TARGETING THE WNT PATHWAY IN OVARIAN CANCER DISSEMINATION

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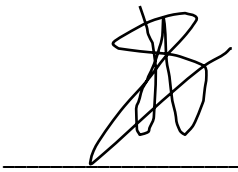
DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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SUMMARY

Epithelial ovarian cancer (EOC) is the most prevalent form of ovarian cancer, with its' asymptomatic nature making early detection difficult, resulting in late-stage diagnosis with poor prognostic outcome. EOC cells metastasize *via* the formation of spheroids that dissociate from the primary tumour to resuspend in the patient ascites, eventually reattaching along the peritoneal cavity. Previously, *in vitro* culture of ovarian cancer spheroids showed up-regulated expression of Frizzled-7 (*FZD7*), a WNT receptor. Furthermore, *FZD7* expression was enriched in EOC cell lines displaying a stem-like molecular expression profile, suggesting a role of FZD7 in the maintenance of spheroids in ovarian cancer. In this study, stable knockdown of FZD7 in spheroid-forming EOC cells disrupted spheroid formation, with cells undergoing *anoikis* when cultured in suspension, which was further substantiated with increased Annexin V population and the up-regulation of apoptotic markers. The findings of this study suggest WNT signalling to be involved in protecting EOC cells from undergoing *anoikis*, thereby promoting the metastatic process of this disease.

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LIST OF SYMBOLS/ ABBREVIATIONS

5-Aza-C	5- Aza-2'deoxyctiydine
ABCG2	ATP-binding cassette G2
ACTB	beta-actin
ADR	Adriamycin resistant
AKT	v-akt murine thymoma viral oncogene homolog
ALDH1A1	Aldehyde dehydrogenase 1A1
ANKRD6	Ankyrin repeat domain 6
AP-1	Activator protein 1 transcription factor
APC	Adenomatous polyposis coli
Arg	Arginine
ATP	Adenosine tri-phosphate
B2M	beta 2 microglobulin
BAD	Bcl2-associated agonist of cell death
BAK	Bcl-2 homologous Antagonist/Killer
BAX	Bcl-2 Associated protein X
BCA	Bicinchoninic acid
BCL2	B-cell CLL lymphoma 2
BCL2L11	BCL2-like 11
BCL9L	B-cell CLL/ Lymphoma-9-Like BIM
BIM	Bcl-2-interacting mediator of cell death
bp	base pairs
BRAF	B-raf proto-oncogene, serine/ threonine kinase
BRCA1	Breast Cancer 1, early onset
BRCA2	Breast Cancer 2, early onset
c-MET	MET proto-oncogene receptor tyrosine kinase
c-MYC	v-myc avian myelocytomatosis viral oncogene homolog
CAF	Cancer associated fibroblasts
CASP3	Caspase 3

CASP8	Caspase 8

LIST OF SYMBOLS/ ABBREVIATIONS

CASP9	Caspase 9
CCLE	Cancer cell line encyclopedia
CCNB1	Cyclin B1
CCND1	Cyclin D1
CCND2	Cyclin D2
CD3	Cluster of Differentiation 3 molecule
CDC2	Cyclin-dependent kinase A1
CDH1	Cadherin-1, type 1, E-cadherin
cDNA	complementary deoxy-ribose nucleic acid
CE	Convergent extension
CELSR1	Cadherin, EGF LAG seven-pass G-type receptor 1
CELSR2	Cadherin, EGF LAG seven-pass G-type receptor 2
CGH	Comparative genomic hybridization
CGI	CpG island
ChIP	Chromatin immunoprecipitation
Chz	Chuzhoi transgenic mice
CK1	Casein kinase 1
CKIδ	Casein kinase 1 delta
CLOVAR	Classification of ovarian cancer
cRNA	complementary ribonucleic acid
CRC	Colorectal cancer
CRD	Cysteine rich domain
CREBBP	cAMP Response Element binding protein-binding protein
CSNK2β	Casein kinase II beta
CST1	Cystatin 1
C_T	Threshold cycle
CTNNB1	beta-catenin, cadherin associated protein, 88kD
CX43	Connexin 43

CYCYS	Cytochrome complex

LIST OF SYMBOLS/ ABBREVIATIONS

Daam1	Dishevelled associated activator of morphogenesis 1
DAPI	4',6-diamidino-2-phenylindole
DEP	Dishevelled, Egl-10 and pleckstrin domain
DIX	Dishevelled axin domain
DKK1	Dickkopf WNT signalling inhibitor 1
DKK2	Dickkopf WNT signalling inhibitor 2
DKK3	Dickkopf WNT signalling inhibitor 3
DMEM	Dubelcco's Modified Eagle's Medium
DMZ	Dorsal marginal zone
dn	dominant negative
DNA	Deoxy-ribonucleic acid
DOC1	Down-regulated in ovarian cancer 1
DS	Dachsous
Dsh	Dishevelled (*invertebrates*)
Dsh-DN	N-terminus truncated Dishevelled
DVL	Dishevelled (mammalian)
EDN3	Endothelin 3
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
EOC	Epithelial ovarian cancer
Epi-A	Epithelial molecular subtype A
Epi-B	Epithelial molecular subtype B
ER	Endoplasmic reticulum
ERK1	Extracellular signal-regulated kinase 1
ERK2	Extracellular signal-regulated kinase 2
ERRB2	erb b2 receptor tyrosine kinase
EV	Empty vector
fab	Fragment antigen binding protein

FACS	Fluorescence activated cell sorting

LIST OF SYMBOLS/ ABBREVIATIONS

FAK	Focal adhesion kinase
FAP	Familial adenomatous polyposis
FAS	Fas cell surface death receptor
FBS	Fetal bovine serum
FGF2	Fibroblast growth factor 2
FGF9	Fibroblast growth factor 9
FGF20	Fibroblast growth factor 20
FILIP1L	Filamin A Interacting Protein 1-Like
FJ	Four-jointed
Flna	Filamin A
fmi	flamingo
FOSL1	FOS-like antigen 1
FRAT1	Frequently Rearranged in Advanced T-cell lymphomas 1
FT	Fat gene
Fz	Frizzled
fzd2	frizzled-2
FZD7	Frizzled-7
FZD7ΔC	Carboxy-terminus truncated FZD7
Fzd8	Frizzled-8
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GOI	Genes of interest
GPCR	G-protein coupled receptor
GSK3	Gycogen synthase kinase 3
GTP	Guanine tri-phosphate
GTPase	GTP hydrolase
HCC	Hepatocellular carcinoma
hECC	human embryonal carcinoma cells

LIST OF SYMBOLS/ ABBREVIATIONS

hESC	human embryonic stem cells
HGF	Hepatocyte growth factor
HGSOA	High grade serous ovarian adenocarcinoma
HGSOC	High grade serous ovarian carcinomas
HKG	Housekeeping genes
HOSE	Human ovarian surface epithelium
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HR	Hazard ratio
hrs	hours
IC_{50}	Inhibitory concentration at 50%
ICAT	Inhibitor of beta-catenin and TCF
iCRT	Inhibitor of beta-catenin responsive transcription
IHC	Immunohistochemistry
Ile	Isoleucine
int-1	first common integration site
ISH	*in situ* hybridization
ITF-2	Immunoglobulin Transcription Factor 2
JNK	Jun N-terminal kinase
kD	kilodaltons
KRAS	Kirsten rat sarcoma oncogene
L1CAM	L1 cell adhesion molecule
LAMC2	Laminin g2
LEF	Lymphoid enhancing factor
Lgr5	Leucine-rich-repeat-containing G-protein-coupled receptor 5
LGSOC	Low grade serous ovarian carcinomas
LiCl	lithium chloride
LMP	Low malignant potential
Lp	Looptail transgenic mice

LIST OF SYMBOLS AND ABBREVIATIONS

Lrp6	Low-density-lipoprotein Receptor-related Protein 6, a single-span transmembrane co-receptor
Lys	Lysine
mAbs	Monoclonal antibodies
MAPK	Mitogen-activated kinase-like protein
MBOAT	Membrane-bound O-acyl transferase
Mcl1	Myeloid cell leukemia 1
MCS	Migrating cancer stem cells
MCST	Microcystic stromal tumour
MEK1	MAP-ERK kinase 1
MEK2	MAP-ERK kinase 2
MET	Mesenchymal-epithelial transition
MFP	Mammary fat pads
miR/ miRNA	micro RNA
miR155	Micro RNA 155
MMP1	Matrix metalloproteinase 1
MMP7	Matrix metalloproteinase 7
MMP9	Matrix metalloproteinase 9
MMP14	Matrix metalloproteinase 14
MMTV	Mouse mammary tumour virus
MOMP	Mitochondrial outer membrane permeabilization
MSP	Methylation specific PCR
MSX2	Muscle segment homeobox 2
MTOR	Mechanistic target of rapamycin
MUC1	Mucin-1
MUC16	Mucin-16
NANOG	Nanog homeobox
NAT10	N-Acetyl Transferase 10
NF-kB	Nuclear factor of kappa light polypeptide gene enhancer of activated B cells

LIST OF SYMBOLS AND ABBREVIATIONS

NFAT	Nuclear factor activated T cell
NOD	non-obese diabetic
NPC	Nasopharyngeal carcinoma
NSCLC	Non small cell lung cancer
NTRK2	Neurotrophic Tyrosine Kinase receptor type 2
OCT4	Oct4 protein, also known as POU5F1
OEA	Ovarian endometrioid adenocarcinoma
OS	Overall survival
OSE	Ovarian surface epithelium
OTIC	Ovarian tumour initiating cells
PARP	Poly-(ADP-ribose) polymerase
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PDZ	post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
PFA	Paraformaldehyde
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase alpha
PFS	Progression free survival
PITX2	Pituitary homeobox 2
pk	Prickle
PKC/ PRKC	Protein kinase C
PKCδ	Protein kinase C delta
PMA	Phorbol-12-myristate-13-acetate
PORCN	Porcupine
POU5F1	POU domain, class 5 transcription factor 1
PPAR-δ	Peroxisome Proliferator-Activated Receptor delta
PRD	Proline rich domain
Pro	Proline

PS	phosphatidylserine

LIST OF SYMBOLS AND ABBREVIATIONS

pSK6	phsopho-ribosomal protein S6 kinase
PTEN	Phosphatase and tensin homolog
PTK7	Protein tyrosine kinase 7
PVDF	polyvinylidene difluoride
PYGO	Pygopus family PHD finger
qPCR	Quantitative real time polymerase chain reaction
RAB25	RAB25, member of RAS oncogene
Rac1	Ras-related C3 botulinum toxin substrate rho family, small GTP binding protein
RhoA	Ras homolog family member A
RIPA	Radio-immunoprecipitation assay
RIPK4	Receptor-Interacting Protein Kinase 4
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
Rok2	Rho-associated kinase 2
ROR2	Receptor tyrosine kinase-like orphan receptor 2
RPL13A	Ribosomal protein, large 13A
RYK	Receptor like tyrosine kinase
SA-β-Gal	Senescence-associated beta-galactosidase
SCID	Severe combined immunodeficient
SCS	Stationary cancer stem cells
Ser	Serine
SFN	Stratifin
Sfrp1	Secreted frizzled related protein 1
Sfrp2	Secreted frizzled related protein 2
Sfrp3	Secreted frizzled related protein 3
Sfrp4	Secreted frizzled related protein 4
SGOCL	Singapore ovarian cancer cell lines
siRNA	short interfering ribonucleic acid

LIST OF SYMBOLS AND ABBREVIATIONS

shRNA	short-hairpin ribonucleic acid
SNAI1	Snail family zinc finger 1
SNAI2	Snail family zinc finger 2
SOX9	Sex determining region Y box 9
Src	SRC proto-oncogene
Stbm	Strabismus
SV40-TAg	Simian Vacuolating virus, large T-antigen
TCF	T-cell factor
TCGA	The cancer genome atlas
TGN	*trans* golgi network
Thr	Threonine
TMA	Tissue microarrays
TNBC	Triple negative breast cancer
TOP	Tcf motif upstream of a minial c-Fox promoter
TP53	Tumour protein p53
TRIB1	tribbles pseudokinase 1
TRITC	Tetra methyl rhodamine C
TSS	Transcriptional start site
TUBGCP4	Tubulin Gamma associated Protein Complex 4
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labeling
ULAS	Ultralow attachment surface
Val	Valine
vang	Van Gogh
VANGL1	Vang-like planar cell polarity protein 1
VANGL2	Vang-like planar cell polarity protein 2
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
VIM	Vimentin

LIST OF SYMBOLS AND ABBREVIATIONS

Wg	Wingless
WIF	Wnt inhibitory factor
WLS	Wntless
Wnt1	Wingless-related MMTV integration site family 1
Wnt2B	Wingless-related MMTV integration site family 2B
WNT3A	Wingless-related MMTV integration site family 3A
Wnt5A	Wingless-related MMTV integration site family 5A
Wnt7A	Wingless-related MMTV integration site family 7A
Wnt8	Wingless-related MMTV integration site family 8

CHAPTER 1.1

Epithelial Ovarian Cancer (EOC)

Ovarian cancer is the seventh most prevalent cancer among women in the world,[1] and the fifth most in industrialized/ resource-rich nations, including the United States[2] and Singapore[3]. It is the most deadly among gynecological malignancies, partly due to the asymptomatic nature of the disease, making early detection difficult. As such, majority of patients (61%) when presented are diagnosed at the later stages of disease progression, with only 15% of patients being diagnosed at stage I (localized).[2] Prognosis of patients with stage I (localized) ovarian cancer is good, with a 5-year survival rate of 92%, drastically falling to just 27% for patients diagnosed at stage IV (distant).[2]

Majority of ovarian cancer display an epithelial morphology (90%) and are generally termed epithelial ovarian cancer (EOC). From a histopathological perspective, EOC is classified into four major histological subtypes: serous, endometrioid, mucinous and clear cell.[4] Ovarian serous carcinoma is the most prevalent within EOC, which can be further classified into either high-grade or low-grade (reviewed in Gilks and Prat, 2009).[5] The prevalence of the major histological subtypes of EOC is as follow: high-grade serous (70%), endometrioid (10%), clear cell (10%), low-grade serous (<5%) and mucinous (3%). Furthermore, depending on the presence of stromal invasion, each histological subtype may be subdivided into benign, borderline (low malignant potential, LMP) or malignant.[6]

Tumours of serous, endometrioid and mucinous histological subtypes resemble the epithelium of the Müllerian duct derivatives during embryonic development, namely: fallopian tubes, endometrium and endocervix

respectively. Clear cell tumours morphologically resemble cells of the gastrointestinal tract and renal cells, suggesting a non-ovarian origin.[7] The heterogeneity within EOC suggests different histological subtypes representing different cell of origins.[7] However, conventional treatment strategies for ovarian cancer does not take this heterogeneity into account, with patients of all histological subtypes receiving a standard mix of platinum-taxane-based chemotherapeutic reagents as the first-line of treatment with variable outcome.[8-11] This underlines a critical lapse in our understanding of ovarian cancer, thus improved therapeutic outcome would require better appreciation of the molecular aspects of the disease.

In attempts to better classify ovarian cancer towards better prediction of clinical response, various groups have analysed the molecular expression profiles of the histological subtypes of ovarian cancer, with an emphasis on the most prevalent and aggressive high-grade serous subtype which accounts for two-thirds of all ovarian cancer mortalities.[12] Mutually exclusive *BRAF* and *KRAS* mutations were found in accumulatively 61% of low-grade serous ovarian carcinomas (LGSOC), whereas high-grade serous ovarian carcinomas (HGSOC) carry wild-type *BRAF* and *KRAS*.[13] However, ~50% of HGSOC carry *TP53* mutations [14] while 14-26% carry germline mutations for *BRCA1/2* tumour suppressor genes.[15] Somatic mutations in sporadic HGSOC are rare, though epigenetic silencing *via* promoter hypermethylation of *BRCA1* has been reported in ~33% of cases.[16] Taken together, these results suggest that HGSOC and LGSOC are mediated by different molecular pathways and may therefore be two distinct subtypes altogether of different origins.[13] Interestingly, classification of 47 ovarian cancer cell lines from the Broad-Novartis Cancer

Cell Line Encyclopedia (CCLE) based on HGSOC & LGSOC molecular subtype expression profiles identified several less commonly used lines (COV318, KURAMOCHI, OVKATE, OVSAHO, SNU119) that show greater genetic similarities to HGSOC than the more commonly used serous lines, such as A2780, HEY A8, OAW42 and SKOV-3.[17]

Another study by the same group proposed a dualistic classification model (type I and type II) based on accumulative clinicopathological and molecular studies reported previously, with type I ovarian carcinoma encompassing the low-grade serous, low-grade endometrioid, mucinous and clear cell carcinomas. Type I ovarian tumours are generally less aggressive, indolent in nature and usually presented at low stages (stages I and II). They resemble benign, borderline and low-grade tumours, suggesting type I tumours to follow a conventional neoplastic-to-tumourigenic progression model.[18] Molecular expression profile of type I ovarian tumours include mutations in specific subset of genes for various histopathologies, including: *BRAF*, *KRAS* and *ERRB2* (low-grade serous), *PTEN*, *CTNNB1* and *PIK3CA* (low-grade endometrioid), *KRAS* (mucinous) and activating *PIK3CA* mutations (clear cell). Type II ovarian tumours encompass high-grade serous, high-grade endometrioid and undifferentiated carcinomas. They are generally characterized to be more aggressive and associated with late stage of disease (stages III and IV) and poor prognosis. Molecular expression profiles of type II ovarian tumours show >80% *TP53* mutations[18,19] Interestingly, type I ovarian tumours show chemoresistance to conventional chemotherapeutic reagents[20-24] and *vice-versa* for type II ovarian tumours,[21,24] indicating that classification of

EOC *via* molecular subtyping may aid in identifying specific patient groups with the more appropriate treatment regimen.[25]

With the advent of microarray-based gene expression profiling technologies, high throughput genome-wide analysis of gene expression profiles of individual patient ovarian tumour samples allow prediction of patients presenting specific gene signatures for clinicopathological features, such as early relapse[26] and chemotherapeutic response.[27] In one such study, gene expression profiling of a cohort of serous, endometrioid and mixed serous-endometrioid ovarian patient samples identified a 14-gene signature list that can predict the likelihood of early recurrence with a high degree of accuracy (24/28 correct predictions, 86%). Remarkably, of the 19 samples predicted for early relapse, 18 patients did eventually relapsed within 21 months post-treatment.[26] Separately, cDNA microarray analysis between two patient groups that show either intrinsic chemoresistance or chemosensitivity to platinum-paclitaxel treatment identified 85 genes differentially expressed between the two groups.[27] Furthermore, cross-analysis between gene expression profiles of patient specimens whom subsequently acquired chemoresistance and the intrinsic chemoresistance group identified overlapping genes that would indicate to be crucial in mediating chemoresistance.

