but most tumors were initiated through several stages from acquired mutations. Many of the mutations are located in the gene loci which encode for tumor suppressors and oncogenes (Table 1) (2). These molecular abnormalities are strong risk factors for ovarian cancer and have contributed to the determination of treatment options. Mutations in the *TP53*, *BRCA1*, and *BRCA2* tumor suppressor genes are frequently detected in HGSCs (2). In addition, several genes are mutated in ECs, such as *CTNNB1*, *PIK3CA*, *KRAS*, *ARID1A*, *PTEN*, and *PPP2R1A* (17). Mutations of *CTNNB1* are commonly observed in EC cases but are rare in all other subtypes EOC. In contrast, *PIK3CA* and *ARID1A* are frequently mutated in both EC and CCC (18,19). Besides, CCCs have a low frequency of mutations in *PPP2R1A*, *PTEN*, *KRAS*, and *TP53* (20-22). *KRAS* and *TP53* mutations were found in 50% of invasive MCs (23) while amplification of *ERBB2* was found in 19% of MCs (24). For the most part, LGSCs and HGSCs have non-overlapping mutational profiling. *KRAS* and *BRAF* are characteristically mutated in LGSCs (25,26).

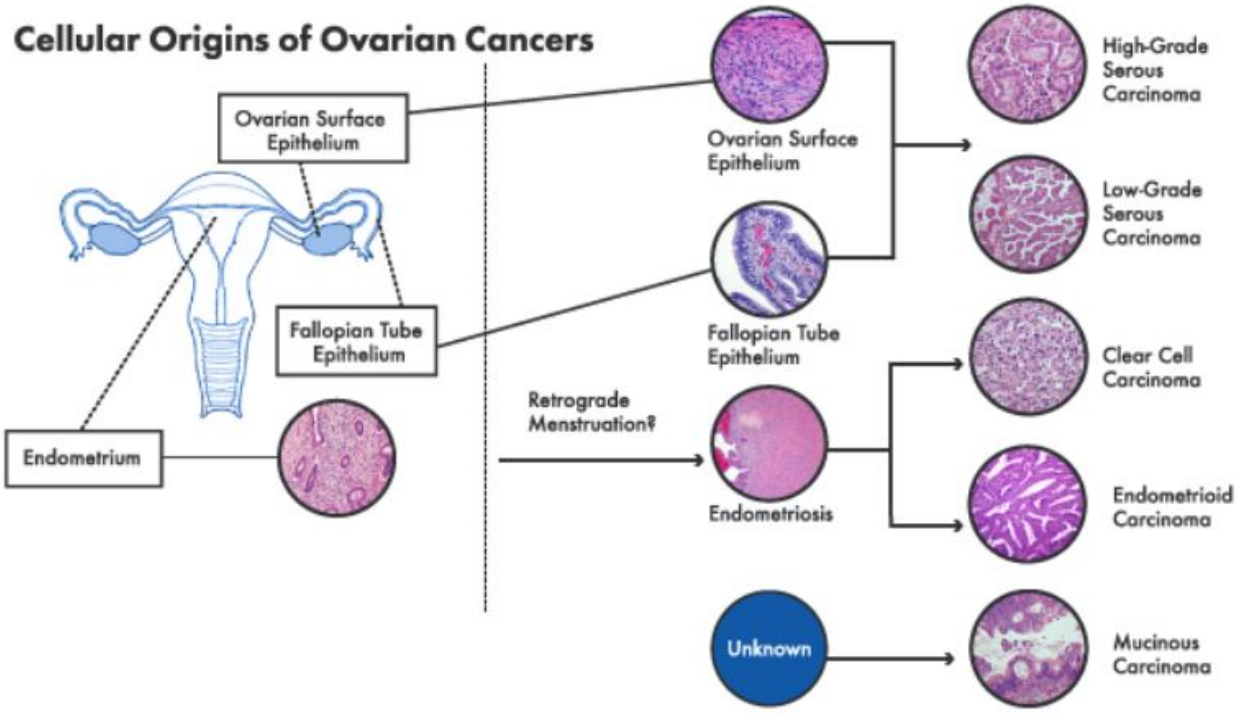



**Figure 1: Potential origins of ovarian carcinomas**. High-grade serous and low-grade serous carcinoma were suggested to originate from OSE or FTE while clear cell and endometrioid carcinoma were suggested to arise from endometriosis. The origin of mucinous carcinoma remains unclear. Figure obtained with permission from (2).

| Table 1: Characterizations of ovarian carcinoma subtypes | | | | | |
|---|---|---|---|---|---|
| **Carcinoma subtype** | High-grade serous | Low-grade serous | Endometrioid | Clear cell | Mucinous |
| **Precursor lesion** | STICs | Serous borderline tumor | Endometriosis<br>Uterine EC | Endometriosis | Borderline tumor |
| **Gene activating mutations** | ND | BRAF, KRAS | CTNNB1, KRAS, PIK3CA, PPP2R1A | KRAS, PIK3CA, PPP2R1A | BRAF, KRAS, ERBB2 |
| **Gene inactivating mutations** | BRCA1, BRCA2, TP53 | ND | ARID1A, BRCA1, BRCA2, PTEN | ARID1A, PTEN, TP53 | CDKN2A, RNF43, TP53 |

*ND: not determined

### 1.1.2. Stage and grade

Ovarian cancer staging is essential for the characterizations and determination of treatment options (2). The stage of EOC is determined by the levels of dissemination of the tumors in the body, as described in table 2. Stage I of EOC is defined when a tumor is limited in the ovary (2). Stage II involves the extension of the tumors into the pelvic area such as the uterus and fallopian tube. Local metastasis to small bowel or omentum is a confined characteristic in stage III, while stage IV is characterized by distant metastasis (2).

EOC is also classified by grades which are based on the degree of differentiation of malignant cells. Grade 1 of EOC is confined as well-differentiated while grade 3 and 4 are poorly differentiated or undifferentiated (27). Recently, the two-tiered system has been introduced for EOC grading as low or high-grade (28). Low-grade usually means the ovarian cancer cells are

well-differentiated and less likely to spread, resembling normal ovarian cells while high-grade cancer cells are poorly differentiated and more likely to spread.

| **Table 2: FIGO stage of ovarian cancer (2)** | |
|---|---|
| Stage I | Tumor confined to one or both ovaries or fallopian tubes<br>IA. Tumor limited to one ovary (capsule intact) or fallopian tube<br>IB. Tumor limited to both ovaries or fallopian tubes<br>IC. Tumor limited to both ovaries or fallopian tubes with any of the following:<br>IC1. Surgical spill intraoperatively<br>IC2. Capsule ruptured before surgery or tumor on ovarian or fallopian tube surface<br>IC3. Malignant cells present in the ascites or peritoneal washings |
| Stage II | Tumor involves one or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or peritoneal cancer<br>IIA. Extension and/or implants on any one or more of the following: uterus, fallopian tube(s), ovaries<br>IIB. Extension to other pelvic intraperitoneal (IP) tissues |
| Stage III | Tumor involves one or both ovaries or fallopian tubes, or primary peritoneal cancer, with cytologically and histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes<br>IIIA. Metastasis to the retroperitoneal lymph nodes with or without microscopic peritoneal involvement beyond the pelvis<br>IIIA1. Positive retroperitoneal lymph nodes only (cytologically or histologically proven)<br>IIIA1(i). Metastasis ≤ 10 mm in greatest dimension (note that this is a tumor dimension and not a lymph node dimension)<br>IIIA1(ii). Metastasis > 10 mm in greatest dimension |

| | |
|---|---|
| | IIIA2. Microscopic extrapelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes<br>IIIB. Macroscopic peritoneal metastases beyond the pelvic brim ≤ 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes<br>IIIC. Macroscopic peritoneal metastases beyond the pelvic brim >2 cm in greatest dimension, with or without metastases to the retroperitoneal nodes |
| Stage IV | Distant metastasis excluding peritoneal metastases<br>IVA. Pleural effusion with positive cytology<br>IVB. Metastases to extra-abdominal organs |

**1.2. Treatments**

The standard chemotherapy for EOC patients is a combination of a platinum product, such as cisplatin or carboplatin, with a taxane, such as paclitaxel or docetaxel (2). However, many patients develop resistance to these therapies and relapse (29,30). Insight into molecular profiling of histological subtypes has led to the development of targeted therapies as third cytotoxic agents beside platinum-taxane treatments. The new targeted therapies focus on targeting pathways which are responsible for survival and apoptosis, proliferation, migration and invasion, angiogenesis, immunomodulation, epigenetics, DNA repair, and stem cell maintenance. Recent research on target therapies has introduced several therapeutic agents that target specific cancer-driven factors to inhibit ovarian cancer development. For example, bevacizumab, an antibody against vascular endothelial growth factor (VEGF)-A, has been approved by the FDA to be used in combination with carboplatin and paclitaxel (31). Moreover, several Poly(ADP-

Ribose) Polymerase (PARP) inhibitors, which target the defective DNA repair pathway in HGSC, have been approved for the treatment of recurrent, BRCA-associated EOC (32).

## 2. Wnt/β-catenin signaling

### 2.1. Overview of the Wnt/β-catenin signaling pathway

Wnt-off: inactivation and degradation of β-catenin

β-catenin is the key mediator of the canonical Wnt pathway, which plays vital roles in directing cell fates and tissue homeostasis in embryonic and adult tissues (33). In the absence of a Wnt ligand, β-catenin is degraded by a destruction complex. The core components of the complex include Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1), glycogen synthase kinase β (GSK3β) proteins and the E3 ligase, bTrCP (Fig. 2A). Protein phosphatase 2A (PP2A) is also associated with the β-catenin destruction complex. Axin is a scaffolding protein that has interaction sites for multiple proteins including PP2A, APC, GSK3β, and CK1 (34). Therefore, the presence of Axin is essential for the assembly of the destruction complex. β-catenin is first phosphorylated by CK1 at the residue S45 and then by GSK3β at S33, S37, and T41 (35,36). GSK3β also phosphorylates Axin, stabilizing it, and enhancing its interaction with β-catenin (37,38). APC, another core member of the destruction complex, contains multiple regions for Axin and β-catenin interaction, enhancing the rate of β-catenin phosphorylation (34). Finally, phosphorylated β-catenin is transferred to βTrCP, which forms a complex with Skp1 and Cullin to facilitate ubiquitylation and degradation of β-catenin (39).

Wnt-on: activation of β-catenin

β-catenin signaling is activated in the presence of Wnt ligands. On the cell surface, the binding of a Wnt ligand induces the heterogeneous dimerization of Frizzled (Fzd) and LRP5/6 receptors, leading to their conformation change (33). Disheveled (Dvl) is then recruited to the membrane through its interaction with the cytoplasmic domain of Fzd (40). Here, Dvl assists in recruiting the scaffold protein of the destruction complex Axin to the membrane. The association between the destruction complex and the membrane is further strengthened following phosphorylation of the cytoplasmic domain of LRP5/6 by kinases such as CDK14, and GSK3β (41). Consequently, the activities of the destruction complex in promoting β-catenin phosphorylation and degradation are inhibited. Unphosphorylated cytoplasmic β-catenin can, therefore, accumulate and translocate to the nucleus. Since β-catenin does not have DNA-binding domain, β-catenin activates transcription through the association with TCF/LEF members, histone modifiers such as CREB-binding proteins (CBP) and other transcription factors (41). Once inside the nucleus, β-catenin displaces the transcriptional repressor, Groucho, which, in the absence of Wnt stimulation, binds the T cell factor/lymphoid enhancer factor (TCF/LEF) (42). The active transcriptional complex β-catenin/TCF can then initiate the transcription of its target genes (42) (Fig. 2B).

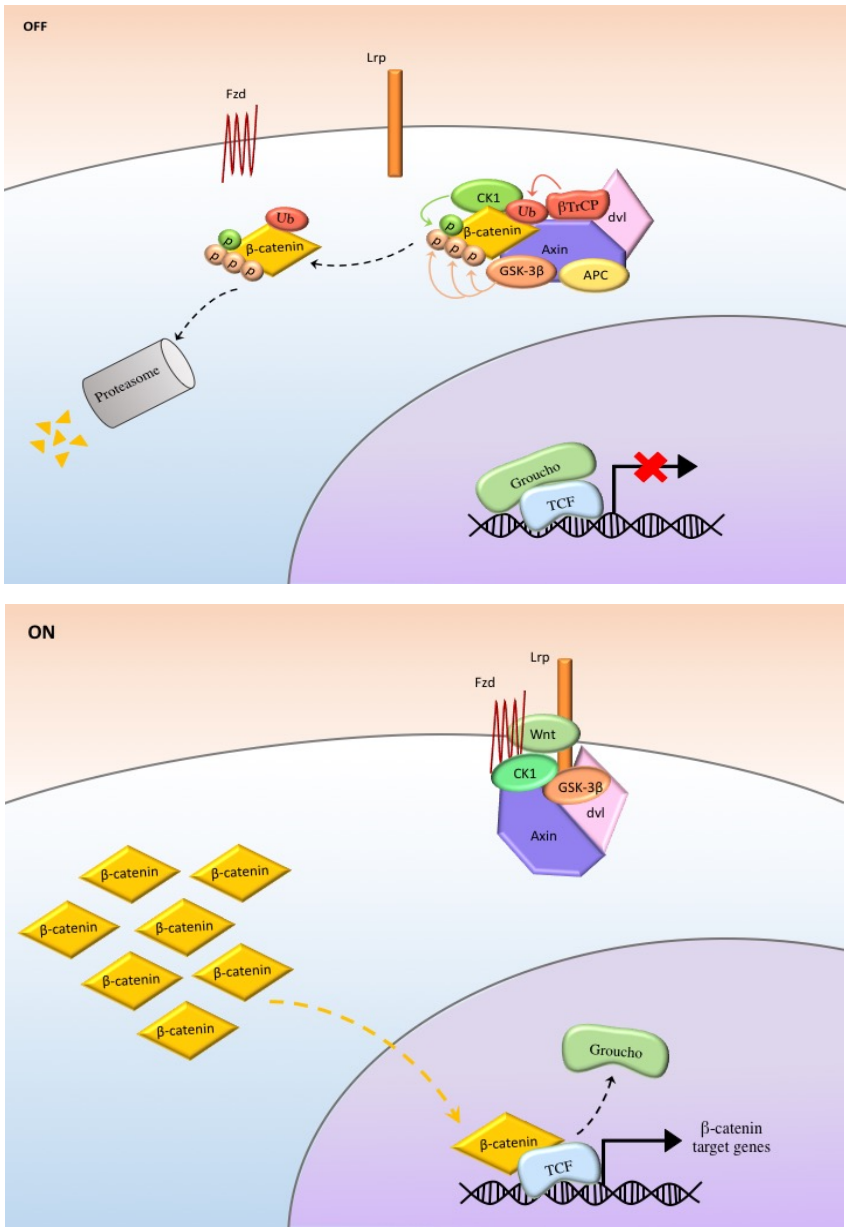



**Figure 2: The Wnt/β-catenin signaling pathway.** A) Wnt signaling OFF. The absence of Wnt ligand binding to the Fzd receptor prevents the interaction between Fzd and LRP5/6. The destruction complex which resides in the cytoplasm binds to and promotes degradation of free cytoplasmic β-catenin. Specifically, CK1 and GSK-3β phosphorylate β-catenin, targeting it for βTrCP-mediated ubiquitination and subsequent proteasome degradation. Within the nucleus, the transcriptional repressor, Groucho binds to TCF and inhibits its transcriptional activity. B) Wnt signaling ON. Binding of the Wnt ligand to Fzd and LRP5/6 promotes the recruitment of Dvl and the destruction complex to the membrane. As a result, the destruction complex's ability to phosphorylate and degrade cytoplasmic β-catenin is inhibited. β-catenin is accumulated and translocated into the nucleus where it displaces Groucho and binds to TCF. Together with co-activators, the transcription of downstream target genes is initiated.

### 2.2. Genetic alteration of the Wnt/β-catenin pathway in ovarian carcinoma

#### 2.2.1. β-Catenin

The most common genetic alteration in the Wnt/β-catenin pathway involved in EOC is in the β-catenin gene, *CTNNB1* (43). Mutations in this gene often result in increased nuclear localization of β-catenin and, subsequently, an increase in transcription of its target genes, including c-myc, and cyclin D1 (44). This is most commonly observed in the EC subtypes, as one study found that activating mutations in *CTNNB1* accounted for up to 54% of the EC cases (43). The same study showed that, in ECs that carried a missense mutation in *CTNNB1*, the mutation was always found within the amino-terminal domain (43). Phosphorylation of this domain by GSK3β is required for degradation of β-catenin; therefore, mutations within this domain would render β-catenin resistant to degradation. Indeed, mutations within the GSK3β phosphorylation domain were positively correlated with the nuclear localization of β-catenin and the level of β-catenin/TCF target genes (43).

#### 2.2.2. Destruction complex

Mutations in several components of the destruction complex, such as Axin, GSK3β and APC, have been reported in EOC. Since these proteins are important for the degradation of β-catenin, genetic alterations that render them less effective or non-functional are likely candidates for driving hyperactive β-catenin signaling and, as a result, oncogenesis.

Although much less common than mutations in *CTNNB1*, mutations in the genes encoding Axin and APC proteins (*AXIN1/2* and *APC*, respectively) have also been reported in EOC (43,44). Axin protein exists in two isoforms: Axin1 and Axin2. A nonsense mutation in *AXIN1* has been

found in one case of EC, while a frameshift mutation in *AXIN2* resulting in truncation has been found in another (43). This finding supports its role as a negative regulator of β-catenin signaling.

Genetic alterations in APC, while frequent in colon cancers, are rarely found in EOC (45,46). There is some contradictory evidence concerning the involvement of *APC* mutations in EOC. For instance, it was reported that the I1307K missense mutation in the *APC* gene conferred a modest increase in the risk of hereditary and sporadic breast/ovarian cancer development through its association with BRCA1/2 mutations. Later analysis, however, concluded that, although there exists a high prevalence of I1307K mutations amongst BRCA1/2 carriers, the I1307K allele confers no additional risk for cancer development (47). In addition, two missense mutations (K90N, S1400L) and one nonsense mutation (R1114) within the *APC* gene were identified in a MC tumor of ovarian cancer (48). While the exact contributions made by these mutations were not examined in this study, they were determined to be likely pathogenic. More research is needed to determine the mechanism underlying *APC* mutations and the frequency at which these mutations occur in EOC.

**2.3. Dysregulation of Wnt/β-catenin signaling in ovarian carcinoma**

Although mutations in *CTNNB1* and components of the β-catenin destruction complex are rare and restricted to only the EC and MC subtypes, higher β-catenin activity is often observed in EOC, especially in HGSC. The mechanisms underlying the hyperactivation of the Wnt/β-catenin pathway in EOC are not entirely clear. However, many studies have reported the aberrant expression or activation of the components and regulators of this pathway. It is therefore highly possible that alterations in these proteins contribute to the higher activity of the Wnt/β-catenin in EOC (Fig. 3).

### 2.3.1 Ligands and receptors

Several Wnt ligands have been reported to be upregulated in EOC and associated with poor prognosis in EOC patients. For example, using immunohistochemistry, Wnt-5A expression was found to be strongly upregulated in EOC tumors when compared with benign epithelial neoplasia and normal ovarian samples and was negatively correlated with patient survival (49). This study also found higher Wnt-1 immunoreactivity in EOC tumors but no significant association between Wnt-1 expression and patient survival (49). Another study, which measured mRNA levels of all Wnt ligands in ovarian tumors revealed that Wnt-7A and Wnt-7B were highly expressed, while Wnt-3 and Wnt-4 were reduced, in malignant ovarian tissues compared with normal ovarian tissues (50). Subsequent analyses of Wnt-7A by *in situ* hybridization confirmed that the up-regulation occurred more frequently in serous carcinoma than in EC, MC, and CCC tumors (50). Overexpression of Wnt-7A has also been identified in EC when compared to normal endometrium and benign endometrial lesions and the immunoreactivity of Wnt7A in tumors was found to be negatively correlated with both overall and disease-free survival (51). *In vitro* functional analysis showed that downregulation of Wnt-7A reduced ovarian cell proliferation, adhesion, invasion and expression of β-catenin target genes, suggesting its crucial role in the activation of Wnt signaling and malignant transformation in ovarian cancer (51). The significance of Wnt-5A, Wnt-1 and Wnt-7B upregulation and Wnt-3 and Wnt-4 downregulation in EOC remain to be determined. Similarly, the causes of aberrant Wnt expression in EOC are not clear at present and require further investigation.

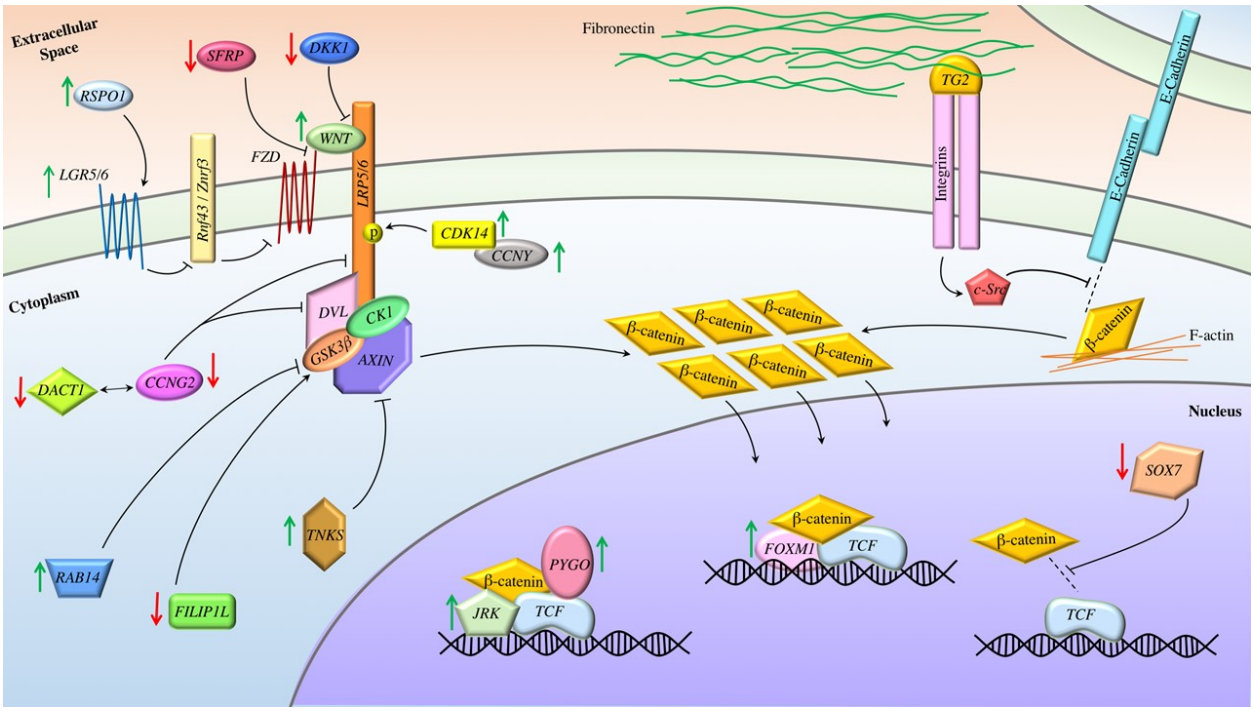



**Figure 3: Proposed mechanisms of Wnt/β-catenin dysregulation in ovarian cancer**. The Wnt/β-catenin pathway is regulated by many factors, whose aberrant expression leads to the hyperactivation of β-cat in the EOC. Note that green arrows indicate proteins whose expression is upregulated in EOC, while red arrows indicate downregulation. DKK1 and SFRP2, which inhibit the dimerization of Fzd and LRP5/6 and directly prevent Fzd activation, respectively, are downregulated in EOC tumors. In contrast, Wnt ligands activate the pathway by forming a receptor complex with Fzd and LRP5/6, while R-spondins bind LGRs and prevent the sequestration of the Fzd. Both Wnt ligands and LGRs are overexpressed EOC. CCNY and CDK14 are also upregulated in EOC and have been suggested to collaborate to promote LRP5/6 phosphorylation and its subsequent activation. CCNG2, which is reported to be downregulated in EOC, decreases LPR6 levels. It may also interact with DACT1 to promote DVL degradation. At the destruction complex, TNKS destabilizes AXIN to increase β-catenin activity and TNKS1 is known to be up-regulated in EOC. RAB14 inhibits the activity of GSK-3β, and its upregulation contributes to higher β-catenin activity in EOC. FLIP1L, whose expression is negatively correlated with EOC progression, enhances GSK-3β activation. This inhibition of the destruction complex results in the accumulation of β-catenin within the cytosol and its translocation into the nucleus. In addition, TG2, which is overexpressed in EOC, binds to integrin and fibronectin. This results in the recruitment of c-Src and subsequent disruption of E-cadherin/β-catenin complex on the membrane and the accumulation of β-catenin within the cytoplasm. Finally, within the nucleus, higher expression of several co-activators of β-catenin/TCF, such as PYGO, JRK, and FOXM1, and lower expression of SOX7, which was reported to inhibit the interaction between β-catenin and TCF, leads to higher transcriptional activity of this complex.

A recent study revealed that abnormal expression of R-spondin 1 also contributes to the dysregulation of the Wnt/β-catenin signaling pathway at the receptor level (52). R-spondin 1 (encoded by *RSPO1*) belongs to the secreted R-spondin protein family, which bind to the LGR4, LGR5, or LGR6 receptors (53). This binding inhibits the sequestration of Fzd by the transmembrane E3 ligases, Rnf43 and Znrf3 (53), thereby enhancing β-catenin activity (54). A genome-wide association study identified SNPs at *RSPO1* as an EOC susceptibility locus (55). RSPO1 was upregulated in all EOC cell lines and in a small number of tissue samples when compared to normal ovarian tissue samples (38). In HSGC subtype ovarian tumors, amplification of RSPO1, RSPO2, LGR5 and LGR6 expression has also been observed; however, this upregulation was observed at low frequency (56). Moreover, overexpression of RSPO1 has been observed to increase EOC cell proliferation, migration, and chemotherapy resistance (52). Furthermore, overexpression of RSPO1 enhanced, whereas deletion of RSPO1 attenuated β-catenin activity (52). Similarly, silencing of LGR6 inhibited β-catenin activity (57). These findings strongly support the role of RSPO1/LGR in enhancing Wnt/β-catenin signaling and suggest that their upregulation during EOC development contributes to the hyperactive β-catenin signaling.

Wnt/β-catenin signaling is tightly controlled by several negative regulators, some of which inhibit activation of Wnt signaling by competing with Wnt ligands for their receptors. Abnormal levels of these regulatory proteins have been associated with the dysregulation of Wnt/β-catenin signaling activity and EOC. For example, Dickkopf (DKK) inhibits Wnt signaling by binding to LRP5/6 and disrupting the dimerization of Fzd and LRP5/6 (58). Interestingly, DKK1 was reported to be downregulated in EOC cells and negatively correlated with the stage of tumor development (59). The expression of DKK2 was significantly lower in EOC tumors than in normal ovary (60).

Secreted Frizzled-related proteins (SFRPs), similarly, interact with Wnt ligands and FZD receptors to abrogate their activations (58). SFRP4 expression has been observed to be significantly downregulated in EOC cell lines and to be negatively correlated with the aggressiveness of EOC tumors and patient survival (61,62). In OVCAR-3, a cell line with no detectable levels of SFRP4 (61), treatment with SFRP4 remarkably inhibited β-catenin activity (63), indicating the important role of SFRP4 in suppressing β-catenin activity.

Downregulation of DKK2 in EOC tumors is believed to be a result of epigenetic silencing. Compared to benign tumors and normal ovarian tissues, DKK2 in EOC tumors was more commonly methylated, and its methylation was increased in higher grades and stages of EOC (60). Similarly, decreases in DKK1 and SFRP2 levels in EOC has been suggested to be induced by DNA methylation (64). TET1, a member of ten-eleven translocation (TET) family, was reported to promote the activation of DKK1 and SFRP2 expression in EOC (64). TET1 plays an essential role in DNA demethylation by catalytically converting 5-methylcytosine to hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine (65). Hypomethylation of the DKK1 and SFRP2 promoters was observed in EOC cells with ectopic TET1 expression (64). Therefore, it is proposed that TET1 may bind to the CpG islands at the promoter regions of DKK1 and SFRP2, reducing the methylation levels and stimulating their expression by the transcriptional machinery. This is further supported by the increase in DKK1 and SFRP2 levels observed in EOC cells with induced expression of TET1 and by undetectable levels of TET1 expression in EOC cell lines including SKOV3, OVCAR-3, and OVSAHO (64).