Another study group identified six distinctively novel molecular subtypes (C1-C6) from a cohort of 285 ovarian tumour samples, mostly representing high-grade serous histological subtype but also includes borderline serous (low malignant potential, LMP) and low-grade endometrioid tumours.[28] Briefly, high-grade serous ovarian tumours mainly clustered into four subtypes (C1, -2, -4 and -5) while the less aggressive borderline serous ovarian tumours and low-

grade endometrioid tumours mainly clustered into subtypes C3 and C6 respectively. Both subtypes (C3 and C6) displayed low mitotic-index, evident by low expression levels of proliferative genes such as cyclin B1 and cyclin-dependent kinase A-1 (*CCNB1*, *CDC2*). Elevated expression of mitogen-activated protein kinase (MAPK)-related genes were present in subtype C3 (borderline serous tumours), supporting previous reports of type I low-grade serous ovarian tumours carrying *BRAF* and *KRAS* mutations.[18] Also, subtype C6 tumours (low-grade endometrioid) showed higher expression of β-catenin/LEF/TCF target genes, a hallmark of ovarian endometrioid adenocarcinomas (OEA, *reviewed in section 1.3: WNT Signalling in EOC*). Subtypes C1 and -5 tumours (high-grade serous) showed low levels of $CD3^+$ tumour-infiltrating T-cells whereas subtypes C2 and -4 displayed higher CD3 expression levels. Subtype C5 tumours (high-grade serous) showed up-regulated expression of mesenchymal-associated genes, including homeobox genes, down-regulated expression of differentiated ovarian cancer markers such as Mucin-1 and Mucin-16 (MUC1 & MUC16), while displaying low membrane E-cadherin (CDH1) protein expression. More interestingly, a distinctive trend in progression-free survival (PFS) and overall survival (OS) was observed: Patients whose tumour specimens fell under C3 (borderline serous) and C6 (low-grade endometrioid) subtypes have markedly better prognosis while C1 (high-grade serous, low intratumoural $CD3^+$ tumour infiltrating T-cells) and C5 (high-grade serous, mesenchymal-like and low intratumoural $CD3^+$ tumour infiltrating T-cells) subtypes showed the worst prognosis. As such, this study shows the example of using gene expression

profiling of ovarian tumours for novel molecular subtype classification to better predict clinical outcome.[28]

Another study by The Cancer Genome Atlas group (TCGA) analysed the expression profiles of ~12,000 genes from 489 high-grade serous ovarian adenocarcinomas (HGSOA) and identified four molecular subtypes within HGSOA, namely: i) immunoreactive, ii) differentiated, iii) proliferative and iv) mesenchymal.[29] Though no significant differences in survival were shown among these four subtypes, this study came out with a 193 genes signature list capable of predicting overall survival, as further validated on three other datasets. Interestingly, patients with tumours carrying *BRCA1/2* mutations (germline or somatic) had better overall survival as compared to those carrying wildtype *BRCA1/2* or epigenetically-silenced *BRCA1/2*, suggesting *BRCA1/2* inactivating mutations act differently from epigenetic silencing mechanisms.[29] In a follow-up study, a patient survival-related gene signature list refined from the 193 genes signature list[29] resulted in a gene expression-based prognostic model (Classification of Ovarian Cancer, CLOVAR).[30] Validation of CLOVAR on a dataset consisting of 879 HGSOC gene expression profiles showed that patient tumours that were double-classified as both 'poor prognosis' and 'mesenchymal' to have the worst outcome, with a median overall survival of 23 months and that 63% of patients in this double-classification, as compared to 23% in all other classifications, to be resistant towards platinum-based chemotherapy.[30]

In an independent study to address the heterogeneity of EOC, meta-analysis of publicly available microarray gene expression profiles of 1538 EOC samples lead to the identification of five distinctive molecular subtypes with different

clinicopathological features, namely: Epi-A, Epi-B, Mesenchymal, Stem-A and Stem-B.[31] Epi-A tumours mostly represent the low-grade, less aggressive LMP/ borderline serous subtype with better prognosis while both Mesenchymal and Stem-A molecular subtype tumours were mainly of high-grade with poorer prognosis. Stem-B tumours encompassed all histological subtypes that may complement the type I classification of the dualistic model.[18] *In vitro* validation of genes affecting cell growth in the Stem-A molecular subtype eventually identified two microtubule-related genes Tubulin Gamma associated Protein Complex 4 (*TUBGCP4*) and N-Acetyl Transferase 10 (*NAT10*) to significantly inhibit cell growth. Of clinical significance, Stem-A molecular subtype ovarian cancer cell lines show significant chemosensitivity towards tubulin polymerization inhibitors (vincristine and vinorelbine) as compared to non Stem-A ovarian cancer cell lines. Furthermore, vincristine treatment of the Stem-A cell lines induced apoptosis after 48hr treatment. In summary, this study showed that molecular subtyping of EOC is of clinical significance in identifying and classifying particular subtypes toward specific clinicopathological phenotypes.[31]

Anoikis: A role in EOC Metastasis

Two hallmarks of cancer include the evasion of apoptosis and acquiring a metastatic phenotype.[32,33] In EOC progression, ovarian cancer cells must overcome an anchorage-independent apoptotic cell death mechanism termed '*anoikis*' for survival when suspending in the ascitic fluid, thereby facilitating eventual metastatic spread to the peritoneum.

Activating BRAF mutations has been previously reported to protect melanoma cells from *anoikis via* inhibition of pro-apoptotic BH3-mimetics BAD (BCL2-associated Agonist of cell Death) and BCL2L11/BIM (BCL2-like 11).[34,35] Interestingly, type I LGSOC commonly carry this activating BRAF mutation,[13] suggesting that LGSOC are inherently 'primed' towards *anoikis*-resistance. Antisense cDNA suppression of the anti-apoptotic effector BCL2-like 1 (BCL2L1/ BCL-X_L) in HEY ovarian serous carcinoma cells induced *anoikis* upon three-dimensional culturing and the capacity for anchorage-independent growth in soft agar was abrogated.[36] Furthermore, down-regulation of BCL2L1 greatly diminished subcutaneous tumourigenic growths *in vivo*. Array comparative genomic hybridization (CGH) analysis of advanced serous EOC identified increased DNA copy number on chromosome 1q22[37] to be associated with elevated expression of the RAB25 small GTPase.[38] Interestingly, high RAB25 expression correlated with poor prognosis. A2780 and T29 ovarian carcinoma cell lines stably overexpressing *RAB25* augmented *anoikis*-resistance in suspension culture, while increasing anchorage-independent growths in soft agar.[38] This was abrogated upon RNAi-mediated knockdown of *RAB25* in A2780 cells. Furthermore, *RAB25* overexpression concurrently increased AKT phosphorylation and down-regulated expression of pro-apoptotic effectors BAK (Bcl-2 homologous Antagonist/Killer) and BAX (Bcl-2 Associated protein X). Taken together, these results suggest that RAB25 down-regulates pro-apoptotic effectors BAK and BAX by the PI3K-AKT pathway to promote *anoikis*-resistance.[38] However, a recent study based on ovarian cancer clinical specimens found no

significant correlation between elevated immunohistochemical-staining of RAB25 and the occurrence of peritoneal metastasis.[39]

Neurotrophic Tyrosine Kinase receptor type 2 (NTRK2/ TrkB) was previously reported to be associated with an aggressive phenotype in various cancer types and elevated NTRK2 expression was found in the *anoikis*-resistant sub-population of OVCAR-3 ovarian serous carcinoma cells.[40] siRNA-mediated knockdown of *NTRK2* inhibited PI3K-AKT pathway and sensitized OVCAR-3 cells towards *anoikis*. Furthermore, *NTRK2* knockdown partially impaired OVCAR-3 cell invasiveness *in vitro* and smaller tumours were formed *in vivo*. These results show that NTRK2 regulates *anoikis* and tumourigenesis *via* the PI3K-AKT pathway. Another study showed that hepatocyte growth factor (HGF) induced *anoikis*-resistance in Caov-3 and SKOV-3 ovarian carcinoma cells when cultured in suspension. Conversely, both siRNA-mediated knockdown and small molecule inhibition of the HGF receptor c-MET (MET proto-oncogene receptor tyrosine kinase) abrogated *anoikis*-resistance.[41] Interestingly, HGF-stimulation activated both the PI3K-AKT and ERK1/2-Mitogen-Activated Protein Kinase (MAPK) pathways. Co-treatment of HGF with either PI3K-inhibitors (wortmannin or LY294002) or *AKT* siRNA or an ERK1/2 inhibitor (PD98059) all abrogated *anoikis*-resistance. Furthermore, PI3K inhibitors also inhibited ERK1/2 phosphorylation. In summary, this study showed a crosstalk between PI3K-AKT and ERK1/2 MAPK pathways upon induction by HGF to mediate *anoikis*-resistance.[41] In a separate study, the use of Foretinib, a small molecule multi-kinase inhibitor of c-MET and Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) induced cell detachment and subsequent *anoikis* in

Caov-3 cells in a caspase-dependent manner *in vitro* and reduced the presence of SKOV-3 metastatic lesions *in vivo*.[42]

In another study, the inhibition of ERK1/2 MAPK pathway with U0126 (small molecular inhibitor of MEK1/2) in IGROV-1 ovarian endometrioid carcinoma cells impaired *anoikis*-resistance, accompanied by an increment in the levels of the apoptotic marker cleaved-PARP (Poly ADP-Ribose Polymerase).[43] Similarly, inhibition of Protein Kinase C (PKC) with calphostin C in IGROV-1 cells during suspension culture increased levels of cleaved-PARP and caspase-3 activity while the reverse trend was observed upon PKC activation with the phorbol ester-based PKC activator PMA. A similar pro-*anoikic* trend was also observed after silencing of αv integrins. Furthermore, a slight decrease in ERK1/2 phosphorylation was observed after αv integrins knockdown, while the opposite was observed after PMA-induced PKC activation. Taken together, these suggest a crosstalk between PKC and αv integrins towards ERK1/2 activation for mediating *anoikis*-resistance in IGROV-1 cells.[43]

In vivo passaging of two ovarian serous carcinoma cell lines SKOV-3 and HEY generated daughter cells that acquired a more aggressive phenotype, termed SKOV-3.ip1 and HEY 1B respectively. Interestingly, these transformed cells displayed enhanced *anoikis*-resistance when cultured in suspension,[44] further evident by the enhancement of anchorage-independent growths of SKOV-3.ip1 cells in soft agar colony formation assays. Of note, these transformed cells displayed increased Src kinase activity relative to the parental cells, while the ectopic overexpression of Src in OVCAR-5 and

OVCA420 ovarian carcinoma cells induced *anoikis*-resistance when cultured in suspension, further showing the role of Src in mediating *anoikis*-resistance.

Following neoplastic formation of the primary tumour, ovarian cancer cells eventually shed off from the primary site to suspend in the ascitic fluid within the peritoneal cavity. Cells that are *anoikis*-resistant form aggregates and multicellular spheroids. While in suspension, single cells from these aggregates and multicellular spheroids may also bud off as precursors,[45] acting as 'seeds' in the ascitic fluid, subsequently re-attaching onto the mesothelial lining within the peritoneal cavity.[46] Spheroid formation may be initiated from monolayer cultures *in vitro*, with several ovarian cancer cell lines-derived spheroids spontaneously budding off thereafter, indicating an alternative route towards EOC metastasis.[47]

The attachment of ovarian carcinoma cells onto the mesothelial lining of the peritoneal cavity marks a critical step in ovarian cancer progression, indicating the onset of invasion through the peritoneum towards organs around the abdomen and eventual distal metastasis. Previous *in vitro* studies has shown that cell-cell and cell-matrix interacting proteins such as CD44 and β1 integrins to mediate ovarian carcinoma-mesothelial cell interactions.[48-52] Similarly, spheroids from both OVCAR-5 and patient ascites display adhesive affinity towards multiple ligands of integrins,[53-55] whereby β1 integrins were shown to mediate spheroid adhesion onto both ECM components and LP9 human mesothelial cells. Furthermore, patient-derived spheroids were able to disseminate onto live mesothelial cells *in vitro*, with some invading into the mesothelial monolayer.[56] Upon re-attachment and dissemination onto the mesothelial layer, these cancer cells generate contractile forces on the

surrounding mesothelial cells, clearing a passage for subsequent invasion. This contractile force is mediated by α5β1-integrin/ myosin II/ talin I between the ovarian cancer cells and the mesothelial cells.[57] Interestingly, α5 and β1 integrins mediate *in vitro* spheroid formation in OVCAR-5 and HEY ovarian carcinoma cell lines, whereby inhibition *via* the respective monoclonal antibodies (mAbs) abrogated spheroid formation.[53,58] In addition, fibronectin and laminin (ligands of integrin receptors) enhanced spheroid formation efficiency, further supporting the role of integrins in EOC spheroid formation.[53] Patient ascitic fluid has been reported to promote spheroid formation and cell invasion *in vitro*, though the study did not find any association between the two phenotypes when treated in the same ascitic fluid.[59] The study identified tribbles pseudokinase 1 (*TRIB1*) expression to be differentially up-regulated in cells treated with ascitic fluid that enhanced cell invasiveness. Furthermore, *TRIB1* expression was found to be associated with poorer patient survival.[59] Of importance, spheroids display enhanced resistance against both radiotherapy[60] and chemotherapeutic reagents[61-63] *in vitro*, further complicating treatment strategies. Of note, pathway and network analyses of oligonucleotide microarray data of ovarian carcinoma cell lines-derived spheroids treated with conventional chemotherapeutic agents (cisplatin, topotecan and paclitaxel) identified five common networks affected, namely i) cell growth/ proliferation, ii) cell cycle, iii) cell death, iv) motility and v) metabolism.[64] These results would allow for future studies to overcome the mechanisms of chemotherapeutic resistance.

CHAPTER 1.2

WNT Signalling: History and Brief Introduction

In the mouse, the proto-oncogene *int-1* (first common integration site) gene was first identified as the proviral integration site of the retroviral mouse mammary tumour virus (MMTV) to induce mammary tumourigenesis.[65] Initial work showed int-1 to contain a secretion signal sequence, suggestive of a growth factor function.[66,67] Subsequent sequencing studies carried out separately[68,69] identified *int-1* to be identical to that of the segment polarity gene *wingless* (*wg*) in Drosophila, mutants of which display a literally wingless phenotype.[70,71] As such, *int-1* was identified as the first mammalian homologue of *Drosophila wg*. Subsequent confusions in the naming nomenclature of *int*-related genes eventually resulted in the *int-1* gene being re-named as *Wnt1* (Wingless-related MMTV integration site family 1), an acknowledgement of the independent studies that resulted in the identification of the gene.[72] In the human genome, a total of 19 *WNT* ligands have been identified to date, all sharing a highly conserved distribution of 22-24 cysteine residues.[73,74]

Synthesis and Secretion of WNT Ligands

WNT ligands are synthesized in the endoplasmic reticulum (ER) and post-translationally palmitoylated on two conserved residues by the membrane bound o-acyltransferase (MBOAT) Porcupine (PORCN),[75,76] crucial for facilitating proper secretion and ligand-receptor binding.[77,78] Palmitoylated WNTs are subsequently transported from the trans Golgi network (TGN) to the cell surface for secretion by Wntless (WLS), a seven transmembrane WNT

protein carrier/ transporter molecule.[79-81] Cells expressing mutant WLS showed an accumulation of cytosolic WNT, without any WNT ligands detectable within the supernatant of the cells.[79] Point mutation of a conserved serine residue (S209) on WNT3A disrupted proper palmitoylation by PORCN, preventing WLS recognition to initiate the transport process.[77] Furthermore, proper secretions of WNTs by WLS require an acidic vacuolar environment.[77] WLS is subsequently endocytosed and carried back to the endoplasmic reticulum (ER)[82] through the TGN by the retromer complex through a retrograde transport mechanism.[83-86] After secretion, WNT ligands may be deacylated by the extracellular deacylase Notum, which acts as a regulator of homeostasis to render the ligands inactive (see Figure A).[87]

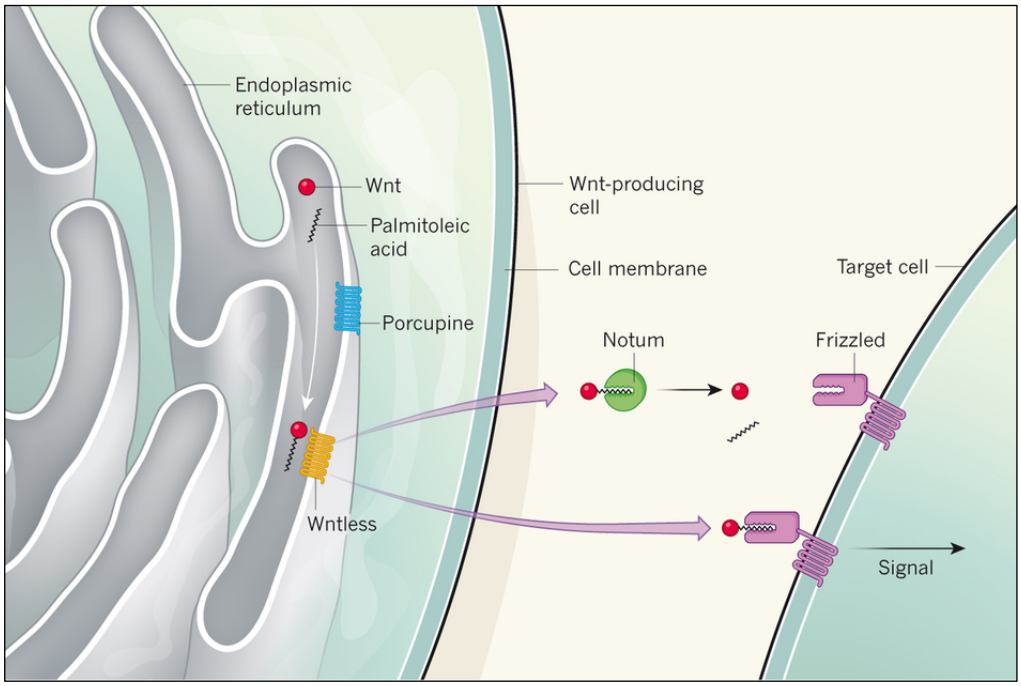


Figure A: Cartoon depicting the process of WNT synthesis, whereby nascent WNTs from the rough endoplasmic reticulum are palmitoylated by Porcupine (PORCN) and transported by Wntless (WLS) to the cell surface. Notum acts to maintain homeostatic levels of WNT activity, deacylating WNT ligands for inactivation. WNT ligands not targeted by Notum proceeds to interact with Frizzled (FZD) receptors on the target cell, activating the Wnt cascade. Image from Nusse (2015).[88] Reproduced with permission from Nature Publishing Group. Refer to Appendix A for the copyrights permission granted.

Subsequently, WNT ligands not affected by Notum interacts with their respective Frizzled-class receptors (FZD), which are a unique group of seven-pass trans-membrane G-protein coupled receptors (GPCR) that share a homologous N-terminal extracellular cysteine-rich domain (CRD) that interacts with WNT ligands. In addition, the C-terminus intracellular domain contains a 'Ser/Thr-Xxx-Val' motif that interacts with the PDZ domain of the cytoplasmic Dishevelled (DVL/Dvl in mammals, Dsh in *Drosophila*) adaptor proteins[89] to mediate different branches of the WNT signalling. FZD was first identified to be a WNT receptor based on *Drosophila* models, whereby S2 cells exogenously expressing *fzd2* showed elevated levels of phosphorylated armadillo (Drosophila homologue of β-catenin) upon addition of wg. Furthermore, immunofluorescence studies showed fzd2 and wg co-localization in both *Drosophila* S2 and human 293 cells.[90] To date, ten known FZD in the human genome have been identified, FZD1-10 (reviewed in Schulte, 2010).[91] X-ray crystallography between *Xenopus* Wnt8 and Fzd8 CRD revealed Wnt8 binding to the Fzd8 CRD on two conserved sites, whereby a post-translationally modified palmitoleic acid group on a conserved serine residue on the amino-terminus (N-terminus) of Wnt8 inserts into the hydrophobic groove within the Fzd8 CRD on one site and hydrophobic interactions between the CRD at the carboxyl-terminus (C-terminus) of Wnt8 and Fzd8 mediates interaction on the second site.[78]

WNT signalling is generally sub-divided into two major categories, the 'canonical' or β-catenin-mediated pathway and the 'non-canonical' β-catenin-independent pathway, which is further sub-divided into the planar cell polarity

(PCP)/ convergent extension (CE) pathway and the calcium Ca^{2+} signalling pathway, which is beyond the scope of the current study and will not be discussed any further. The following will attempt to review the current understanding of the β-catenin-mediated and also the PCP/CE pathways instead. In the past, WNT ligands have been classified into being either canonical or non-canonical. However, numerous recent studies have shown that WNT ligands work in a context-dependent manner, with some activating the β-catenin-mediated pathway in one system while activating the PCP/CE pathway in another system, further adding to the complexity of WNT signalling. For a summary of the WNT ligands and FZD receptors involved in the various pathways, please refer to Tables 1A and 1B.

WNT Signalling (*β*-Catenin-mediated)

In the absence of WNTs, *β*-catenin (CTNNB1) is phosphorylated by glycogen synthase kinase 3 (GSK3) and Casein Kinase 1 (CK1), which together with AXIN (a scaffolding molecule), Adenomatous Polyposis Coli (APC) and the protein Dishevelled forms the '*β*-catenin destruction complex'. Phosphorylated *β*-catenin molecules are eventually ubiquitin-tagged for proteosomal degradation. During WNT signalling, WNT ligands interact with FZD receptors and also with co-receptor Lrp6 (Low-density-lipoprotein Receptor-related Protein 6, a single-span transmembrane co-receptor)[92,93] *via* a linker region between the N/C-terminus of Wnt ligands and the extracellular domain of Lrp6.[94,95] As such, Wnt-binding induces a ternary complex formation between Fzd-Wnt-Lrp6 while Wnt-Fzd binding induces Fzd-Dvl interactions *via* the PDZ[96] and DEP[97] domains of Dvl. This allows for Dvl

Table 1A: Classification of WNT ligands into the respective pathways

	β-Catenin- TCF/LEF	PCP/CE	Ca^{2+} Flux
WNT1	Gazit *et al*., 1999[98] He *et a*l., 2004[99] Korinek *et al*., 1998[100] Mizushima *et al*., 2002[101] Papkoff *et al*., 1996[102] Rhee *et al*., 2002[103] Shimizu *et al*., 1997[104]		
WNT2	Basu and Roy, 2013[105] Bravo *et al*., 2013[106] Fu *et al*., 2011[107] Gazit *et al*., 1999[98] Goss *et al*., 2009[108] Karasawa *et al*., 2002[109] Pu *et al*., 2009[110] Shi et al., 2007[111] Shimizu *et al*., 1997[104] Wang *et al*., 2013[112] Xu *et al*., 2015[113] You *et al*., 2004[114,115]	Le Floch *et al*., 2005[116]	Wayman *et al*., 2006[117]
WNT2B	Cho *et al*., 2006[118] Kobayashi *et al*., 2012[119] Li *et al*., 2015[120] Liu *et al*., 2012[121]		
WNT3	Gazit *et al*., 1999[98] Lu *et al*., 2004[122] Shimizu *et al*., 1997[104]		
WNT3A	Boland *et al*., 2004[123] Gazit *et al*., 1999[98] Nakamura *et al*., 2003[124] Samarzija *et al*., 2009[125] Shimizu *et al*., 1997[104] Yokoyama *et al*., 2007[126]	Simonetti *et al*., 2014[127]	Samarsija et al., 2009[125] Simonetti *et al*., 2014[127]
WNT4	Guo *et al*., 2004[128] Lyons *et al*., 2004[129] Tsaousi *et al*., 2011[130]	Chang *et al*., 2007[131] Du *et al*., 1995[132] Maurus *et al*., 2005[133] Torban *et al*., 2006[134]	
WNT5A	Mikels and Nusse, 2006[135]	Andre *et al*., 2012 20[136] Fenstermaker *et al*., 2010[137] Gao *et al*., 2011[138] Moon *et al*., 1993[139] Qian *et al*., 2007[140] Yamamoto *et al*., 2007[141]	Saneyoshi *et al*., 2002[142] Slusarski *et al*., 1997[143,144]
WNT5B		Hayes *et al*., 2013[145] Lin *et al*., 2010[146] Matthews *et al*., 2008[147] Westfall *et al*., 2003[148]	
WNT6	Cawthorn *et al*., 2012[149] Krawetz and Kelly, 2008[150]	Li *et al*., 2014[151] Schmidt *et al*., 2007[152]	

Table 1A (continued): Classification of WNT ligands into the respective pathways

	β-Catenin- TCF/LEF	PCP/CE	Ca^{2+} Flux
WNT7A	Daneman *et al.*, 2009[153] King *et al.*, 2014[154] Posokhova *et al.*, 2015[155] Yoshioka *et al.*, 2012[156]	Bentzinger *et al.*, 2013[157] Dabdoub *et al.*, 2003[158] Le Grand *et al.*, 2009[159] van den Berg and Sasson, 2009[160]	
WNT7B	Arensman *et al.*, 2014[161] Daneman *et al.*, 2009[153] Pietilä *et al.*, 2011[162] Posokhova *et al.*, 2015[155] Wang *et al.*, 2005[163] Yu *et al.*, 2009[164]	Fenstermaker *et al.*, 2010[137] Zheng *et al.*, 2013[165]	
WNT8A	Kimura-Yoshida *et al.*, 2005[166] So *et al.*, 2013[167]		
WNT8B	Kelly *et al.*, 1995[168,169] Kim *et al.*, 2002[170] Lee *et al.*, 2006[171]		
WNT9A	Guo *et al.*, 2004[128]	Curtin *et al.*, 2011[172] Dougherty *et al.*, 2013[173] Kan and Tabin, 2013[174]	
WNT9B	Boivin *et al.*, 2015[175] Carroll *et al.*, 2005[176] Karner *et al.*, 2011[177] Kiefer *et al.*, 2012[178] Lan *et al.*, 2006[179]	Karner *et al.*, 2009[180]	
WNT10A	Cawthorn *et al.*, 2012[149] Hsu *et al.*, 2012[181]		
WNT10B	Cawthorn *et al.*, 2012[149] Li *et al.*, 2011[182] Wend *et al.*, 2012[183] Wend *et al.*, 2013[184] Yoshikawa *et al.*, 2007[185]		
WNT11	Tao *et al.*, 2005[186] Ye *et al.*, 2011[187]	Bai *et al.*, 2014[188] Du *et al.*, 1995[132] Hayes *et al.*, 2013[145] Heisenberg *et al.*, 2000[189] Kim *et al.*, 2008[190] Marlow *et al.*, 2002[191] Tada and Smith, 2000[192]	Ouko *et al.*, 2004[193]
WNT16	Guo *et al.*, 2004[128] Wergedal *et al.*, 2015[194]	Kan and Tabin, 2013[174]	
WNT16B	Mazieres *et al.*, 2005[195] Moveraré-Skrtic *et al.*, 2014[196] Sun *et al.*, 2012 2012[197]	Teh *et al.*, 2007[198] Moveraré-Skrtic *et al.*, 2014[196]	

Table 1B: Classification of FZD receptors into the respective pathways

	β-Catenin- TCF/LEF	PCP/CE	Ca^{2+} Flux
FZD1	Doi *et al*., 2014[199] Flahaut *et al*., 2009[200] Gao and Wang, 2006[201] Liu *et al*., 2001[202] Neumann *et al*., 2010[203] Planutis *et al*., 2013[204] Trowe *et al*., 2012[205] Wang *et al*., 2005[163] Zhang *et al*., 2012[206]	Hung *et al*., 2014[207] Yu *et al*., 2010[208]	
FZD2	Li *et al*., 2008[209] Verkaar *et al*., 2009[210]	Gujral *et al*., 2014[211] Kadzik *et al*., 2014[212] Li *et al*., 2013[213] Lin *et al*., 2010[146] Yu *et al*., 2010[208] Yu *et al*., 2012[214]	Ahumada *et al*., 2002[215] Ma and Wang, 2006[216] Zhou *et al*., 2012[217]
FZD3		Chai *et al*., 2015[218] Cizelsky *et al*., 2014[219] Hua *et al*., 2014[220,221] Lyuksyutova *et al*., 2003[222] Montcouquiol *et al*., 2006[223] Onishi *et al*., 2013[224] Simonetti *et al*., 2014[127]	Simonetti *et al*., 2014[127]
FZD4	Birdsey *et al*., 2015[225] Jin *et al*., 2011[226] Tickenbrock *et al*., 2008[227] Ye *et al*., 2011[187]	Bian *et al*., 2014[228] Descamps *et al*., 2012[229] Yu *et al*., 2010[230]	
FZD5	Liu *et al*., 2008[231]	Lee and Heur, 2014[232] Slater *et al*., 2013[233]	Arderiu *et al*., 2014[234]
FZD6		Cantilena et al., 2012[235] Devenport and Fuchs, 2008[236] Golan *et la*., 2004[237] Guo *et al*., 2004[238] Hua et al., 2014[221]	
FZD7	Chakrabarti *et al*., 2014[239] Kim *et al*., 2008[240] Mei *et al*., 2014[241] Zhang *et al*., 2013[242]	Asad *et al*., 2014[243] Bentzinger *et al*., 2013[157] Kim *et al*., 2008[190] (JCB) Kraft *et al*., 2012[244] Yu *et al*., 2012[214] Yuan *et al*., 2015[245] Zhang *et al*., 2013[242]	
FZD8	Albers et al., 2013[246] Bourhis *et al*., 2010[94] Bravo *et al*., 2013[106] Kim *et al*., 2002[170] Miao *et al*., 2015[247] Yin *et al*., 2013[248]	Moon *et al*., 1993[139]	
FZD9	Fujimoto *et al*., 2009[249] Karasawa *et al*., 2002[109] Wang *et al*., 2009[250]		
FZD10	Galli *et al*., 2014[251] Terasaki *et al*., 2002[252] Wang *et al*., 2005[163]	Fukukawa *et al*., 2009[253]	

recruitment of the Axin-Gsk3 complex *via* a homologous DIX domain between Dvl- Axin.[254,255] As such, recruitment of the Axin-Gsk3 complex by Dvl within close proximity of Lrp6 facilitates Gsk3 phosphorylation of multiple P-P-P-S/T-P proline- rich motifs on the cytoplasmic domain of Lrp6,[256] thereby promoting Lrp6-Axin interactions[257-259] that disrupt proper formation of the β-catenin destruction complex.[260] It should also be noted that CK1 has also been implicated in Lrp6 phosphorylation to mediate Wnt pathway activation as well, reportedly in a membrane-bound manner independent of Axin and GSK-3β.[257] As such, unphosphorylated *β*-catenin molecules accumulate in the cytoplasm, eventually translocating to the cell nucleus by a yet unclear mechanism to activate Tcf/LEF transcription factors (reviewed in Clevers & Nusse, 2012).[261] Some of the target genes include: c-MYC,[262] cyclin D1 (CCND1),[263,264] fibronectin (Gradl *et. al*, 1999),[265] matrix metalloproteinase (MMP) 1/ 7/ 14,[266-269] Slug/ SNAI2,[270] survivin[271,272] and ATP-binding Cassette G2 (ABCG2) transporter efflux pump.[273] For an overview of the β-catenin-mediated pathway, refer to Figure B.

WNT Signalling (Non β-catenin-mediated/ β-catenin-independent)

Planar cell polarity (PCP) describes the pathway regulating the axial positioning of a body plane; aligning cells/structure/organ/body with reference to the plane towards specific orientations, resulting in coherent group of cells with uniformed polarity. In *Drosophila*, this involves two subsets of PCP-related genes expressed asymmetrically in either the proximal- or distal-end at the cellular level (refer to Table 1). Furthermore, gradient expression of *Fat* (*FT*), *Dachsous* (*DS*) and *Four-jointed* (*FJ*) provide cues to promote FT-DS

heterodimer towards a particular orientation. These asymmetric expression patterns stimulate downstream effector molecules, providing positional cues to orientate cells or tissue towards a particular polarity (reviewed in Bayly & Axelrod, 2011).[274]One such example is the development of the *Drosophila* wing, whereby hair cuticles orientate uniformly towards the distal end (reviewed in Adler, 2002).[275]

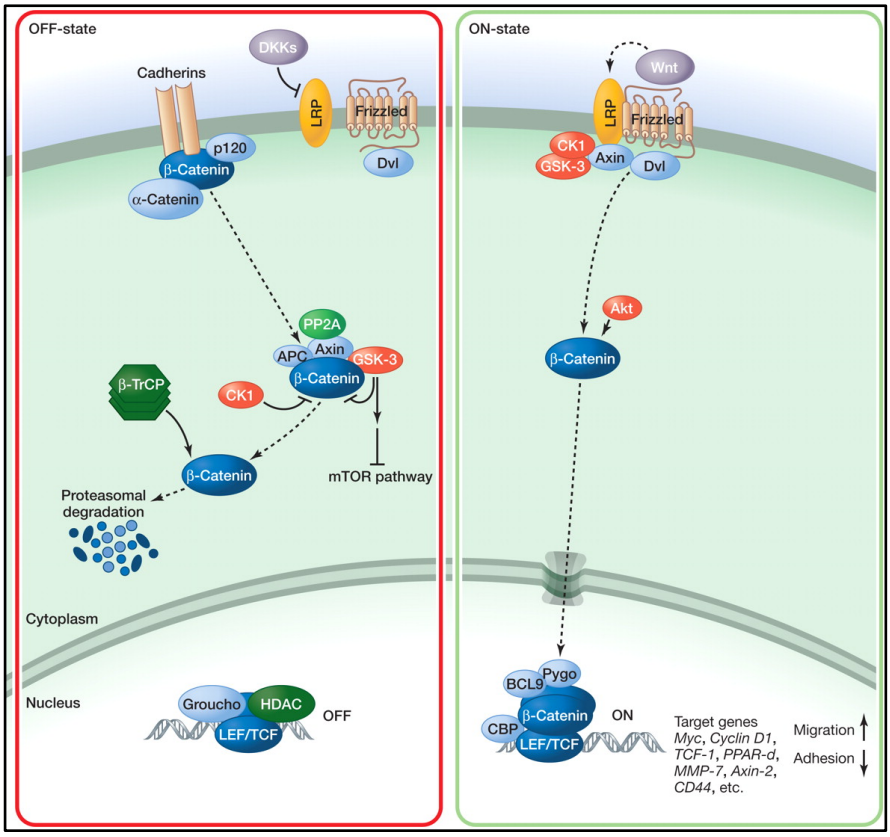


Figure B: Overview of WNT/β-catenin-mediated pathway. Image from Nusse, 2012.[276]