Cyclin G2 (CCNG2) is an unconventional cyclin which has been shown to inhibit cell proliferation, migration and invasion in EOC cells (66,67). CCNG2 was downregulated in EOC

tissues compared to tumors with low malignant potential or normal ovarian tissues (67). The inhibitory effects of cyclin G2 on EOC cell proliferation and invasion are mediated, at least in part, by the inhibition of β-catenin. Specifically, CCNG2 has been found to reduce LRP6, DVL2, and β-catenin levels in EOC (67). While the mechanism by which CCNG2 inhibits LPR6 and Dvl2 in EOC is not known, a recent report in gastric cancer indicated that CCNG2 downregulated DVL2 through the interaction with Dapper1 (DACT1) (68), a Wnt signaling antagonist that has been shown to promote DVL2 degradation (69). Gao *et al.* showed that there was a direct interaction of CCNG2 and DACT1 via co-immunoprecipitation analysis, and that overexpression of DACT1 decreased DVL2 and β-catenin levels in gastric cancer cells (68). While unphosphorylated DACT1 inhibited Wnt/β-catenin pathway, its phosphorylated form promoted Wnt/β-catenin signaling (70). Remarkably, overexpression of CCNG2 inhibited phosphorylation of DACT1 by CK1, suggesting CCNG2 exhibits the inhibitory effects on canonical Wnt signaling by suppressing DACT1 phosphorylation and promoting DVL2 degradation by unphosphorylated DACT1 (68). Recently, it was reported that DACT1 was downregulated in EOC samples derived from LGSC, EC, CC, and MC, when compared with ovarian tissues collected from patients with benign gynecological disorders (71). Thus, down-regulation of CCNG2 and DACT1 could contribute to the hyperactivation of the Wnt/β-catenin pathway. However, whether or not there is an interaction between CCNG2 and DACT1 in EOC requires further investigation.

Another cell cycle regulator, cyclin Y (CCNY), also regulates β-catenin signaling. CCNY has been found to be upregulated in EOC tissues and its expression to be positively correlated with the clinicopathological stage (72). The study found that overexpression of CCNY increased cell proliferation, migration and invasion, which was mediated by the Wnt/β-catenin pathway.

Ectopic expression of CCNY increased nuclear β-catenin levels and its transcriptional activity, leading to the upregulation of downstream target genes (72). A previous study indicated that cyclin Y and CDK14 could interact at the membrane level to modulate LRP6 activation through phosphorylation (73). Notably, the expression of CDK14 was also upregulated in clinical EOC samples and its expression was found to enhance the accumulation of nuclear β-catenin (74). Therefore, the upregulation of both cyclin Y and CDK14 in EOC may enhance their association and in turn, promote canonical Wnt signaling.

### 2.3.2. The β-catenin destruction complex

Components of the destruction complex play an important role in maintaining cytoplasmic β-catenin proteins at basal levels in the absence of Wnt ligands. Decreases in the expression of certain components of the destruction complex are frequently observed in EOC (75). For example, several studies have reported significantly higher methylation rates in the promoter region of APC in EOC tumors when compared to benign ovarian tumors or normal ovarian tissue samples (76–78). However, the mechanisms underlying the hypermethylation which regulates APC suppression in EOC are not clear.

Tankyrases (TNKS), which belong to the poly (ADP-ribose) polymerase (PARP) family, are positive regulators of Wnt/β-catenin signaling (79,80). TNKS catalyzes ADP-ribosylation of AXIN and destabilizes the protein. Upregulation of TNKS1 expression was observed in EOC tissues and the immunoreactivity of TNKS1 was positively correlated with tumor size and stage (81). Furthermore, inhibition or knockdown of TNKS1 resulted in inhibition of EOC cell proliferation, migration, invasion, and colony formation *in vitro* and tumor growth in nude mice, as well as

aerobic glycolysis. Further studies confirmed that TNKS1 exerts these effects by promoting Wnt/β-catenin signaling (81).

Inhibition of GSK3β has also been observed in EOC (82,83). Initially, it was reported that GSK3β was overexpressed in EOC and was positively regulated the proliferation of ovarian cancer cells (75,84). However, further analyses revealed that GSK3β is frequently phosphorylated and thereby inactivated in EOC (12). It was further postulated that this GSK3β phosphorylation might be linked to the high frequency of activating mutations in PI3K in ovarian cancers (85). The PI3K/AKT pathway is known to inhibit GSK3β activity through phosphorylation at S9 (86). This increase in PI3K results in higher levels of active AKT, which in turn inactivates GSK3β and thus downregulates β-catenin signaling. In addition, Filamin A interacting protein 1-like (FILIP1L), which was reported to be down-regulated in EOC and negatively correlated with EOC tumor stages, chemoresistance, and patient survival (83), has been found to induce β-catenin degradation (83,87). While the underlying mechanisms by which FILIP1L inhibits β-catenin has not been determined in EOC, knockdown of FILIP1L in colon cancer cell lines led to an increase in phosphorylated AKT and GSK-3β and a decrease in phosphorylated β-catenin levels, suggesting that FILIP1L may promote β-catenin degradation by inhibiting AKT and thereby increasing GSK3β activity (88). Finally, RAB14, a GTPase protein belonging to the RAS small G-protein superfamily (82,89), has also been reported to be upregulated in EOC tissues and cell lines (82). Overexpression of RAB14 increased GSK3β phosphorylation at residue S9 and enhanced β-catenin activity (82), suggesting that higher expression of RAB14 in EOC tumors contributes the hyperactivation of β-catenin by inhibiting GSK3β activity.

### 2.3.3. Regulation of β-catenin subcellular localization

β-catenin is a dynamic protein that can function as a component of adherens junctions or as a transcription factor depending on its subcellular localization. At the adherens junctions, β-catenin interacts with the cytoplasmic tail of E-cadherins and links E-cadherins to actin filaments through its interaction with α-catenin to maintain the dynamics of the cytoskeleton (90,91). Dissociation of the adherens junctions results in the accumulation of β-catenin in the cytoplasm and its nuclear translocation to promote transcription of target genes (92). The dissociation between β-catenin and E-cadherin is mediated by tyrosine phosphorylation at the C-terminal of β-catenin, decreasing its binding affinity to E-cadherin and α-catenin (93). In contrast, serine phosphorylation of E-catenin at its cytoplasmic tail increases the binding between E-cadherin and β-catenin (90), stabilizing the adherens junctions complex.

During EOC development, the membrane-associated β-catenin is dysregulated. Tissue transglutaminase 2 (TG2) has been shown to promote the dissociation of E-cadherin and β-catenin in EOC cells. TG2 was found to be overexpressed in EOC tumors and positively correlated with β-catenin levels in ovarian cancer cell lines (94,95). TG2 formed a complex with fibronectin (FN) and β1-integrin, enhancing the binding of FN to its cognate receptor, leading to the activation of c-Src. It has been proposed that, at the plasma membrane, activated c-Src could phosphorylate β-catenin at Y654, inhibiting the interaction between E-cadherin and β-catenin (80). Similarly, activation of lysophosphatidic acid receptors (LPAR) by its ligand, lysophosphatidic acid (LPA) also contributes to the aggregation and subsequent activation of β1-integrin in ovarian cancer cells (96). Immunofluorescence results revealed that E-cadherin was colocalized with β1 integrin clusters, suggesting the recruitment of E-catenin to the β1 clusters, which allows the

release of β-catenin from the junctional complex (96). This would lead to the accumulation of cytoplasmic β-catenin proteins and increase its nuclear activity.

### 2.3.4. Regulation of β-catenin in the nucleus

Several proteins are believed to modulate β-catenin activity within the nucleus. SOX7, a member of the Sox transcription factor family, was demonstrated to suppress Wnt signaling in ovarian cancer cells harboring either wildtype or mutant β-catenin (97). This was proposed to be accomplished through direct binding of SOX7 to β-catenin to disrupt its activity. Ectopic expression of SOX7 in TOV-112D cells significantly inhibited β-catenin transcriptional activity with downregulation of β-catenin/TCF target genes. Immunofluorescence and co-immunoprecipitation analysis indicated that SOX7 mainly localized in the nucleus where it interacted with β-catenin and TCF4 (97). Furthermore, SOX7 expression was found to be significantly reduced in EOC tumors and negatively correlated with tumor progression (98). An additional study that utilized bioinformatic analysis predicted that another member of the Sox family, SOX17, was involved in the development of ovarian cancer through its interaction with β-catenin and regulation of the Wnt pathway (99). However, this has not yet been proven experimentally.

Multiple proteins have been reported to form a complex with β-catenin and TCF/LEF and increase the stability of the transcriptional complex. Pygopus (PYGO), a co-activator, binds β-catenin directly in the nucleus and assists in transcription of its target genes (32). PYGO2 has been detected in the all histological subtypes of EOC tumors, and its expression was higher in EOC tissues than in benign ovarian tumors (100). Suppression of PYGO2 inhibited cell proliferation, colony formation, and tumor growth, suggesting that it may play an important role in ovarian

cancer progression (100). However, whether or not PYGO2 exerts these tumor-promoting effects by promoting β-catenin/TCF activity requires confirmation. In addition, JRK, which interacts directly with β-catenin through its N-terminal, stabilizes the transcriptional complex consisting of β-catenin, LEF1, and PYGO2 (101,102). Mining the TCGA database revealed copy-number gains in *JRK* and higher JRK mRNA levels in some serous tumors (102). Furthermore, knockdown or deletion of the N-terminal of JRK decreased the activity of β-catenin, downregulated β-catenin target genes, and inhibited cell proliferation (102). These findings suggest that JRK plays a role in EOC development by enhancing β-catenin activity.

FOXM1, a member of forkhead transcription factors, plays important roles in EOC development in part by regulating β-catenin signaling. High FOXM1 levels were found to be correlated with EOC tumor grade and stage, and to predict poor prognosis and chemoresistance (103–106). Interestingly, high FOXM1 immunoreactivity was found to be significantly associated with high β-catenin staining (107). FOXM1 has been shown to induce β-catenin transcription EOC cells (106,107). On the other hand, activation of Wnt/β-catenin signaling also increased mRNA and protein levels of FOXM1 (106). In addition, FOXM1 has also been reported to promote β-catenin nuclear translocation and to form a complex with β-catenin and TCF4 to induce target gene expression in glioma cells (108). This suggests that FOXM1 may also function as a co-activator of β-catenin to promote EOC development.

### 2.4. Role of Wnt/β-catenin in ovarian cancer development

It is well established that the Wnt/β-catenin pathway exerts tumor-promoting effects in EOC (85,109,110). This pathway has been shown to promote cell proliferation, survival, migration, and invasion, maintain cancer stem cells, induce resistance to therapeutic agents, and may also be involved in the tumor angiogenesis (111) and immune suppression (112).

#### 2.4.1. Stemness

It is now widely accepted that tumors are made up of a heterogeneous population of cancer cells, a small portion of which is characterized as cancer stem cells (CSCs) (113). Like normal stem cells, CSCs possess self-renewal and differentiation potential which contribute to the heterogeneity of cancer cell populations. CSCs have high tumorigenic potential and mediate the resistance to chemotherapy, driving tumor initiation, metastasis, and cancer recurrence (113). Multiple studies on intratumoral heterogeneity enable the identification of CSCs in EOC. Ovarian CSCs have been characterized by functional and phenotypic expression of surface markers such as CD24, CD44, CD117, ALDH, CD133, SOX2, NANOG, OCT4, and EPCAM (106,114,115). Ovarian tumor-isolated mesenchymal stem cells were identified to exhibit high levels of CD133 and ALDH expression (115). Additionally, the increase in stem cell marker expression in ovarian CSCs was detected together with the ability to form spheroids *in vitro* and tumors *in vivo*, contributing to the initiation and progression of EOC (106,114,116,117). These cells are resistant to chemotherapy and capable of giving rise to progenitor tumor cells, leading to tumor progression, metastasis, and recurrence (118-123).

Accumulating evidence points toward the Wnt/β-catenin pathway in playing an important role in the acquisition of stem-like properties in ovarian cancer cells (106,114,124). Among stem

cell markers, ALDH1A1 has been found to be a direct transcriptional target of β-catenin (125). In addition, silencing of β-catenin strongly reduced the stem-like properties (125,126). These findings provide direct evidence that β-catenin is involved in promoting EOC stemness. Several studies have found that modulation of β-catenin activity altered the CSC-like properties. For example, suppression of SFRP1, AXIN2, and ICAT, three important negative regulators of the Wnt/β-catenin signaling pathway, by miR-1207 activated β-catenin signaling and induced the expression of CSC markers (114). Besides, Wnt positive regulators LGR5 and LGR6 have been recognized as markers for ovarian cancer stem cells (57,127,128). The high expression of LGR5 and LGR6 was positively correlated with poor patient survival and was observed predominantly in high-grade tumors (57,127). Silencing of LGR6 significantly inhibited stemness, and the effects of LGR6 were demonstrated to be mediated by the β-catenin activity (57). Moreover, overexpression of FOXM1 promoted β-catenin activity and the formation of spheroids, while knockdown of FOXM1 had the opposite effects (106). Finally, several β-catenin inhibitors were documented to exert inhibitory effects on ovarian CSCs. Theaflavin-3, 3'-digallate (TF3), a black tea polyphenol, was found to inhibit EOC stemness by blocking Wnt/β-catenin signaling (129). Ginsenoside-Rb1, a natural saponin isolated from the rhizome of *Panax quinquefolius* and *notoginseng*, and its metabolite, compound K, suppressed CSC self-renewal and inhibited β-catenin activity (130). Together, these studies strongly support the critical role of the Wnt/β-catenin pathway in maintaining stemness in EOC.

### 2.4.2. Chemoresistance

Chemoresistance is one of the major challenges in developing treatments for ovarian cancer. Recent studies encompassing ovarian CSCs and their involvement in OC tumorigenesis reveal the association of CSCs and chemoresistance (106,116,131,132). $ALDH^+$, $CD44^+$ ovarian CSCs exhibited higher levels of resistance to paclitaxel and carboplatin (132). Additionally, an increase in cisplatin and paclitaxel resistance was observed in IGROV1 sublines and was associated with elongated mesenchymal-like morphology and a decrease in cell-cell interactions (106). In association with the upregulation of stem cell markers, a prominent increase in nuclear β-catenin levels and its activities were reported in multiple studies, indicating the promoting role of the Wnt/β-catenin pathway in chemoresistance (106,116,131,132). Since CSCs are linked to chemoresistance, many of the studies described above also reported the chemosensitizing effects of β-catenin silencing (133) or inhibition (52,81,106) on EOC cells.

One of the mechanisms that are responsible for chemoresistance in CSCs is the deregulation of membrane transporters, which is mediated at least in part by Wnt/β-catenin signaling (106,114,134). The study by Chau *et al.* (2013) identified the involvement of c-kit (also known as CD177), a stem cell-associated factor (SCF) receptor, in promoting ovarian stem-like phenotypes and chemoresistance via the Wnt/β-catenin/ABCG2 axis (134). c-kit and its ligand SCF were upregulated in ovarian tumor-initiating cells. Knockdown of c-kit reduced the numbers of spheroids formed *in vitro* and rendered the cells more susceptible to chemotherapeutic reagents, including cisplatin and paclitaxel (134). In addition, increased c-kit transcriptional level led to an increase in Wnt/β-catenin signaling pathway and mRNA levels of ABCG2 transporter which promoted the efflux of chemotherapeutic drugs as the results (134). Besides, Wnt/β-

catenin pathway was reported to indirectly modulate the expression of human copper transporter 1 (hCRT1) via FOXM1 (106). hCRT1 is a transmembrane transporter which allows the passage of copper and cisplatin through the membrane barrier into cells (106). In cisplatin-resistant EOC cells, upregulation of FOXM1 inhibited the expression of human copper transporter 1 (hCTR1) and SP1, a transcription factor that induces hCTR1 expression (106). It has been demonstrated that FOXM1 promotes β-catenin nuclear localization, while β-catenin activation promotes FOXM1 expression as a positive feedback loop (106,108). In response to Wnt3A, FOXM1 expression was upregulated in TOV-21G cells in a dose-dependent manner (106). Thus, induced expression of FOXM1 by Wnt/β-catenin signaling would impair cisplatin uptake in EOC cells.

In addition to promoting resistance to conventional chemotherapies, a recent study provided evidence to support the activation of the Wnt/β-catenin signaling in inducing resistance to a PARP inhibitor, olaparib (135). Activators and target genes of the Wnt/β-catenin pathway were found to be induced, while inhibitors of this pathway were suppressed in olaparib-resistant HGSC cell lines. Overexpression of Wnt3A reduced the sensitivity of EOC cells to olaparib. Conversely, inhibition of Wnt/β-catenin signaling enhanced the anti-tumor effects of olaparib both *in vitro* and *in vivo* (135). These results and studies discussed above support the potential of Wnt/β-catenin inhibitors for the management of EOC patients with drug resistance.

**2.4.3. EMT and metastasis**

Epithelial to mesenchymal transition (EMT) is a cellular process in which epithelial cells lose cell-cell adhesion and acquire mesenchymal characteristics, including migration and invasion (136). The attainment of invasiveness allows cells to break through the basement membrane,

which eventually results in metastasis in ovarian cancer (85,136). Many studies examining invasive characteristics of ovarian cancer suggest that activation of EMT is a critical step in acquiring malignant phenotypes, especially in high-grade serous ovarian carcinoma (137,138).

Recent evidence indicates that the activity and expression levels of E-cadherin and β-catenin are critical in the initiation of EMT in ovarian cancer cells (137). Loss of E-cadherin has been observed in ovarian cancer cell lines with increased invasion and migration phenotypes (139,140). E-cadherin assists in keeping a low cytosolic/nuclear β-catenin level by forming a complex with β-catenin at the adherens junctions; therefore, the decrease in E-cadherin would involve in the promotion of β-catenin signaling. The accumulation of nuclear β-catenin levels was detected together with an increase in cancer cell motility (139,141). Moreover, the Wnt/β-catenin pathway modulates the expression of E-cadherin through upregulation of key transcription factors, whether directly or indirectly. These transcription factors are known as mesenchymal inducers, including Twist, Snail, and Slug (139,141-146). Twist, Snail, and Slug bind to specific E-boxes located proximal to the E-cadherin promoter and suppress its expression (147). In addition, Snail can form a transcriptional complex with β-catenin, providing a positive regulatory feedback to enhance its expression through the transcriptional activity of β-catenin (147,148).

Additionally, Wnt/β-catenin signaling is involved in the remodeling of the extracellular tumor matrix in EOC, which is suggested to be mediated by the activities of matrix metallopeptidases (MMP). MMPs are proteolytic enzymes that act on diverse extracellular matrix (ECM) components such as fibronectin, gelatins, collagens, and laminins (149). MMP-2, MMP-7, and MMP-9 have all been shown to be upregulated in Wnt-activating cells and were reported as

direct transcriptional targets of β-catenin (50,140,150,151). Reporter analysis indicated that Wnt-1 stimulation activated the promoter activity of MMP-2 and MMP-9 proximal promoters in T cells (151). The Wnt-dependent expression of MMP-7 was promoted by the presence of a TCF-binding site at the gene promoter in Wnt-7A-induced EOC cells (50). Dysregulation of these MMPs was frequently observed in EOC (152–154). The upregulation of MMP-9 was suggested to contribute to lymph node metastasis in ovarian cancer (155). In addition, MMP-2 has been reported to be activated in ovarian cancer invasion - the first step of metastasis (139) while matrilysin MMP-7 was reported to activate progelatinases MMP-2 and MMP-9 *in vitro* (156). Furthermore, increased β-catenin levels have been detected in tumor samples from orthotopic xenograft mice implanted with high metastatic EOC cells (157). Silencing of β-catenin displayed a significant reduction in the ability to form primary tumors and ascites in the mouse model, providing direct evidence for an essential role of β-catenin in EOC metastasis.

**2.4.4. Tumor angiogenesis**

Angiogenesis, wherein tumors promote blood vessel formation around to provide themselves with nutrients and oxygen, is one of the hallmarks of cancer (158). There are multiple steps involved in angiogenesis including vasculature disruption, cell migration, cell proliferation, and vessel formation (159). While studies in other cancers have provided strong evidence that the Wnt/β-catenin pathway is an important player in tumor angiogenesis (158, 160), very few studies have been done in EOC. A recent study by Tang *et al.* (2018) examined the role of soluble E-cadherin in EOC and revealed that it interacted with VE-cadherin to induce angiogenesis (161). Interestingly, soluble E-cadherin containing exosomes induced strong β-catenin accumulation in the nucleus. Importantly, silencing of β-catenin expression attenuated the effect of soluble E-

cadherin containing exosomes on the formation of network-like structure (161). These findings suggest that β-catenin may induce tumor angiogenesis. However, more studies, especially involving *in vivo* mouse models, is required to confirm the role of β-catenin in ovarian tumor angiogenesis.

### 2.4.5. Immune suppression

Ovarian cancer has been reported to evade the immune system by multiple mechanisms, including the recruitment of regulatory T cells (Treg) and the promotion of T cell apoptosis via PD-L1 (162,163). The presence of Treg in ovarian tumors increases immune tolerance and is correlated with poor patient prognosis (162). In addition, IL-10 and indoleamine 2, 3-dioxygenase (IDO) were reported to promote immune evasion by ovarian tumor-associated macrophages (164). Notably, expression of IDO is associated with poor prognosis in ovarian cancer (165,166). In the presence of ovarian tumor ascites $CD14^+$ cells, which expressed IDO and IL-10, CD4+ T cells showed inhibition in responsiveness to antigen stimulation, suggesting IDO and IL-10 might be involved in the regulation of the immune response in OC (164). The same study suggests that IDO may induce Treg differentiation and apoptosis of T-cells, regulating the balance of Treg and effector T cells Th17. IDO promoter contains TCF/LEF binding domains, which was reported to be activated by Wnt/β-catenin signaling (167). However, there is no direct evidence indicating the Wnt/β-catenin pathway promotes immune evasion of EOC cells.

## 3. Small molecule inhibitors targeting the Wnt/β-catenin signaling pathway

The involvement of Wnt signaling in stem cell biology and human diseases has attracted considerable amounts of interest in developing therapeutic strategies targeting the pathway. Mutations of the components and abnormal expression of regulators of the canonical Wnt signaling pathway are frequently detected in EOC, resulting in hyperactivity of β-catenin (85). Over the past decade, a number of small molecule inhibitors have been identified through high-throughput screening and characterized with potential anti-tumor effects by targeting different components of the Wnt/β-catenin pathway as listed in Table 3. Among small molecule inhibitors have been developed, several Porcupine inhibitors which inhibit palmitoylation of Wnt ligands and their secretion have entered clinical trials. While the targets of most of the inhibitors are identified or confirmed, some of them remain to be investigated.

**Table 3: Small molecule inhibitors of the Wnt/β-catenin signaling pathway**

| **Small molecule inhibitors** | Mechanism of actions | Developmental stage | Condition | Reference |
|---|---|---|---|---|
| **Porcupine inhibitors** | | | | |
| **Wnt-C59 (C59)** | Inhibits PORCN activity | Preclinical | Nasopharyngeal carcinoma | 168 |
| **IWP-L6** | Inhibits PORCN activity and suppresses phosphorylation of Dishevelled 2 | Preclinical | Kidney tubule formation | 169 |
| **IWP-2** | Inhibits PORCN activity | Preclinical | Cancers | 170 |
| **IWP-O1** | Inhibits PORCN activity and suppresses phosphorylation of Dishevelled 2/3 | Preclinical | NA | 171 |
| **LGK-974** | Inhibits PORCN activity | Phase 1 | Pancreatic Cancer, BRAF Mutant Colorectal Cancer, Melanoma, Triple Negative Breast | 172,173 |

| | | | | |
|---|---|---|---|---|
| | | | Cancer, Head and Neck Squamous Cell Cancer, Cervical Squamous Cell Cancer, Esophageal Squamous Cell Cancer, Lung Squamous Cell Cancer | |
| **ETC-1922159 (ETC-159)** | Inhibits PORCN activity | Phase 1A/B | Solid Tumors | 174 |
| **CGX1321** | Inhibits PORCN activity | Phase 1 | Solid Tumors, Gastrointestinal Cancer | 175 |
| **RXC004** | Inhibits PORCN activity | Phase 1 | Cancer, Solid Tumor | 176 |
| **Tankyrase inhibitors** | | | | |
| **XAV-939** | Regulates Axin levels by inhibiting the activity of tankyrase 1 and 2 | Preclinical | Hepatocellular carcinoma, colon cancer, neuroblastoma, breast cancer and ovarian cancer | 146, 177-181 |
| **WIKI4** | Inhibits the activity of tankyrase 2 | Preclinical | Embryonic stem cells | 182,183 |
| **MSC2504877** | Inhibits the activity of tankyrase 1/2 | Preclinical | Colon cancer | 184 |
| **RK-287107** | Inhibits the activity of tankyrase 1/2 | Preclinical | Colon cancer | 185 |
| **C44** | Inhibits tankyrase activity by disrupting the interaction between TNKS and USP25 | Preclinical | Prostate cancer | 186 |
| **β-catenin inhibitors** | | | | |
| **iCRT14** | Binds to β-catenin and inhibits β-catenin/TCF interaction | Preclinical | Leukemia, renal cell carcinoma, lymphoma, breast cancer | 187-190 |
| **iCRT3** | Binds to β-catenin and inhibits β-catenin/TCF interaction | Preclinical | Colon cancer, Sepsis | 191,192 |
| **LF3** | Inhibits β-catenin and TCF interaction | Preclinical | Colon cancer | 193 |
| **PNU-74654** | Binds to β-catenin and inhibits β-catenin/TCF interaction | Preclinical | Colon cancer, adrenocortical cancer | 194-196 |

| | | | | |
|---|---|---|---|---|
| **PKF115-584** | Disrupts the formation of β-catenin/TCF complex | Preclinical | Leukemia, endometriosis, colon cancer, melanoma, adrenocortical cancer, multiple myeloma | 197-203 |
| **PKF118-310** | Disrupts the formation of β-catenin/TCF complex | Preclinical | Hepatocellular carcinoma, breast cancer, osteosarcoma, prostate cancer | 204-207 |
| **Others** | | | | |
| **PRI-724** | Binds to CBP and inhibits CBP/β-catenin interaction | Phase 2<br><br>Phase 1/2A | Colorectal Adenocarcinoma, Stage IVA Colorectal Cancer, Stage IVB Colorectal Cancer<br>Hepatitis C, Hepatitis B, Liver Cirrhoses | 209,209 |
| **ICG-001** | Binds to CBP and inhibits CBP/β-catenin interaction | Preclinical | Colon cancer and breast cancer | 210,211 |
| **KY02111** | Inhibits Wnt/β-catenin signaling. The mechanism is unclear | Preclinical | Human stem cell differentiation, and glaucoma | 212,213 |
| **SM08502** | Inhibits Wnt/β-catenin signaling. The mechanism is unclear | Phase 1 | Solid tumors | 214 |
| **KYA1797K** | Binds to Axin and enhances the binding of Axin to β-catenin | Preclinical | Colon cancer and lung cancer | 215,216 |
| **FH535** | Inhibits Wnt/β-catenin signaling and recruitment of β-catenin to PPARδ and PPARγ | Preclinical | Liver cancer, hepatocellular cancer, colon cancer and osteosarcoma | 217-220 |

NA: not available

## 4. Rationale, Hypothesis, and Objectives

Accumulating evidence showed that aberrant Wnt signaling is associated with a number of diseases and tumor types. Wnt signaling has been showed to regulate cell viability, apoptosis and migration, initiating cancer progression, and metastasis (43,137,138,221-223). Deregulation of Wnt/β-catenin signaling was found in many carcinomas, including EOC (224,225). One of the mechanisms of Wnt/β-catenin hyperactivation in ovarian cancer is the activating mutations of the β-catenin gene (*CTNNB1*), which has been identified in the endometrioid subtype of ovarian cancer (43,225).

Besides, recent studies indicated Wnt/β-catenin signaling plays an essential role in the function of ovarian CSCs. The self-renewal potential of CSCs is maintained through the abnormal activation of Wnt signaling, inducing cancer stemness features (118,226). CSCs are subpopulations of cancer cells with high tumorigenic potential (226). They are capable of giving rise to transformed progenitor cells which form highly aggressive tumors *in vivo* (226). In addition, CSCs have been showed to be resistant to chemotherapies and radiation (226). Therefore, inhibition of CSC survival and self-renewal capacity would improve treatment outcomes.

Canonical Wnt signaling is mediated through the translocation of β-catenin to the nucleus and subsequent expression of β-catenin/TCF target genes (224). The β-catenin/TCF complex is a critical mediator in transducing Wnt signaling to downstream effectors. Therefore, targeting the complex would efficiently eradicate Wnt signaling. *In-silico* screening, a computational-based drug-repositioning approach, has been an innovative approach which significantly speeds up the drug discovery process (227). The method automatically integrates and identifies compounds

which are promising candidates for disease treatments. We performed the analysis in the library of 200,000 natural products. We further limited the number of β-catenin inhibitors by functional assays. Our lab has identified two novel promising small inhibitors, referred to as compound 2 (C2) and compound 10 (C10). We hypothesize that C2 and C10 would bind to the TCF-binding pocket on β-catenin, inhibiting the formation of the transcriptional complex β-catenin/TCF. This would lead to the inhibition of downstream Wnt target gene expression and cancer progression as a consequence.

Aim 1: To determine the anti-tumorigenic effects of C2 and C10 on ovarian cancer.

Aim 2: To determine if C2 and C10 bind directly to β-catenin and disrupt its signaling.

# CHAPTER 2

# INVESTIGATION OF THE ANTI-TUMOR EFFECTS OF TWO NOVEL β-CATENIN INHIBITORS IN OVARIAN CANCER CELLS

**ABSTRACT**

Ovarian cancer is the leading cause of death among gynecologic malignancies. Clinical management of ovarian cancer remains a major challenge due to the development of chemoresistance. Recent studies reveal that the Wnt/β-catenin pathway plays critical roles in ovarian cancer progression, metastasis, and cancer stemness. When β-catenin is activated by a Wnt ligand, it translocates into the nucleus and interacts with TCF transcription factors, such as TCF4, to regulate target gene expression. We aim to develop a potent β-catenin inhibitor that disrupts the interaction between β-catenin and TCF4. A druggable pocket on the β-catenin was predicted using the crystal structure of β-catenin. Through *in-silico* screening and functional assays, we identified two compounds, C2 and C10, that have strong anti-tumor effects. C2 and C10 inhibited the formation of colonies in soft agar colony formation assays and suppressed spheroid formation of ovarian cancer cell lines under stem cell culture conditions. Furthermore, C2 and C10 also reduced the expression of cancer stem cell markers. Using TOPFlash reporter assays, we showed that C2 and C10 inhibited the transcriptional activity of β-catenin/TCF4 complex. Furthermore, Western blot results indicated that there was a downregulation of β-catenin/TCF4 target gene expression after C2 and C10 treatment. Utilizing biolayer interferometry binding assay, we confirmed that C2 and C10 bind to β-catenin protein. Taken together, these findings suggest that C2 and C10 inhibit the Wnt/β-catenin signaling to suppress ovarian cancer proliferation and stemness and they may potentially be further developed as a targeted therapy to treat cancer patients with hyperactive Wnt/β-catenin signaling.

## INTRODUCTION

Ovarian cancer is the most lethal gynecologic malignancy and is ranked as the fifth leading cause of cancer deaths in females (1). It is estimated that there were approximately 22,530 new cases with a mortality rate of approximately 13,980 deaths per year in the United States in 2019 (1). Ovarian cancers are grouped into three categories due to the cell of origin: epithelial, stromal, and germ cell cancer (2). Epithelial ovarian cancer (EOC) is the most common subtype of ovarian cancer, accounting for approximately 90% of cases (3). EOC is difficult to treat due to the lack of specific symptoms and effective screening methods at early stages when the disease is still curable. In addition, treating ovarian cancer remains a challenge due to the development of chemoresistance, metastasis, and cancer relapse (3,4). Most of the patients were diagnosed with stage III or IV, and the survival rate was less than five years in these patients (5). Therefore, the development of new therapeutics is needed to improve patient outcome.

Canonical Wnt signaling (or Wnt/β-catenin signaling) is essential for early embryonic development and post-embryonic physiological processes (6). Canonical Wnt signaling is activated by the binding of Wnt ligands to its surface receptors, resulting in the stabilization of cytosolic β-catenin (6). In the absence of Wnt ligands, β-catenin is targeted for ubiquitylation-proteasomal degradation by a destruction complex which consists of adenomatous polyposis coli (APC), the scaffold Axin, casein kinase-1 (CK1) and glycogen synthase kinase 3 (GSK-3) (6). Accumulating evidence showed that abnormal activation of Wnt signaling has an implication in ovarian cancer tumorigenesis in various aspects (11-15). Wnt signaling has been reported to promote cell viability, proliferation, and migration through EMT, resulting in the more advanced stages of ovarian cancer and metastasis (14,16). In addition, Wnt/β-catenin signaling is

associated with cancer stem cell maintenance, contributing to chemoresistance and cancer relapse in ovarian cancer patients (17-20). Finally, an activating mutation of *CTNNB1* was detected in endometrioid subtypes of EOC (16, 21). Although less common than *CTNNB1*, inactivating mutations at *AXIN* and *APC* were also reported in patients with endometrioid EOC (21,22).

The Wnt/β-catenin pathway has been recognized as one of potential therapeutic targets for cancer treatment. Over the past decade, researchers have introduced a number of small molecule inhibitors which target different components of canonical Wnt signaling to downregulate the signaling. While some molecules inhibit the Wnt receptor complex (23), there are small molecules that bind to Porcupine (IWPs) to suppress Wnt ligand secretion (24,25). In addition, other compounds have been developed to promote the stability of the destruction complex such as tankyrase inhibitors (26,27). Several small molecule inhibitors that disrupt the interaction between β-catenin and its co-activator, CBP (ICG-001, PRI-724) (28,29), β-catenin and TCF (iCRT3, LF3) (30,31), or β-catenin and BCL9 (32), have also been reported. Among these inhibitors, PRI-724 had entered clinical trials for treating metastatic colorectal cancer and hepatitis B and hepatitis C (29,33). PRI-724 was withdrawn at randomized phase II trial for patients with metastatic colorectal cancer (NCT02413853) (33) while the small molecule is at the recruitment stage for phase I/IIa trial for hepatitis B or C (NCT03620474) (29). Therefore, the development of a potent small molecule inhibitor for this pathway is still underway.

Recent studies in our lab strongly suggest that Wnt/β-catenin plays important roles in EOC development (34-36). We demonstrated that cyclin G2, an unconventional cyclin, inhibited key components of the Wnt signaling pathway, including Dvl and LRP6, to reduce β-catenin

activity (34). On the other hand, miR-590-3p promoted EOC development (35) in part, by targeting cyclin G2 and its transcription factor FOXO3 to enhance Wnt/β-catenin signaling (36). The results prompted us to search for inhibitors of this pathway. Since it has been suggested that disruption of β-catenin/TCF interaction could result in higher specificity (37), we collaborated with Dr. Yi Sheng (Department and Biology, York University) and Dr. Yong Zhao (Beijing Computing Center), to predict a druggable target on β-catenin that would disrupt the interaction between β-catenin and TCF4. We performed *in-silico* screening on 200,000 small molecules, and preliminary functional assays revealed that two of the molecules, referred to as compound (C) 2 and C10, were most effective in suppressing colony formation. In this study, we further examined the anti-tumor effects of C2 and C10 on EOC cell proliferation, anchorage-independent growth, and spheroid formation. In addition, we investigated the molecular mechanisms underlying the actions of C2 and C10 in EOC cells.

## METHODS AND MATERIALS

### Cell lines and cell cultures

Four different epithelial ovarian cancer cell lines were used. HEY (38) and OVCAR-3 (3) cells are used as models of high-grade serous ovarian cancer (HGSC), while TOV-112D (3) and SKOV3.ip1 cells which is derived from SKOV3 (39), are models of endometrioid ovarian cancer. In addition, HCT-116, a colorectal cancer cell line harboring a hyperactive *CTNNB1* mutation and commonly used in studies involving Wnt/β-catenin (40), was included in some of the experiments. TOV-112D, HCT-116 and OVCAR-3 cells were purchased from American Type Culture Collection (Manassas, VA, USA). SKOV3.ip1 and HEY cells were kindly provided by Dr. Mien-Chie Hung (University of Texas M.D. Anderson Cancer Center, Huston, Texas) and Dr. Theodore Brown (Mount Sinai Hospital, Toronto, Canada) respectively. The cells were maintained in media supplemented with 10% fetal bovine serum (FBS) as follow: TOV-112D cells were maintained in MCDB105/M199, SKOV3.ip1 and HCT-116 cells were maintained in McCoy's 5A, and HEY and OVCAR-3 were cultured in DMEM media.

### Proliferation and Cytotoxicity assays

IncuCyte proliferation assay was used to examine the effects of C2 and C10 on cell proliferation. TOV-112D, SKOV3.ip1, HEY, and OVCAR-3 cells were seeded onto 96-well plates with different seeding densities (TOV-112D: 2500 cells/well; SKOV3.ip1: 3000 cells/well; HEY: 2000 cells/well; OVCAR3: 3000 cells/well). Cells were treated with either C2 or C10 with different concentrations ranging from 0-50μM, and the plates were placed in the IncuCyte for imaging every 3 hours. Proliferation curves were generated using Incucyte proliferation analysis with confluency as the parameter. For CCK-8 assay, cells were treated in a similar way as for

proliferation assay. After 24 hours with C2, C10 or PRI-724 treatment, cells were treated with 10µl of tetrazolium salt solution which was provided from CCK-8 kit in each well (10µl of substrate per 100µl cell media). The tetrazolium salt was reduced by dehydrogenase enzymes from viable cells, giving a yellow-color formazan dye. The colorimetric assay was detected at the wavelength of 450nm, which indirectly reflects the number of viable cells.

**Cell fractionation and Immunoblotting**

To separate the cytoplasmic and nuclear extracts from cultured cells, the Thermo Scientific NE-PER Extraction kit was used. Cells were cultured in 10 cm dishes and trypsinized. 500,000 cells were obtained from each cell line and proceeded for protein fractionation. Collected cells were then pelleted by centrifugation at 500 x g for 5 minutes at 4°C and washed with 1×PBS. Cellular fractions were prepared according to the manufacturer's protocol. Cytoplasmic and nuclear fractions were mixed with SDS-sample buffer, and proteins were analyzed using Western blot.

Western blot samples were prepared as being described previously (35). After treatments, cells were lyzed in Radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10 mm NaF, 0.1% SDS, 1% Nonidet P-40, and 1× protease inhibitor cocktail) (Pierce, IL, USA). Cell lysates were collected by centrifugation at 12,000×rpm for 15 minutes at 4°C. Total protein was quantified by the Pierce BCA Protein Assay Kit, and equal amounts of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis. The proteins were then transferred from the SDS-PAGE gel onto Immuno-Blot polyvinylidene difluoride (PVDF) membranes (BioRad). The membranes were then blocked with 5% milk for 1 hour at room temperature and incubated with primary antibodies (as listed in Table 1) overnight at 4°C.

Subsequently, the membranes were probed with secondary HRP-coupled antibodies. Proteins were visualized by HRP Substrate (Millipore or Bio-Rad) according to the manufacturer's protocols.

| **Table 1: Antibody for Western blot** | | | |
|---|---|---|---|
| **Antibody** | Company | Species | Dilution/Concentration |
| **β-catenin** | Cell signaling | Rabbit | 1:1000 |
| **p-β-catenin (S33/37)** | Cell signaling | Rabbit | 1:1000 |
| **Lamin B** | Santa Cruz | Goat | 1:500 |
| **Axin 2** | Cell signaling | Rabbit | 1:1000 |
| **c-myc** | Santa Cruz | Mouse | 1:250 |
| **Cyclin D1** | Santa Cruz | Mouse | 1:250 |
| **GAPDH** | Santa Cruz | Mouse | 1:5000 |

**Spheroid formation**

To examine the effect of C2 and C10 on self-renewal ability of ovarian stem cells, viable cells were counted and seeded onto ultra-low attachment 96-well plates in stem cell culture medium which contains DMEM/F12, B27, 20ng/ml EGF, 20ng/ml bFGF and 4µl/mL heparin. At a cell density of 500 cells/100µl/well, EOC cells were treated with various concentrations of C2 and C10 ranging from 0-50µM. The images of spheroids in each well were obtained from the Incucyte. After 7 days, spheroids with the diameter ≥ 50µm were counted.

**Soft agar colony formation assay**

To investigate C2 and C10 effects on tumor formation *in vitro*, soft agar colony formation assay was performed. The assay characterizes the ability of cancer cells to growth and form

tumors in an anchorage-independent way (41). 1% agar and 2X culture media with 20% FBS were pre-incubated in 40°C using a water bath and then mixed at equal volumes to form the 0.5% base agar. In a 6-well plate, 1.5 mL of base agar was added to each well and set aside for at least 15 minutes to solidify. Next, 0.6% agarose was heated to 40°C, and adherent cells were trypsinized for counting. After mixing with 2X culture media with 20% FBS to make 0.3% agarose, the cell suspension was added to the mixture which was then added on top of the base layer. The cell density was seeded on each well was 5000 cells/well. The plates were incubated at 37°C in a humidified incubator until colonies formed were visible (approximately 28 days). Colonies were stained with 0.005% crystal violet for an hour and then counted using ImageJ. Colonies which had diameter ≥70μM were counted. Before performing the soft agar formation assay, the cells were pre-treated with C2, C10, or iCRT3 for 48 hours.

**RNA extraction, Reverse transcription and Real time-PCR**

Total RNA was extracted using Ribozol reagent (Invitrogen, Life Technologies) and the obtained RNAs were converted into complementary DNA (cDNA) using M-MuLV Reverse transcriptase (New England Biolabs) following the manufacturer's protocol. RT-PCR was carried out in 20μL volume contained 1X reaction buffer, M-MuLV Reverse transcriptase, RNase inhibitor, oligoT, and dNTP. The expression of genes of interest was quantified using Qiagen Rotorgene Q. qPCR was carried out in 20μl volumes containing 1X EvaGreen qPCR master mix (Invitrogen), 300nM of forward and reverse primers. The sequences of primers are listed in Table 2. Amplification was performed with an annealing temperature of 60° C with 40 cycles. The expression levels of β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used

as internal controls. The relative levels of mRNA were quantified using the comparative Ct (ΔΔCt) method.

| **Table 2: PCR primers** | |
|---|---|
| **AXIN2** | F: 5’ – CTCCTTATCGTGTGGGCAT – 3’<br>R: 5’ – CTTCATCCTCTCGGATCTGA – 3’ |
| **MYC** | F: 5’ – AAACACAAACTTGAACAGCTAC – 3’<br>R: 5’ – ATTTGAGGCAGTTTACATTATGG – 3’ |
| **CCND1** | F: 5’ – ACAAACAGATCATCCGCAAACAC – 3’<br>R: 5’ – TGTTGGGGCTCCTCAGGTTC – 3’ |
| **ALDH1A1** | F: 5’ – TGCGCTACTGTGCAGGTTGGG – 3’<br>R: 5’ – CCACAGCTCAGTGCAGGCCC – 3’ |
| **CD24** | F: 5’ – CTCCAAGCACCCAGCATCCTGTAGA – 3’<br>R: 5’ – TAGAAGACGTTTCTTGGCCTGAGTCT – 3’ |
| **CD44** | F: 5’ – TCCAACACCTCCCAGTATGACA – 3’<br>R: 5’ – GGCAGGTCTGTGACTGATGTACA – 3’ |
| **CD133** | F: 5' – GAGTCGGAAACTGGCAGATAGCA – 3’<br>R: 5' – ACGCCTTGTCCTTGGTAGTGTTG – 3’ |
| **EPCAM** | F: 5’ – CGCAGCTCAGGAAGAATGTG – 3’<br>R: 5’ – TGAAGTACACTGGCATTGACG – 3’ |
| **SOX2** | F: 5’ – GCTAGTCTCCAAGCGACGAA – 3’<br>R: 5’ – GCAAGAAGCCTCTCCTTGAA – 3’ |
| **OCT4** | F: 5’ – CCAGGTTGGAGTGGGGCTAGT – 3’<br>R: 5’ – GGAGGCCCCATCGGAGTTGCTC – 3’ |
| **NOTCH3** | F: 5’ – TCTTGCTGCTGGTCATTCTC – 3’<br>R: 5’ – TGCCTCATCCTCTTCAGTTG – 3’ |
| **B-actin** | F: 5’ – AAACTGGAACGGTGAAGGTG – 3’<br>R: 5’ – AGAGAAGTGGGGTGGCTTTT – 3’ |
| **GAPDH** | F: 5’ – AAGGTCATCCCTGAGCTGAAC – 3’<br>R: 5’ – ACGCCTGCTTCACCACCTTCT – 3’ |

**ALDEFLOUR Assay**

The ALDEFLUOR kit (StemCell Technologies) was used to determine the population of ovarian cancer stem cells with high ALDH activity. After treatment, cells were labeled with ALDEFLOUR reagent according to the manufacturer's instructions. Briefly, cells were trypsinized and resuspended into the culture medium. 5 µL of the activated ALDEFLUOR™ Reagent per milliliter of the sample was added into "test" tubes. 1mL of cell suspension was added to the "test" tube and 0.5mL of the mixture was immediately transferred to the DEAB "control" tube containing 5 µL of ALDEFLUOR™ DEAB Reagent ("control" tubes). "Test" and "Control" samples were incubated at 37°C for 45 minutes. Cells were then centrifuged for 5 mins at 250×g and resuspended in ALDEFLUOR™ Assay Buffer. The samples were analyzed using cell sorter Sony SH800z.

**TOPflash assay**

TOPFlash reporter assay was used to detect the transcriptional activity of β-catenin/TCF complex. HCT-116, HEY, SKOV3.ip1, and OVCAR-3 were cotransfected with 1µg M50 Super 8X TOPFlash reporter construct containing 7 TCF/LEF consensus binding sites which are upstream of firefly luciferase cDNA and 25ng Renilla internal control plasmid. After 4-6 hours of transfection, cells were recovered by adding the culture media containing C10 in various concentrations ranging from 1-20µM. For SKOV3.ip1, HEY, and OVCAR-3 cells, the media were supplemented with Wnt3A (Wnt3A conditioned media) to induce Wnt/β-catenin signaling activation. The cells were lysed after 24 hours of treatments, and the signals were obtained using Luc-Pair Dual Luciferase Kit (Gene Copoeia).

**Purification of recombinant β-catenin protein**

To examine the binding of C2 and C10 to β-catenin protein directly using bio-layer interferometry (BLI) binding assays, recombinant 6xHis-tag β-catenin proteins were purified. pET28a$^{+}$ plasmid containing full-length of human *CTNNB1* cDNA was transformed into *E. coli* strain BL21(DE3). The transformed bacterial cells were selected and cultured in lysogeny broth (LB) medium containing 50mg/ml kanamycin at 37°C shaker. When $OD_{600}$ reached 0.6-0.8, the bacterial cells were induced with 0.5mM of isopropyl-beta-D-thiogalactopyranoside (IPTG) at 16°C for approximately 18-20 hours. Cells were then harvested by centrifugation and proceeded to the purification process. The bacterial pellet was suspended in the lysis buffer (20mM Tris, pH 8.0, 500mM NaCl, 10% glycerol, 0.1% Triton X-100, 1mM β-mercaptoethanol (BME), 10mM imidazole and protease inhibitors including 0.5mM PMSF, 1mM benzamidine) and sonicated to lyse the cells. After centrifugation, the supernatant was obtained and applied to NiNTA column which had been pre-equilibrated with the lysis buffer. His-tag β-catenin protein was eluted from the column after adding elution buffer (lysis buffer with 400mM Imidazole). The samples were then dialyzed in Tris buffer (20mM Tris, pH 8.0, 100mM NaCl) and stored. Eluents collected from the affinity column were analyzed and validated using SDS-PAGE and Western blot.

***In vitro* kinetic binding assay using FortieBio Octet instrument**

The kinetics and affinities of the molecular interactions between C2 and C10 with β-catenin protein were detected and quantified using OctetRED384 system. The interaction was conducted at 25°C in HEPES-buffer saline (HBS) buffer supplemented with 0.001g/ml BSA, which acted as blocking buffer. The Anti-Penta-His Biosensors were prewet in the HBS buffer prior usage. Samples were added to fluorescence 96-well plates with the volume as 200µl per well.

The sensor tips were wet in the blocking buffer for baseline establishment. The biosensor tips were then moved to the wells containing 1mg/ml of purified full-length his-tag β-catenin proteins to load the proteins onto the sensor tips. This was followed by a washing step to remove excess amounts of β-catenin proteins which bound onto the sensor tips. A new baseline was then established in the HBS buffer. C2 and C10 were prepared as a serial dilution as 20, 50, and 100μM. After the protein was loaded on the sensor tips and new baseline was established, the sensor tips were dipped into wells containing a single concentration of C2 or C10 to detect whether there was any binding between the protein and the small molecule inhibitors. Washing steps were carried out to observe the dissociation from the binding and the sensor tips proceeded to other concentrations of C2 and C10. New baselines were established between dissociation and association steps. Negative control of 0.1% DMSO was included in the experiment. Data were processed using reference subtraction (negative control) in the ForteBio Octet Data Analysis software.

**Statistical analysis**

All *in vitro* experiments were performed with as least triplicates in each group. The results were expressed as mean ±SEM. Statistical analysis and graphs were done using GraphPad Prism 8.0 with statistical significance were defined as $p<0.05$. $IC_{50}$ values were determined using nonlinear regression analysis with dose-response inhibition model. In addition, one-way ANOVA and Student's t-test were used for comparisons among multiple groups and comparisons between two groups respectively.

## RESULTS

### β-catenin levels among EOC cell lines

Recent mutational analysis showed that TOV-112D and HCT-116 cells harbor *CTNNB1* mutation at GSK3β phosphorylation site (S37) (21,40). In addition, Wnt-7A ligand was identified to be highly expressed in SKOV3.ip1 (42). The data suggested the Wnt/β-catenin signaling pathway might be hyperactivated in these cell lines. While most of the endometrioid subtype has been shown to possess mutations on *CTNNB1*, mutations on this gene have not been found on the serous subtype. Recent studies revealed secreted frizzled receptor protein-4 (SFRP4), a Wnt inhibitor, was not detected in OVCAR-3 cell lines (43, 44). SFRP4 contains a frizzled-like cysteine-rich domain, which allows it to bind directly to Wnt ligands or Frizzled receptors, inhibiting the binding of Wnt ligands to Frizzled receptors (44). The downregulation of SFRP4 was reported together with the increase in nuclear β-catenin in OVCAR-3 cells. In addition, induced expression of recombinant SFRP4 decreased the expression of β-catenin, indirectly suggesting Wnt signaling might upregulate in OVCAR-3 cell line (44). These cell lines, alone with HEY, which represents the HGSC subtype (38) and has not been reported to have alteration in the Wnt/β-catenin pathway, were used in this study. To elucidate the relative activity of Wnt/β-catenin signaling in EOC cell lines, I examined the levels of total β-catenin and phospho-β-catenin using western blot analysis.

Total β-catenin was detected in all 5 cell lines tested and the level was highest in TOV-112D, followed by HCT-116, SKOV3.ip1 and OVCAR-3, while HEY had the lowest levels (Fig. 1A). To confirm that TOV-112D has the highest β-catenin activity, total and phospho-β-catenin were measured in both nuclear and cytoplasmic fractions of the four EOC cells and the β-catenin activity was quantified as the ratio of total and phospho-β-catenin, and then normalized to

internal controls. TOV-112D had the highest nuclear β-catenin activity among all four cell lines, followed by OVCAR3 and SKOV3.ip1, while HEY cells had the lowest nuclear β-catenin activity (Fig. 1B). Similarly, in cytoplasmic fractions, TOV-112D had the highest levels of β-catenin proteins, followed by OVCAR-3, SKOV3.ip1, and HEY. The results suggest that the activity of Wnt/β-catenin signaling would be highest in TOV-112D in EOC cell lines, following by OVCAR-3, SKOV3.ip1, and HEY respectively. Since the small molecule inhibitors were predicted to inhibit β-catenin activity, we hypothesized that C2 and C10 would exhibit the inhibitory effects on cell lines depending on their β-catenin levels or activities.

**C2 and C10 inhibit EOC proliferation**

To determine the effects of C2 and C10 on cell proliferation *in vitro*, cells were treated with different concentrations of C2 or C10, and cell confluency was monitored for 3 days using the IncuCyte. C2 and C10 exhibited a dose-dependent inhibitory effect on cell proliferation in all EOC cell lines tested (Fig. 2). There was a decrease in cell proliferation with increasing concentrations of C2 and C10. In addition, the inhibitory effect of C2 and C10 on cell proliferation were varied among cell lines. While TOV-112D cells proliferated significantly slower (~ 50%) at 10μM of either C2 or C10 treatment (Fig. 2A), the proliferation rate of SKOV3.ip1 and OVCAR-3 was reduced more dramatically with either C2 or C10 treatment at the concentration of 20μM or higher (Fig. 2B,C). Among the four cell lines, HEY cells were less responsive to the small molecule inhibitors, especially with C10 than other cell lines ( Fig. 2D). C2 and C10 had a similar potency in inhibiting cell proliferation at the concentration of 20μM or lower. However, at higher concentrations, C2 was more effective than C10 (Fig. 2).

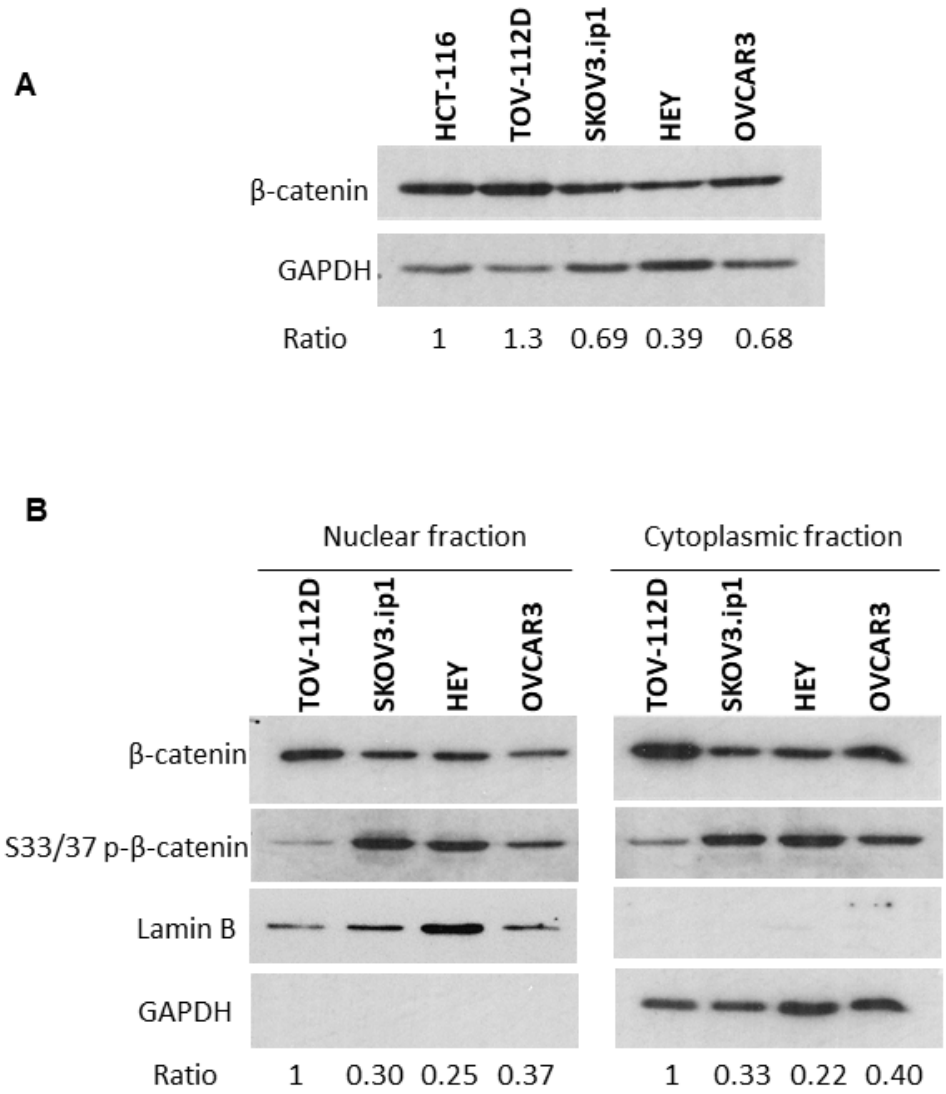



Fig. 1: β-catenin expression among different EOC cell lines. A. Total β-catenin levels in cell lines HCT-116, TOV-112D, SKOV3.ip1, HEY, and OVCAR3. The ratio between β-catenin and GAPDH in each cell line is indicated. B. Nuclear and cytoplasmic fractions of total and phospho-β-catenin in EOC cell lines. Nuclear and cytoplasmic fractions were separated, and western blotting was performed. Lamin B and GAPDH were used as the internal controls for nuclear and cytoplasmic β-catenin. The ratio of total and phospho-β-catenin was calculated and normalized to the internal controls. The experiments were repeated once and similar results were obtained.