Table 2: Asymmetric distribution of core components of the Frizzled/PCP pathway

Proximal		Distal	
Drosophila	Vertebrate	*Drosophila*	Vertebrate
Flamingo (fmi)	CELSR1/2	Flamingo (fmi)	CELSR1/2
Prickle (pk)	PRICKLE1/2/3	Frizzled (fz)	Frizzled (FZD1-10)
Van Gogh (vang)/ Strabismus (stbm)	Vang-like (VANGL1/2)	Dishevelled (dsh)	Dishevelled (DVL1/2/3)
		Diego (dgo)	Ankyrin repeat domain 6 (ANKRD6)

Drosophila carrying mutant *frizzled* (*fz*) display defective cuticular polarity with hair cuticles being non-uniformly patterned along the proximal-distal axis in multiple regions including the wings and legs, suggesting the involvement of Wnt signalling in mediating PCP.[277-279] In *mus musculus*, presence of either Wnt7a or Wnt-antagonists Sfrp1 (Secreted Frizzled Related Protein 1) or Wif1 (Wnt Inhibitory Factor 1) inhibited proper orientation of hair cell stereociliary bundles in the sensory epithelium of the cochlea[158] while *Ryk*$^{-/-}$ mice (receptor related to tyrosine kinase, a Wnt receptor tyrosine kinase with a Wnt Inhibitory Factor/WIF-domain),[280,281] *Ptk7* mutant mice (Protein tyrosine kinase 7, a pseudokinase) and *Wnt5a*$^{-/-}$ embryos all displayed a similar phenotype.[140,282,283] Knock-out of the *Wnt* receptor *Frizzled-6* (*Fzd6*) has been shown to regulate hair-patterning growth independent of Wnt/β-catenin signalling,[238] displaying hair whorl patterning similar to that of *Fz*$^{-/-}$ *Drosophila* wings, demonstrating the conservation of PCP-pathway genes between invertebrates and vertebrates. Furthermore, the asymmetric localization of Fzd6 in the embryonic epidermis of mice was shown to mediate hair follicle polarity during development.[236]

Convergent extension (CE) describes the intercalation of cells towards a single plane (convergence) that results in a collective directional elongation out towards the perpendicular plane (extension) during development, usually involving the convergence of cells in the mediolateral and dorsal-ventral axes and extension of the anterior-posterior axis. Examples of CE movements include mesodermal cells along the archenteron resulting in notochord formation during *Xenopus* and zebrafish (*danio rerio*) gastrulation and neurulation,[284] and elongation of presomitic mesodermal cells during

neurulation in mice.[285] Of note, the genes regulating PCP in invertebrates also regulate CE in vertebrates, showing that PCP and CE share a conserved pathway across species and more importantly, that cell polarity mediates the onset of motility.[286,287] During the collective migration of cells observed during CE, migratory cells at the leading edge adopt a front-back planar polarity, whereby active protrusions at the frontal edge and a retractive area at the back are formed to mediate motility (reviewed in Mayor and Theveneau, 2014).[288] Wnt/PCP mediate CE upon Wnt ligand binding to Fzd receptors, recruiting Dishevelled (Dsh/ DVL) and Dishevelled Associated Activator of Morphogenesis 1 (Daam1, a Formin-homology containing cytoplasmic adaptor protein) *via* both PDZ and DEP domains[289,290] to activate RhoA and Rac1 small GTPases to induce cytoskeletal remodeling, acquiring cell polarity to mediate cellular migration. Rac1 induces actin polymerization required for the formation of active protrusions (lamellipodia, filopodia and invadopodia) to facilitate motility at the leading edge while RhoA regulates Rho associated kinase (Rok2) activation to induce stress fiber formation and actomyosin contractility at the back-end through cell-cell 'contact inhibition of locomotion' mechanisms.[288] The involvement of Wnts in convergent extension movements during vertebrate development was demonstrated after overexpression of *Wnt5A* in *Xenopus* embryos resulted in a neural ectoderm defective phenotype (incomplete anterior neural tube closure), manifesting itself with the compression of the anterior-posterior body axis and widening of the medial-lateral axis, appearing 'shorter and fatter' than wildtype embryos.[139] Furthermore, *Wnt5A* overexpressing *Xenopus* embryos did not undergo morphogenetic elongation after activin-mediated mesoderm

induction[139] while antagonism of *Wnt5A* and *Fzd8* disrupted convergent extension during *Xenopus* gastrulation.[291] A subsequent follow-up study in *Xenopus* identified overexpression of either *Wnt4* or *Wnt11* to induce a similar phenotype *ex vivo*.[132] In developing mice embryos, graded expression of *Wnt5a* in the palate was observed in an anterior-posterior distribution and mesenchymal palatal cells migrated directionally towards the Wnt5a gradient.[292] *Wnt5a*$^{-/-}$ embryos displayed a defective migratory phenotype in the palatal mesenchyme, resulting in cleft palate.[292] Furthermore, *Wnt5a*$^{-/-}$ embryos impaired CE movements, evident by shortened anatomical features including body length and tail during anterior-posterior extension, forelimbs at the distal end with loss of digital differentiation, frontal-nasal development and cochlear duct extension.[136,138,140] Morpholino-induced silencing of *Wnt5b* in zebrafish embryos impaired posterior extension accompanied by dense somitic distribution.[146] Furthermore, expression of mutant Dishevelled (*Dsh*) in *Xenopus* Keller explants disrupted cell polarity and stability of cell protrusions, subsequently impairing regular convergent extension movements during gastrulation and neurulation.[293] Interestingly, this defective phenotype was shown to be mediated by the PDZ and DEP but not the DIX domains of *Dsh*,[293] implicating the former two domains to mediate Wnt/PCP pathway, as has been previously reported.[294,295] In addition, phosphorylation of Dsh is mediated by Wnt11 and expression of a dominant-negative *Wnt11* (*dnWnt11*, C-terminus truncation) impaired Dsh phosphorylation, accompanied by disruption of convergent extension during *Xenopus* gastrulation.[192] Rescue of the phenotype was achieved by expressing a *Dsh-ΔN* (N-terminus truncation) construct comprising of the PDZ and DEP but not the DIX domains,

demonstrating Wnt-induced CE movements is β-catenin-independent[192] as the DIX domain is required for interacting with Axin to disrupt subsequent β-catenin degradation.[254,296,297] Similarly, loss-of-function mutation of *Wnt11* in zebrafish impaired regular convergent extension during gastrulation, even resulting in *cyclopia*.[189] Furthermore, constitutive activation of β-catenin (ΔN β-catenin) in *Wnt11*$^{-/-}$ embryos failed to rescue the phenotype, neither did expression of a dominant-negative Tcf-3 induce the *Wnt11*$^{-/-}$ phenotype,[189] providing further evidence that CE movements is not mediated by Wnt/β-catenin pathway. In addition, morpholino-silencing of *Ryk* displayed the same phenotype as *Wnt11*$^{-/-}$ in zebrafish[282] while impairing neural tube closure and Keller sandwich explant elongation during embryonic development in *Xenopus*.[190] *Ptk7* mutant embryos of both mice and zebrafish displayed hallmarks of PCP-defects: shortened anterior-posterior body length and extended medio-lateral body axis[145,283,285] accompanied by shorter and wider posterior notochord.[145,285] Interestingly, *Ptk7* potentiated the Wnt/PCP pathway in zebrafish embryos, enhancing the phenotypes induced by *Wnt5b* or *Wnt11* overexpression.[145] Furthermore, *chuzhoi* (*Chz*) mutant mice carry a splicing mutation in *Ptk7*, resulting in an addition of three amino acids that disrupted proper function.[298] *Ptk7*$^{Chz/Chz}$ homozygous embryos display impaired PCP/CE movements, evident by severe neural tube closure defects and mis-orientation of stereociliary hair bundles within the cochlear epithelium.[298] Also, embryos of double heterozygous mice carrying either, *Ptk7*$^{-/+}$;*Vangl2*$^{Lp/+}$, *Ror2*$^{-/-}$;*Vangl2*$^{Lp/+}$ or *Ryk*$^{-/+}$;*Vangl2*$^{Lp/+}$ mutations displayed enhanced severity in neural tube closure defects as compared to the single heterozygous mutants (Lp: *Looptail* transgenic mice carrying *Vangl2* G1391A

point mutation, resulting in a serine → asparagine mutation at amino acid 464 of the protein).[138,282,283] Taken together, these results further show that Ptk7, Ror2 and Ryk mediate PCP and CE through the Wnt/PCP pathway.[138,282,283] Similarly, transgenic mice models carrying mutations in several of the PCP homologs disrupted mesodermal CE movements during neurulation, displaying severe defects in neural tube closure.[299-304]

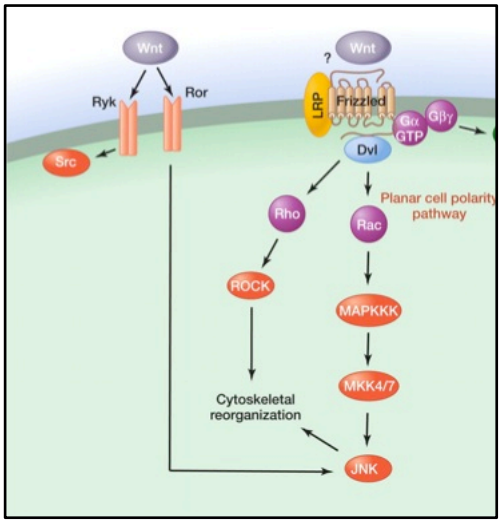


Figure C: Overview of the PCP pathway. Image taken from Nusse, 2012.[276]

Other WNT co-receptors mediating PCP

ROR2 (Receptor tyrosine kinase-like orphan receptor 2) is a single transmembrane receptor that was first identified through cDNA PCR screening of a human neuroblastoma cell line (SH-SY5Y) with an intracellular tyrosine kinase domain[305] and a conserved extracellular cysteine-rich domain (CRD) homologous to that of the FZD receptors.[305,306] Subsequent *in vitro* binding and immunoprecipitation assays identified this CRD of Ror2 to bind to Wnt5a and Fzd2 in rats (*rattus norvegicus,* note that rat *Fzd2* is homologous to human *Fzd5*),[307] Wnt5a, Wnt8 and Wnt11 in *Xenopus*,[308] Wnt11 in zebrafish (*danio rerio*)[188] and Fzd7 in *mus musculus*,[309] demonstrating Ror2 to

act as a receptor/ co-receptor for Wnt ligands. Perturbed fore- and hindlimb cartilage growths at the distal end displayed by *Ror2* homozygous-deletion mutant mice (*Ror2*$^{-/-}$)[310,311] is due to the disruption of Wnt5a-induced Ror2-Vangl2 heterodimer receptor complex formation and subsequent Vangl2 phosphorylation by casein kinase I delta (CKIδ) to induce PCP/CE directional growths.[138] Dominant-negative Ror2 in zebrafish (dnRor2) lacking the cytoplasmic domain displayed defective CE movements during gastrulation, evident by shorter embryos in the anterior-posterior axis, wider somites in the mediolateral axis and cyclopia,[188] which could be partially rescued by exogenous introduction of either *Dsh* or *RhoA*.[188] Of interest, several *in vivo* studies have shown similar phenotypes between either *Ror2*$^{-/-}$/*Wnt5a*$^{-/-}$ or *Ror2*$^{-/-}$/*Wnt11*$^{-/-}$ models[138,188,292,307,312] while *Ror2* siRNA-silencing impaired NIH3T3 cellular response to Wnt5a-induced cell migration/ wound healing *in vitro*.[313] Ror2 mediates Wnt5a-induced activation of JNK/AP-1 to promote filopodia formation for polarized cell motility by forming a Wnt5a-Fzd7-Ror2 complex that promotes Dvl2 phosphorylation and polymerization, which recruits Rac1 to induce cytoskeletal reorganization *via* the JNK/AP-1 pathway.[309,312] Furthermore, the C-terminus proline-rich domain (PRD) at the cytoplasmic end of Ror2 recruits Filamin A *alpha* (Flna, an actin-binding protein involved in cytoskeletal remodeling) to initiate the formation of active protrusions (filopodia and lamellipodia) at the leading edge to facilitate cell motility.[313,314] In addition, Ror2 can activate both Rho small GTPase and JNK pathway *via* Wnt5-a induced Ror2-Vangl2 receptor complex formation.[138] Furthermore, Ror2$^{-/-}$ palatal cells displayed defective cell migration in palate development even in the presence of Wnt5a *ex vivo*.[292] In contrast, ectopic

expression of Ror2 in *Xenopus* and *danio rerio* inhibited CE movements during gastrulation, neurulation and anterior-posterior elongating growths.[188,307,308]

RYK (receptor-like tyrosine kinase) was identified in mice by selective PCR amplification of receptor tyrosine kinase-related domains[315,316] while the human homolog was sequenced from a hepatoma cDNA library shortly after.[317,318] RYK is a single transmembrane receptor carrying a Wnt Inhibitory Factor (WIF) extracellular domain[319] and a catalytically inactive tyrosine kinase domain in the cytoplasmic region with a PDZ-domain at the carboxyl-terminus (reviewed in Halford and Stacker, 2001).[280] The WIF-domain of Ryk mediates binding to several Wnt ligands, including: Wnt1,[320,321] -3,[322,323] -3a,[320] 5a,[190,281,282] -5b[146] and -11[190,282] while the PDZ domain has been reported to interact with Dvl/Dsh in mice[320] and *Xenopus*.[190]

Ryk$^{-/-}$ mice displayed similar phenotypes to PCP-impairment, including craniofacial defects, impaired anterior-posterior extension and cleft palate.[281,324] Ryk was subsequently identified to mediate Wnt-induced repellent signals during corticospinal tract axon guidance of neonatal mice.[321] Similarly, Ryk mediated Wnt3-induced retinal ganglion cell axonal repulsion[322] and Wnt5a-indcued repulsion of callosal axons to guide contralateral crossing.[281]

Interestingly, Ryk acts as a co-receptor with Fzd7 to mediate Wnt11-induced endocytic-internalization of Fzd7-Dsh and Dsh hyperphosphorylation in *Xenopus* during CE movements in the dorsal marginal zone (DMZ).[190] Expression of *Ryk* morpholino disrupted Dsh internalization and promoted

Dsh co-localization to the cell membrane.[190] Also, Ryk mediates RhoA and JNK activation, translocating RhoA to the cell membrane,[190] supporting another later study whereby Ryk activates RhoA small GTPase in CHO-K1 cells.[282]

Furthermore, treatment of SN4741 murine cells with a RYK WIF-domain human monoclonal antibody (RWD1 mAb) impaired Wnt5a-induced Dvl2/3 phosphorylation *in vitro* and antagonized Wnt5a-induced axonal neurite outgrowths by murine embryonic cortical neurons.[325] However, it should be noted that Ryk has previously been reported to mediate Wnt3a/β-catenin pathway to induce murine neurite outgrowths by forming a ternary complex between Wnt3a and Fzd8 through the extracellular WIF-domain and with Dvl through the cytoplasmic PDZ-domain to promote Tcf transcriptional activity.[320] Furthermore, Ryk siRNA-silenced murine dorsal root ganglion cells did not respond to Wnt3a-induced neurite outgrowths *ex vivo*.[320]

Antisense morpholino silencing of either *Wnt5b* or *Ryk* in zebrafish embryos induced similar phenotypes with impaired anterior-posterior extension and wider somites in the mediolateral axis indicative of disrupted CE movements, suggesting Ryk to mediate Wnt5b-induced CE.[146] Furthermore, double knockdown of *Wnt5b* and *Ryk* enhanced CE defective phenotypes as compared to just single knockdown, indicating Wnt5b-Ryk genetic interaction in zebrafish during gastrulation.[146] Genetic interactions between *Wnt 11* and *Ryk* were also shown with double knockdown zebrafish embryos displaying a more severe ocular developmental defect.[282]

Wnt5b-Ryk expression induced polarized active protrusion formation (lamellipodia and filopodia) in embryonic hypoblast cells, which was

abolished when a dominant-negative Ryk (dnRyk) was introduced.[146] Interestingly, the protrusions of Ryk-expressing cells were oriented away from Wn5b expression, indicating that Wnt5b acts as a directional cue for Ryk-expressing cells.