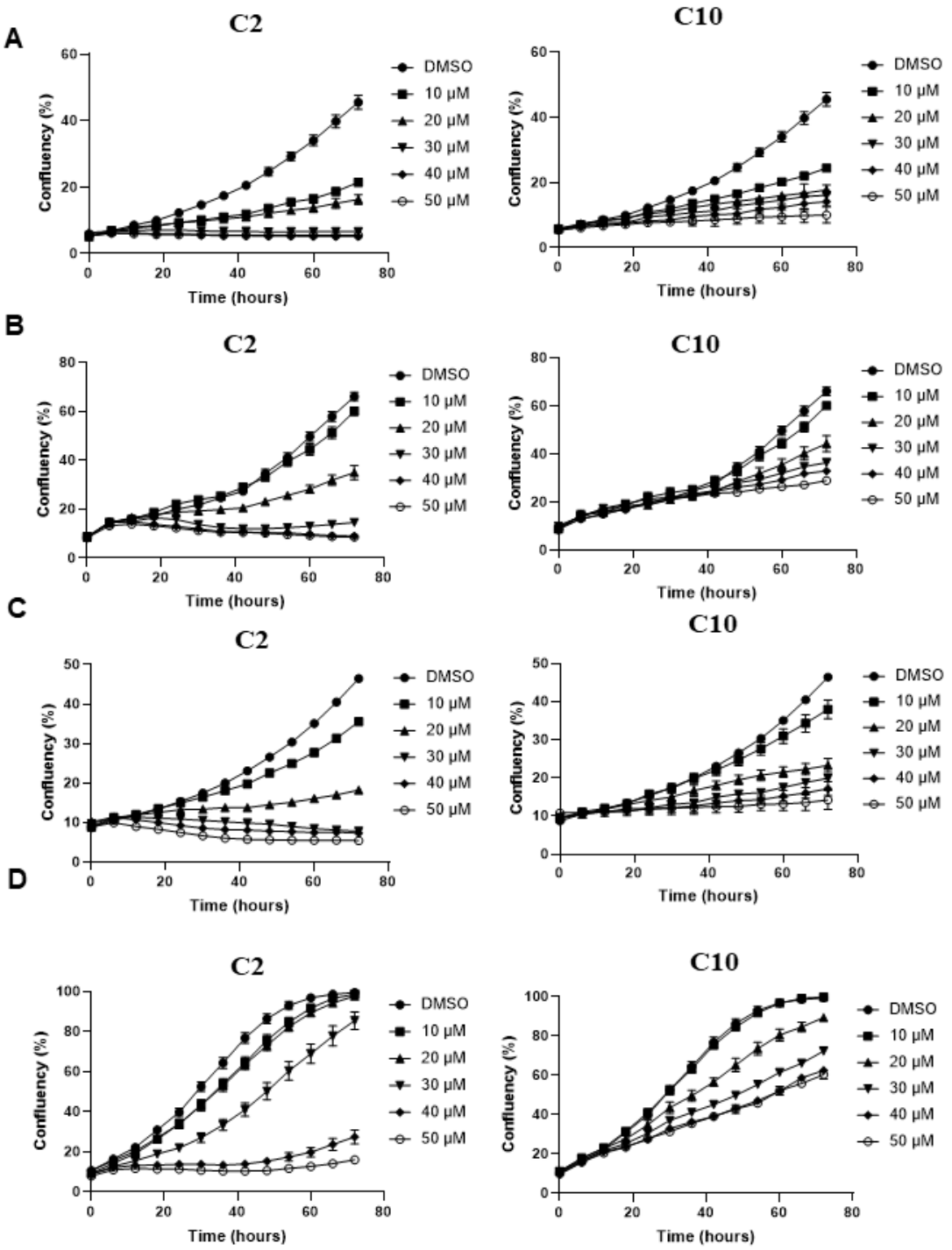


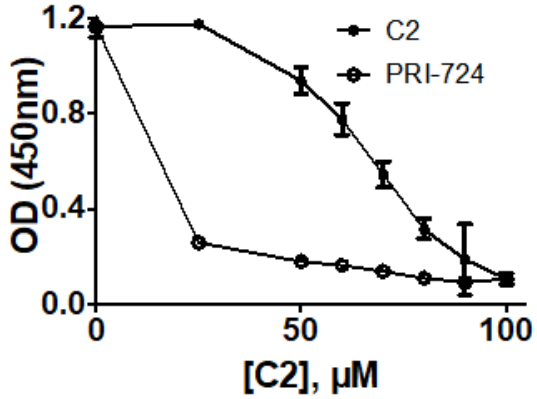

Fig. 2: Effects of C2 (left panels) and C10 (right panels ) on TOV-112D (A), SKOV3.ip1 (B), OVCAR-3 (C) and HEY (D) cell proliferation. Cells were seeded onto 96-well plates and treated with different concentrations of C2 and C10 ranging from 10-50µM and monitored for 3 days in the IncuCyte. DMSO (vehicle for C2 and C10) was used as the negative control. Data represent Mean ± SEM (*n* = 4).

We also measured the cytotoxic effect of C2 and C10 and compared them with the β-catenin/CBP inhibitor PRI-724 in a noncancerous trophoblast cell line, HTR-8/SVneo (30) using the CCK-8 assay. Both C2 (Fig. 3A) and C10 (Fig. 3B) had much lower cytotoxic effects on HTR-8/SVneo cells than PRI-724. At the dose of 25μM, approximately 30% of cells survived in PRI-724 treatment, while more than 80% of cells were still viable after C2 and C10 treatments. At the concentration of 100μM, C2 and PRI-724 significantly induced cell death on HTR-8/SVneo cells while there was no significant change in HTR-8/SVneo cell viability in C10 treatment from 60μM-100μM.

**C2 and C10 inhibit colony formation**

Anchorage-independent growth is one of the characteristics of cancer cells, which allows the cancer cells to grow independently of solid surface (41). Unlike normal cells, cancer cells are resistant to an apoptotic process called anoikis and do not require the adhesion of extracellular matrix to proliferate (45). It has been shown previously from our lab that C2 could strongly inhibit anchorage-independent growth of two cell lines HCT-116 and SKOV3.ip1 at the concentration of 10μM. In this study, we examined the effects of C10 on colony formation of HCT-116 cell line. The results showed that C10 inhibited the colony-forming ability of HCT-116 cells in a dose-dependent manner (Fig. 4). A significant decrease in the numbers of colonies formed in soft agar was observed in HCT-116 cells treated with C10 compared to the control (Fig. 4A). At 10μM, C10 almost abolished the colony-forming ability of HCT-116 completely. iCRT-3 is a β-catenin inhibitor that has been reported to disrupt the interaction between β-catenin and TCF4 (30). We compared the effects of C10 with iCRT-3 on colony formation in the experiment and C10 showed stronger effects than iCRT-3 (Fig. 4).

A




B

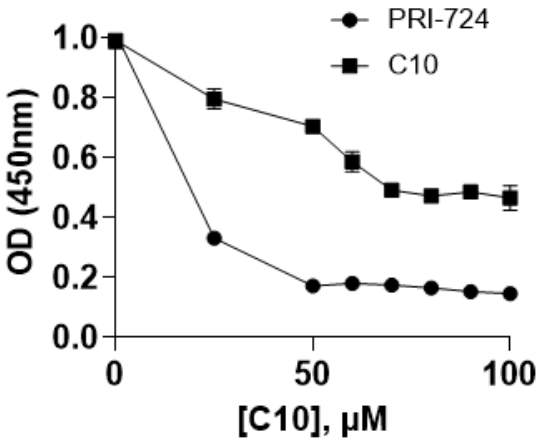



Fig. 3: Cytotoxic effects of C2 (A), C10 (B) and PRI-724 on a trophoblast cell line, HTR-8/SVneo. Cells were treated C2 or C10 and PRI-724 for 24 hours and the number of viable cells was measured using CCK-8 kit. Cytotoxicity of C2 was performed by Benedikt Wimmer. Data represent Mean ± SEM ($n$ = 4).

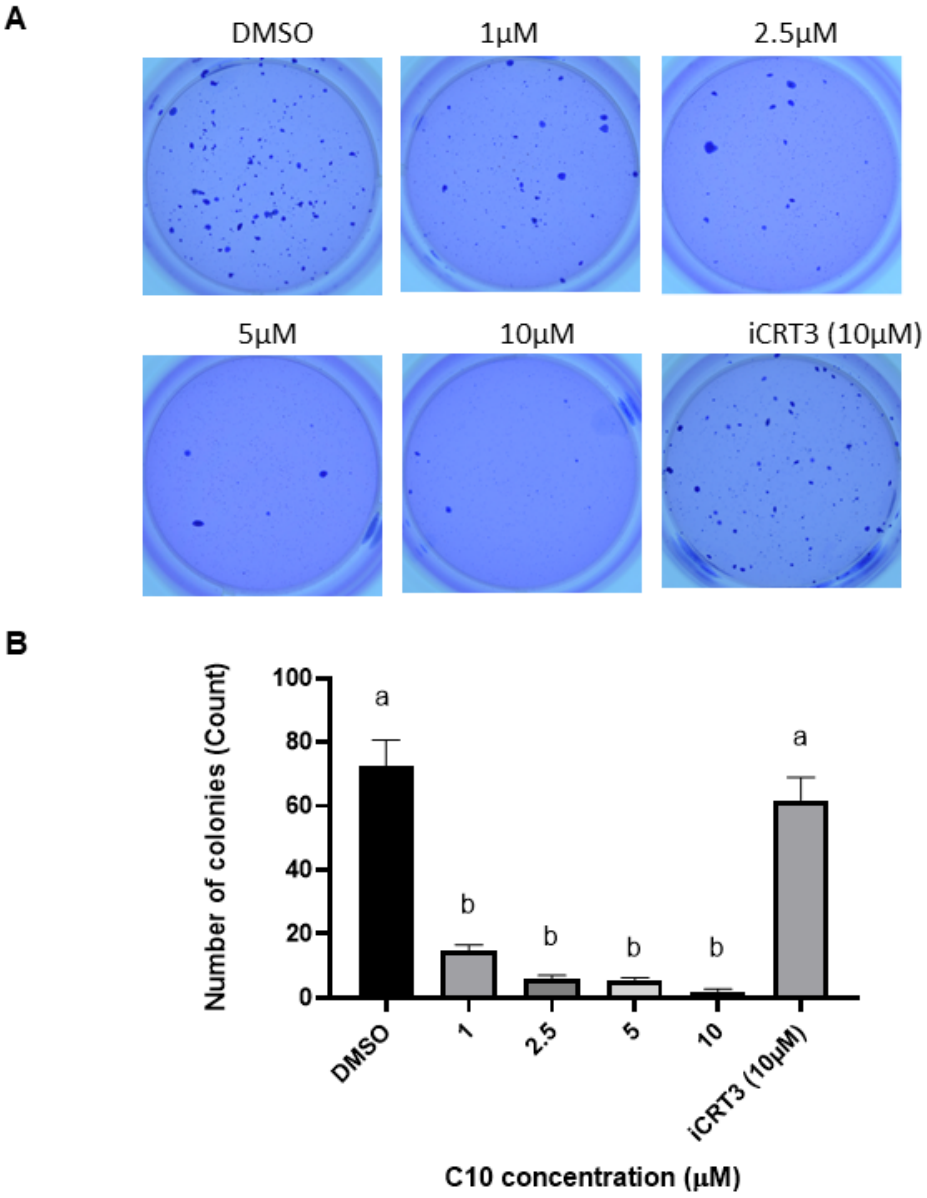



Fig. 4: Effects of C10 on anchorage-independent growth. A) Representative images of soft agar colony formation of HCT-116 cells which were treated with C10 and iCRT3.
B) Quantification of colony formation of HCT-116 cells. The cells were pre-treated with C10 and iCRT3 in normal culture conditions for 48-hours before proceeded to soft agar. Cells were suspended in 0.3% agarose and were seeded at the density of 5000 cells/well onto 6-well plates. The treatments were refreshed twice a week for 28 days. DMSO (vehicle of C2/C10) was used as the negative control. Colonies with a diameter ≥70µM were quantified. Data represent Mean ± SEM ($n$ = 3; $P$ < 0.05). Statistical analyses were performed using ANOVA/Tukey test and different letters above bars denote statistical significance.

**C2 and C10 inhibit ovarian cancer stemness**

Stemness is characterized by the expression of stem marker genes and its ability to form spheroids when cells are cultured in stem cell medium (46). To examine the effect of C2 and C10 on self-renewal of ovarian CSCs, EOC cells were seeded onto ultra-low attachment 96-well plates in stem cell culture medium at the density of 500 cells/100µl/well with C2 and C10 concentrations of 0-50µM. Both C2 (Fig. 5) and C10 (Fig. 6) inhibited spheroid formation in a dose-dependent manner. As shown in Table 3, TOV-112D cell line had the lowest $IC_{50}$ for C2 and C10 as 8.54µM and 6.85µM respectively, indicating both inhibitors were most effective in this cell line (Table 3). SKOV3.ip1 and OVCAR-3 had relative the same $IC_{50}$ at approximately 10µM, while HEY cells were more resistant to C2 and C10 treatments (Table 3). $IC_{50}$ of C2 among EOC was slightly higher than $IC_{50}$ of C10 (Table 3), suggesting C10 might be more potent to CSCs compared to C2.

**Table 3: Half maximal inhibitory concentration ($IC_{50}$) of C2 and C10 on EOC spheroid formation**

Numbers of spheroids were counted after 7 days of treatments with C2 and C10

| Cell line | C2 (µM) | C10 (µM) |
|---|---|---|
| HEY | 17.77±0.41 | 13.71±0.54 |
| SKOV3.ip1 | 11.13±0.22 | 9.18±0.60 |
| OVCAR3 | 10.31±0.35 | 9.50±0.53 |
| TOV-112D | 8.54±0.87 | 6.85±0.43 |

* Spheroid with diameter ≥50µM was counted

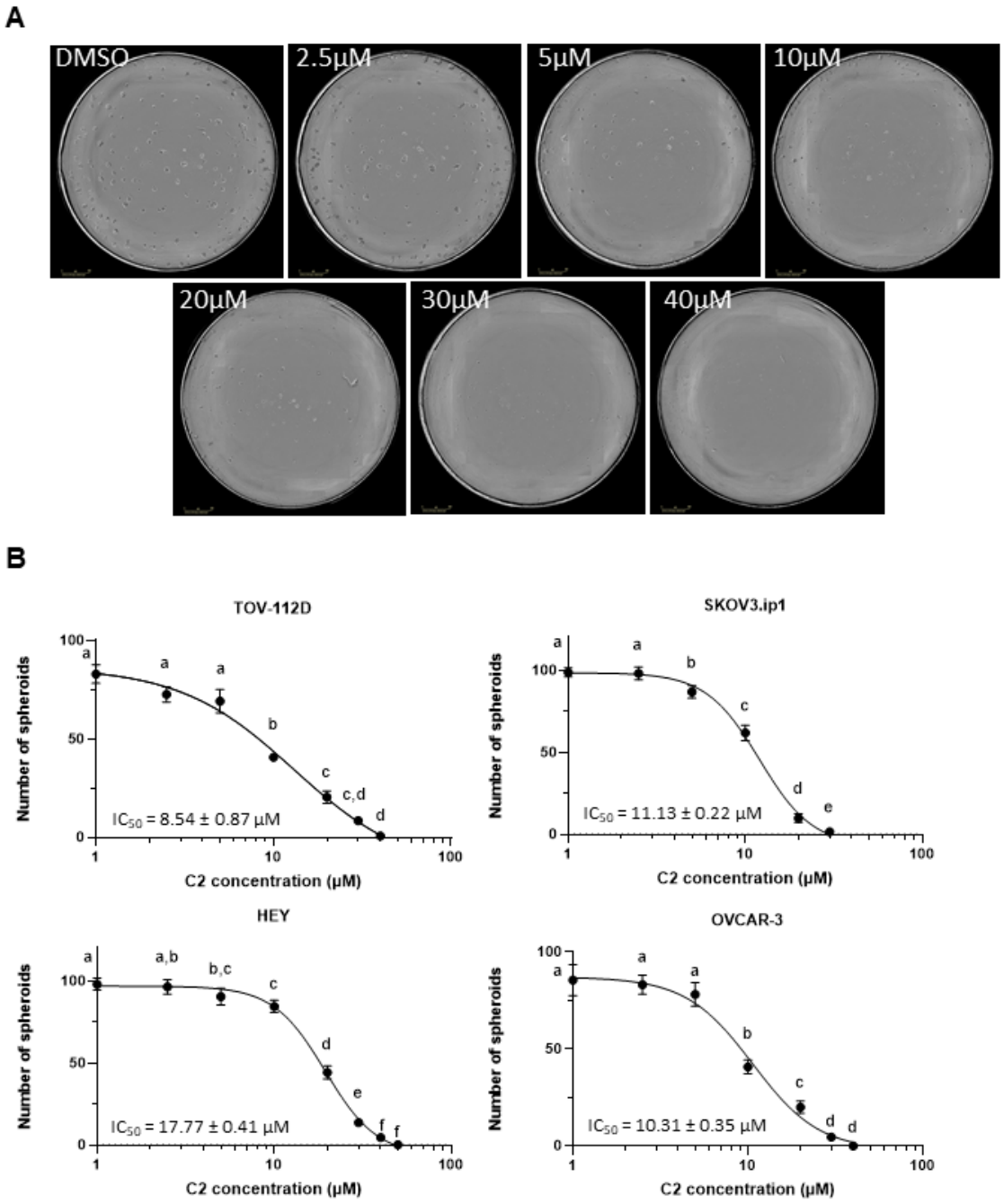



Fig. 5: Effects of C2 on spheroid formation of EOC cell lines. A) Representative images of spheroid formation of TOV-112D cells after being treated with C2 for 7 days.
B) Dose-dependent curves of spheroid formation after C2 treatment of TOV-112D , SKOV3.ip1, HEY, and OVCAR-3 cells. Cells were seeded onto ultra-low attachment 96-well plate in stem cell culture medium at the density of 5 cells/μl and treated with different doses of C2 for 7 days. DMSO (vehicle for C2) was used as the negative control. The numbers of spheroids with diameter ≥50μM were quantified. Data represent Mean ± SEM ($n$ = 8; $P$ < 0.05). Different letters above data points denote statistical significance.

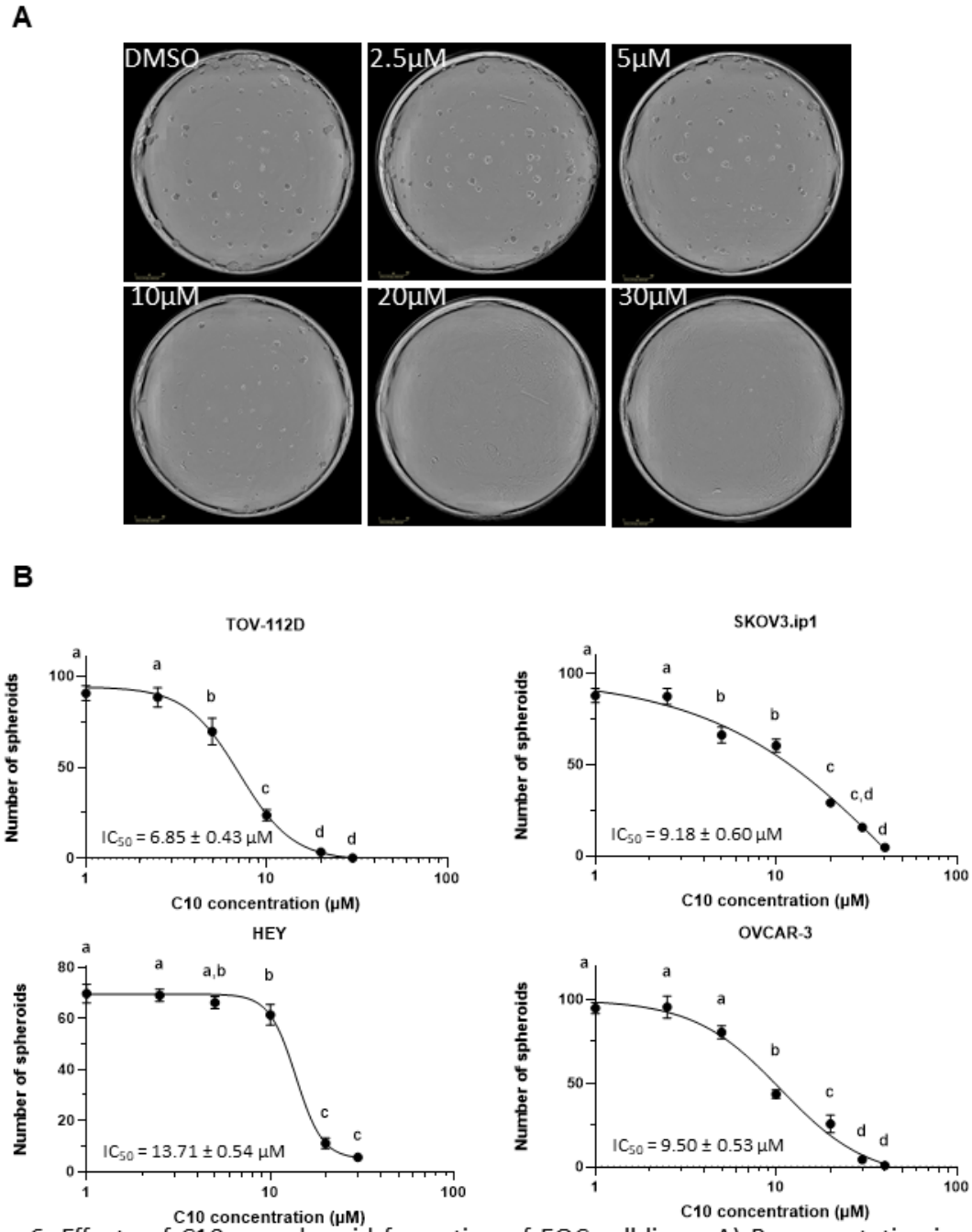



Fig. 6: Effects of C10 on spheroid formation of EOC cell lines. A) Representative images of spheroid formation of TOV-112D cells after being treated with C10 for 7 days.

B) Dose-dependent curves of spheroid formation after C10 treatment of TOV-112D, SKOV3.ip1, HEY, and OVCAR-3 cells. Cells were seeded onto ultra-low attachment 96-well plate in stem cell culture medium at the density of 5 cells/µl and treated with different doses of C10 for 7 days. DMSO (vehicle for C10) was used as the negative control. The numbers of spheroids with diameter ≥50µM were quantified. Data represent Mean ± SEM ($n$ = 8; $P$ < 0.05). Different letters above data points denote statistical significance.

Activation of the Wnt/β-catenin signaling promotes the expression of various stem cell markers including Aldehyde dehydrogenase (ALDH), cluster of differentiation (CD) proteins, SOX2, NANOG and OCT4 (47,48). To determine which genes can be used as stem cell markers, we first examined the expression of several genes in both stem-cell enriched spheroids and in whole population. qRT-PCR results showed that *ALDH1A1* and *EpCAM* expression were upregulated in all EOC stem cell-enriched samples (Fig. 7). In addition, SKOV3.ip1 and OVCAR-3 spheroids expressed higher levels of *CD24*, *CD44*, *CD133* and *OCT4* compared to whole cell population. The increase in *CD24*, *CD133* and *SOX2* expression was also observed in TOV-112D spheroids while *CD44* and *OCT4* expression was elevated in HEY spheroids.

To further confirm the effect of C2 and C10 on CSC self-renewal, we used RT-qPCR to measure the mRNA levels of stem cell markers which were selected based on the results presented in Fig. 7. C10 significantly decreased the mRNA levels of *ALDH1A1*, *CD24*, and *SOX2* in TOV-112D and *EpCAM*, *CD44,* and *OCT4* in HEY (Fig. 8A) stem cell-enriched samples. Since *ALDH1A1* levels were upregulated in spheroid populations of all cell lines, *ALDH1A1* activity was examined using flow cytometry after C10 treatment. The results from flow cytometer revealed that there was a decrease in $ALDH^{br}$ (ALDH bright) population of SKOV3.ip1 which was treated with C10 compared to the control (Fig. 8B).

**C2 and C10 inhibit β-catenin/TCF transcriptional activity**

To elucidate whether C10 downregulates Wnt/β-catenin signaling, TOPFlash reporter assay was first used to measure β-catenin/TCF transcriptional activity. HCT-116, HEY, SKOV3.ip1, and OVCAR-3 cells were cotransfected with the TOPFlash reporter construct containing 7 TCF/LEF consensus binding sites upstream of firefly luciferase cDNA and a Renilla plasmid as an internal

control for transfection efficiency. Work done by others in the lab had shown that C2 inhibited TOPflash signals in transfected EOC cell lines. Similarly, there was a decrease in normalized luciferase signal in HCT-116, HEY and SKOV3.ip1 and OVCAR-3 cells after being treated with increasing concentrations of C10 (Fig. 9).

To further confirm that C2 and C10 inhibit the activity of β-catenin/TCF complex, cells were treated with different concentrations of C2 or C10 and Western blot analyses were performed at 6h or 24h after treatment to determine the protein levels of several β-catenin/TCF target genes. In TOV-112D cells, protein levels of β-catenin target genes were downregulated in a dose-dependent and time-dependent manner (Fig. 10). There was a decrease in protein levels of Axin2, c-Myc, and cyclin D1 at 50μM at 6 hours after C2 treatments (Fig. 10A). The downregulation of target genes sustained at 24 hours after treatments with a more inhibitory effect on Axin2 protein levels. For C10, the downregulation of β-catenin target genes was observed at 25μM and higher concentrations at both time points (Fig. 10B). At 24 hours, Axin2 and c-myc protein levels were reduced in a dose-dependent manner while there was no change in cyclin D1 protein levels with C10 treatment.

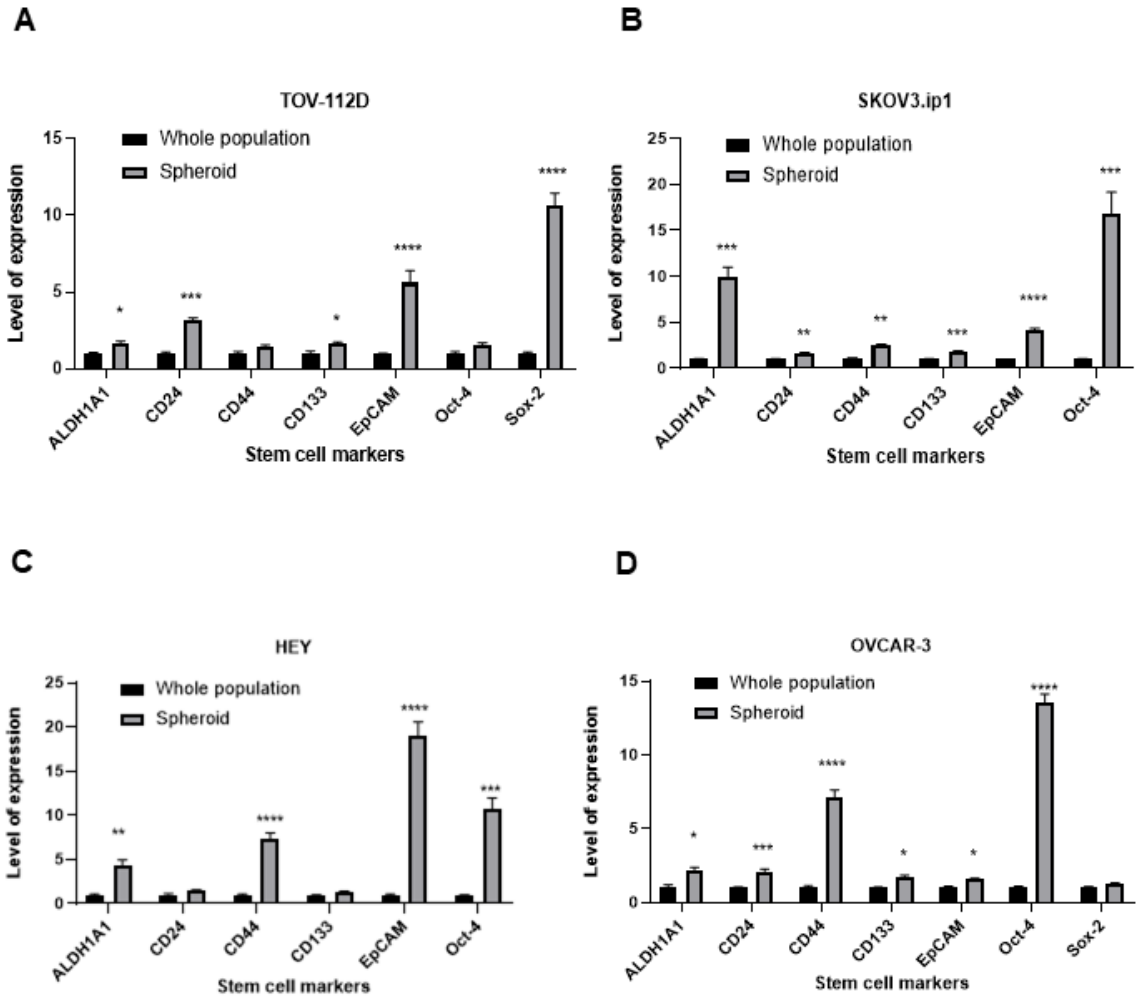



Fig. 7: RT-qPCR analysis of stem cell markers in whole cell population and in stem cell-enriched spheroids of TOV-112D(A), SKOV3.ip1 (B), HEY (C), and OVCAR3 (D). Cells were cultured either on regular culture dishes (whole cell population) or on ultra-low attachment plates with stem cell culture medium (spheroids) and collected after 7 days. Total RNA was extracted, and RT-qPCR was performed using various primers for reported EOC stem cell markers in spheroids vs whole population. Data represent Mean ± SEM ($n$ = 4). * $p < 0.05$, ** $p < 0.01$ , *** $p < 0.001$ and **** $p<0.0001$ ( Student's t-test).

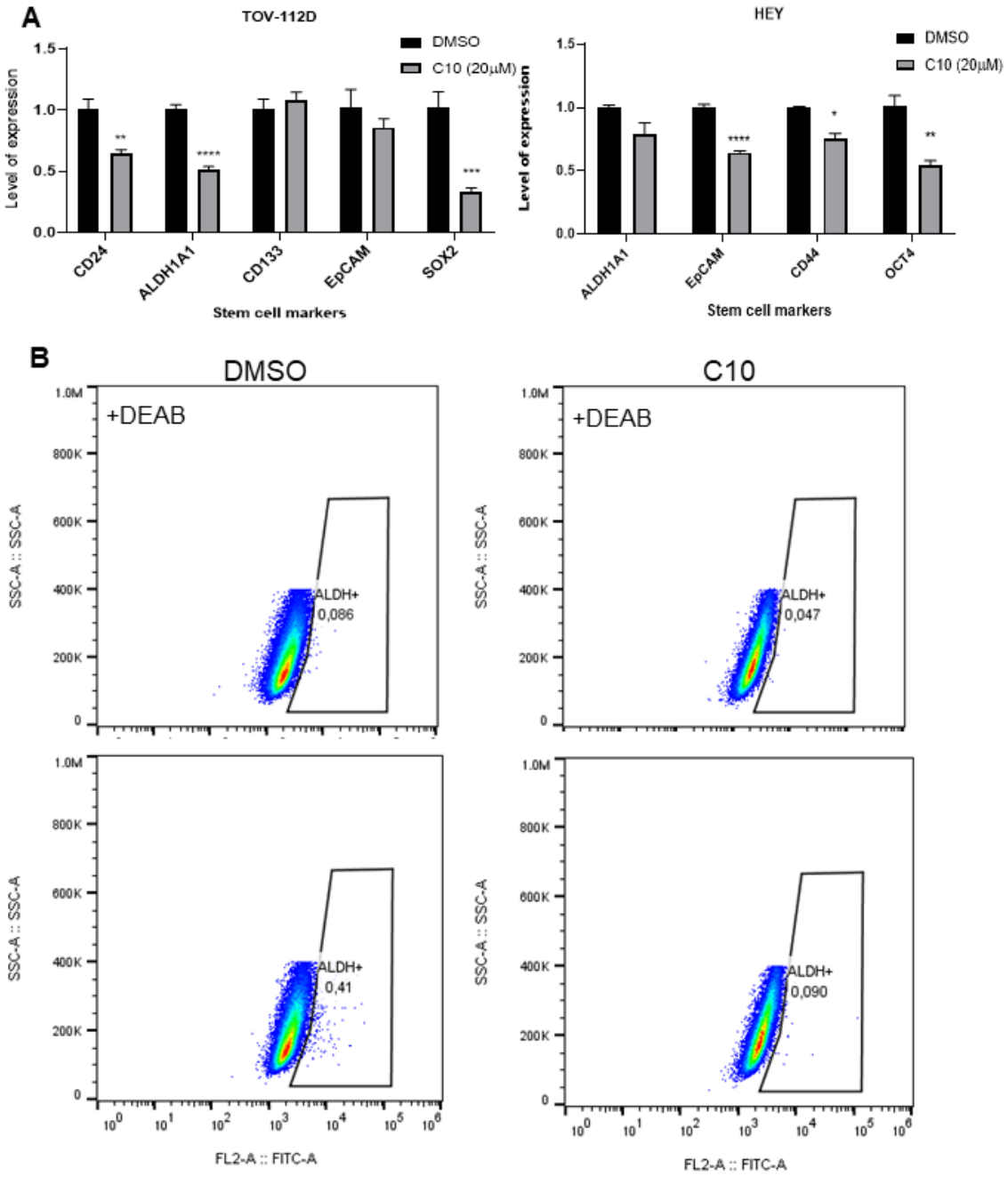



Fig. 8: Inhibitory effects of C10 on stem cell marker expression. A) mRNA levels of CSC markers in TOV-112D and HEY cells. Cells were cultured on ultra-low attachment plates and treated with C10 treatment (20 μM) or its vehicle (DMSO) as the negative control for 48h. Total RNA was extracted, reversed transcribed, and qPCR was performed. Data represent Mean ± SEM ($n$ = 4). * $p < 0.05$, ** $p < 0.01$ , and **** $p<0.0001$ (Student's t test). B) ALDH activity. SKOV3.ip1 cells were treated with DMSO or 30μM of C10 for 48 hours. Cells were then stained for stem cell marker ALDH using ALDEFLUOR (ALDH) kit and sorted using Sony SH800. C10 treatment reduced the portion of ALDH positive cells. (+DEAB: control).

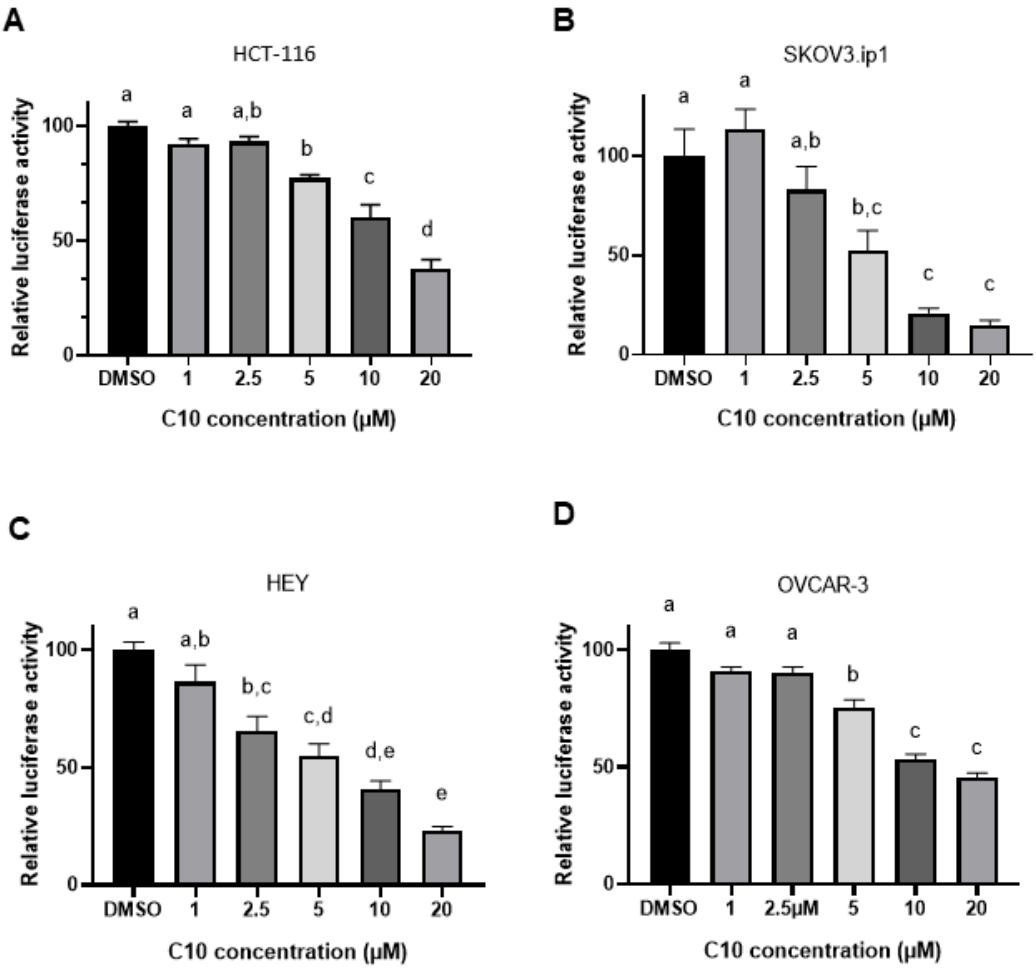



Fig. 9: Effects of C10 on the transcriptional activity of β-catenin/TCF4. HCT-116 (A), SKOV3.ip1 (B), HEY (C ) and OVCAR-3 (D) were cotransfected with the plasmids encoding TOPFlash and Renilla reporters. Cells were treated with conditioned media containing Wnt3A and different concentrations of C10, or its vehicle control (DMSO). Firefly luciferase and Renilla luciferase activities were measured at 24h after treatments. Relative luciferase activity was calculated by normalizing firefly luciferase to Renilla signal in the same sample. Data represent Mean ± SD ($n = 4$, $P < 0.05$). Statistical analyses were performed using ANOVA/Tukey test and different letters above bars denote statistical significance.

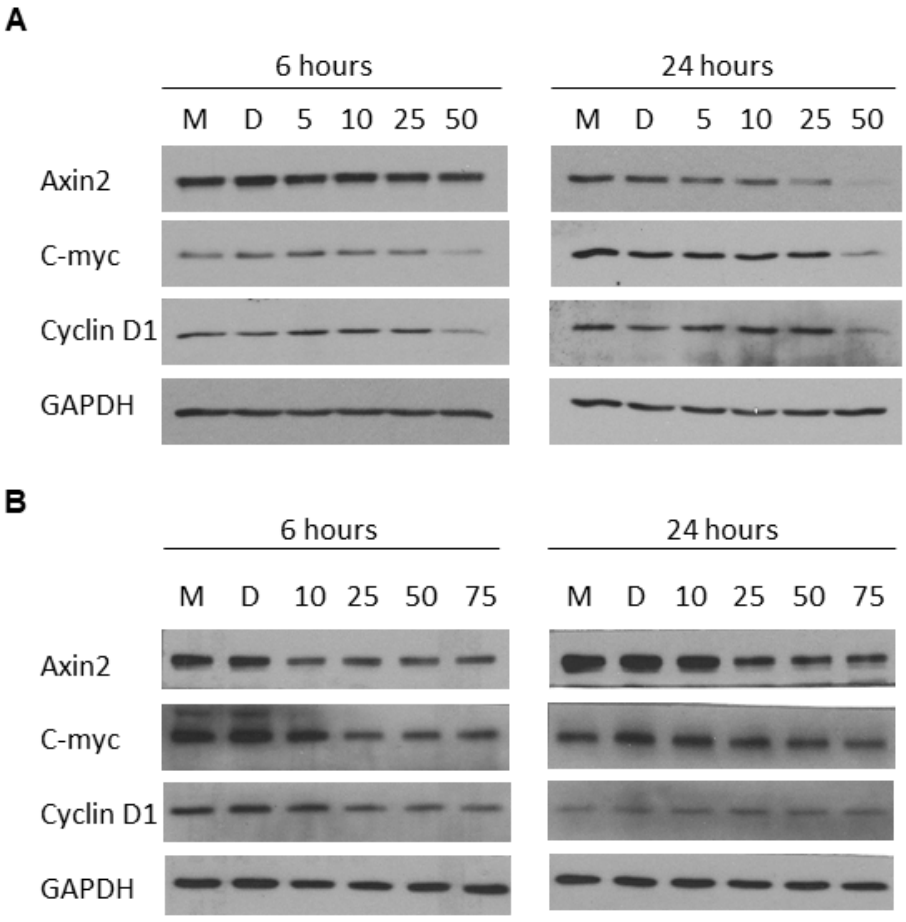



Fig. 10: Effects of C2 (A) and C10 (B) on the protein levels of β-catenin target genes in TOV-112D cells. Cells were mock treated (M, media only), or treated with DMSO (D, vehicle control) or different concentrations of C2 of C10. Cells were then collected at 6 hours and 24 hours after treatment and proceeded with Western blotting. The experiments were repeated and similar results were obtained.

**C2 and C10 bind to β-catenin**

The recombinant full-length β-catenin proteins were purified from *E.coli* BL21 (DE3) and further analyzed using Western blot prior binding assay (Fig. 11A,B). The purified recombinant β-catenin protein obtained from elution fractions had the molecular weight of approximately 90kDa as predicted (Fig. 11A). The identity of the protein collected from the elution fractions was further determined using Western blotting with Anti-6X His tag antibody. The results showed the detection of his-tagged β-catenin protein at the relative same molecular weight, confirming the identity of the protein (Fig. 11B). The binding of C2 and C10 to β-catenin protein was investigated by bio-layer interferometry (BLI) approach using the Forte Octet system. BLI is a label-free technique which detects biomolecular interactions by measuring the difference of the interference patterns of white light between the reference and samples. BLI results showed that there were spectral shifts in the presence of C2 and C10 ( Fig.11 C, D, Fig. S1 A,B) while the spectral shift was not observed in the control sample containing buffer or DMSO (D1) (Fig.S1 C,D), indicating C2 and C10 bind to the recombinant β-catenin protein. In addition, the wavelength shift increased with increasing concentrations of the inhibitor C10. Furthermore, the binding profiles of C2/β-catenin and C10/β-catenin provided information about the kinetics of the interactions. The binding kinetic sensorgrams showed that the interactions exhibited fast association and dissociation rate ( Fig. 11C,D, Fig. S1 A,B).

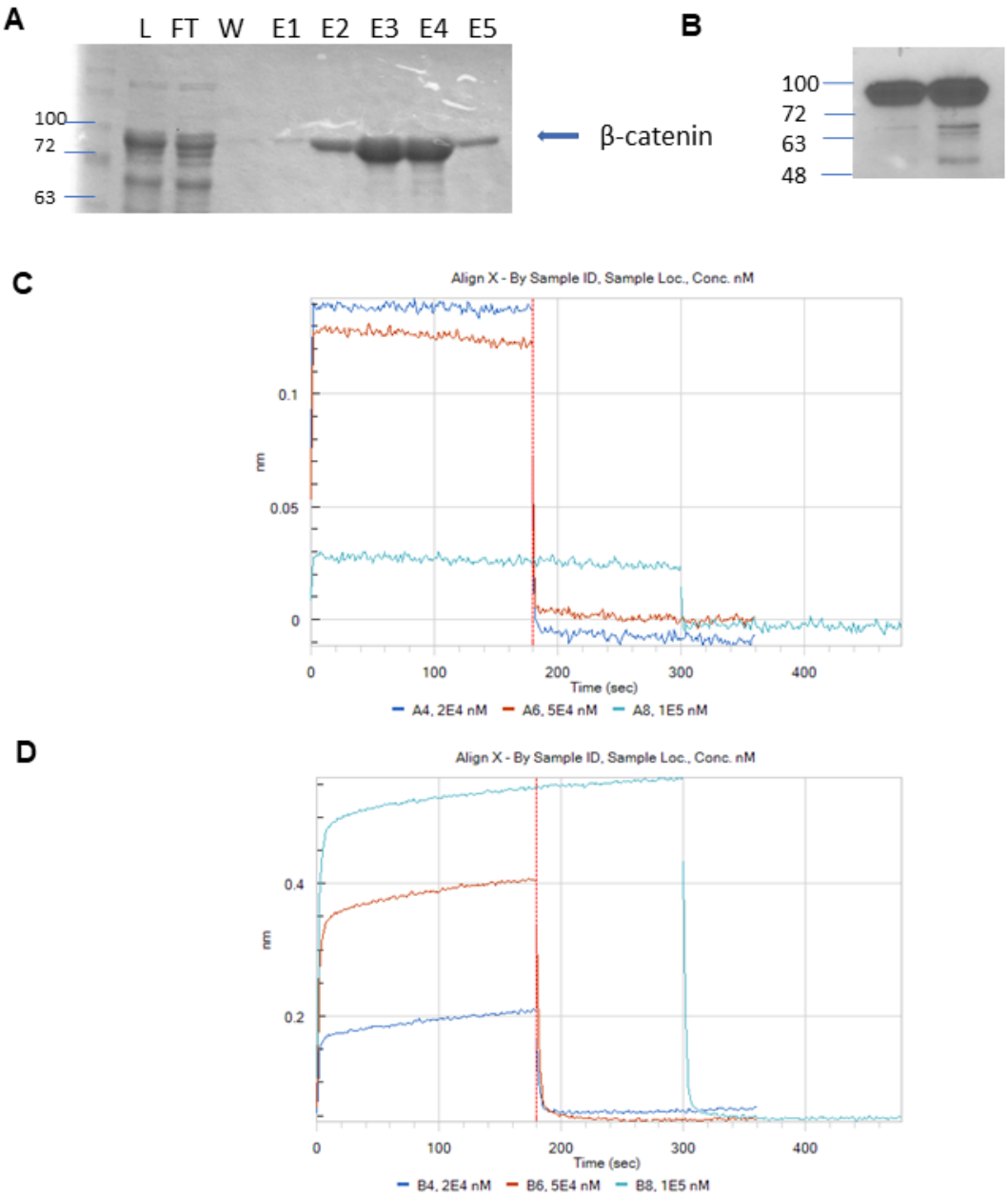



Fig. 11: Interactions between C2, C10 and recombinant β-catenin. A). His-tagged β-catenin was purified from *E.coli* BL21 which was transformed with pET28a+ vector carrying the full-length human β-catenin cDNA (L: lysate; FT: Flow through; W: Wash; E: Elution). Eluent samples were loaded on 8% SDS-PAGE gel and visualized by Coomassie stain. B). Confirmation of purified β-catenin protein using Western blot. The purified proteins were confirmed by Western blotting using anti-6X His tag antibody. Samples were run on 15% SDS-PAGE gel. Binding sensorgrams showing the interactions between C2 and β-catenin (C) and between C10 and β-catenin (D). The binding sensorgrams were generated by normalizing to the negative control (DMSO sample). There are three curves on the binding sensorgrams corresponding to different concentrations of C2 and C10: 20μM (blue), 50μM (red) and 100μM (green).

**DISCUSSION**

Wnt signaling plays critical roles in the carcinogenesis of EOC. Genetic mutations in the β-catenin pathway have been found frequently on the endometrioid subtypes of EOC, which leads to the hyperactivation of the signaling pathway (16,36). In addition, the dysregulation of interacting partners which assist in fine-tuning the signaling is detected in different subtypes of EOC, further increasing β-catenin stability and formation of the transcription complex (42,47-49). Although a number of small molecules have been developed and introduced to target Wnt components and β-catenin, none of them has been approved as targeted therapies for EOC or other types of cancer with hyperactive Wnt signaling.

In this study, we identified two novel β-catenin inhibitors, C2 and C10, that have strong anti-tumor effects. C2 and C10 exhibited more significant inhibitory effects on TOV-112 cell growth and spheroid formation compared to other cell lines. Between OVCAR-3 and SKOV3.ip1 cells, C2 and C10 were slightly more effective in suppressing cell proliferation and spheroid formation in OVCAR-3 cells than in SKOV3.ip1 cells. Among examined EOC cells, C2 and C10 were least effective on HEY cells. Consistent with the results of these functional studies, we found that TOV-112D had the highest levels of total β-catenin and nuclear β-catenin, followed by OVCAR-3 and SKOV3.ip1 cells, while HEY cells expressed the lowest total β-catenin levels. The results suggest the effectiveness of C2 and C10 are positively correlated with β-catenin activity.

It is commonly believed that β-catenin is phosphorylated and degraded in the cytoplasm (10). In this study, we detected phospho-β-catenin in the nuclear fraction of EOC cell lines. This is unlikely due to the cytoplasmic contamination as cytoplasmic marker GAPDH was not detected in the nuclear fraction. Previous studies also reported the presence of phospho-β-catenin in the

nucleus in other cells, such as colorectal cells (50,51). Interestingly, inhibition of proteasomal activity led to nuclear translocation of phospho-β catenin (51). It has been reported that phosphorylated β-catenin could form a complex with LEF-1 but failed to bind to DNA (51), suggesting phosphorylated β-catenin remains functionally inactive in the nuclear.

Ovarian CSCs are implicated in chemoresistance, metastasis, and cancer relapse and therefore represent a major challenge in the treatment of EOC (16,17,49,52-54). The Wnt/β-catenin signaling has been reported to play a crucial role in CSC self-renewal (16,49,54). We examined the effects of C2 and C10 on CSCs by performing spheroid formation assays and by measuring stem cell marker expression in several EOC cell lines. We showed that both C2 and C10 significantly inhibited the ability of EOCs to form spheroids, when plated in low density under stem cell conditions. These results suggest that C2 and C10 suppress the self-renewal ability of ovarian CSCs. Consistent with this notion, we also found that mRNA levels of several CSC markers were significantly inhibited by C10. We observed that C10 reduced the mRNA levels of *CD24*, *ALDH1A1,* and *SOX2* in TOV-112D spheroids and *EpCAM*, *CD44*, and *OCT4* in HEY spheroids. Preliminary studies also revealed a decrease in $ALDH^{br}$ population in C10-treated SKOV3.ip1 cells. *ALDH1A1* was identified as a direct target of β-catenin in ovarian cancer spheroids (55), while *CD24* (56), *CD44* (57), *CD133* (58) and *EpCAM* (59) were reported as direct target genes of β-catenin/TCF signaling in other cancer types. In addition, TCF3 has been reported to bind to the promoter regions of *OCT4, SOX2* and *NANOG* in mouse embryonic stem cells (60), and it was suggested that the interaction between β-catenin and TCF3 altered repressive chromatin state to activate transcription of the target genes (61). Therefore, it is possible that these stem cell markers are direct transcriptional targets of β-catenin/TCFs in EOC cells and the inhibitory effects

of C2 and C10 on these markers are due to the inhibition of β-catenin/TCF interaction. The stem cell markers have been reported to involve in ovarian cancer tumorigenesis such as stemness, chemoresistance, and metastasis (62). In addition, Oct4 and Sox2 are pluripotency core transcription factors which enhance expression of a subset of genes to promote cell programming to a pluripotent state (60). Thus, the inhibition of spheroid formation and downregulation of stem cell markers after C2 and C10 treatment indicated C2 and C10 target and inhibit the tumorigenic potential of CSCs.

Previous studies have reported different CSC markers in different EOC cell lines (63-65). Similarly, we found the upregulation of *ALDH1A1*, *CD44* and *CD133* mRNA levels in SKOV3 and OVCAR-3 spheroids compared to the conventional cultured cells (63-65). In contrast to the previous finding which showed that *CD44* expression was upregulated in TOV-112D CSCs (63), we did not observe a significant increase in *CD44* mRNA levels in TOV-112D spheroids. The difference might be stemming from different approaches. While stem cell markers were examined by flow cytometer at protein levels (63,65), we only measured mRNA levels of these marker genes. More studies are required to determine if CD44 is a CSC marker in TOV-112D cells.

Several lines of evidence support the role of C2 and C10 as β-catenin inhibitors. Luciferase reporter assays revealed that C2 and C10 inhibited β-catenin/TCF transcriptional activity. In addition, they downregulated the β-catenin/TCF target genes, including Axin2, c-myc, and cyclin D1. Among β-catenin direct target genes, Axin2 expression is a more immediate indicator of β-catenin activity. Axin2 plays a role in forming the β-catenin destruction complex, and it is rapidly induced by β-catenin/TCF transcriptional complex to exert negative feedback on Wnt/ β-catenin signaling (66). In addition, c-Myc and cyclin D1 have been reported to involve in cancer

progression (67). Furthermore, preliminary studies suggest that C2 and C10 can directly bind to recombinant β-catenin *in vitro*. Finally, work done by others in our lab indicates that both C2 and C10 decreased the interaction between β-catenin and TCF4, in co-immunoprecipitation assays. However, more studies are required to determine that C2 and C10 exert antitumor effects specifically by inhibiting β-catenin/TCF4 interaction.

We found that C2 and C10 had potent antitumor effects on suppressing cell proliferation, anchorage-independent growth and spheroid formation of several cell lines representing two different subtypes of ovarian cancer, endometrioid carcinoma, and high-grade serous carcinoma. Comparing to the recent developed inhibitors, C2 and C10 exhibit lower cell toxicity compared to PRI-724 and more effective than iCRT3 in suppressing anchorage-independent growth of cancer cells. Additional examinations are underway to further define the anti-tumor effects of C2 and C10 on cell migration and invasion *in vitro* and *in vivo* and their specificity in inhibiting abnormal activation of Wnt/β-catenin signaling. Since Wnt/β-catenin is implicated in many types of cancer, development of C2 and C10 into a targeted therapy will benefit cancer patients with hyperactive Wnt/β-catenin signaling.