RYK interacts with VANGL2 through the PDZ domain at the cytoplasmic region in HEK293T cells[282] and co-immunoprecipitation between Ryk-Vangl2 was enhanced in the presence of Wnt5a.[136] Furthermore, Vangl2 protein levels increased in the presence of either Wnt5a or Ryk, suggesting that Ryk mediates Vangl2 stability through Wnt5a.[136]

WNT Signalling in Cancer and Stem Cells

The proto-oncogenic role of WNT was established when MMTV proviral-integration into *Wnt1* (*int1*) promoter region drove mammary tumour formation in mice.[65] Subsequently, *APC* germline mutations were reported in patients with familial adenomatous polyposis (FAP),[326,327] while *CTNNB1* mutations in other cancers, including endometrioid ovarian cancer, showed varying frequencies[328-333] (reviewed in Bell, 2005)[334]. These mutations on conserved N-terminus serine/ threonine residues confer resistance against GSK-3β-mediated phosphorylation, protecting *β*-catenin from proteosomal degradation.[335,336]

The involvement of Wnt/ *β*-catenin pathway in maintaining stemness was demonstrated when non-functional, unpalmitoylated Wnt-3A diminished self-renewal of hematopoietic stem cells (HSC),[75] while the constitutive activation of *β*-catenin maintained self-renewal.[337] Furthermore, activation of WNT signalling by GSK-3 inhibition in human embryonic stem cells (hESC)

maintained pluripotency.[338] β-catenin has also been implicated in maintaining colonic progenitor cells by activating c-MYC to suppress p21-mediated G1 cell cycle arrest and differentiation.[339] In addition, the Wnt target gene *leucine-rich-repeat-containing G-protein-coupled receptor 5* (Lgr5) is a marker for intestinal stem cells in the small intestinal and colonic crypt base.[340] Activation of the Wnt pathway in $Lgr5^+$ intestinal stem cells *via* conditional-deletion of Apc transformed these cells to become tumourigenic, a proof-of-concept for cancer stem cells.[341] Previously, overexpression of *LGR5* has been reported in ovarian cancer as well.[342] More recently, expression of $Lgr5^+$ cells was found on the OSE and oviduct of the mouse ovaries.[343] *In vivo* lineage tracing of $Lgr5^+$ ovarian stem/ progenitor cells of both embryonic and neonate mice were shown to undergo consistent clonal expansion to regenerate and maintain the ovarian surface epithelium (OSE) during pre-puberty.[343] Furthermore, adult $Lgr5^+$ cells displayed variable rates of expansion post-puberty, with a higher density of $Lgr5^+$ cells located around either sites of rupture during ovulatory repair or the *corpus luteum*, providing evidence that the pool of $Lgr5^+$ stem/ progenitor cells' recruitment to sites of ovulatory rupture mediated regenerative healing.[343]

β-catenin-mediated activation of Survivin expression confers anti-apoptotic resistance, maintains stem cell survival in the basal colonic crypt and has been suggested to promote stemness in cancers.[271] Survivin is also highly expressed in leukemic stem/ progenitor cells[344] and confers drug-resistance.[345] The exogenous co-expression of Slug (SNAI2) and sex determining region Y box 9 (SOX9) in differentiated mammary luminal cells was able to revert these luminal cells back into a mammary stem cell state.[346] Furthermore, induced

co-expression of SNAI2 and SOX9 augmented micrometastases in MCF7 breast cancer cells. Interestingly, *β*-catenin is known to positively regulate transcriptional activation of both SOX9[347] and Slug/ SNAI2.[270] Heterogeneous *β*-catenin expression in colorectal cancer (CRC) demonstrates the functional multiplicity of *β*-catenin: membranous *β*-catenin expression in well-differentiated/ epithelial-like cells of the tumour core mediates cellular adhesion within the adherens junctions, while predominant nuclear *β*-catenin expression in the dedifferentiated/ mesenchymal-like cells at the invasive front indicates activated Wnt signalling.[348,349] Nuclear β-catenin expression at the invasive front activated the expression of L1 cell adhesion molecule (L1CAM) and laminin γ2 (LAMC2), both of which mediates cellular motility and invasion.[350,351] In cancer progression, invasive cancer cells from the primary site undergo a morphological transformation from a differentiated, epithelial phenotype into a more de-differentiated/ mesenchymal phenotype, a process described by the developmental process epithelial-mesenchymal transition (EMT). Upon completion of the metastatic cascade, these invasive cancer cells revert back to the epithelial morphology through mesenchymal-epithelial transition (MET) to drive tumour growth in the secondary site.[352] β-catenin has been reported to induce EMT[353] and to promote a more stem-like phenotype in primary cultures.[354] Taken together, the distinctive expression profile of nuclear β-catenin at the invasive front led to the suggestion that Wnt signalling is involved in mediating cancer progression between stationary and migrating cancer stem cells (SCS/ MCS cells).[355]

The WNT/PCP pathway has also been shown to mediate cancer progression. Wnt5a overexpression in gastric cancer cells promoted cell migration *in vitro*

by activating focal adhesion kinase (FAK) and Rac while RNAi-mediated silencing of *Wnt5a* inhibited cell migration by disrupting the turnover of paxillin focal adhesions.[356] Furthermore, *in vitro* Wnt5a-treatment of MDA-MB-231 triple negative breast cancer (TNBC) cells activated RHOA to stimulate cell migration in a WNT5A-DVL2-DAAM1-dependent manner, while constitutive activation of DAAM1 further enhanced cell migration in MCF-7 breast cancer cells.[357] Similarly, exogenous overexpression of *WNT5A* in both T21PT (atypical ductal hyperplasia) and T21NT (ductal carcinoma *in situ*) cells increased cellular migration in transwell migration assays while shRNA-mediated knockdown of *RHOA* diminished this increment.[358] Furthermore, stable knockdown of *VANG1* curtailed cell invasion in both *WNT5A*-transfectants, further implicating the WNT/PCP pathway in mediating cell motility and invasion in breast cancer.

The BRAF serine/ threonine kinase proto-oncogene is frequently mutated ($BRAF^{V600E/K}$) in melanomas, resulting in constitutive activation. These BRAF-mutant melanomas initially respond to BRAF-inhibitors (BRAFi) such as PLX4270, though subsequent acquired resistance has been associated with WNT5A up-regulation while siRNA-mediated knockdown of *WNT5A* impaired PLX4720-resistance.[359] Furthermore, WNT5A expression activated the PI3K/AKT pathway while suppressing WNT/β-catenin pathway, suggesting that the mechanisms involved in acquiring BRAFi-resistance is β-catenin-independent.

In another study, exosomes secreted by breast cancer associated fibroblasts (CAF) induced formation of active protrusions in MDA-MB-231 cells that enhanced cell motility *via* autocrine WNT11-mediated PCP pathway, whereby

WNT11 secreted by MDA-MB-231 cells tethers to the exosomes secreted from CAF, eventually being endocytosed by MDA-MB-231 cells to activate autocrine PCP pathway.[360] PCP core components displayed asymmetric distribution during Wnt11-mediated PCP signalling, whereby Fzd6 and Dvl1 expression co-localized at the leading edge of cell protrusions while Pk1 and Vangl1 co-localized at the non-protrusive rear cortex. Of relevance, siRNA-mediated knockdown of each of these PCP-core genes, including Wnt11 abrogated cell protrusions and significantly reduced cell motility even when in the presence of CAF. Furthermore, shRNA-mediated knockdown of Pk1 in MDA-MB-231 cells impaired micrometastases formation when injected in mice mammary fat pads (MFP). [360]

In synovial sarcoma, FZD10 mediates the PCP pathway through activating the DVL2/3-RAC1-JNK cascade.[253] Ectopic overexpression of FZD10 in synovial sarcoma cell lines induced actin reorganization through RAC1 activity (GTP-bound) and JNK1/2 phosphorylation while siRNA-mediated knockdown of either *DVL2/3* suppressed RAC1 activity and inhibited JNK1/2 phosphorylation. Furthermore, FZD10 overexpression inhibited RHOA-mediated actin stress fiber formation, demonstrating that FZD10 in synovial sarcoma mediates RHAOA and RAC1 activity in a mutually exclusive manner. Interestingly, FZD10 overexpression in synovial sarcoma cell lines promoted *anoikis*-resistance in soft colony formation assays while siRNA-mediated knockdown of *FZD10* abrogated anchorage-independent cell growth.[253]

FZD7 in Cancer

FZD7 (FzE3) expression was initially identified in poorly differentiated human squamous cell esophageal carcinoma with high metastatic potential.[361] Ectopic overexpression of *FZD7* (*FzE3*) promoted cytoplasmic and nuclear β-catenin accumulation in KYSE150 esophageal carcinoma cells,[361] indicating the capability of FZD7 (FzE3) in promoting WNT/β-catenin signalling. A comparative genomic hybridization (CGH) study of 46 sporadic and familial ovarian cancer samples showed one of the minimal region of interest most commonly amplified is within chromosome 2q32.1-34.[362] Interestingly, this region includes the locus of two *FZD* genes: *FZD5* (2q33.3) and *FZD7* (2q33). cDNA microarray analysis of primary cultures of EOC and OSE revealed ~43% (9/21) of EOC cultures overexpress *FZD7*, while *FZD7* transcript expression in most OSE cultures were lower.[363] While screening through the transcript expression profile of EMT-related genes on a panel of ovarian cancer cell lines (SGOCL-series), *Frizzled-7* (*FZD7*) expression was identified to be up-regulated in SKOV-3 ovarian cancer cells when cultured as spheroids in suspension.[243] Previously, it has been reported that the formation of spheroids from primary cultures resulted in the enrichment of stem-like/ progenitor populations,[364,365] whereby single cells dissociated from non-adherent mammospheres formed colonies of mixed lineages (ductal and myoepithelial) at a higher frequency than freshly dissociated mammary tissues.[365] Interestingly, FZD7 was reported to regulate hESC pluripotency by regulating POU5F1/OCT4, a key component involved in maintaining stem cell pluripotency.[366] Withdrawal of the self-renewing factor Fibroblast Growth Factor 2 (FGF2) from culture media induced stem cell differentiation,

accompanied by a marked reduction in *FZD7* transcript expression.[367] Inhibition of *FZD7* in hESC through either short hairpin RNA (shRNA) or a FZD7-specific fragment antigen binding protein (FZD7-Fab) down-regulated expression of pluripotency markers *NANOG* and *POU5F1*/*OCT4*, indicating a role that FZD7 has in maintaining hESC pluripotency. Furthermore, pre-treatment of FZD7-Fab to hESC cells prior to mouse Wnt3a stimulation (highly homologous to WNT3) reduced TOP-GFP fluorescence readout, suggesting WNT3 to be a ligand for FZD7.[367] cDNA microarray analysis showed *FZD7* to be overexpressed in both hESCs and human embryonal carcinoma cells (hECCs).[368] Furthermore, *FZD7* transcript expression in hECC was up-regulated in the presence of Wnt-3A.[369] Another study reported that the knockdown of *FZD7* in hECC reduced proliferation.[370] In contrast, a plasma membrane-based study reported FZD7 to be differentially expressed in hESC but not hECC.[371]

cDNA microarray analysis of nasopharyngeal carcinoma (NPC) in two studies both identified *FZD7* to be differentially expressed in the tumour compartment.[372,373] Expression was further validated using *in situ* hybridization (ISH) staining.[373] Interestingly an association was found between EBV-infection and expression of genes in WNT signalling, including: *FZD7*, CREB-binding protein (*CREBBP*) and casein kinase II β (*CSNK2β*), suggesting a potential role of WNT signalling in mediating EBV-infection.[373] In a microarray study of breast cancer patient samples, FZD7 was more highly expressed in the triple negative breast cancer (TNBC) subtype. *FZD7* short hairpin RNA-mediated (shRNA) knockdown in MDA-MB-231 cells (TNBC-subtype) reduced cell proliferation *in vitro* and formed smaller

tumours *in vivo*.[374] Short interfering RNA (siRNA)-mediated knockdown of *FZD7* in colorectal cancer (CRC) cell lines reduced invasion and formed less liver metastases *in vivo*.[375,376] Of clinical relevance, CRC patients with higher *FZD7* expression showed poorer survival rates.[376] Similarly, SK-CO-1 colorectal adenocarcinoma cell line ectopically expressing FZD7 ecto-domain (C-terminus truncated FZD7, FZD7ΔC) induced EMT-like morphological changes and abrogated tumour formation in severe combined immunodeficient (SCID) mice.[377] sh*FZD7*-mediated knockdown in LIM1863-*Mph* (human CRC cell line) maintained a mesenchymal phenotype with reduced proliferative rates and abrogated carcinoid formation *in vitro* by inhibiting mesenchymal-epithelial transition (MET).[378]

In melanoma, FZD7 acts as a receptor for WNT5A to mediate β-catenin-independent signalling to mediate drug resistance against BRAFi PLX4720.[359] siRNA-mediated knockdown of *FZD7* reduced cell viability and abrogated colony and melanosphere formation *in vitro* in two melanoma cell lines (A375 and MEL624). In addition, loss of FZD7 chemosensitized these two cell lines towards PLX4720 by disrupting FZD7-mediated PI3K/AKT activation of proliferative/ survival signals.

FZD7 was found to be overexpressed in hepatocellular carcinoma (HCC) in both transcript and protein levels.[379] Introduction of a dominant negative construct of FZD7 into primary HCC cultures reduced *β*-catenin phosphorylation and nuclear accumulation, demonstrating that FZD7 can activate the *β*-catenin pathway in HCC.[379] WNT3 was subsequently reported to be the ligand responsible for activating FZD7 in HCC.[240] Interestingly, stable over-expression of WNT3/FZD7 transformed non-tumourigenic hepatic

cells, acquiring enhanced capabilities in proliferation, migration, invasion and anchorage-independent growths *in vitro*, though the transformed cells failed to form tumours *in vivo*,[380] suggesting that WNT3/FZD7-mediated WNT signalling to be an early driver of HCC development. Pharmacological inhibition of FZD7 amino-terminus-DVL protein-protein interactions *via* a small molecule peptide RHPD-P1 induced β-catenin degradation and eventual apoptotic cell death in Huh7 HCC cells in a PKCδ-dependent manner *in vitro*.[381] Furthermore, tumourigenic impairment was observed after injection of RHPD-P1 into SV40-TAg mice predisposed of hepatocarcinogenesis, demonstrating RHPD-P1 potency against HCC both *in vitro* and *in vivo*.[381] Similarly, a small molecule inhibitor (FJ9) antagonising FZD7 carboxyl-terminus-DVL1 PDZ domain protein-protein interactions was previously reported to inhibit β-catenin/ TCF transcriptional activity in HCT116 CRC cells.[382] Furthermore, FJ9 also inhibited tumourigenic growths of LOX melanoma cells, H460 and H1073 non-small cell lung carcinoma (NSCLC) cells *in vitro* and H460 cells *in vivo*.[382] Competitive inhibition of FZD7 by sFZD7 (soluble extracellular domain of FZD7) sensitized HCC cells towards doxorubicin treatment, showing that combinatorial treatment of a conventional chemotherapeutic agent with a WNT inhibitor (sFZD7) is possible.[383]

In samples of Wilm's Tumour, certain cell populations were reported to undergo apoptosis when treated with a FZD7 antibody.[384] These cells, when xenografted into mice models showed reduced cell proliferation, evident by smaller tumour growths with weaker Ki-67 immunostaining. The authors also reported that FZD7-treatment within this cell population eliminated their ability to form spheroid in suspension, an indication of loss of self-renewal in

the stem/ progenitor population, suggesting that FZD7 plays a role in maintaining the stem/ progenitor-like properties of the cancer cells. Recently, one FZD7-based recombinant antibody, OMP18R5 (Vantictumab, developed by OncoMed Pharmaceuticals Inc.) showed significant reduction in tumour growths in xenograft models, especially in pancreatic, breast and lung tumour models.[385] However, this antibody also interacts with four other FZD receptors, namely FZD1/2/5/8. Phase 1 dose escalation trial against solid tumours began in May 2011 but was temporarily halted as 13% of patients receiving treatment displayed 'on-target mild-to-moderate bone-related adverse effects' (Oncomed Pharmaceutical website). Recruitment is currently underway for phase 1b clinical trials of Vantictumab in combination with other standard-of-care chemotherapeutic agents: stage IV pancreatic cancer (with nanoparticle albumin-bound (Nab)-Paclitaxel and Gemcitabine), previously treated non-small cell lung carcinoma (NSCLC, with Docetaxel) and metastatic breast cancer (with Paclitaxel). These clinical trials will be completed by the late 2015 and results are highly anticipated (Clinical Trials.gov website).