# CHAPTER 3

# SUMMARY AND FUTURE DIRECTIONS

Ovarian cancer is the leading cause of death among gynecologic malignancies (1). Clinical management of ovarian cancer remains a major challenge due to the development of chemoresistance. Recent studies reveal cancer stem cells, a subpopulation of cancer cells, are linked to chemoresistance, cancer metastasis, and cancer relapse (2,3). In addition, Wnt signaling is highly active in these cancer stem cells and is involved in maintaining cancer stemness, cancer progression, and relapse (4). Wnt signaling is mediated by the translocation of β-catenin to the nucleus and its interaction with TCF transcription factors to regulate target gene expression (2). We aim to develop a potent β-catenin inhibitor that disrupts its interaction with TCF4. Using *in-silico* screening and functional assays, we found two novel β-catenin inhibitors, referred to as C2 and C10, exhibited promising anti-tumor effects on EOC cell lines *in vitro.*

In this dissertation, I investigated the antitumor effects of C2 and C10 on four EOC cell lines which represented two subtypes of EOC: endometrioid and serous ovarian carcinoma. I found that C2 and C10 inhibited cell proliferation and the anchorage-independent growth of these cells. I also performed spheroid formation assays and measured CSC markers and observed that C2 and C10 reduced the numbers of spheroids formed and the mRNA levels of CSC markers. These findings suggest that C2 and C10 inhibit CSC self-renewal. However, it has been suggested that only low numbers of CSCs are isolated in spheroid formation assays (5). Therefore, future studies will use a cell sorter to select CSCs and directly test the effects of C2 and C10 in these cells. Stem cell markers such as ALDH1A1, CD133, and CD44 are well-studied markers which have been shown to be involved in ovarian cancer stemness and carcinogenesis (6,7). We will isolate CSCs that express these markers and treat them with C2 and C10 to confirm that C2 and C10 target CSCs and suppress their self-renewal and tumorigenicity.

Wnt signaling is also involved in cell migration and invasion through EMT, and metastasis in ovarian cancer (2). Work done by others in the lab has shown that C2 significantly inhibited tumor burden *in vivo*. We will perform the same experiment to examine the anti-metastatic effect of C10. In addition, we will further determine the anti-tumor effects of C2 and C10 on cell migration, and invasion *in vitro*. Wound-healing assays will be performed to determine the effects of C2 and C10 on migration. In addition, the effect of C2 and C10 on the invasive potential of ovarian cancer cells to neighboring tissues will be measured by Transwell Invasion Assay. Treatments with C2 and C10 are expected to disrupt Wnt signaling and inhibit migration and invasion of ovarian cancer.

The involvement of Wnt signaling in angiogenesis and immune suppression has been suggested recently (8,9). Since these are important aspects of cancer development, it would be interesting to determine if C2 and C10 reduce tumor angiogenesis and immune suppression. Endothelium cells will be co-cultured with EOC cells treated with control or C2/C10 (10). The assessment of C2 and C10 ability to promote immune response can be obtained by determining the ratio of $CD8^+$ T cell: Treg in tumors collected from *in vivo* mouse model which are treated with control or C2/C10. The increase in helper T cells enhances cancer immune surveillance and is associated with better outcome (11).

In this study, I provided evidence that C2 and C10 inhibit β-catenin/TCF signaling. I showed that C2 and C10 inhibited the transcriptional activity of β-catenin/TCF, as evidenced by the decreased in TOPFlash reporter activity and protein levels of β-catenin/TCF target genes. In addition, the effect of C2 and C10 appears to be positively correlated with β-catenin levels. Preliminary kinetic binding studies suggest that C2 and C10 bind to β-catenin directly. This assay

can be repeated with the addition of TCF4 peptide. TCF4 peptide will be purified and added with C2 and C10 in the same wells for competitive binding assays. The biosensor will be loaded with full-length β-catenin, and the association with either the small inhibitor or TCF will be observed on the sensorgrams. We will be able to determine the concentrations at which C2 and C10 completely inhibit the interactions between β-catenin and TCF. Furthermore, we will investigate whether the anti-tumor effects of C2 and C10 are specific to hyperactive Wnt/β-catenin phenotypes. We will generate β-catenin knockout cell lines and perform functional assays. Due to the loss of β-catenin, we expect that C2 and C10 exhibit no effects in these cell lines.

Lastly, we will modify the structures of C2 and C10 to improve their efficacy and potency. C2 and C10 are readily dissolved in DMSO but are insoluble in most solvents at high concentrations. This incurs a problem when C2 and C10 are administered into mouse model since C2 and C10 are dissolved in a high percentage of DMSO which could be toxic to cells. In addition, the solubility of C2 and C10 is a challenge when dissolving the inhibitors in the same buffer as for the recombinant β-catenin proteins for biophysics binding assays. Therefore, modifying the structures of C2 and C10 would improve its solubility, which will help determine the toxicity and efficacy of C2 and C10 more accurately.

## CONCLUSION

Extensive research provides a better understanding of the molecular mechanisms of ovarian carcinogenesis and therapeutic targets for ovarian cancer treatment. The Wnt/β-catenin signaling pathway has been shown to has a great implication in ovarian cancer carcinogenesis. In this study, we present two novel β-catenin inhibitors which exhibit potent inhibitory effects on ovarian cancer cell proliferation, anchorage-independent growth, and stem cell renewal. Further examinations on their anti-tumor effects and molecular mechanisms would help elucidate their potentials in therapeutic development which will benefit ovarian cancer patients with abnormal Wnt/β-catenin signaling.

# SUPPLEMENTARY DATA

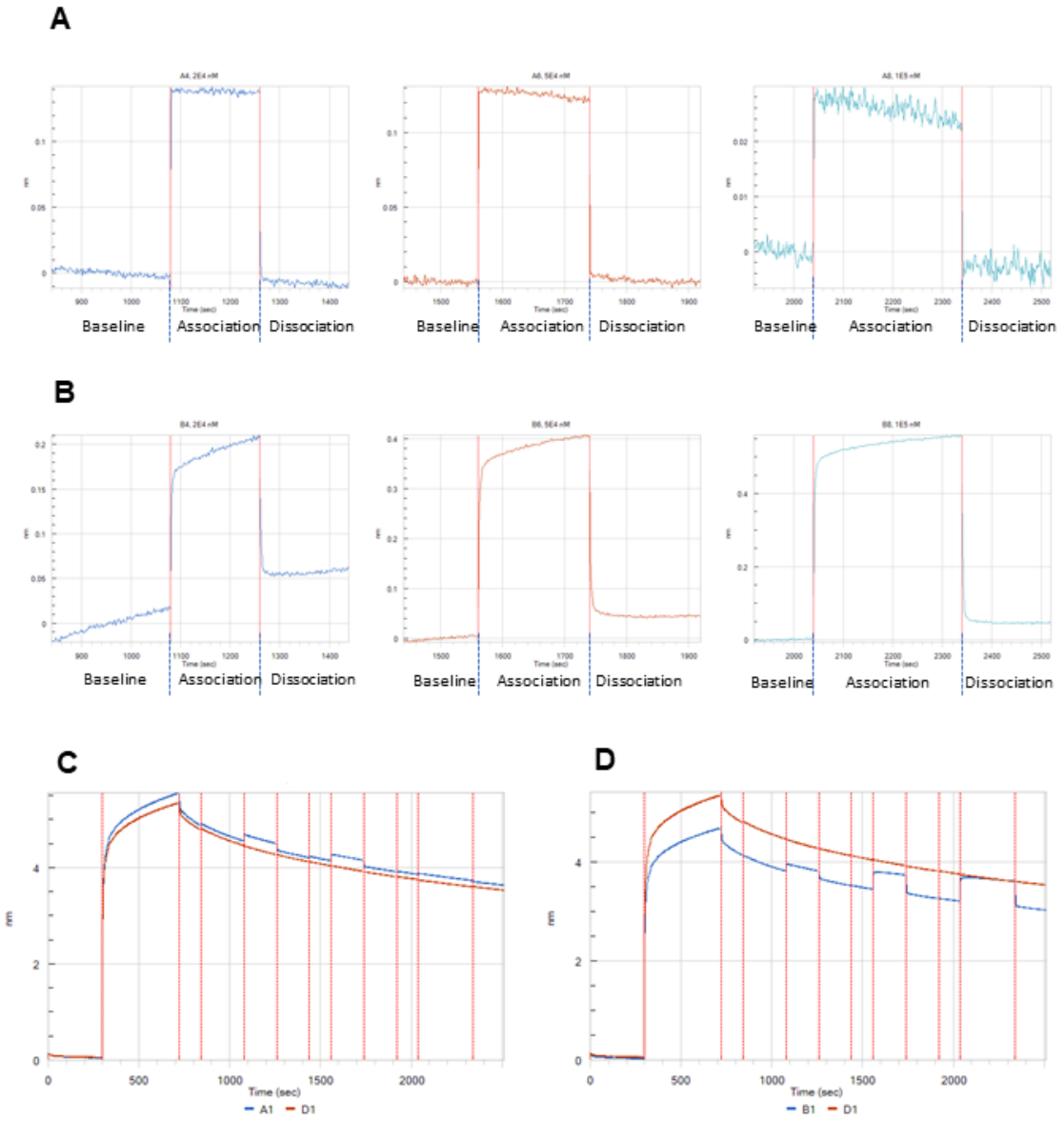



Figure S1: BLI results showing the interactions between C2 and β-catenin and between C10 and β-catenin. Binding kinetic sensorgrams of C2/β-catenin (A) and C10/β-catenin (B) interaction. The binding sensorgrams were generated by normalizing to the negative control (A1: C2, B1: C10, D1: negative control). The three curves on the binding sensorgrams corresponding to different concentrations of C2 and C10: 20μM (blue), 50μM (red) and 100μM (green). Raw binding curves of C2/β-catenin (C) and C10/β-catenin (D) interaction.

# APPENDIX

# REFERENCES

## Chapter 1


1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA: A Cancer Journal for Clinicians. 2019;69(1):7-34.
2. Medicine Io, National Academies of Sciences E, Medicine. Ovarian Cancers: Evolving Paradigms in Research and Care. Washington, DC: The National Academies Press; 2016. 396 p.
3. Kurman RJ, Shih IM. The origin and pathogenesis of epithelial ovarian cancer: A proposed unifying theory. Am J Surg Pathol. 2010; 34(3):433-443.
4. Marquez RT, Baggerly KA, Patterson AP, Liu J, Broaddus R, Frumovitz M, et al. Patterns of gene expression in different histotypes of epithelial ovarian cancer correlate with those in normal fallopian tube, endometrium, and colon. Clin Cancer Res. 2005; 11(17):6116-6126.
5. Kindelberger DW, Lee Y, Miron A, Hirsch MS, Feltmate C, Medeiros F, et al. Intraepithelial carcinoma of the fimbria and pelvic serous carcinoma: Evidence for a causal relationship. Am J Surg Pathol. 2007; 31(2):161-169.
6. Y. L, F. M, D. K, M.J. C, M.G. M, Lee Y, et al. Advances in the recognition of tubal intraepithelial carcinoma: Applications to cancer screening and the pathogenesis of ovarian cancer. Adv Anat Pathol. 2006; 13(1):1-7.
8. Przybycin CG, Kurman RJ, Ronnett BM, Shih IM, Vang R. Are all pelvic (Nonuterine) serous carcinomas of tubal origin? Am J Surg Pathol. 2010; 34(10):1407-1416.
9. Brinton LA, Sakoda LC, Sherman ME, Frederiksen K, Kjaer SK, Graubard BI, et al. Relationship of benign gynecologic diseases to subsequent risk of ovarian and uterine tumors. Cancer Epidemiol Biomarkers Prev. 2005; 14(12):2929-2935.
10. DePriest PD, Banks ER, Powell DE, van Nagell JR, Gallion HH, Puls LE, et al. Endometrioid carcinoma of the ovary and endometriosis: The association in postmenopausal women. Gynecol Oncol. 1992; 47(1):71-75.
11. Eržen M, Rakar S, Klančar B, Syrjänen K. Endometriosis-associated ovarian carcinoma (EAOC): An entity distinct from other ovarian carcinomas as suggested by a nested case-control study. Gynecol Oncol. 2001; 83(1):100-108.
12. Forte A, Cipollaro M, Galderisi U. Genetic, epigenetic and stem cell alterations in endometriosis: new insights and potential therapeutic perspectives. Clin Sci. 2014; 126(2):123-138.
13. Pavone ME, Lyttle BM. Endometriosis and ovarian cancer: Links, risks, and challenges faced. International Journal of Women's Health. 2015. 7:663-672.
14. Fukunaga M, Nomura K, Ishikawa E, Ushigome S. Ovarian atypical endometriosis: Its close association with malignant epithelial tumours. Histopathology. 1997; 30(3):249-255.
15. Sainz De La Cuesta R, Eichhorn JH, Rice LW, Fuller AF, Nikrui N, Goff BA. Histologic transformation of benign endometriosis to early epithelial ovarian cancer. Gynecol Oncol. 1996; 60(2):238-244.

16. Veras E, Mao TL, Ayhan A, Ueda S, Lai H, Hayran M, et al. Cystic and adenofibromatous clear cell carcinomas of the ovary: Distinctive tumors that differ in their pathogenesis and behavior: A clinicopathologic analysis of 122 cases. Am J Surg Pathol. 2009; 33(6):844-853.
17. McConechy MK, Ding J, Senz J, Yang W, Melnyk N, Tone AA, et al. Ovarian and endometrial endometrioid carcinomas have distinct CTNNB1 and PTEN mutation profiles. Mod Pathol. 2014; 27(1):128-134.
18. Jones S, Wang TL, Shih IM, Mao TL, Nakayama K, Roden R, et al. Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. Science (80- ). 2010; 330(6001):228-231.
19. Matsumoto T, Yamazaki M, Takahashi H, Kajita S, Suzuki E, Tsuruta T, et al. Distinct β-catenin and PIK3CA mutation profiles in endometriosis-associated ovarian endometrioid and clear cell carcinomas. Am J Clin Pathol. 2015; 144(3):452-463.
20. Cho KR, Shih I-M. Ovarian cancer. Annu Rev Pathol [Internet]. 2009; 4:287–313. Available from: https://www.ncbi.nlm.nih.gov/pubmed/18842102
21. McConechy MK, Anglesio MS, Kalloger SE, Yang W, Senz J, Chow C, et al. Subtype-specific mutation of PPP2R1A in endometrial and ovarian carcinomas. J Pathol. 2011; 223(5):567-573.
22. Shih IM, Panuganti PK, Kuo KT, Mao TL, Kuhn E, Jones S, et al. Somatic mutations of PPP2R1A in ovarian and uterine carcinomas. Am J Pathol. 2011; 178(4):1442-1447.
23. Rechsteiner M, Zimmermann AK, Wild PJ, Caduff R, von Teichman A, Fink D, et al. TP53 mutations are common in all subtypes of epithelial ovarian cancer and occur concomitantly with KRAS mutations in the mucinous type. Exp Mol Pathol. 2013; 95(2):235-241.
24. Anglesio MS, Kommoss S, Tolcher MC, Clarke B, Galletta L, Porter H, et al. Molecular characterization of mucinous ovarian tumours supports a stratified treatment approach with HER2 targeting in 19% of carcinomas. J Pathol. 2013; 229(1):111-120.
25. Tone AA, McConechy MK, Yang W, Ding J, Yip S, Kong E, et al. Intratumoral heterogeneity in a minority of ovarian low-grade serous carcinomas. BMC Cancer. 2014; 14:982.
26. Vang R, Shih IM, Kurman RJ. Ovarian low-grade and high-grade serous carcinoma: Pathogenesis, clinicopathologic and molecular biologic features, and diagnostic problems. Advances in Anatomic Pathology. 2009; 16(5):267-282.
27. Williams TI, Toups KL, Saggese DA, Kalli KR, Cliby WA, Muddiman DC. Epithelial ovarian cancer: Disease etiology, treatment, detection, and investigational gene, metabolite, and protein biomarkers. Journal of Proteome Research. 2007; 6(8), 2936-62.
28. Shih IM, Kurman RJ. Ovarian Tumorigenesis: A Proposed Model Based on Morphological and Molecular Genetic Analysis. American Journal of Pathology. 2004;164(5): p. 1511-8.
29. Luvero D, Milani A, Ledermann JA. Treatment options in recurrent ovarian cancer: Latest evidence and clinical potential. Vol. 6, Therapeutic Advances in Medical Oncology. 2014. p. 229–39.
30. Coleman RL, Monk BJ, Sood AK, Herzog TJ. Latest research and treatment of advanced-stage epithelial ovarian cancer. Nature Reviews Clinical Oncology. 2013;10:211.
31. Cortez AJ, Tudrej P, Kujawa KA, Lisowska KM. Advances in ovarian cancer therapy. Cancer

Chemotherapy and Pharmacology. 2018; 81:17-38.
32. Parkes EE, Kennedy RD. Clinical Application of Poly(ADP-Ribose) Polymerase Inhibitors in High-Grade Serous Ovarian Cancer. Oncologist. 2016; 21:586-593.
33. Logan CY, Nusse R. The Wnt Signaling pathway in development and disease. Annu Rev Cell Dev Biol. 2004;20:781–810.
34. Stamos JL, Weis WI. The β-catenin destruction complex. Cold Spring Harb Perspect Biol. 2013;5(1):a007898.
35. Liu C, Li Y, Semenov M, Han C, Baeg G-H, Tan Y, et al. Control of β-Catenin Phosphorylation/Degradation by a Dual-Kinase Mechanism. Cell. 2002;108(6):837-47.
36. Ha NC, Tonozuka T, Stamos JL, Choi HJ, Weis WI. Mechanism of phosphorylation-dependent binding of APC to β-catenin and its role in β-catenin degradation. Mol Cell. 2004;15(4):511–21.
37. Jho E-h, Lomvardas S, Costantini F. A GSK3β Phosphorylation Site in Axin Modulates Interaction with β-Catenin and Tcf-Mediated Gene Expression. Biochemical and Biophysical Research Communications. 1999;266(1):28-35.
38. Kim SE, Huang H, Zhao M, Zhang X, Zhang A, Semonov M V, et al. Wnt stabilization of β-catenin reveals principles for morphogen receptor-scaffold assemblies. Science. 2013 May;340(6134):867–70.
39. Wu G, Xu G, Schulman BA, Jeffrey PD, Harper JW, Pavletich NP. Structure of a β-TrCP1-Skp1-β-Catenin Complex: Destruction Motif Binding and Lysine Specificity of the SCFβ-TrCP1 Ubiquitin Ligase. Molecular Cell. 2003;11(6):1445-56.
40. Tauriello DVF, Jordens I, Kirchner K, Slootstra JW, Kruitwagen T, Bouwman BAM, et al. Wnt/β-catenin signaling requires interaction of the Dishevelled DEP domain and C terminus with a discontinuous motif in Frizzled. Proceedings of the National Academy of Sciences. 2012;109(14):E812.
41. Nusse R, Clevers H. Wnt/β-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. Cell. 2017;169(6):985-99.
42. Clevers H, Nusse R. Wnt/β-catenin signaling and disease. Vol. 149, Cell. 2012. p. 1192–205.
43. Wu R, Zhai Y, Fearon ER, Cho KR. Diverse Mechanisms of β-Catenin Deregulation in Ovarian Endometrioid Adenocarcinomas. Cancer Res. 2001;61(22):8247–55.
44. Filho LB, Oshima CTF, Lima F de O, Costa H de O, Damaão R de S, Gomes TS, et al. Canonical and noncanonical Wnt pathway: A comparison among normal ovary, benign ovarian tumor and ovarian cancer. Oncol Rep. 2009;21(2):313–20.
43. Gamallo C, Palacios J, Moreno G, De Mora JC, Suárez A, Armas A. β-Catenin expression pattern in stage I and II ovarian carcinomas: Relationship with β-catenin gene mutations, clinicopathological features, and clinical outcome. Am J Pathol. 1999;155(2):527–36.
44. Sarrio D, Moreno-bueno G, Sa C, Ban I, Hardisson D. Expression of cadherins and catenins correlates with distinct histologic types of ovarian carcinomas. 2006;1042–9.
45. Polakis P. The many ways of Wnt in cancer. Vol. 17, Current Opinion in Genetics and Development. 2007. p. 45–51.
46. MacDonald BT, Tamai K, He X. Wnt/β-Catenin Signaling: Components, Mechanisms, and Diseases. Dev Cell. 2009;17(1):9–26.

47. Gershoni-Baruch R, Patael Y, Dagan, Figer A, Kasinetz L, Kadouri E, et al. Association of the I1307K APC mutation with hereditary and sporadic breast/ovarian cancer: more questions than answers. British Journal of Cancer. 2000;83(2):153-5.
48. Mueller JJ, Schlappe BA, Kumar R, Olvera N, Dao F, Abu-rustum N, et al. Massively parallel sequencing analysis of mucinous ovarian carcinomas: genomic profiling and differential diagnoses. Gynecol Oncol [Internet]. 2018;150(1):127–35. Available from: https://doi.org/10.1016/j.ygyno.2018.05.008
39. Filho LB, Oshima CTF, Lima F de O, Costa H de O, Damaão R de S, Gomes TS, et al. Canonical and noncanonical Wnt pathway: A comparison among normal ovary, benign ovarian tumor and ovarian cancer. Oncol Rep. 2009;21(2):313–320.
50. Yoshioka S, King ML, Ran S, Okuda H, MacLean JA, McAsey ME, et al. WNT7A Regulates Tumor Growth and Progression in Ovarian Cancer through the WNT/ β-Catenin Pathway. Mol Cancer Res. 2012;10(3):469–83.
51. Liu Y, Meng F, Xu Y, Yang S, Xiao M, Chen X, et al. Overexpression of Wnt7a Is Associated With Tumor Progression and Unfavorable Prognosis in Endometrial Cancer. International Journal of Gynecologic Cancer. 2013;23(2):304.
52. Liu Q, Zhao Y, Xing H, Li L, Li R, Dai J, et al. The role of R-spondin 1 through activating Wnt/β-catenin in the growth, survival and migration of ovarian cancer cells. Gene. 2019;689:124–30.
53. de Lau W, Peng WC, Gros P, Clevers H. The R-spondin/Lgr5/Rnf43 module: Regulator of Wnt signal strength. Genes and Development. 2014; 28: 305-316
54. Kim KA, Zhao J, Andarmani S, Kakitani M, Oshima T, Binnerts ME, et al. R-spondin proteins: A novel link to β-catenin activation. Cell Cycle. 2006;5(1):23–6.
55. Kuchenbaecker KB, Ramus SJ, Tyrer J, Lee A, Shen HC, Beesley J, et al. Identification of six new susceptibility loci for invasive epithelial ovarian cancer. Nature Genetics. 2015;47:164.
56. Schindler AJ, Watanabe A, Howell SB. LGR5 and LGR6 in stem cell biology and ovarian cancer. Oncotarget. 2018;9(1):1346–55.
57. Ruan X, Liu A, Zhong M, Wei J, Zhang W, Rong Y, et al. Silencing LGR6 Attenuates Stemness and Chemoresistance via Inhibiting Wnt/β-Catenin Signaling in Ovarian Cancer. Mol Ther - Oncolytics. 2019;14:94–106.
58. Cruciat CM, Niehrs C. Secreted and transmembrane Wnt inhibitors and activators. Cold Spring Harb Perspect Biol. 2013; 5(3): a015081.
59. Barbolina M V, Liu Y, Gurler H, Kim M, Kajdacsy-Balla AA, Rooper L, et al. Matrix rigidity activates wnt signaling through down-regulation of dickkopf-1 protein. J Biol Chem. 2013; 288(1): 141–151.
60. Zhu J, Zhang S, Gu L, Di W. Epigenetic silencing of DKK2 and Wnt signal pathway components in human ovarian carcinoma. Carcinogenesis. 2012;33(12):2334–43.
61. Jacob F, Ukegjini K, Nixdorf S, Ford CE, Olivier J, Caduff R, et al. Loss of secreted frizzled-related protein 4 correlates with an aggressive phenotype and predicts poor outcome in ovarian cancer patients. PLoS One. 2012; 7(2): e31885.
62. Saran U, Arfuso F, Zeps N, Dharmarajan A. Secreted frizzled-related protein 4 expression is positively associated with responsiveness to Cisplatin of ovarian cancer cell lines in vitro and with lower tumour grade in mucinous ovarian cancers. BMC Cell Biology.

2012;13(1): 25.
63. Ford CE, Jary E, Ma SSQ, Nixdorf S, Heinzelmann-Schwarz VA, Ward RL. The Wnt Gatekeeper SFRP4 Modulates EMT, Cell Migration and Downstream Wnt Signaling in Serous Ovarian Cancer Cells. PLOS ONE. 2013;8(1):e54362.
64. Duan H, Yan Z, Chen W, Wu Y, Han J, Guo H, et al. TET1 inhibits EMT of ovarian cancer cells through activating Wnt/β-catenin signaling inhibitors DKK1 and SFRP2. Gynecologic Oncology. 2017;147(2):408-17.
65. Kohli RM, Zhang Y. TET enzymes, TDG and the dynamics of DNA demethylation. Vol. 502, Nature. 2013. p. 472–9.
66. Xu G, Bernaudo S, Fu G, Lee DY, Yang BB, Peng C. Cyclin G2 Is Degraded through the Ubiquitin-Proteasome Pathway and Mediates the Antiproliferative Effect of Activin Receptor-like Kinase 7. Mol Biol Cell. 2008;19(11):4968–79.
67. Bernaudo S, Salem M, Qi X, Zhou W, Zhang C, Yang W, et al. Cyclin G2 inhibits epithelial-to-mesenchymal transition by disrupting Wnt/β-catenin signaling. Oncogene. 2016;35(36):4816–27.
68. Gao J, Zhao C, Liu Q, Hou X, Li S, Xing X, et al. Cyclin G2 suppresses Wnt/β-catenin signaling and inhibits gastric cancer cell growth and migration through Dapper1. Journal of Experimental & Clinical Cancer Research. 2018;37(1):317.
69. Ma B, Liu B, Cao W, Gao C, Qi Z, Ning Y, et al. The Wnt signaling antagonist dapper1 accelerates dishevelled2 degradation via promoting its ubiquitination and aggregate-induced autophagy. J Biol Chem. 2015; 290(19): 12346–12354.
70. Teran E, Branscomb AD, Seeling JM. Dpr Acts as a Molecular Switch, Inhibiting Wnt Signaling when Unphosphorylated, but Promoting Wnt Signaling when Phosphorylated by Casein Kinase Iδ/ε. PLOS ONE. 2009;4(5):e5522.
71. Li RN, Liu B, Li XM, Hou LS, Mu XL, Wang H, et al. DACT1 Overexpression in type I ovarian cancer inhibits malignant expansion and cis-platinum resistance by modulating canonical Wnt signaling and autophagy. Sci Rep. 2017;7(1):9285.
72. Liu H, Shi H, Fan Q, Sun X. Cyclin Y regulates the proliferation, migration, and invasion of ovarian cancer cells via Wnt signaling pathway. Tumor Biol. 2016;37(8):10161–75.
73. Davidson G, Niehrs C. Emerging links between CDK cell cycle regulators and Wnt signaling. Trends in Cell Biology. 2010;20(8):453-60.
74. Ou-Yang J, Huang LH, Sun XX. Cyclin-Dependent Kinase 14 Promotes Cell Proliferation, Migration and Invasion in Ovarian Cancer by Inhibiting Wnt Signaling Pathway. Gynecologic and Obstetric Investigation. 2017;82(3):230-9.
75. Rask K, Nilsson A, Brännström M, Carlsson P, Hellberg P, Janson PO, et al. Wnt-signaling pathway in ovarian epithelial tumours: Increased expression of β-catenin and GSK3β. Br J Cancer. 2003;89(7):1298–304.
76. Shen C, Sheng Q, Zhang X, Fu Y, Zhu K. Hypermethylated APC in serous carcinoma based on a meta-analysis of ovarian cancer. Journal of Ovarian Research. 2016;9(1):60.
77. Tam KF, Liu VWS, Liu SS, Tsang PCK, Cheung ANY, Yip AMW, et al. Methylation profile in benign, borderline and malignant ovarian tumors. J Cancer Res Clin Oncol. 2007;133(5):331–41.
78. De Caceres II, Battagli C, Esteller M, Herman JG, Dulaimi E, Edelson MI, et al. Tumor cell-specific BRCA1 and RASSF1A hypermethylation in serum, plasma, and peritoneal fluid

from ovarian cancer patients. Cancer Res. 2004;64(18):6476–81.
79. Haikarainen T, Krauss S, Lehtio L. Tankyrases: Structure, Function and Therapeutic Implications in Cancer. Curr Pharm Des. 2014; 20(41):6472-88.
80. Bao R, Christova T, Song S, Angers S, Yan X, Attisano L. Inhibition of Tankyrases Induces Axin Stabilization and Blocks Wnt Signaling in Breast Cancer Cells. PLoS One. 2012;7(11):e48670.
81. Yang H-Y, Shen J-X, Wang Y, Liu Y, Shen D-Y, Quan S. Tankyrase Promotes Aerobic Glycolysis and Proliferation of Ovarian Cancer through Activation of Wnt/β -Catenin Signaling. Biomed Res Int. 2019;2019:14.
82. Hou R, Jiang L, Yang Z, Wang S, Liu Q. Rab14 is overexpressed in ovarian cancers and promotes ovarian cancer proliferation through Wnt pathway. Tumor Biology. 2016;37(12):16005-13.
83. Kwon M, Kim J-H, Rybak Y, Luna A, Choi CH, Chung J-Y, et al. Reduced expression of FILIP1L, a novel WNT pathway inhibitor, is associated with poor survival, progression and chemoresistance in ovarian cancer. Oncotarget. 2016; 7(47): 77052–77070.
84. Luo J. Glycogen synthase kinase 3β (GSK3β) in tumorigenesis and cancer chemotherapy. Cancer Letters. 2009;273(2):194-200.
85. Arend RC, Londono-Joshi AI, Straughn JM, Buchsbaum DJ. The Wnt/beta-catenin pathway in ovarian cancer: a review. Gynecol Oncol [Internet]. 2013;131. Available from: https://doi.org/10.1016/j.ygyno.2013.09.034
86. Yan X, Lyu T, Jia N, Yu Y, Hua K, Feng W. Huaier Aqueous Extract Inhibits Ovarian Cancer Cell Motility via the AKT/GSK3β/β-Catenin Pathway. PLOS ONE. 2013;8(5):e63731.
87. Kwon M, Lee SJ, Wang Y, Rybak Y, Luna A, Reddy S, et al. Filamin A interacting protein 1-like inhibits WNT signaling and MMP expression to suppress cancer cell invasion and metastasis. International Journal of Cancer. 2014;135(1):48-60.
88. Park Y-L, Park S-Y, Lee S-H, Kim R-B, Kim J-K, Rew S-Y, et al. Filamin A interacting protein 1-like expression inhibits progression in colorectal cancer. Oncotarget. 2016; 7(44): 72229–72241.
89. Takai Y, Sasaki T, Matozaki T. Small GTP-Binding Proteins. Physiological Reviews. 2001;81(1):153-208.
90. Huber AH, Weis WI. The structure of the β-catenin/E-cadherin complex and the molecular basis of diverse ligand recognition by β-catenin. Cell. 2001;105(3):391–402.
91. Xu W, Kimelman D. Mechanistic insights from structural studies of beta-catenin and its binding partners. J Cell Sci. 2007;120(19):3337–44.
92. Brembeck FH, Rosário M, Birchmeier W. Balancing cell adhesion and Wnt signaling, the key role of β-catenin. Curr Opin Genet Dev. 2006;16(1):51–9.
93. Kajiguchi T, Katsumi A, Tanizaki R, Kiyoi H, Naoe T. Y654 of β-catenin is essential for FLT3/ITD-related tyrosine phosphorylation and nuclear localization of β-catenin. European Journal of Haematology. 2012;88(4):314-20.
94. Satpathy M, Cao L, Pincheira R, Emerson R, Bigsby R, Nakshatri H, et al. Enhanced peritoneal ovarian tumor dissemination by tissue transglutaminase. Cancer Res. 2007;67(15):7192–202.
95. Condello S, Cao L, Matei D. Tissue transglutaminase regulates β-catenin signaling through a c-Src-dependent mechanism. The FASEB Journal. 2013;27(8):3100-12.