CHAPTER 1.3

WNT Signalling in Epithelial Ovarian Cancer (EOC)

Deregulated β-catenin Expression in EOC

In a small pilot study, immunohistochemical analysis of 40 primary epithelial ovarian tumours showed that a majority of the endometrioid subtype (5/6) displayed β-catenin cytoplasmic and nuclei staining.[386] DNA sequencing of β-catenin (encoded by the *CTNNB1* gene) in these 5 endometrioid ovarian lesions (one borderline tumour and four stage I and II carcinomas) showed activating missense mutations within exon 3 in 3/5 of the endometrioid ovarian lesions with nuclei expression. Interestingly, the two codons mutated (S37 and T41) fall within the consensus phosphorylation site by glycogen synthetase kinase-3 (GSK-3), a serine/ threonine kinase.[335,387,388] These mutations disrupted the GSK-3 consensus recognition sites on β-catenin, preventing regular GSK-3 phosphorylation-induced degradation of β-catenin, eventually resulting in aberrant β-catenin-mediated WNT signalling in this subtype of ovarian cancer. In a follow-up study on 69 stage I and II ovarian carcinomas, 11 samples showed nuclear β-catenin staining, of which 9 were of the endometrioid subtype, the other 2 being serous.[389] Among these 9 endometrioid subtypes, 7 showed *β-catenin* gene missense mutations within the exon 3 region. Unfortunately, *β-catenin* sequencing analysis was not carried out on the 2 serous subtypes. Separate studies carried out by various groups subsequently identified *β-catenin* activating mutations around the exon 3 region, between codons 32 to 45.[328,329,386,389-397] All studies found only missense mutations in the endometrioid subtype, except for one study that

Table 3: List of β-catenin (*CTNNB1*) mutations (exon 3: GSK-3 phosphorylation site) in Ovarian Cancer

Codon Mutation	Histological Subtype [Reference]
D32G (GAC → GGC)	Borderline endometrioid tumour[394] Endometrioid carcinoma[389,395]
D32H (GAC → CAC)	Endometrioid carcinoma[329]
D32Y (GAC → TAC)	Borderline endometrioid tumour[394] Endometrioid carcinoma[329,389,392,393]
S33A (TCT → GCT)	Endometrioid carcinoma[397]
S33C (TCT → TGT)	Borderline endometrioid tumour[394] Endometrioid carcinoma[328,329,389,391-393,395-398] Ovarian microcystic stromal tumour (MCST)[399]
S33F (TCT → TTT)	Borderline endometrioid tumour[394] Endometrioid carcinoma[328,391,396,397]
S33P (TCT → CCT)	Endometrioid[393]
S33Y (TCT → TAT)	Endometrioid carcinoma[329,397]
G34E (GGA → GAA)	Endometrioid carcinoma[329,391]
G34R (GGA → CGA)	Endometrioid carcinoma[329,397]
G34R (GGA → AGA)	Endometrioid carcinoma[397]
G34V (GGA → GTA)	Endometrioid carcinoma[328,329,395,397]
H36P (CAT → CCC)	Endometrioid carcinoma[397]
S37A (TCT → GCT)	Endometrioid carcinoma[397] TOV112D ovarian endometrioid adenocarcinoma cells[329]
S37C (TCT → TGT)	Borderline endometrioid tumour[386] Endometrioid carcinoma[328,329,386,391-393,395,397] Mucinous carcinoma[390]
S37F (TCT → TTT)	Borderline endometrioid tumour[394] Endometrioid carcinoma[386,392,393,397]
S37Y (TCT TAT)	Endometrioid carcinoma[395]
A39T (GCC → ACC)	Endometrioid carcinoma[393]
T41A (ACC → GCC)	Endometrioid carcinoma[386,390,392,395,396]
T41I (ACC → ATC)	Endometrioid carcinoma[390,393,397]
S45P (TCT → CCT)	Endometrioid carcinoma[396]
S45Y (TCT → TAT)	Endometrioid carcinoma[329]
S45S (Silent mutation) (TCT → TCG)	Endometrioid carcinoma (squamous metaplasia)[391]

found a missense mutation in one tumour of mucinous histological subtype (S37C, refer to table 3).[390] Of note, no interstitial deletions were identified on β-catenin in ovarian tumours. Among the studies cited here, the frequency of *β-catenin* gene missense mutations in the endometrioid subtype varies between ~10 to 55% (refer to table 4).[328,329,386,389-393,395-397] It should be noted that most of the ovarian endometrioid tumours carrying activating mutations of *CTNNB1* displayed strong nuclear and membranous β-catenin staining, suggesting that these activating mutations help tumour cells to offset availability of β-catenin for either nuclear translocation or adherent junctions formation.[328] However, one study reported that endometrioid tumours with squamous metaplasia and nuclear β-catenin staining showed reduced membranous staining intensity when compared to surrounding ovarian carcinoma cells without nuclear β-catenin staining.[391] A similar observation was made in ovarian serous adenocarcinoma samples (nuclear accumulation with weaker membranous staining).[400] Interestingly, patients with ovarian endometrioid tumours carrying nuclear β-catenin expression correlated with a better prognosis, either relapse-free survival[389,393,401] or better overall patient survival, suggesting β-catenin to be a potential prognostic biomarker in ovarian endometrioid cancer.[389] In addition, it was observed that many low malignant potential (LMP) endometrioid tumours carry *β-catenin* missense mutations, suggesting that β-catenin may drive ovarian tumourigenesis from LMP precursors.[386,389] Consistent with these observations, ovarian endometrioid tumours with nuclear β-catenin staining are generally characterized to be well differentiated[329] (with regions of squamous metaplasia displaying nuclear β-catenin)[391,402], of low-grade and in the early

Table 4: Frequency of nuclear staining of β-catenin and *CTNNB1* gene mutations in Ovarian Carcinomas

Study	Endometrioid		Others
	Nuclear Staining	β-catenin Mutation	Nuclear Staining
Palacios and Gamallo, 1998. *Can. Res.* **58** (7): 1344-7[386]	4/5 (80%)	3/5 (60%)	-
Gamallo *et al.*, 1999. *Am. J. Pathol.* 155 (2): 526-37[389]	9/13 (69%)	7/13 (54%)	Serous 2/16 (12.5%)
McConechy *et al.*, 2014. *Mod. Pathol.* **27** (1): 128-34[397]	N.A.	25/50 (50%)[§] 1/3 (33%)[‡]	N.A.
Moreno-Bueno *et al.*, 2001. *Diagn. Mol. Pathol.* **10** (2): 116-22[392]	21/25 (84%)	11/25 (44%)	-
Zhai *et al.*, 2002. *Am. J. Pathol.* **160** (4): 1229-38[403]		13/32 (41%)	N.A.
Sarrió *et al.*, 2006. *Hum. Pathol.* **37** (8): 1042-9[395]	9/13 (69%)	7/19 (37%)	-
Sagae *et al.*, 1999. *Jpn. J. Can. Res.* **90** (5): 510-5[390]	3/11 (27%)	4/12 (33%)	Mucinous 1/7 (14%)
Wu *et al.*, 2001. *Can. Res.* **61** (22): 8247-55[329]	14/45 (31%)	14/45 (31%)	N.A.
Saegusa and Okayasu, 2001. *J. Pathol.* **194** (1): 59-67[391]	8/23 (35%)	6/23 (26%)	N.A.
Catasús *et al.*, 2004. *Hum. Pathol.* **35** (11): 1360-8[393]	8/22 (38%)	5/22 (23%)	Clear Cell 1/18 (5.5%) Mixed[†] 2/15 (13%)
Willner *et al.*, 2007. *Hum. Pathol.* **38** (4): 607-13[396]	N.A.	6/26 (23%)	N.A.
Wright *et al.*, 1999. *Int. J. Can.* **82** (5): 625-9[328]	11/61 (18%)	10/63 (16%)	Serous* 2/30 (7%)
Shedden *et al.*, 2005. *Clin. Can. Res.* **11** (6): 2123-31[404]	12/33 (36%)	N.A.	N.A.
Kildal *et al.*, 2005. *Eur. J. Can. Res.* **41** (8): 1127-34[401]	27/51 (53%)	N.A.	Serous 1/59 (2%) Clear Cell 2/55 (4%) Mucinous 2/55 (4%) Unclassified 1/15 (6%)
Chou *et al.*, 2003. *Histopathol.* **43** (2): 151-6[405]	2/8 (25%)	N.A.	Serous 1/20 (5%) Mucinous 4/43 (9%)
Logani *et al.*, 2005. *Mod. Pathol.* **18** (1): 19-25[402]	2/10 (20%)	N.A.	Mucinous 3/13 (23%)

Table 4 (continued): Frequency of nuclear staining of β-catenin and *CTNNB1* gene mutations in Ovarian Carcinomas

Study	Endometrioid		Others
	Nuclear Staining	β-catenin Mutation	Nuclear Staining
Wang *et al*., 2006. *Br. J. Can.* **94** (5): 686-91[400]	5/5 (100%)[Δ]	N.A.	Serous[Δ] 24/41 (59%) Clear Cell[Δ] 4/11 (36%)
Oliva *et al*., 2006. *J. Pathol.* **208** (5): 708-13[394]	8/8 (100%)[#]	7/8 (88%)[#]	N.A.
Lee et al., 2003. *Gyn. Oncol.* **88** (3): 363-8[406]	N.A.	N.A.	Serous 13/105 (12%) LMP 1/10 Lo-Grade 1/47 Hi-Grade 11/48
Yoshioka & King *et al*., 2012. *Mol. Can. Res.***10** (3): 469-82[156]	N.A.	N.A.	Serous 25/36 (69%)

§ Low-grade (Grades 1 & 2) ovarian endometrioid tumours
‡ High-grade (Grade 3) ovarian endometrioid tumours

† β-catenin (*CTNNB1*) mutation and nuclear staining was only found in the endometrioid compartment
* Less than 5% of total cells stained positive for nuclear β-catenin
Δ This study takes into account of both nuclear and cytoplasmic immunostaining as positives
Ovarian endometrioid tumour of borderline malignancy

stages.[393,398] However, it should be highlighted that a contrasting trend was observed in separate studies on ovarian serous tumours: both low- and high-grade tumours with nuclear β-catenin staining showed shorter patient survival as compared to tumours without nuclear β-catenin expression. Also, majority of the serous tumours with nuclear β-catenin immuno-reactivity were classified as high-grade tumours (11/48, 23%).[406,407] Interestingly, a separate study found nuclear β-catenin staining in 25/36 (69%) of ovarian serous

carcinoma samples tested, with none of the samples analyzed carrying *β-catenin* gene mutations.[156] Of note, 7 of these samples were also used in a separate panel for WNT7A *in situ* hybridization analysis. 5/7 (71%) of these samples showed strong positivity for WNT7A and nuclear β-catenin, suggesting that an alternative route for activating WNT/β-catenin pathway in serous carcinomas.[156] In ovarian mucinous carcinoma, the frequency of nuclear β-catenin varies between 4-23%,[390,401,402,405] though only one missense mutation in the *β-catenin* gene has been reported.[390] As such, the only consensus conclusion for β-catenin activating missense mutations in EOC is that it is histotype-specific, predominantly found in OEAs of low grade and early stage, with better prognosis.

One observation that arose from these studies was that not all tumours with nuclear β-catenin immunostaining carried β-catenin mutations. This would indicate the involvement of other components of the WNT/β-catenin signalling pathway that can contribute to β-catenin constitutive activation. Aside from β-catenin itself, mutations have been reported in other genes encoding the β-catenin destruction complex, including APC, AXIN1 and AXIN2.[329,395] Briefly, a primary ovarian endometrioid adenocarcinoma (OEA) with nuclear β-catenin harbouring wildtype β-catenin was identified to carry two truncating nonsense mutations (Arg554Stop and Arg1114Stop) of the *APC* gene.[329] These premature stop codons effectively inactivated APC functions, promoting stabilized β-catenin accumulation. In another study, APC expression was found to be absent in 46/113 (41%) primary and metastatic serous ovarian tumour biopsies. Interestingly, 3 samples stained for

nuclear β-catenin, all devoid of APC expression.[408] However, it should be pointed out that the other 43 samples negative for APC expression has no detectable nuclear β-catenin expression. This would indicate that either serous ovarian carcinomas seldom express β-catenin, or β-catenin phosphorylation might not solely depend on the β-catenin destruction complex itself. In an epigenetic study, APC promoter hypermethylation was present in 22% of invasive ovarian carcinoma (5/23) but not in benign cystadenomas.[409] Interestingly, APC promoter hypermethylation was only present in serous, mucinous and clear cell histological subtypes but not in the endometrioid subtype.

AXIN1 truncating nonsense mutation (Lys416Stop) was identified in a primary OEA sample.[329] However, this OEA only expressed weak cytoplasmic β-catenin but not nuclear β-catenin. Similarly, one OEA sample with missense mutation (Thr520Pro) showed membranous β-catenin staining.[395] In contrast, a human OEA-derived cell line (MDAH2774) carrying an *AXIN1* missense mutation (Val555Ile) stained positively for nuclear β-catenin and displayed enhanced TCF transcriptional activity. This amino acid change falls within the AXIN1: β-catenin interaction domain, thereby preventing proper formation of the β-catenin destruction complex.[329] In another primary OEA sample, an interstitial deletion of a guanine (G) base resulted in a frameshift mutation at codon 665 of *AXIN2*.[329] This stable form of mutant AXIN2 was previously reported in colorectal cancer to enhance TCF-dependent transcription activity, indicating the mutant to be dominant-negative.[410] Of note, the frequency of these mutations is relatively low as compared to β-catenin mutations.

In an animal study, mice OSE cells with aberrant β-catenin expression were generated by conditional deletion of β-catenin (*Ctnnb1*$^{\Delta(ex3)/+}$).[411] Pre-tumoural nests of cells with nuclear β-catenin expression were detected in the ovarian *bursa* and OSE of 12 week-old mice. No pre-tumoural nests were observed in control mice. Interestingly, upregulated expression of the tumour suppressor Phosphate Tension and homolog (Pten) was observed in the pre-tumoural lesions. Half of these β-catenin mutant mice developed tumours between 8 – 12 months, of which most were of the undifferentiated histological subtype, positively staining for the mesenchymal marker vimentin. 5/6 mice examined displayed epithelial glands, a hallmark of OEAs. To determine if Pten mediated the latent tumourigenic phenotype, mice with aberrant β-catenin expression in the OSE with Pten deletion (*Ctnnb1*$^{\Delta(ex3)/+}$;*Pten*$^{\Delta/\Delta}$) were generated. OSE from these mice developed gross tumours within 6 weeks, as compared to 8 – 12 months in the *Ctnnb1*$^{\Delta(ex3)/+}$ mice. In summary, the result from this animal study demonstrates a proof-of-concept that dysregulated Wnt/β-catenin signalling drives ovarian tumourigenesis formation when devoided of Pten/PI3K signalling.[411] Another finding from this study was that tumours derived from both *Ctnnb1*$^{\Delta(ex3)/+}$ and *Ctnnb1*$^{\Delta(ex3)/+}$;*Pten*$^{\Delta/\Delta}$ mice showed elevated phospho-ribosomal protein S6 Kinase (pS6K) expression, suggesting the Mechanistic Target of Rapamycin (MTOR) pathway to be involved as S6K is a downstream target of mTOR. Interestingly, human OEAs with nuclear β-catenin expression were found to stain strongly for MTOR, pMTOR and pS6K, indicating activated MTOR pathway in these human OEA cells. To further test this, *Ctnnb1*$^{\Delta(ex3)/+}$;*Pten*$^{\Delta/\Delta}$ ovarian tumour cells were allotransplanted into non-obese diabetic/ severe combined immunodeficient

(NOD/SCID) mice and received rapamycin treatment for 12 weeks. Rapamycin-treated mice showed significantly smaller tumour growths compared to vehicle control-treated mice. Furthermore, ovarian tumour cells from rapamycin-treated mice had a lower mitotic index and increased Caspase 3 (Casp3) activation, indicative of reduced proliferation and active apoptosis respectively. In addition, pS6K expression was abrogated in the rapamycin-treated group, further supporting that β-catenin-induced activation MTOR drove murine ovarian tumourigenesis.[411]

WNT Target Genes in EOC

To better understand how the WNT/β-catenin signalling pathway drives ovarian carcinogenesis, several studies sought to identify target genes regulated by the pathway. Based on previously reported β-catenin target genes in other cancer models,[263,264,268,412-414] comparison of transcript expression between OEAs with aberrant β-catenin expression and intact β-catenin regulation identified significantly upregulated expression of five target genes: i) Cyclin D1 (*CCND1*), ii) Matrix Metalloproteinase 7 (*MMP7*), iii) Peroxisome Proliferator-Activated Receptor delta (*PPAR-δ*), iv) Connexin 43 (*CX43*) and v) Immunoglobulin Transcription Factor 2 (*ITF-2*).[403] Validation *via* immunohistochemical staining of CCND1, MMP7 and ITF-2 in the OEAs showed a significant correlation between nuclear β-catenin immunoreactivity and higher protein expression levels of CCND1, MMP7 or ITF-2.[403,414] More importantly, statistical significance was found in the expression of the three target genes between OEAs with deregulated β-catenin and OEAs carrying wildtype β-catenin. These results were supported by an

immunohistochemical-based study, whereby CCND1 expression was found to be present in 3/8 (37.5%) of OEAs with nuclear β-catenin staining. It was also found that patients co-expressing MMP7 and nuclear β-catenin had a better survival outcome than patients expressing MMP7 alone (mean patient follow-up of 8.5 years).[393] Subsequent oligonucleotide gene expression microarray analysis between the two groups of OEAs found 81 candidate target genes regulated by the WNT/β-catenin/TCF pathway, of which 7 were previously reported to be direct β-catenin gene targets in other models.[415] Validation *via* transcript expression profiling of the top five candidate target genes with the highest upregulation, including: *CCND1*, Cystatin 1 (*CST1*), Endothelin 3 (*EDN3*), Fibroblast Growth Factor 9 (*FGF9*) and Stratifin (*SFN*/*14-3-3σ*), all showed significant correlation with the microarray data.[415] Furthermore, putative TCF-binding sites were identified between 500 to 2000 basepairs (bp) upstream of the transcription start sites (TSS) of both *CST1* (four sites) and *EDN3* (three sites). Mutations of all these sites carried out separately in HEK293 cells with constitutive β-catenin activation nullified both *CST1* and *EDN3* gene expression respectively, indicating that these TCF-binding sites act as proximal promoters for both *CST1* and *EDN3*, both of which can be regulated by β-catenin/TCF transcription factors. In addition, stable expression of a dominant-negative TCF (dnTCF) construct in TOV112D (OEA cell line carrying a constitutive β-catenin mutation[329]) reduced luciferase reporter activity of both CST1 and EDN3, further showing that these two genes are direct targets of the β-catenin/TCF signalling.[415] Following up on this study, the same group subsequently showed that both the transcript and protein expression of FGF9 to be upregulated in OEAs with

defective β-catenin regulation.[416] A similar trend was also observed in two OEA cell lines with deregulated β-catenin expression: MDAH2774 and TOV112D. Moreover, GSK-3β inhibition *via* lithium chloride (LiCl) treatment in OVCA420 serous ovarian carcinoma cells with wildtype β-catenin increased *FGF9* transcript expression. Furthermore, TOV112D-dnTCF-4 cells (TOV112D cells stably expressing a dominant-negative TCF-4 construct) showed a marked reduction in *FGF9* transcript expression.[416] Interestingly, stable knockdown of *FGF9 via* short hairpin RNA (shRNA) in TOV112D OEA cells abrogated anchorage-independent growth in soft agar, indicating FGF9 protects TOV112D cells from undergoing *anoikis*. However, it should be noted that *FGF9* was previously reported to be preferentially expressed in OSE than in EOC cultures.[363]