96. Burkhalter RJ, Westfall SD, Liu Y, Stack MS. Lysophosphatidic acid initiates epithelial to mesenchymal transition and Induces β-Catenin-mediated transcription in epithelial ovarian carcinoma. J Biol Chem. 2015;290(36):22143–54.
97. Chan DW, Mak CSL, Leung THY, Chan KKL, Ngan HYS. Down-regulation of Sox7 is associated with aberrant activation of Wnt/β-catenin signaling in endometrial cancer. Oncotarget. 2012; 3(12):1546-56.
98. Liu H, Yan Z-Q, Li B, Yin S-Y, Sun Q, Kou J-J, et al. Reduced expression of SOX7 in ovarian cancer: a novel tumor suppressor through the Wnt/β-catenin signaling pathway. Journal of Ovarian Research. 2014;7(1):87.
99. Du L, Qian X, Dai C, Wang L, Huang D, Wang S, et al. Screening the Molecular Targets of Ovarian Cancer Based on Bioinformatics Analysis. Tumori Journal. 2015;101(4):384-9.
100. Popadiuk CM, Xiong J, Wells MG, Andrews PG, Dankwa K, Hirasawa K, et al. Antisense Suppression of Pygopus2 Results in Growth Arrest of Epithelial Ovarian Cancer. Clinical Cancer Research. 2006;12(7):2216.
101. Benchabane H, Xin N, Tian A, Hafler BP, Nguyen K, Ahmed A, et al. Jerky/Earthbound facilitates cell-specific Wnt/Wingless signaling by modulating β-catenin-TCF activity. EMBO J. 2011;30(8):1444–58.
102. Pangon L, Ng I, Giry-Laterriere M, Currey N, Morgan A, Benthani F, et al. JRK is a positive regulator of β-catenin transcriptional activity commonly overexpressed in colon, breast and ovarian cancer. Oncogene. 2016;35(22):2834–2841.
103. Tassi RA, Todeschini P, Siegel ER, Calza S, Cappella P, Ardighieri L, et al. FOXM1 expression is significantly associated with chemotherapy resistance and adverse prognosis in non-serous epithelial ovarian cancer patients. J Exp Clin Cancer Res [Internet]. 2017 May 8;36(1):63. Available from: https://www.ncbi.nlm.nih.gov/pubmed/28482906
104. Zhao F, Siu MKY, Jiang L, Tam KF, Ngan HYS, Le XF, et al. Overexpression of Forkhead Box Protein M1 (FOXM1) in Ovarian Cancer Correlates with Poor Patient Survival and Contributes to Paclitaxel Resistance. PLoS One [Internet]. 2014 Nov 20;9(11):e113478. Available from: https://doi.org/10.1371/journal.pone.0113478
105. Wen N, Wang Y, Wen L, Zhao S-H, Ai Z-H, Wang Y, et al. Overexpression of FOXM1 predicts poor prognosis and promotes cancer cell proliferation, migration and invasion in epithelial ovarian cancer. J Transl Med [Internet]. 2014 May 20;12:134. Available from: https://www.ncbi.nlm.nih.gov/pubmed/24885308
106. Chiu W-T, Huang Y-F, Tsai H-Y, Chen C-C, Chang C-H, Huang S-C, et al. FOXM1 confers to epithelial-mesenchymal transition, stemness and chemoresistance in epithelial ovarian carcinoma cells. Oncotarget. 2015;6(4):2349–65.
107. Pratheeshkumar P, Divya SP, Parvathareddy SK, Alhoshani NM, Al-Badawi IA, Tulbah A, et al. FoxM1 and β-catenin predicts aggressiveness in Middle Eastern ovarian cancer and their co-targeting impairs the growth of ovarian cancer cells. Oncotarget [Internet]. 2017 Dec 16;9(3):3590–604. Available from: https://www.ncbi.nlm.nih.gov/pubmed/29423068
108. Zhang N, Wei P, Gong A, Chiu WT, Lee H Te, Colman H, et al. FoxM1 Promotes β-Catenin Nuclear Localization and Controls Wnt Target-Gene Expression and Glioma Tumorigenesis. Cancer Cell. 2011;20(4):427–42.

109. Zhan L, Li J, Wei B. Long non-coding RNAs in ovarian cancer. Journal of Experimental & Clinical Cancer Research. 2018;37(1):120.
110. Deb B, Uddin A, Chakraborty S. miRNAs and ovarian cancer: An overview. J Cell Physiol. 2018;233(5):119–23.
111. Tang MKS, Yue PYK, Ip PP, Huang R-L, Lai H-C, Cheung ANY, et al. Soluble E-cadherin promotes tumor angiogenesis and localizes to exosome surface. Nature Communications. 2018;9(1):2270.
112. Cannon M, Ghosh D, Gujja S. Signaling Circuits and Regulation of Immune Suppression by Ovarian Tumor-Associated Macrophages. Vaccines. 2015 May;3(2):448–66.
113. Ahmed N, Abubaker K, Findlay JK. Ovarian cancer stem cells: Molecular concepts and relevance as therapeutic targets. Molecular Aspects of Medicine. 2014;39:110-25.
114. Wu G, Liu A, Zhu J, Lei F, Wu S, Zhang X. MiR-1207 overexpression promotes cancer stem cell – like traits in ovarian cancer by activating the Wnt / β-catenin signaling pathway. 2015; 6(30): 28882–28894.
115. Parte SC, Batra SK, Kakar SS. Characterization of stem cell and cancer stem cell populations in ovary and ovarian tumors. J Ovarian Res. 2018; 11(1):69
116. Chen M-W, Yang S-T, Chien M-H, Hua K-T, Wu C-J, Hsiao SM, et al. The STAT3-miRNA-92-Wnt Signaling Pathway Regulates Spheroid Formation and Malignant Progression in Ovarian Cancer. Cancer Research. 2017;77(8):1955–68.
117. Silva IA, Bai S, McLean K, Yang K, Griffith K, Thomas D, et al. Aldehyde dehydrogenase in combination with CD133 defines angiogenic ovarian cancer stem cells that portend poor patient survival. Cancer Res. 2011; 71(11):3991-4001.
118. Beck B, Blanpain C. Unravelling cancer stem cell potential. Nat Rev Cancer. 2013;13(10):727–38.
119. Kang HG, Kim D-H, Kim S-J, Cho Y, Jung J, Jang W, et al. Galectin-3 supports stemness in ovarian cancer stem cells by activation of the Notch1 intracellular domain. Oncotarget. 2016;7(42):68229–68241.
120. Wang YC, Yo Y Te, Lee HY, Liao YP, Chao TK, Su PH, et al. ALDH1-bright epithelial ovarian cancer cells are associated with CD44 expression, drug resistance, and poor clinical outcome. Am J Pathol. 2012;180(3):1159–69.
121. Burgos-Ojeda D, Rueda BR, Buckanovich RJ. Ovarian cancer stem cell markers: Prognostic and therapeutic implications. Cancer Lett. 2012;322(1):1–7.
122. Garson K, Vanderhyden BC. Epithelial ovarian cancer stem cells: Underlying complexity of a simple paradigm. Reproduction. 2015; 149(2):R59-70.
123. Ffrench B, Gasch C, O'Leary JJ, Gallagher MF. Developing ovarian cancer stem cell models: laying the pipeline from discovery to clinical intervention. Mol Cancer. 2014;13(262):1–15.
124. Shah MM, Landen CN. Ovarian cancer stem cells: Are they real and why are they important? Gynecologic Oncology. 2014; 132(2):483-9.
125. Condello S, Morgan CA, Nagdas S, Cao L, Turek J, Hurley TD, et al. β-Catenin-regulated ALDH1A1 is a target in ovarian cancer spheroids. Oncogene [Internet]. 2014/06/23. 2015 Apr 30;34(18):2297–308. Available from: https://www.ncbi.nlm.nih.gov/pubmed/24954508

126. Nagaraj AB, Joseph P, Kovalenko O, Singh S, Armstrong A, Redline R, et al. Critical role of Wnt/β-catenin signaling in driving epithelial ovarian cancer platinum resistance. Oncotarget. 2015;6(27):23720-34.
127. Sun Y, Jia X, Wu X. High Expressions of Lgr5 and ALDH1 in Primary Epithelial Ovarian Cancer Correlate with Advanced Tumor Stage and Grade as well as Poor Prognosis of the Patients. Gynecol Obstet Invest. 2016; 81:162-168.
128. Flesken-Nikitin A, Hwang C Il, Cheng CY, Michurina T V, Enikolopov G, Nikitin AY. Ovarian surface epithelium at the junction area contains a cancer-prone stem cell niche. Nature. 2013;495(4770):241.
129. Pan H, Kim E, Rankin GO, Rojanasakul Y, Tu Y, Chen YC. Theaflavin-3,3'-digallate inhibits ovarian cancer stem cells via suppressing Wnt/β-Catenin signaling pathway. Journal of Functional Foods 2018;50(Complete):1-7
130. Deng S, Wong CKC, Lai H-C, Wong AST. Ginsenoside-Rb1 targets chemotherapy-resistant ovarian cancer stem cells via simultaneous inhibition of Wnt/β-catenin signaling and epithelial-to-mesenchymal transition. Oncotarget [Internet]. 2017 Apr 18;8(16):25897–914. Available from: https://www.ncbi.nlm.nih.gov/pubmed/27825116
131. Rosen DG, Mercado-Uribe I, Yang G, Bast RC, Amin HM, Lai R, et al. The role of constitutively active signal transducer and activator of transcription 3 in ovarian tumorigenesis and prognosis. Cancer. 2006;107(11):2730–40.
132. Mariya T, Hirohashi Y, Torigoe T, Tabuchi Y, Asano T, Saijo H, et al. Matrix metalloproteinase-10 regulates stemness of ovarian cancer stem-like cells by activation of canonical Wnt signaling and can be a target of chemotherapy-resistant ovarian cancer. Oncotarget. 2016;7(18):26806–26822.
133. Wang J, Zhou D, He X, Wang Y, Hu W, Jiang L, et al. Effect of downregulated β-catenin on cell proliferative activity, the sensitivity to chemotherapy drug and tumorigenicity of ovarian cancer cells. Cell Mol Biol (Noisy-le-grand). 2011; 57 Suppl OL1606-13.
134. Chau WK, Ip CK, Mak ASC, Lai HC, Wong AST. C-Kit mediates chemoresistance and tumor-initiating capacity of ovarian cancer cells through activation of Wnt/β-catenin-ATP-binding cassette G2 signaling. Oncogene. 2013; 32(22):2767-81.
135. Yamamoto TM, McMellen A, Watson ZL, Aguilera J, Ferguson R, Nurmemmedov E, et al. Activation of Wnt signaling promotes olaparib resistant ovarian cancer. Mol Carcinog [Internet]. 2019 Jun 10;0(0). Available from: https://doi.org/10.1002/mc.23064
136. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. Journal of Clinical Investigation. 2009; 119(6):1420-8.
137. Al-Alem L, Curry TE. Ovarian cancer: involvement of the matrix metalloproteinases. REPRODUCTION. 2015;150(2):R55–64.
138. Davidson B, Tropé CG, Reich R. Epithelial–Mesenchymal Transition in Ovarian Carcinoma. Front Oncol. 2012; 2:33. doi: 10.3389/fonc.2012.00033.
139. Kenny HA, Lengyel E. MMP-2 functions as an early response protein in ovarian cancer metastasis. Cell Cycle. 2009;8(5):683–8.
140. Lou Y, Jiang H, Cui Z, Wang L, Wang X, Tian T. Linc-ROR induces epithelial-to-mesenchymal transition in ovarian cancer by increasing Wnt/β-catenin signaling. Oncotarget. 2017;8(41):69983.
141. Zhang Y, Dun Y, Zhou S, Huang X. LncRNA HOXD-AS1 promotes epithelial ovarian cancer

cells proliferation and invasion by targeting miR-133a-3p and activating Wnt / β -catenin signaling pathway. Biomed Pharmacother [Internet]. 2017;96(August):1216–21. Available from: https://doi.org/10.1016/j.biopha.2017.11.096
142. Yu R, Cai L, Chi Y, Ding X, Wu X. miR-377 targets CUL4A and regulates metastatic capability in ovarian cancer. Int J Mol Med. 2018;41(6):3147–56.
143. Wang W-S, Yang X-S, Xia M, Jiang H-Y, Hou J-Q. Silencing of Twist Expression by RNA Interference Suppresses Epithelial-mesenchymal Transition, Invasion, and Metastasis of Ovarian Cancer. Asian Pacific J Cancer Prev. 2013; 13(9):4435-9.
144. Zhang LY, Chen Y, Jia J, Zhu X, He Y, Wu LM. MiR-27a promotes EMT in ovarian cancer through active Wnt/β-catenin signaling by targeting FOXO1. Cancer Biomarkers. 2019;24(1):31–42.
144. Li N, Yang L, Sun Y, Wu X. MicroRNA-16 inhibits migration and invasion via regulation of the wnt/β-catenin signaling pathway in ovarian cancer. Oncol Lett. 2019; 17(3):2631-2638. doi: 10.3892/ol.2019.9923.
145. Wei C, Zhang X, He S, Liu B, Han H, Sun X. MicroRNA-219-5p inhibits the proliferation, migration, and invasion of epithelial ovarian cancer cells by targeting the Twist/Wnt/β-catenin signaling pathway. Gene. 2017; 637:25-32.
146. Sun J, Yang X, Zhang R, Liu S, Gan X, Xi X, et al. GOLPH3 induces epithelial–mesenchymal transition via Wnt/β-catenin signaling pathway in epithelial ovarian cancer. Cancer Med. 2017;6(4):834–44.
147. Russell H, Pranjol MZI. Transcription factors controlling E-cadherin down-regulation in ovarian cancer. Biosci Horizons Int J Student Res. 2018; 11:hzy010. https://doi.org/10.1093/biohorizons/hzy010
148. Stemmer V, De Craene B, Berx G, Behrens J. Snail promotes Wnt target gene expression and interacts with β-catenin. Oncogene. 2008; 27(37):5075-80.
149. Curry TE, Osteen KG. The matrix metalloproteinase system: Changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. Endocrine Reviews. 2003; 24(4):428-65.
150. Hou R, Yang Z, Wang S, Chu D, Liu Q, Liu J, et al. miR-762 can negatively regulate menin in ovarian cancer. Onco Targets Ther. 2017; 10: 2127–37.
151. Wu B, Crampton SP, Hughes CCW. Wnt signaling induces matrix metalloproteinase expression and regulates T cell transmigration. Immunity [Internet]. 2007 Feb;26(2):227–39. Available from: https://www.ncbi.nlm.nih.gov/pubmed/17306568
152. Wang L, Jin X, Lin D, Liu Z, Zhang X, Lu Y, et al. Clinicopathologic significance of claudin-6, occludin, and matrix metalloproteinases -2 expression in ovarian carcinoma. Diagn Pathol [Internet]. 2013 Nov 19;8:190. Available from: https://www.ncbi.nlm.nih.gov/pubmed/24245968
153. Zohny SF, Fayed ST. Clinical utility of circulating matrix metalloproteinase-7 (MMP-7), CC chemokine ligand 18 (CCL18) and CC chemokine ligand 11 (CCL11) as markers for diagnosis of epithelial ovarian cancer. Med Oncol [Internet]. 2010;27(4):1246–53. Available from: https://doi.org/10.1007/s12032-009-9366-x
154. Hu X, Li D, Zhang W, Zhou J, Tang B, Li L. Matrix metalloproteinase-9 expression correlates with prognosis and involved in ovarian cancer cell invasion. Arch Gynecol Obstet [Internet]. 2012;286(6):1537–43. Available from: https://doi.org/10.1007/s00404-

012-2456-6
155. Li L-N, Zhou X, Gu Y, Yan J. Prognostic Value of MMP-9 in Ovarian Cancer: A Meta-analysis. Asian Pacific J Cancer Prev. 2013; 14(7):4107-13.
156. Wang FQ, So J, Reierstad S, Fishman DA. Matrilysin (MMP-7) promotes invasion of ovarian cancer cells by activation of progelatinase. Int J Cancer. 2005;114(1):19–31.
157. To SKY, Mak ASC, Eva Fung YM, Che C-M, Li S-S, Deng W, et al. β-catenin downregulates Dicer to promote ovarian cancer metastasis. Oncogene [Internet]. 2017 Jun 26;36:5927. Available from: https://doi.org/10.1038/onc.2017.185
158. Olsen JJ, Pohl S öther G, Deshmukh A, Visweswaran M, Ward NC, Arfuso F, et al. The role of Wnt signaling in angiogenesis. Clinical Biochemist Reviews. Australasian Association of Clinical Biochemists. 2017; 38(3): 131–142.
159. Chung AS, Ferrara N. Developmental and Pathological Angiogenesis. Annual Review of Cell and Developmental Biology. 2011; 27:563-84. doi: 10.1146/annurev-cellbio-092910-154002.
160. Rapp J, Jaromi L, Kvell K, Miskei G, Pongracz JE. WNT signaling - lung cancer is no exception. Respiratory Research. 2017. 169. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004;10(9):942–9.
161. Tang MKS, Yue PYK, Ip PP, Huang RL, Lai HC, Cheung ANY, et al. Soluble E-cadherin promotes tumor angiogenesis and localizes to exosome surface. Nat Commun. 2018;9(1):2270.
162. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004; 10(9):942–9.
163. Hamanishi J, Mandai M, Iwasaki M, Okazaki T, Tanaka Y, Yamaguchi K, et al. Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. Proc Natl Acad Sci. 2007;104(9):3360–5.
164. Goyne HE, Stone PJB, Burnett AF, Cannon MJ. Ovarian tumor ascites CD14+ cells suppress dendritic cell-activated CD4+ T-cell responses through IL-10 secretion and indoleamine 2,3-dioxygenase. J Immunother. 2014;37(3):163–9.
165. Okamoto A, Nikaido T, Ochiai K, Takakura S, Saito M, Aoki Y, et al. Indoleamine 2,3-dioxygenase serves as a marker of poor prognosis in gene expression profiles of serous ovarian cancer cells. Clin Cancer Res. 2005; 11(16):6030-9.
166. Inaba T, Ino K, Kajiyama H, Yamamoto E, Shibata K, Nawa A, et al. Role of the immunosuppressive enzyme indoleamine 2,3-dioxygenase in the progression of ovarian carcinoma. Gynecol Oncol. 2009;115(2):185–92.
167. Soichot M, Hennart B, Al Saabi A, Leloire A, Froguel P, Levy-Marchal C, et al. Identification of a variable number of tandem repeats polymorphism and characterization of LEF-1 response elements in the promoter of the IDO1 gene. PLoS One. 2011; 6(9):e25470. doi: 10.1371/journal.pone.0025470.
168. Cheng Y, Phoon YP, Jin X, Chong SYS, Ip JCY, Wong BWY, et al. Wnt-C59 arrests stemness and suppresses growth of nasopharyngeal carcinoma in mice by inhibiting the Wnt pathway in the tumor microenvironment. Oncotarget. 2015;6(16):14428-39.

169. Wang X, Moon J, Dodge ME, Pan X, Zhang L, Hanson JM, et al. The development of highly potent inhibitors for porcupine. J Med Chem. 2013;56(6):2700-4.
170. García-Reyes B, Witt L, Jansen B, Karasu E, Gehring T, Leban J, et al. Discovery of Inhibitor of Wnt Production 2 (IWP-2) and Related Compounds As Selective ATP-Competitive Inhibitors of Casein Kinase 1 (CK1) δ/ε. J Med Chem. 2018;61(9):4087-102.
171. You L, Zhang C, Yarravarapu N, Morlock L, Wang X, Zhang L, et al. Development of a triazole class of highly potent Porcn inhibitors. Bioorg Med Chem Lett. 2016;26(24):5891-5.
172. Jiang X, Hao H-X, Growney JD, Woolfenden S, Bottiglio C, Ng N, et al. Inactivating mutations of RNF43 confer Wnt dependency in pancreatic ductal adenocarcinoma. Proc Natl Acad Sci U S A. 2013;110(31):12649-54.
173. Guimaraes PPG, Tan M, Tammela T, Wu K, Chung A, Oberli M, et al. Potent in vivo lung cancer Wnt signaling inhibition via cyclodextrin-LGK974 inclusion complexes. Journal of Controlled Release. 2018;290(Complete):75-87.
174. Madan B, Ke Z, Harmston N, Ho SY, Frois AO, Alam J, et al. Wnt addiction of genetically defined cancers reversed by PORCN inhibition. Oncogene. 2015;35:2197.
175. Jiang J, Lan C, Li L, Yang D, Xia X, Liao Q, et al. A novel porcupine inhibitor blocks WNT pathways and attenuates cardiac hypertrophy. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2018;1864(10):3459-67.
176. Bhamra I, Adams N, Armer R, Bingham M, McKeever H, Phillips C, et al. Novel porcupine (PORCN) inhibitor RXC004: Evaluation in models of RNF43 loss of function cancers. Journal of Clinical Oncology. 2017;35(15_suppl):e14094-e.
177. Huang S-MA, Mishina YM, Liu S, Cheung A, Stegmeier F, Michaud GA, et al. Tankyrase inhibition stabilizes axin and antagonizes Wnt signaling. Nature. 2009;461:614.
178. Jia J, Qiao Y, Pilo MG, Cigliano A, Liu X, Shao Z, et al. Tankyrase inhibitors suppress hepatocellular carcinoma cell growth via modulating the Hippo cascade. PLoS ONE. 2017;12(9).
179. Emons G, Spitzner M, Reineke S, Möller J, Auslander N, Kramer F, et al. Chemoradiotherapy Resistance in Colorectal Cancer Cells is Mediated by Wnt/β-catenin Signaling. Molecular Cancer Research. 2017;15(11):1481.
180. TIAN X, HOU W, BAI S, FAN J, TONG H, XU HE. XAV939 inhibits the stemness and migration of neuroblastoma cancer stem cells via repression of tankyrase 1. Int J Oncol 2014;45(1):121.
181. Bao R, Christova T, Song S, Angers S, Yan X, Attisano L. Inhibition of Tankyrases Induces Axin Stabilization Blocks Wnt Signaling in Breast Cancer Cells. PLoS ONE. 2012;7(11).
182. Haikarainen T, Venkannagari H, Narwal M, Obaji E, Lee H-W, Nkizinkiko Y, et al. Structural Basis and Selectivity of Tankyrase Inhibition by a Wnt Signaling Inhibitor WIKI4. PLoS ONE. 2013;8(6).
183. James RG, Davidson KC, Bosch KA, Biechele TL, Robin NC, Taylor RJ, et al. WIKI4, a Novel Inhibitor of Tankyrase and Wnt/ß-Catenin Signaling. PLoS ONE. 2012;7(12).
184. Menon M, Elliott R, Bowers L, Balan N, Rafiq R, Costa-Cabral S, et al. A novel tankyrase inhibitor, MSC2504877, enhances the effects of clinical CDK4/6 inhibitors. Scientific Reports. 2019;9.