Using microarray gene expression profiling as before,[415] gene expression profiles of two sets of data were compared i) OEAs with wildtype β-catenin against OEAs carrying β-catenin activating mutations, ii) human embryonic kidney HEK293 cells stably expressing a β-catenin mutant construct (HEK293$^{CTNNB1\text{-S37A}}$) against HEK293 cells stably expressing the empty vector with GFP construct (HEK293$^{\text{GFP-control}}$). From these analyses, two more β-catenin/TCF candidate target genes were identified: Fibroblast Growth Factor 20 (*FGF20*) and Dickkopf WNT signalling inhibitor 1 (*DKK1*).[417] Subsequent validation work carried out in the HEK293 cells included chromatin immunoprecipitation (ChIP) and analysis of proximal promoter regions upstream of the respective genes' TSS for TCF-binding sites. β-catenin immunoprecipitates from chromatin of HEK293$^{CTNNB1\text{-S37A}}$ cells were enriched for DNA regulatory sequences for both *FGF20* and *DKK1*, as

compared to chromatin from HEK293$^{GFP\text{-}control}$ cells. Also, TCF-binding sites were identified as proximal promoters upstream of the TSS for both FGF20 (two sites) and DKK1 (four sites). Co-transfection of a constitutively active β-catenin mutant construct with a luciferase reporter construct containing the putative promoter regions of either FGF20 or DKK1 in HEK293 cells showed increased luciferase activity from both FGF20 and DKK1 reporters. This luciferase reporter activity was lost upon co-transfecting an inhibitor of β-catenin and T-cell factor (ICAT) construct. Complete loss of DKK1 reporter luciferase activity was also achieved when all putative TCF-binding sites were either deleted or point-mutated *via* site-directed mutagenesis.[417] Interestingly, *DKK1* expression was found to be downregulated >20-fold in an oxaliplatin-resistant derivative of A2780 ovarian carcinoma cell line.[418]

Following up on the microarray analysis of OEA samples carried out previously,[415] Muscle Segment homeobox-2 (MSX2) overexpression was found in OEA samples with defective WNT signalling regulation. Subsequent validation showed *MSX2* transcript expression to be nine-fold higher when compared against OEA samples with wildtype β-catenin regulation.[419] Immunohistochemical staining showed 13/17 (76%) of OEA samples with defective WNT signalling regulation to stain positively for MSX2 (moderate to strong intensity). Of the 13 OEA samples with intact WNT signalling regulation, only one sample was shown to stain positively for MSX2.[419] Also, murine OEA tumours derived from *APC*$^{-}$/*PTEN*$^{-}$ background showed >200-fold higher *Msx2* transcript expression than murine *KRAS*^{mut}/*PTEN*$^{-}$ driven OEA tumours, suggesting that aberrant Wnt signalling in mice activates *Msx2* expression. Furthermore, activation of WNT signalling either by i) treatment

with recombinant mouse Wnt3a in human OSE cell line IOSE80 or ii) inhibiting GSK-3β *via* the small molecule inhibitor SB216763 in a human OEA-derived cell line MDAH2774, both increased *MSX2* transcript expression after treatment. In addition, TOV112D-dnTCF-4 cells (TOV112D cells stably expressing a dominant-negative TCF-4 construct) showed a marked reduction in both the transcript and protein expression of MSX2. Nine consensus TCF/LEF binding sites were located around the *MSX2* gene locus, suggesting *MSX2* to be a direct target gene of β-catenin/TCF/LEF transcription. ChIP of TOV112D cells against TCF4 antibody showed chromatin DNA to be enriched for three of the MSX2 TCF/LEF consensus binding sites, confirming *MSX2* to be a direct β-catenin target gene.[419] TOV112D and TOV21G cells stably expressing short hairpins of *MSX2* displayed *in vitro* growth impairment. Anchorage-independent growth was also impaired after *MSX2* knockdown in TOV112D cells, evident by fewer colonies formed in soft agar. Subcutaneous injection of TOV112D cells after *MSX2* knockdown into mice formed significantly smaller tumours than the control after 3 weeks, demonstrating that MSX2 acts as an oncogene in EOC. Ectopic overexpression of *MSX2* in IOSE80 cells promoted *in vitro* cell invasion through Matrigel-coated transwells. Furthermore, *in vivo* cell invasion assay showed PEO4 human ovarian carcinoma cells ectopically overexpressing MSX2 displayed enhanced invasiveness into chick chorioallantoic membrane, mimicking stromal invasion. In summary, *MSX2* was shown to be a direct target oncogene for β-catenin/TCF/LEF transcription, evident by influencing cell proliferation, anchorage-independent growth, *in vivo* tumourigenicity and cell invasion.[419]

In a study investigating the enhanced chemoresistance of spheroid-forming ovarian tumour initiating cells (OTIC), transcript expression of ATP-binding Cassette G2 (*ABCG2*), an efflux transporter mediating drug-resistance was shown to be up-regulated in spheroids derived from SKOV-3 and HEY A8 ovarian serous carcinoma cell lines.[273] Interestingly, either siRNA-mediated down-regulation of *β-catenin* (*CTNNB1*) or ectopic expression of the dominant-negative TCF (dnTCF) construct down-regulated *ABCG2* expression and chemosensitized SKOV-3 and HEY A8 OTICs towards cisplatin and paclitaxel. Moreover, siRNA-silencing of *ABCG2* also displayed chemosensitivity. Furthermore, suppressed TOPflash luciferase activities (β-catenin-TCF reporter construct) were shown in cells carrying the dnTCF construct. Taken together, the suppression of β-catenin/TCF down-regulated *ABCG2* expression, indicating *ABCG2* to be a gene target of β-catenin/TCF transcription.[273] Microarray analysis of spheroid and monolayer cultures of both IGROV-1 and SKOV-3 ovarian carcinoma cell lines identified expression of Aldehyde Dehydrogenase 1A1 (*ALDH1A1*), a stem cell marker, to be up-regulated in ovarian carcinoma spheroids.[420] Pathway network analysis identified *ALDH1A1* to be a central node directly connected to *CTNNB1*. Moreover, spheroids showed up-regulated *β-catenin* and *ALDH1A1* transcript expression, further accompanied by increased TCF/LEF promoter activity. Conversely, *ALDH1A1* expression was down-regulated after *β-catenin* siRNA silencing, indicating *ALDH1A1* to be involved in the WNT/β-catenin pathway. In addition, amplicons of the *TCF*/*LEF* binding motif specific to the *ALDH1A1* promoter region was amplified out from chromatin

DNA immunoprecipitated by a β-catenin antibody, proving *ALDH1A1* to be a direct target of the β-catenin/ TCF/LEF transcription complex.[420]

WNT Regulators in EOC

Several studies also reported identifying regulators of the WNT/β-catenin pathway in EOC.[105,400,421-424] Frequently Rearranged in Advanced T-cell lymphomas 1 (FRAT1) and Axin has previously been reported to share the same binding region to GSK-3, suggesting that FRAT1 can negatively regulate WNT/β-catenin signalling by competing with Axin for binding.[425-427] In a separate immunohistochemical-based study on tissue microarrays (TMA) of human EOC samples, significant correlation was found between FRAT1 overexpression and accumulation of cytoplasmic/ nuclear β-catenin expression within the same samples, especially in the serous subtype.[400] It should be noted that no molecular studies were provided in this study. In other previous studies, the disruption of E-cadherin/ β-catenin adhesion complex increased subcellular localization of β-catenin, thereby promoting WNT/β-catenin activity.[428,429] This would implicate E-cadherin to be a regulator of the WNT/β-catenin pathway. In particular, this disruption was induced by interaction with collagen type I in pancreatic cancer cells.[429] During ovarian cancer metastasis, ovarian cancer spheroids/ multicellular aggregates reattach to the mesothelial peritoneal cavity lining *via* integrin-collagen type I mediated adhesion.[54-57] To test if this interaction disrupts E-cadherin/ β-catenin adhesion to activate WNT/β-catenin signalling, OVCA429 and OVCA433 ovarian cancer cell lines were cultured on collagen type I surface and E-cadherin distribution was analyzed thereafter *via* immunofluorescence

staining.[421] Junctional E-cadherin localization was significantly redistributed to the cytoplasm after culturing in collagen type I surfaces. This interaction was mediated by integrins α2, α3 and β1, but not α5 and αv.[421] This in turn lead to an accumulation of nuclear of β-catenin (validated by immunoblotting of collagen type I-treated nuclear extracts), suggesting active WNT/β-catenin signalling. In addition, immunoblotting of whole cell lysates against active β-catenin (dephosphorylated β-catenin at Ser37 and Thr41) showed an increase in the active form after integrin β1 engagement. Interestingly, GSK-3 was inactivated (Ser 9 phosphorylation) after integrin β1 interaction, suggesting that cytoplasmic β-catenin was not targeted for degradation by GSK-3. Furthermore, TOPflash luciferase reporter assays showed increased luciferase readout after integrin β1 engagement, indicating activation of β-catenin/LEF/TCF transcription. This was further validated by increased transcript expression of several WNT target genes.

Pituitary homeobox 2/ Paired-like homeodomain 2 (*PITX2*) is a transcription factor of the bicoid homeodomain family that has been previously reported to regulate left/ right asymmetry determination in vertebrates.[430] PITX2 has also been reported to act as an effector and regulator of the Wnt/β-catenin pathway to mediate cell proliferation during development.[431,432] Interestingly, PITX2 overexpression was reported in high-grade ovarian cancer.[433] Furthermore, overexpression of PITX2 in mouse models enhanced ovarian cancer cells proliferation, with an increased expression of WNT/β-catenin target genes *CCND1* and *c-MYC*.[433] Microarray analysis showed upregulated *PITX2* and *WNT5A* expression by ~20-fold in an oxaliplatin-resistant derivative of A2780 ovarian carcinoma cell line.[418] Taken

together, PITX2 may play a role in mediating WNT/β-catenin signalling in driving ovarian carcinogenesis and mediating drug resistance. In a separate study using ChIP-on-Chip assays, wildtype *PITX2* in the human epithelial ovarian adenocarcinoma cell line SKOV-3 was shown to bind to the promoter region of several *WNT* genes, including *WNT2, -2B, -5A, -6* and *-9*.[105] Further validation using promoter-driven luciferase-reporter constructs in Chinese Hamster Ovary cells (CHO) showed enhanced *WNT2* and *WNT5A* promoter activity (10 and 8-fold respectively) in the presence of PITX2, indicating PITX2 to regulate the expression of *WNT2* and *WNT5A*. Moreover, SKOV-3 cells carrying overexpressing constructs of each of the three PITX2 isoforms (*PITX2A, -B, -C*) all showed increased transcript levels of four WNT ligands: *WNT2, -5A, -6* and *-9*. Conversely, RNA-silencing of *PITX2* in SKOV-3 cells *via* small-interfering RNA (siRNA) markedly reduced transcript expression of the four *WNT* ligands. Interestingly, an increase in the pool of activated β-catenin (unphosphorylated) was observed in SKOV-3 cells overexpressing PITX2 isoforms. Furthermore, these cells showed increased transcript expression of β-catenin target genes *CCND1* and *c-MYC*. A similar finding was made when SKOV-3 cells were treated in PITX2-conditioned-media enriched for the PITX2 isoforms. Also, PITX2 overexpression unexpectedly reduced the expression of several Frizzled receptors (*FZD2, -3, -4, -9*) and low density lipoprotein related protein co-receptors (LRP5/6), suggesting that PITX2 may act as a negative feedback-loop regulator of the WNT/β-catenin pathway.

Transcript of FILamin A Interacting Protein 1-Like (*FILIP1L*, formerly called Downregulated in Ovarian Cancer 1, *DOC1*) is preferentially expressed

in human OSE (HOSE) cells but not in ovarian carcinoma cell lines.[434] Previously, FILIP1L was shown to inhibit endothelial cell migration[435] and downregulated FILIP1L expression *via* promoter hypermethylation in ovarian cancer cell lines induced cell invasiveness,[436] demonstrating FILIP1L to be an inhibitor of cell invasion. In a study to better understand the mechanistic pathway that FILIP1L employs to inhibit cell invasion and metastasis, ES2 ovarian carcinoma cells stably expressing a doxycycline-inducible FLIP1L-mCherry tagged construct were orthotopically injected into ovaries of mice, which generated aggressive tumours with the presence of lung micrometastases within 14 days.[422] Upon doxycycline treatment, cell invasion was greatly diminished, resulting in significant reduction of micrometastases formed. Interestingly, a similar phenotype was observed between doxycycline-treated FILIP1L clones and FILIP1L clones treated with a pan-MMP inhibitor (GM6001) in the absence of doxycycline. This shared phenotype would suggest that FILIP1L might target MMP expression to cause impaired cell invasion. Of note, MMPs are known to be WNT/β-catenin target genes.[403] True enough, transcript expressions of *MMP3, -7, -9* were found to be downregulated in the FILIP1L clones. This was further validated by immunofluorescence and immunoblotting experiments. However, FILIP1L clones invasiveness dramatically increased upon transfection of a *MMP9* cDNA construct, indicating that the FILIP1L-induced impairment of cell invasiveness may be overcome with ectopic expression of *MMP9*. Furthermore, FILIP1L clones-derived tumours showed downregulated transcript expression of six *WNT* ligands, including *WNT2*, *-3A*, *-4*, *-5A*, *-7A* and *-11*. This was further demonstrated when treatment of FILIP1L clone

cultures with WNT3A induced *MMP3*, *-7* and *-9* transcript expression, whereas *MMP* transcript expression decreased upon co-treatment with doxycycline and WNT3A. This confirms that FILIP1L does indeed down-regulate WNT ligand expression. In addition, FILIP1L clone cultures showed reduced TOPflash luciferase activity when treated with WNT agonists (pan-WNT agonist and GSK-3β inhibit, BIO), as compared to the uninduced control. Also, immunoblots of FILIP1L clones showed higher levels of phosphorylated β-catenin, while immunofluorescence staining showed reduced levels of nuclear β-catenin as compared to the uninduced controls. Taken together, these results indicate that FILIP1L inhibits WNT/β-catenin signalling pathway, thus reducing expression levels of WNT target genes, such as MMPs, resulting in the impairment of cell invasiveness of ovarian cancer cells to prevent metastasis.

In the β-catenin/TCF transcription complex, two nuclear co-factors Pygopus family PHD finger (PYGO) and B-cell CLL/ Lymphoma-9-Like (BCL9L), were identified to be required for mediating proper β-catenin/TCF transcription activity.[437-440] In a study, immunohistochemical analysis of one OSE cell line, three EOC cell lines and 125 patient tumour samples representing all histological subtypes showed nuclear PYGO2 immunoreactivity in all three EOC cell lines and majority of EOC tumour samples (113/118, 96%) but not in the OSE cell line or in all seven benign tumour samples.[441] Interestingly, only 8 EOC tumour samples showed nuclear immunoreactivity for both β-catenin and PYGO2, suggesting that PYGO2 may also act independently of β-catenin. Antisense-mediated knockdown of *PYGO2* in four EOC cell lines (SKOV-3, OVCAR-3, TOV21G and

TOV112D) all reduced cell proliferation over a 72hr period. Conversely, rescue experiments by exogenous expression of *PYGO2* in *PYGO2*-knocked-down SKOV-3 and OVCAR-3 serous ovarian carcinoma cell lines restored cell proliferation rates similar to that of control conditions. Strikingly, parental SKOV-3, OVCAR-3 and TOV21G (clear cell-derived, β-catenin-null) cells showed negligible TOPflash luciferase activity, indicating that PYGO2 possess functional effects in EOC even in the absence of β-catenin/TCF transcriptional activity. Furthermore, *in vitro* soft agar colony formation assays of SKOV-3 cells after *PYGO2* knockdown reduced colony formation efficiency. Also, colonies formed were smaller, indicating PYGO2 affects anchorage-independent growth in EOC. In addition, subcutaneous implantation of these cells into SCID mice displayed smaller and slower tumourigenic growths, with a significant difference in tumour mass between *PYGO2* knockdown and control mice 18 weeks post-implantation. Taken together, PYGO2 affects both anchorage-dependent and anchorage-independent growths of EOC cells *in vitro* and *in vivo*, independent of β-catenin/TCF status. In another study, *BCL9L* transcript expression was found to have increased two-fold in persistent tumour samples matched against primary tumour samples.[442] Of note, persistent tumour samples displayed higher expression profile of stem-like markers including ALDH1A, CD44 and CD133, indicating the presence of ovarian cancer stem cells (CSC). This may suggest upregulated β-catenin/TCF transcriptional activity in ovarian CSCs.

Receptor-Interacting Protein Kinase 4 (RIPK4) is a serine/ threonine kinase interacting with protein kinase C delta (PRKCδ) that was previously reported to activate NFκB and JNK.[443,444] RIPK4 overexpression in human embryonic

kidney 293T cells (HEK293T) upregulated WNT/β-catenin target genes transcript expression (*CCND1*, *LEF1*, *JUN*, *c-MYC*, *TCF7*), elevated TOPbrite TCF-luciferase reporter activity and increased the pool of cytosolic β-catenin, indicating that RIPK4 is an activator of WNT/β-catenin pathway.[423] Conversely, siRNA-mediated knockdown of *RIPK4* in Wnt3a-treated A2780 and COV434 ovarian carcinoma cells abrogated TOPbrite TCF-luciferase activity and reduced cytosolic β-catenin accumulation. Furthermore, RIPK4 was shown to interact with and directly phosphorylate DVL2.[423] RIPK4 was also shown to bind with LRP6 after Wnt3a stimulation, suggesting that upon Wnt3a ligand binding, LRP6 co-receptor recruits RIPK4 to phosphorylate DVL2, which then associates with AXIN-GSK3 to disrupt formation of the β-catenin destruction complex, promoting cytosolic β-catenin accumulation.[423] Interestingly, human ovarian adenocarcinomas showed significantly higher RIPK4 transcript and protein expression accompanied by the presence of cytosolic β-catenin, as compared to normal ovarian tissue. This would suggest that RIPK4 mediates activation of WNT/β-catenin signalling in ovarian cancer and thus RIPK4 may be a potential therapeutic target in treating ovarian cancer.[423]

WNTLESS (WLS) is a seven-transmembrane protein involved in the effective secretion of WNT ligands from the signalling cell to activate downstream WNT signalling.[79-81] Immunohistochemical analysis of WLS expression in a panel of EOC and OSE patient specimens found WLS expression in 137/201 (68%) of EOC specimen but none of the OSE.[424] Of note, a significant majority of OEA samples stained positively for WLS (19/23, 83%), supporting previous findings of aberrant WNT/β-catenin

signalling in OEAs (as described earlier). Also, significant majority of high-grade serous ovarian carcinoma samples (89/116, 77%) express WLS, as compared to just 25% (5/20) of borderline and low-grade serous tumour samples, suggesting WNT as one of the drivers of EOC. Interestingly, 7/201 (4%) of the EOC samples showed HER2 expression, all of which express WLS. Furthermore, 5/7 (71%) of the HER2-expressing tumours are of the serous histological subtype, showing a strong correlation between WLS and HER2 expression in serous ovarian carcinoma. This correlation may further suggest WLS/HER2 co-expression as a prognostic marker in serous ovarian carcinoma.