185. Mizutani A, Yashiroda Y, Muramatsu Y, Yoshida H, Chikada T, Tsumura T, et al. RK-287107, a potent and specific tankyrase inhibitor, blocks colorectal cancer cell growth in a preclinical model. Cancer Science. 2018;109(12):4003-14.
186. Cheng H, Li X, Wang C, Chen Y, Li S, Tan J, et al. Inhibition of tankyrase by a novel small molecule significantly attenuates prostate cancer cell proliferation. Cancer Letters. 2019;443(Complete):80-90.
187. Dandekar S, Romanos-Sirakis E, Pais F, Bhatla T, Jones C, Bourgeois W, et al. Wnt inhibition leads to improved chemosensitivity in paediatric acute lymphoblastic leukaemia. British Journal of Haematology. 2014;167(1):87-99.
188. Dere R, Perkins AL, Bawa-Khalfe T, Jonasch D, Walker CL. Wnt/β-Catenin Links von Hippel-Lindau to Aurora Kinase A and Loss of Primary Cilia in Renal Cell Carcinoma. Journal of the American Society of Nephrology. 2015;26(3):553.
189. Mathur R, Sehgal L, Braun FK, Berkova Z, Romaguerra J, Wang M, et al. Targeting Wnt pathway in mantle cell lymphoma-initiating cells. Journal of Hematology & Oncology. 2015;8.
190. Zhang JL, Liu Y, Yang H, Zhang HQ, Tian XX, Fang WG. ATP-P2Y2-β-catenin axis promotes cell invasion in breast cancer cells. Cancer Science. 2017;108(7):1318-27.
191. Gonsalves FC, Klein K, Carson BB, Katz S, Ekas LA, Evans S, et al. An RNAi-based chemical genetic screen identifies three small-molecule inhibitors of the Wnt/wingless signaling pathway. Proc Natl Acad Sci U S A. 2011;108(15):5954-63.
192. Sharma A, Yang W-L, Ochani M, Wang P. Mitigation of sepsis-induced inflammatory responses and organ injury through targeting Wnt/β-catenin signaling. Scientific reports. 2017;7(1):9235-.
193. Fang L, Zhu Q, Neuenschwander M, Specker E, Wulf-Goldenberg A, Weis WI, et al. A Small-Molecule Antagonist of the β-Catenin/TCF4 Interaction Blocks the Self-Renewal of Cancer Stem Cells and Suppresses Tumorigenesis. Cancer Research. 2016;76(4):891.
194. Trosset JY, Dalvit C, Knapp S, Fasolini M, Veronesi M, Mantegani S, et al. Inhibition of protein–protein interactions: The discovery of druglike β-catenin inhibitors by combining virtual and biophysical screening. Proteins: Structure, Function, and Bioinformatics. 2006;64(1):60-7.
195. Fasihi A, M. Soltani B, Atashi A, Nasiri S. Introduction of hsa-miR-103a and hsa-miR-1827 and hsa-miR-137 as new regulators of Wnt signaling pathway and their relation to colorectal carcinoma. Journal of Cellular Biochemistry. 2018;119(7):5104-17.
196. Leal LF, Bueno AC, Gomes DC, Abduch R, de Castro M, Antonini SR. Inhibition of the Tcf/beta-catenin complex increases apoptosis and impairs adrenocortical tumor cell proliferation and adrenal steroidogenesis. Oncotarget. 2015;6(40):43016-32.
197. Gekas C, D'Altri T, Aligué R, González J, Espinosa L, Bigas A. β-Catenin is required for T-cell leukemia initiation and MYC transcription downstream of Notch1. Leukemia. 2016;30:2002.
198. Matsuzaki S, Darcha C. In Vitro Effects of a Small-Molecule Antagonist of the Tcf/ß-Catenin Complex on Endometrial and Endometriotic Cells of Patients with Endometriosis. PLoS ONE. 2013;8(4).
199. Mologni L, Brussolo S, Ceccon M, Gambacorti-Passerini C. Synergistic Effects of Combined Wnt/KRAS Inhibition in Colorectal Cancer Cells. PLoS ONE. 2012;7(12).

200. Yaguchi T, Goto Y, Kido K, Mochimaru H, Sakurai T, Tsukamoto N, et al. Immune Suppression and Resistance Mediated by Constitutive Activation of Wnt/β-Catenin Signaling in Human Melanoma Cells. The Journal of Immunology. 2012;189(5):2110.
201. Durand J, Lampron A, Mazzuco TL, Chapman A, Bourdeau I. Characterization of Differential Gene Expression in Adrenocortical Tumors Harboring β-Catenin (CTNNB1) Mutations. The Journal of Clinical Endocrinology & Metabolism. 2011;96(7):E1206-E11.
202. Doghman M, Cazareth J, Lalli E. The T cell factor/β-Catenin Antagonist PKF115–584 Inhibits Proliferation of Adrenocortical Carcinoma Cells. The Journal of Clinical Endocrinology & Metabolism. 2008;93(8):3222-5.
203. Sukhdeo K, Mani M, Zhang Y, Dutta J, Yasui H, Rooney MD, et al. Targeting the β-catenin/TCF transcriptional complex in the treatment of multiple myeloma. Proceedings of the National Academy of Sciences. 2007;104(18):7516.
204. Wei W, Chua M-S, Grepper S, So S. Small molecule antagonists of Tcf4/β-catenin complex inhibit the growth of HCC cells in vitro and in vivo. International Journal of Cancer. 2010;126(10):2426-36.
205. Huang X, Zhong L, Hendriks J, Post JN, Karperien M. The Effects of the WNT-Signaling Modulators BIO and PKF118-310 on the Chondrogenic Differentiation of Human Mesenchymal Stem Cells. International Journal of Molecular Sciences. 2018;19(2).
206. Leow P-C, Tian Q, Ong Z-Y, Yang Z, Ee P-L. Antitumor activity of natural compounds, curcumin and PKF118-310, as Wnt/β-catenin antagonists against human osteosarcoma cells. Investigational New Drugs. 2010;28(6):766-82.
207. Lu W, Tinsley HN, Keeton A, Qu Z, Piazza GA, Li Y. Suppression of Wnt/β-catenin signaling inhibits prostate cancer cell proliferation. European Journal of Pharmacology. 2009;602(1):8-14.
208. Osawa Y, Oboki K, Imamura J, Kojika E, Hayashi Y, Hishima T, et al. Inhibition of Cyclic Adenosine Monophosphate (cAMP)-response Element-binding Protein (CREB)-binding Protein (CBP)/β-Catenin Reduces Liver Fibrosis in Mice. EBioMedicine. 2015;2(11):1751-8.
209. Kimura K, Ikoma A, Shibakawa M, Shimoda S, Harada K, Saio M, et al. Safety, Tolerability, and Preliminary Efficacy of the Anti-Fibrotic Small Molecule PRI-724, a CBP/β-Catenin Inhibitor, in Patients with Hepatitis C Virus-related Cirrhosis: A Single-Center, Open-Label, Dose Escalation Phase 1 Trial. EBioMedicine. 2017;23:79-87.
210. Emami KH, Nguyen C, Ma H, Kim DH, Jeong KW, Eguchi M, et al. A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected]. Proc Natl Acad Sci U S A. 2004;101(34):12682-7.
211. Yan D, Avtanski D, Saxena NK, Sharma D. Leptin-induced epithelial-mesenchymal transition in breast cancer cells requires β-catenin activation via Akt/GSK3- and MTA1/Wnt1 protein-dependent pathways. J Biol Chem. 2012;287(11):8598-612.
212. Minami I, Yamada K, Otsuji Tomomi G, Yamamoto T, Shen Y, Otsuka S, et al. A Small Molecule that Promotes Cardiac Differentiation of Human Pluripotent Stem Cells under Defined, Cytokine- and Xeno-free Conditions. Cell Reports. 2012;2(5):1448-60.
213. Morgan JT, Raghunathan VK, Chang Y-R, Murphy CJ, Russell P. Wnt inhibition induces persistent increases in intrinsic stiffness of human trabecular meshwork cells. Experimental Eye Research. 2015;132(Complete):174-8.

214. Sheridan C. Wnt is back in drugmakers' sights, but is it druggable? Nature Biotechnology. 2018;36:1028.
215. Cha P-H, Choi K-Y. Simultaneous destabilization of β-catenin and Ras via targeting of the axin-RGS domain as a potential therapeutic strategy for colorectal cancer. BMB Reports [Internet]. 2016 Sep 30;49(9):455–6. Available from: http://dx.doi.org/10.5483/BMBRep.2016.49.9.125
216. Park J, Cho Y-H, Shin W-J, Lee S-K, Lee J, Kim T, et al. A Ras destabilizer KYA1797K overcomes the resistance of EGFR tyrosine kinase inhibitor in KRAS-mutated non-small cell lung cancer. Scientific Reports. 2019;9.
217. Handeli S, Simon JA. A small-molecule inhibitor of Tcf/β-catenin signaling down-regulates PPARγ and PPARδ activities. Molecular Cancer Therapeutics. 2008;7(3):521.
218. Gedaly R, Galuppo R, Daily MF, Shah M, Maynard E, Chen C, et al. Targeting the Wnt/β-catenin signalinpathway in liver cancer stem cells and hepatocellular carcinoma cell lines with FH535. PloS one. 2014;9(6):e99272-e.
219. Chen Y, Rao X, Huang K, Jiang X, Wang H, Teng L. FH535 Inhibits Proliferation and Motility of Colon Cancer Cells by Targeting Wnt/β-catenin Signaling Pathway. J Cancer. 2017;8(16):3142-53.
220. Gustafson CT, Mamo T, Shogren KL, Maran A, Yaszemski MJ. FH535 Suppresses Osteosarcoma Growth In Vitro and Inhibits Wnt Signaling through Tankyrases. Frontiers in Pharmacology. 2017;8(285).
221. Gatcliffe TA, Monk BJ, Planutis K, Holcombe RF. Wnt signaling in ovarian tumorigenesis. International Journal of Gynecological Cancer. 2008; 18(5):954-62.
222. Burkhalter RJ, Symowicz J, Hudson LG, Gottardi CJ, Stack MS. Integrin regulation of β-catenin signaling in ovarian carcinoma. J Biol Chem. 2011; 286(26), 23467–75. https://doi.org/10.1074/jbc.M110.199539'
223. Su HY, Lai HC, Lin YW, Liu CY, Chen CK, Chou YC, et al. Epigenetic silencing of SFRP5 is related to malignant phenotype and chemoresistance of ovarian cancer through Wnt signaling pathway. Int J Cancer. 2010; 127(3), 555–567. https://doi.org/10.1002/ijc.25083
224. Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. Oncogene. 2017; 36, 1461–1473 https://doi.org/10.1038/onc.2016.304
225. Wright K, Wilson P, Morland S, Campbell I, Walsh M, Hurst T, et al. β-catenin mutation and expression analysis in ovarian cancer: Exon 3 mutations and nuclear translocation in 16% of endometrioid tumours. Int J Cancer. 1999; 82(5), 625–629. https://doi.org/10.1002/(SICI)1097-0215(19990827)82:5<625::AID-IJC1>3.0.CO;2-2 [pii]
226. Magee JA, Piskounova E, Morrison SJ. Cancer Stem Cells: Impact, Heterogeneity, and Uncertainty. Cancer Cell. 2012; 21(3):283-96. https://doi.org/10.1016/j.ccr.2012.03.003
227. Hurle MR, Yang L, Xie Q, Rajpal DK, Sanseau P, Agarwal P. Computational drug repositioning: From data to therapeutics. Clinical Pharmacology and Therapeutics. 2013; ; 93: 335–341.

**Chapter 2**


1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA: A Cancer Journal for Clinicians. 2019;69(1):7-34.
2. Gilks CB, Prat J. Ovarian carcinoma pathology and genetics: recent advances. Hum Pathol. 2009;40(9):1213–23.
3. Medicine Io, National Academies of Sciences E, Medicine. Ovarian Cancers: Evolving Paradigms in Research and Care. Washington, DC: The National Academies Press; 2016. 396 p.
4. Luvero D, Milani A, Ledermann JA. Treatment options in recurrent ovarian cancer: Latest evidence and clinical potential. Therapeutic Advances in Medical Oncology. 2014; 6: 29–39.
5. Howlader N, Noone AM, Krapcho M, Garshell J, Miller D, Altekruse S, et al. National Cancer Institute SEER Cancer Statistics Review 1975-2012. Natl Cancer Inst. 2015;
6. Logan CY, Nusse R. The Wnt Signaling pathway in development and disease. Annu Rev Cell Dev Biol. 2004;20:781–810.
7. Tauriello DVF, Jordens I, Kirchner K, Slootstra JW, Kruitwagen T, Bouwman BAM, et al. Wnt/β-catenin signaling requires interaction of the Dishevelled DEP domain and C terminus with a discontinuous motif in Frizzled. Proceedings of the National Academy of Sciences. 2012;109(14):E812.
8. Mao J, Wang J, Liu B, Pan W, Farr GH, Flynn C, et al. Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. Mol Cell. 2001; 7(4):801-9.
9. Behrens J, Von Kries JP, Kühl M, Bruhn L, Wedlich D, Grosschedl R, et al. Functional interaction of β-catenin with the transcription factor LEF- 1. Nature. 1996; *382*(6592), 638–642. https://doi.org/10.1038/382638a0
10. Clevers H, Nusse R. Wnt/β-catenin signaling and disease. Vol. 149, Cell. 2012. p. 1192–205.
11. Gao C, Wang Y, Broaddus R, Sun L, Xue F, Zhang W. Exon 3 mutations of *CTNNB1* drive tumorigenesis: a review. Oncotarget. 2018; 9(4):5492-5508.
12. Pai SG, Carneiro BA, Mota JM, Costa R, Leite CA, Barroso-Sousa R, et al. Wnt/beta-catenin pathway: Modulating anticancer immune response. Journal of Hematology and Oncology. BioMed Central Ltd. 2017; 10(1):101. doi: 10.1186/s13045-017-0471-6.
13. Tang MKS, Yue PYK, Ip PP, Huang R-L, Lai H-C, Cheung ANY, et al. Soluble E-cadherin promotes tumor angiogenesis and localizes to exosome surface. Nature Communications. 2018;9(1):2270.
14. Nagaraj AB, Joseph P, Kovalenko O, Singh S, Armstrong A, Redline R, et al. Critical role of Wnt/β-catenin signaling in driving epithelial ovarian cancer platinum resistance. Oncotarget. 2015;6(27):23720-34.

15. Cannon M, Ghosh D, Gujja S. Signaling Circuits and Regulation of Immune Suppression by Ovarian Tumor-Associated Macrophages. Vaccines. 2015 May;3(2):448–66.
16. Arend RC, Londono-Joshi AI, Straughn JM, Buchsbaum DJ. The Wnt/beta-catenin pathway in ovarian cancer: a review. Gynecol Oncol [Internet]. 2013;131. Available from: https://doi.org/10.1016/j.ygyno.2013.09.034
17. Beck B, Blanpain C. Unravelling cancer stem cell potential. Nat Rev Cancer. 2013;13(10):727–38.
18. Ahmed N, Abubaker K, Findlay JK. Ovarian cancer stem cells: Molecular concepts and relevance as therapeutic targets. Molecular Aspects of Medicine. 2014;39:110-25.
19. Magee JA, Piskounova E, Morrison SJ. Cancer Stem Cells: Impact, Heterogeneity, and Uncertainty. Cancer Cell. 2012;21(3):283-296.
20. Milas L, Hittelman WN. Cancer Stem Cells and Tumor Response to Therapy: Current Problems and Future Prospects. Seminars in Radiation Oncology. 2009. *19*(2), 96–105. https://doi.org/10.1016/j.semradonc.2008.11.004
21. Wu R, Zhai Y, Fearon ER, Cho KR. Diverse Mechanisms of β-Catenin Deregulation in Ovarian Endometrioid Adenocarcinomas. Cancer Res. 2001;61(22):8247–55.
22. Sarrio D, Moreno-bueno G, Sa C, Ban I, Hardisson D. Expression of cadherins and catenins correlates with distinct histologic types of ovarian carcinomas. 2006;37(8): 1042–9.
23. Cortez AJ, Tudrej P, Kujawa KA, Lisowska KM. Advances in ovarian cancer therapy. Cancer Chemotherapy and Pharmacology. 2018; 81:17-38.
24. Chen B, Dodge ME, Tang W, Lu J, Ma Z, Fan CW, et al. Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. Nat Chem Biol. 2009; 5(2):100-7.
25. Grandy D, Shan J, Zhang X, Rao S, Akunuru S, Li H, et al. Discovery and characterization of a small molecule inhibitor of the PDZ domain of dishevelled. J Biol Chem. 2009; 284(24):16256-63.
26. Waaler J, Machon O, Tumova L, Dinh H, Korinek V, Wilson SR, et al. A novel tankyrase inhibitor decreases canonical Wnt signaling in colon carcinoma cells and reduces tumor growth in conditional APC mutant mice. Cancer Res. 2012; 72(11):2822-32.
27. Huang SMA, Mishina YM, Liu S, Cheung A, Stegmeier F, Michaud GA, et al. Tankyrase inhibition stabilizes axin and antagonizes Wnt signaling. Nature. 2009; 461(7264):614-20.
28. Eguchi M, Nguyen C, Lee S, Kahn M. ICG-001, A Novel Small Molecule Regulator of TCF/β-Catenin Masakatsu Transcription. Med Chem (Los Angeles). 2005; 1(5):467-72.
29. Kimura K, Ikoma A, Shibakawa M, Shimoda S, Harada K, Saio M, et al. Safety, Tolerability, and Preliminary Efficacy of the Anti-Fibrotic Small Molecule PRI-724, a CBP/β-Catenin Inhibitor, in Patients with Hepatitis C Virus-related Cirrhosis: A Single-Center, Open-Label, Dose Escalation Phase 1 Trial. EBioMedicine. 2017; 23:79-87. doi: 10.1016/j.ebiom.2017.08.016.

30. Gonsalves FC, Klein K, Carson BB, Katz S, Ekas LA, Evans S, et al. An RNAi-based chemical genetic screen identifies three small-molecule inhibitors of the Wnt/wingless signaling pathway. Proc Natl Acad Sci. 2011; 108(15):5954-63.
31. Fang L, Zhu Q, Neuenschwander M, Specker E, Wulf-Goldenberg A, Weis WI, et al. A small-molecule antagonist of the β-catenin/TCF4 interaction blocks the self-renewal of cancer stem cells and suppresses tumorigenesis. Cancer Res. 2016; 76(4):891-901.
32. De La Roche M, Rutherford TJ, Gupta D, Veprintsev DB, Saxty B, Freund SM, et al. An intrinsically labile α-helix abutting the BCL9-binding site of β-catenin is required for its inhibition by carnosic acid. Nat Commun. 2012; 3:680. doi: 10.1038/ncomms1680.
33. University of Southern California. Combination Chemotherapy and Bevacizumab With or Without PRI-724 in Treating Patients With Newly Diagnosed Metastatic Colorectal Cancer (PRIMIER). In: ClinicaltrialsGov. 2015.
34. Bernaudo S, Salem M, Qi X, Zhou W, Zhang C, Yang W, et al. Cyclin G2 inhibits epithelial-to-mesenchymal transition by disrupting Wnt/β-catenin signaling. Oncogene. 2016;35(36):4816–27.
35. Salem M, O'Brien JA, Bernaudo S, Shawer H, Ye G, Brkić J, et al. MiR-590-3p promotes ovarian cancer growth and metastasis via a novel FOXA2-versican pathway. Cancer Res. 2018 Aug 1;78(15):4175–90.
36. Salem M, Shan Y, Bernaudo S, Peng C. miR-590-3p Targets Cyclin G2 and FOXO3 to Promote Ovarian Cancer Cell Proliferation, Invasion, and Spheroid Formation. Int J Mol Sci. 2019; 20(8): 1810.
37. Yan M, Li G, An J. Discovery of small molecule inhibitors of the Wnt/β-catenin signaling pathway by targeting β-catenin/Tcf4 interactions. Experimental Biology and Medicine. 2017; 242(11):1185-1197.
38. Lengyel E, Burdette JE, Kenny HA, Matei D, Pilrose J, Haluska P, et al. Epithelial ovarian cancer experimental models. Oncogene. 2014; 33(28):3619-33.
39. Wolf JK, Scanlon M, Hung MC. Enhanced c-erbB-2/neu Expression in Human OvaRIan Cancer Cells Correlates with More Severe Malignancy That Can Be Suppressed by E1A. Cancer Res. 1993; 53(4):891-8.
40. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, et al. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science (80- ). 1997; 275(5307):1787-90.
41. Borowicz S, Van Scoyk M, Avasarala S, Karuppusamy Rathinam MK, Tauler J, Bikkavilli RK, et al. The Soft Agar Colony Formation Assay. J Vis Exp. 2014; (92):e51998. doi: 10.3791/51998.
42. Yoshioka S, King ML, Ran S, Okuda H, MacLean JA, McAsey ME, et al. WNT7A Regulates Tumor Growth and Progression in Ovarian Cancer through the WNT/ β-Catenin Pathway. Mol Cancer Res. 2012;10(3):469–83.
43. Jacob F, Ukegjini K, Nixdorf S, Ford CE, Olivier J, Caduff R, et al. Loss of secreted frizzled-related protein 4 correlates with an aggressive phenotype and predicts poor outcome in ovarian cancer patients. PLoS One. 2012; 7(2): e31885.

44. Ford CE, Jary E, Ma SSQ, Nixdorf S, Heinzelmann-Schwarz VA, Ward RL. The Wnt Gatekeeper SFRP4 Modulates EMT, Cell Migration and Downstream Wnt Signaling in Serous Ovarian Cancer Cells. PLOS ONE. 2013;8(1):e54362.
45. Taddei ML, Giannoni E, Fiaschi T, Chiarugi P. Anoikis: An emerging hallmark in health and diseases. Journal of Pathology. 2012; 226(2):380-93.
46. Chen SF, Chang YC, Nieh S, Liu CL, Yang CY, Lin YS. Nonadhesive culture system as a model of rapid sphere formation with cancer stem cell properties. PLoS One. 2012; 7(2):e31864. doi: 10.1371/journal.pone.0031864.
47. Yang HY, Shen JX, Wang Y, Liu Y, Shen DY, Quan S. Tankyrase Promotes Aerobic Glycolysis and Proliferation of Ovarian Cancer through Activation of Wnt/ β -Catenin Signaling. Biomed Res Int. 2019; 2019:2686340. doi: 10.1155/2019/2686340.
48. Ying X, Li-ya Q, Feng Z, Yin W, Ji-hong L. MiR-939 promotes the proliferation of human ovarian cancer cells by repressing APC2 expression. Biomed Pharmacother. 2015;71(651):64–9.
49. Sun Y, Jia X, Wu X. High Expressions of Lgr5 and ALDH1 in Primary Epithelial Ovarian Cancer Correlate with Advanced Tumor Stage and Grade as well as Poor Prognosis of the Patients. Gynecol Obstet Invest. 2016; 81:162-168.
50. Maher MT, Mo R, Flozak AS, Peled ON, Gottardi CJ. β-catenin phosphorylated at serine 45 is spatially uncoupled from β-catenin phosphorylated in the GSK3 domain: Implications for signaling. PLoS One. 2010; 5(4):e10184. doi: 10.1371/journal.pone.0010184.
51. Sadot E, Conacci-Sorrell M, Zhurinsky J, Shnizer D, Lando Z, Zharhary D, et al. Regulation of S33/S37 phosphorylated beta-catenin in normal and transformed cells. J Cell Sci. 2002; 115(Pt 13):2771-80.
52. Parte SC, Batra SK, Kakar SS. Characterization of stem cell and cancer stem cell populations in ovary and ovarian tumors. J Ovarian Res. 2018; 11(1):69. doi: 10.1186/s13048-018-0439-3.
53. Ffrench B, Gasch C, O'Leary JJ, Gallagher MF. Developing ovarian cancer stem cell models: laying the pipeline from discovery to clinical intervention. Molecular Cancer. 2014; 13:262. doi: 10.1186/1476-4598-13-262.
54. Ruan X, Liu A, Zhong M, Wei J, Zhang W, Rong Y, et al. Silencing LGR6 Attenuates Stemness and Chemoresistance via Inhibiting Wnt/β-Catenin Signaling in Ovarian Cancer. Mol Ther - Oncolytics. 2019;14:94–106.
55. Condello S, Morgan CA, Nagdas S, Cao L, Turek J, Hurley TD, et al. β-Catenin-regulated ALDH1A1 is a target in ovarian cancer spheroids. Oncogene [Internet]. 2014/06/23. 2015 Apr 30;34(18):2297–308. Available from: https://www.ncbi.nlm.nih.gov/pubmed/24954508
56. Shulewitz M, Soloviev I, Wu T, Koeppen H, Polakis P, Sakanaka C. Repressor roles for TCF-4 and Sfrp1 in Wnt signaling in breast cancer. Oncogene. 2006; 25(31):4361-9.

57. Wielenga VJM, Smits R, Korinek V, Smit L, Kielman M, Fodde R, et al. Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. Am J Pathol. 1999; 154(2):515-23.
58. Katoh Y, Katoh M. Comparative genomics on PROM1 gene encoding stem cell marker CD133. Int J Mol Med. 2007; 19(6):967-70.
59. Munz M, Baeuerle PA, Gires O. The emerging role of EpCAM in cancer and stem cell signaling. Cancer Research. 2009; 69(14):5627-9. doi: 10.1158/0008-5472.CAN-09-0654.
60. Cole MF, Johnstone SE, Newman JJ, Kagey MH, Young RA. Tcf3 is an integral component of the core regulatory circuitry of embryonic stem cells. Genes Dev. 2008; 22(6):746-55. doi: 10.1101/gad.1642408.
61. Lien WH, Polak L, Lin M, Lay K, Zheng D, Fuchs E. In vivo transcriptional governance of hair follicle stem cells by canonical Wnt regulators. Nat Cell Biol. 2014; 16(2):179-90. doi: 10.1038/ncb2903.
62. Klemba A, Purzycka-Olewiecka JK, Wcisło G, Czarnecka AM, Lewicki S, Lesyng B, et al. Surface markers of cancer stem-like cells of ovarian cancer and their clinical relevance. Wspolczesna Onkologia. 2017; 22(1A): 48–55.
63. Nam EJ, Lee M, Yim GW, Kim JH, Kim S, Kim SW, et al. MicroRNA profiling of a CD133+ spheroid-forming subpopulation of the OVCAR3 human ovarian cancer cell line. BMC Med Genomics. 2012; 5:18. doi: 10.1186/1755-8794-5-18.
64. Chen YF, Wang SY, Shen H, Yao XF, Zhang FL, Lai D. The marine-derived fungal metabolite, terrein, inhibits cell proliferation and induces cell cycle arrest in human ovarian cancer cells. Int J Mol Med. 2014; 34(6):1591-8. doi: 10.3892/ijmm.2014.1964.
65. Ward Rashidi MR, Mehta P, Bregenzer M, Raghavan S, Fleck EM, Horst EN, et al. Engineered 3D Model of Cancer Stem Cell Enrichment and Chemoresistance. Neoplasia [Internet]. 2019;21(8):822–36. Available from: http://www.sciencedirect.com/science/article/pii/S1476558618305657
66. Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. Oncogene. 2017;36:1461–1473
67. Liao DJ, Thakur A, Wu J, Biliran H, Sarkar FH. Perspectives on c-Myc, Cyclin D1, and Their Interaction in Cancer Formation, Progression, and Response to Chemotherapy. Crit Rev Oncog. 2012; 13(2):93-158.

**Chapter 3**


1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA: A Cancer Journal for Clinicians. 2019;69(1):7-34.
2. Arend RC, Londono-Joshi AI, Straughn JM, Buchsbaum DJ. The Wnt/beta-catenin pathway in ovarian cancer: a review. Gynecol Oncol [Internet]. 2013;131. Available from: https://doi.org/10.1016/j.ygyno.2013.09.034
3. Ruan X, Liu A, Zhong M, Wei J, Zhang W, Rong Y, et al. Silencing LGR6 Attenuates Stemness and Chemoresistance via Inhibiting Wnt/β-Catenin Signaling in Ovarian Cancer. Mol Ther - Oncolytics. 2019;14:94–106.
4. Beck B, Blanpain C. Unravelling cancer stem cell potential. Nat Rev Cancer. 2013;13(10):727–38.
5. Zhu ZW, Chen L, Liu JX, Huang JW, Wu G, Zheng YF, et al. A novel three-dimensional tumorsphere culture system for the efficient and low-cost enrichment of cancer stem cells with natural polymers. Exp Ther Med. 2018; 15(1):85-92.
6. Sun Y, Jia X, Wu X. High Expressions of Lgr5 and ALDH1 in Primary Epithelial Ovarian Cancer Correlate with Advanced Tumor Stage and Grade as well as Poor Prognosis of the Patients. Gynecol Obstet Invest. 2016; 81:162-168.
7. Parte SC, Batra SK, Kakar SS. Characterization of stem cell and cancer stem cell populations in ovary and ovarian tumors. J Ovarian Res. 2018; 11(1):69. doi: 10.1186/s13048-018-0439-3.
8. Tang MKS, Yue PYK, Ip PP, Huang R-L, Lai H-C, Cheung ANY, et al. Soluble E-cadherin promotes tumor angiogenesis and localizes to exosome surface. Nature Communications. 2018;9(1):2270.
9. Cannon M, Ghosh D, Gujja S. Signaling Circuits and Regulation of Immune Suppression by Ovarian Tumor-Associated Macrophages. Vaccines. 2015 May;3(2):448–66.
10. Wan X, Bovornchutichai P, Cui Z, O'Neill E, Ye H. Morphological analysis of human umbilical vein endothelial cells co-cultured with ovarian cancer cells in 3D: An oncogenic angiogenesis assay. PLoS One. 2017; 12(7):e0180296. doi: 10.1371/journal.pone.0180296.
11. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci. 2005; 102(51):18538-43.