WNT Ligands in EOC

In EOC, several reports have shown WNT ligands expression to be involved, mainly WNT2B,[445,446] WNT5A[363,418,447-450] and WNT7A.[154,156,451] *WNT2B* transcript expression was initially reported to be present in four ovarian carcinoma cell lines, namely: Caov-3, HEY, OVCAR-3 and SKOV-3,[445] while protein expression was reported in A2780 cells.[446] siRNA-mediated knockdown of *WNT2B* in A2780 cells (A2780:si*WNT2B*) reduced cell proliferation in colony formation assays. Also, A2780:si*WNT2B* cells were less invasive in Matrigel. These findings suggest WNT2B to play a role in mediating ovarian cancer metastasis. Furthermore, A2780:si*WNT2B* cells showed increased chemosensitivity to paclitaxel in a dose-dependent manner (between 30-1000μM concentration), evident by an increase in propidium iodide positivity and the number of cells undergoing apoptosis (Annexin V positivity). Interestingly, A2780:si*WNT2B* cells showed elevated levels of

Caspase 9 (CASP9) expression, accompanied by reduced expression levels of anti-apoptotic BCL2 and BCL2L1, suggesting that the chemosensitivity phenotype observed is mediated by the CASP9/BCL2/BCL2L1 apoptotic machinery.

Microarray data showed WNT5A overexpression in EOC cultures as compared to OSE cells.[363] In a separate microarray study, analysis showed upregulated *WNT5A* expression in A2780/C10B, an oxaliplatin-resistant derivative of parental A2780 human ovarian cancer cell line.[418] Similarly, patient samples of melanoma cells gained resistance to the BRAF-inhibitor PLX4720 in a PI3K/AKT-dependent manner that is mediated by WNT5A.[359] Independently, an immunohistochemical study in primary patient samples showed WNT5A overexpression in the EOC samples. Furthermore, EOC patients overexpressing WNT5A showed poorer prognosis.[447] Similar results were obtained from another independent study of similar design, whereby samples from EOC patients showed stronger WNT5A immunoreactivity and patients with WNT5A expression showed lower progression-free and overall survival.[448] In addition, ectopic overexpression of WNT5A in SKOV-3 cells enhanced chemoresistance (increased IC_{50} values) to conventional chemotherapeutic reagents, including paclitaxel, oxaliplatin, 5-fluorouracil (5-FU), epirubicin and etoposide. Further supporting this, *WNT5A* knockdown in SKOV-3 cells *via* RNA-interference (RNAi) enhanced chemosensitivity (decreased IC_{50} values) towards the aforementioned chemotherapeutic reagents,[448] supporting a previous finding that WNT5A mediates chemoresistance.[418] However, contradicting results were observed in another study.[449] Immunohistochemical staining of WNT5A showed a majority of

EOC samples (81/130, 62%) to display lower WNT5A expression as compared to hOSE samples (13/31, 42%). Of note, a majority of the EOC samples are of the Type II high-grade serous classification. Also, lower WNT5A expression correlated to shorter overall survival. Both these findings contradict what was previously reported.[363,447,448] It has been suggested that this discrepancy may be due to a difference in sample size (130[449] vs. 38[447] and 63[448]). Also, it may be due to the difference in number of Types I and II tumour samples present in the other two studies. Of interest, *WNT5A* promoter regions in OVCAR-5 and PEO1 ovarian carcinoma cell lines were found to be hypermethylated. Treatment of PEO1 cells with a DNA demethylating agent 5-Aza-2'deoxyctiydine (5-Aza-C) increased WNT5A transcript and protein expression, demonstrating that WNT5A expression in EOC is epigenetically suppressed by gene promoter hypermethylation.[449] Furthermore, ectopic expression of *WNT5A* in the two cell lines inhibited both anchorage-dependent and anchorage-independent growths, with a marked reduction in the transcription expression levels of β-catenin target genes *CCND1*, *c-MYC* and *FOSL1* (FOS-like antigen 1). Also, immunoblotting showed a reduction of soluble (thus active) β-catenin after WNT5A ectopic expression. These results indicate that WNT5A antagonizes the WNT/β-catenin pathway. In addition, OVCAR-5 and PEO1s cells expressing WNT5A was shown to undergo cellular senescence, as shown by positive staining for the senescence marker SA-β-gal (senescence-associated beta-galactosidase). Furthermore, orthotopic injection of the WNT5A-expressing OVCAR-5 cells into the ovarian *bursa* of immunocompromised mice showed smaller tumour growths after 30 days as compared to the control. Further analysis showed

these WNT5A-expressing tumours to stain weakly for the proliferative marker Ki-67 but positively for SA-β-gal, indicating that WNT5A inhibited *in vivo* EOC tumourigenesis by promoting cellular senescence. In another immunohistochemical study involving a patient cohort of 623 samples (230 benign controls, 86 borderline LMP tumours and 307 EOC samples), EOC samples displayed significantly higher WNT5A expression when compared against the benign controls and borderline tumours.[450] Furthermore, reclassification of the EOC samples into either Type I or Type II groups all showed higher WNT5A expression against fallopian tubal epithelium, OSE and benign controls. However, no significant difference was observed when WNT5A expression status was measured against disease-free or relapse-free survival, suggesting that WNT5A is not a predictor of clinical outcome, as opposed to what was previously reported.[448] *In vitro* treatment of an OSE cell line (HOSE6-3) with a human recombinant WNT5A stimulated WNT5A expression, followed by significant downregulated expression of β-catenin target genes *CCND1*, *c-MYC* and *AXIN2*. Concurrently, upregulation of non-β-catenin pathway target genes was observed: Nuclear Factor of Activated T-cells (*NFAT*), c-Jun N-terminal Kinase (*JNK*) and Protein Kinase C Alpha (*PRKCA*). This supports previous findings whereby WNT5A negatively regulates WNT/β-catenin pathway.[449] Furthermore, WNT5A expression in HOSE6-3 drove cell migration and reduced cell-matrix adhesion towards collagen and fibronectin. In addition, E-cadherin (CDH1) protein expression was downregulated in WNT5A-expressing HOSE6-3 cells, accompanied by upregulation of the mesenchymal marker vimentin (VIM) and EMT drivers SNAIL1 (SNAI1) and SNAIL2 (SNAI2), suggesting that WNT5A can drive

EMT in EOC. siRNA-knockdown of *WNT5A* in OVCAR-3 serous ovarian adenocarcinoma cell line also reduced protein expression of VIM and SNAI2, suggesting an early stage of MET.[450] However, transcript expression levels of the aforementioned target genes of both the WNT/β-catenin and the non-β-catenin pathways showed no significant differences. It should be highlighted that parental OVCAR-3 displays a highly epithelial morphology and that using this cell line as a model for driving MET (knocking down WNT5A, a putative EMT driver) might prove difficult as the results here describes MET at a molecular rather than a cellular context.

Microarray analysis of tumour samples showed *WNT7A* expression to be higher in serous ovarian tumours compared to normal/benign samples.[451] This is supported by transcript expression profiling of 300 tumours and normal ovarian samples, whereby *WNT7A* and *WNT7B* overexpression was observed in malignant samples when compared against normal ovarian specimens.[156] *WNT3* and *WNT4* were found to be downregulated while no significant difference was found between malignant and normal ovarian samples for the other WNT ligands. It should be highlighted here that expression of *WNT5A* in this study did not show any significant difference. *In situ* hybridization of the patient samples against *WNT7A* cRNA probes showed a significant majority of serous adenocarcinomas (65%) to have a higher expression profile. Also, SKOV-3.ip1 cells (derived from ascites of nude mice after intraperitoneal injection of parental SKOV-3 cells) showed elevated endogenous *WNT7A*, *MMP7* and *MMP9* expression as compared to the parental cells. *WNT7A* shRNA knockdown in SKOV-3.ip1 cells (SKOV-3.ip1:sh*WNT7A*) reduced expression of WNT/β-catenin target genes *CCND1*

and *MMP7*. Likewise, ectopic overexpression of *WNT7A* in parental SKOV-3 cells showed upregulated expression of *CCND1* and *MMP7*. Consistently, *WNT7A* knockdown in SKOV-3.ip1 cells reduced proliferation and WNT7A overexpression in SKOV-3 cells increased proliferative rates. Results from cell invasion assays show that overexpression of WNT7A in SKOV-3 cells increased cell invasiveness, whereas SKOV-3.ip1: sh*WNT7A* cells displayed a less invasive phenotype. In agreement with this finding, 5 weeks post-intraperitoneal injection of SKOV-3.ip1 cells into nude mice formed multiple tumour masses with implants around the peritoneal cavity, with some tumour masses invading into the bowels. In contrast, SKOV-3.ip1: sh*WNT7A* cells developed one large tumour in the retroperitoneal space with less implants and no bowel-invasion observed. The *vice versa* effect was observed when WNT7A was overexpressed in SKOV-3 cells as compared to control conditions: larger tumour masses with evidence of tumour implants, bowel invasion and upregulated *CCND1* expression. Furthermore, TOPflash luciferase assays show that WNT7A activates the WNT/β-catenin signalling pathway in this system. Interestingly, co-transfection of WNT7A and FZD5 overexpressing constructs into both SKOV-3 and HEY cell lines containing TOPflash luciferase constructs further elevated luciferase activity as compared to just transfection of WNT7A construct alone, indicating that WNT7A is a ligand for FZD5 in EOCs. In addition, deletion of TCF/LEF binding sites on the promoter region of MMP7 in HEY ovarian papillary cystadenocarcinoma cells ectopically overexpressing WNT7A abrogated MMP7 luciferase reporter activity, indicating that WNT7A can target MMP7 expression *via* the WNT/β-catenin-mediated pathway.[156] Separately,

overexpression of WNT7A in OVCAR-3 ovarian adenocarcinoma cells enhanced cell migration and invasion properties *in vitro* (wound-healing assay and Matrigel invasion chamber assay).[451]

A follow-up study found *WNT7A* transcript expression to correlate with that of *FGF1* in serous and clear cell carcinoma samples.[154] Furthermore, correlation in protein expression levels was observed in SKOV-3.ip1 cells after *WNT7A* knockdown and in SKOV-3 cells after *WNT7A* overexpression. This correlation was also observed after these cells generated tumours *in vivo*. Analysis of the *FGF1* promoter region identified 8 TCF/LEF consensus binding sites and ChIP assays of both SKOV-3.ip1 and WNT7A-overexpressing A2780 cells showed enrichment of FGF1 in chromatin DNA pulled down by a TCF4 antibody. Furthermore, SKOV-3.ip1 cells carrying a dominant-negative TCF4 construct (dnTCF4) reduced *FGF1* transcript expression levels. As expected, A2780 cells stably overexpressing either WNT7A (A2780:*WNT7A*^{OE}) or FGF1 (A2780:*FGF1*^{OE}) both displayed enhanced cell proliferation and adhesion to plastics. However, shRNA-mediated knockdown of *FGF1* in A2780 cells (A2780:WNT7^{OE}/sh*FGF1*) stably overexpressing WNT7A abrogated the enhancements observed. In addition, orthotopic tumours formed *in vivo* by A2780:*WNT7A*^{OE}/sh*FGF1* cells were significantly smaller than those of A2780:*WNT7A*^{OE} and A2780:*FGF1*^{OE} cells. Taken together, these results strongly support FGF1 as a WNT/β-catenin target gene in serous ovarian cancers.[154]

Frizzled (FZD) Receptors and other co-receptors in EOC

Immunohistochemical analysis of patient specimens showed FZD1 expression in a majority of both malignant and benign tumours (97% and 90% respectively), while significantly less in normal ovarian tissues (55%).[447] In contrast, a cDNA array hybridization study found *FZD3* expression to be downregulated in ovarian adenocarcinoma samples as compared to a benign adenoma sample.[452] Furthermore, *FZD3* expression was downregulated in advanced ovarian adenocarcinomas (more aggressive) as compared to highly differentiated ovarian adenocarcinomas (less aggressive).[452] *FZD4 loci* hypermethylation in ovarian serous and ovarian endometrioid adenocarcinoma specimens showed association with disease progression (Hazard Ratio (HR): 2.5), higher risk of disease recurrence (HR: 0.8) and poorer overall survival (HR: 49.4), indicating that epigenetic silencing of *FZD4* expression *via* promoter hypermethylation inversely correlated with prognosis and survival.[453] However, it should be noted that Spearman's correlation did not show statistical significance.[453] FZD5 has been shown to be a receptor for WNT7A, whereby ectopic co-expression of WNT7A and FZD5 into both SKOV-3 and HEY ovarian carcinoma cell lines greatly increased TOPflash luciferase activity, as compared to just WNT7A overexpression alone.[156] Comparative genomic hybridization (CGH) analysis of 46 sporadic and familial ovarian cancers showed one of the minimal region of interest commonly amplified to be within chromosome 2q32.1-34, which includes the *loci* for *FZD5*.[362] However, immunohistochemical analysis of FZD5 expression showed no significant difference on prevalence between ovarian tumours and normal ovarian tissue specimens.[447] Aside from the FZD

receptors, two co-receptors have also been implicated in ovarian cancer. *In vitro* culture of OVCA429 and OVCA433 ovarian carcinoma cells in type I collagen resulted in aggregation of integrin, inducing β-catenin/LEF/TCF transcriptional activity, including upregulated *LRP6* expression.[421] RYK is an atypical receptor tyrosine kinase demonstrated to act as a FZD co-receptor for the WNT/PCP pathway to mediate neurite outgrowths and axonal guidance during mammalian development.[281,320-322,454] In normal OSE tissue, mRNA *in situ* hybridization (ISH) did not detect *RYK* expression. However, stromal regions surrounding benign and borderline tumours showed *RYK* expression while strong *RYK* expression was detected in the epithelium of malignant EOC tumours.[455] In a follow-up study, EOC patient samples with high expression levels of RYK correlated with poorer outcome for overall survival.[456] Interestingly, strong RYK expression in the endothelial and smooth muscle layer of blood vessels was also found to correlate with shorter overall survival, suggesting that RYK expression may potentially be used as a prognostic marker for EOC.[456] Furthermore, *in vitro* functional studies showed overexpression of RYK in PEO4 ovarian carcinoma cells and NIH3T3 mouse fibroblast cells to enhance cell proliferation and anchorage independent growth in soft agar colony formation assays. Nude mice subcutaneously injected with NIH3T3 cells overexpressing RYK (with Matrigel) showed aggressive tumourigenic growths within 2 weeks, evident by peripheral skeletal muscle invasion and high mitotic index. In contrast, no tumourigenic growth was detected in control mice even after 6 months.[457] Taken together, RYK drives tumourigenesis and overexpression in EOC is indicative of poor prognosis.

Inhibitors of WNT Signalling (DKKs & SFRPs) in EOC

As described earlier, Dickkopf WNT signalling inhibitor 1 (DKK1), a member of the Dickkopf (DKK) secreted proteins, was shown to be a target gene of β-catenin in EOC[417] and is involved in enhancing chemosensitivity.[418] Another study found transcript expression profile of *DKK2* in primary EOC tumours to be significantly lower than that of benign tumours and normal ovarian specimens.[458] *DKK2* expression was also found to be very low in two EOC cell lines, SKOV-3 and ES-2. Similarly, immunohistochemical staining of DKK2 in tissue specimens showed EOC samples to display significantly reduced immunoreactivity compared to normal ovarian tissue and benign tumours. Methylation-specific PCR (MSP) analysis subsequently showed *DKK2* promoter hypermethylation in SKOV-3, ES-2 EOC cells and 27/50 (54%) of EOC samples. In contrast, *DKK2* promoter hypermethylation was undetectable in all of the normal ovarian tissue and benign tumour samples. Exogenously overexpressed *DKK2* in both SKOV-3 and ES-2 cells negatively regulates cell proliferation, migration and invasion. In agreement with the *in vitro* data, SKOV-3:*DKK2*^{OE} cells formed significantly smaller tumours after subcutaneous injection into nude mice. Furthermore, treatment of SKOV-3 and ES-2 cells with the demethylating agent 5-Aza-C showed similar effects to DKK2 exogenous overexpression: cell proliferation impairment *in vitro* and reduced tumourigenic potential *in vivo*. Interestingly SKOV-3:*DKK2*^{OE} cells show downregulated transcript expression for *c-MYC* and *CCND1*, target genes of β-catenin/TCF transcription, accompanied by downregulated protein levels for other β-catenin/TCF transcription targets FAK and MMP2, which may explain the invasion-impaired phenotype.

Mean serum DKK3 expression was reported to be lower in 36 ovarian cancer samples (~26 pg/mL) as compared to that of 30 normal healthy controls (42 pg/mL).[459] Also, a Japanese study reported that serum DKK3 reduced slightly in late stage ovarian cancer when compared against the early stages,[460] suggesting DKK3 to be a tumour suppressor in ovarian cancer. In another study, 35/56 (63%) EOC samples showed reduced *DKK3* transcript expression when compared to that of normal ovarian tissue.[461] Further immunohistochemical analysis of the 56 EOC samples showed 37 (66%) with either negative or weak membranous staining. In a recent study, DKK3 protein expression was detected in 4 ovarian mucinous carcinoma cell lines (RMUG-S, RMUG-L, OMC and MCAS) and 1 ovarian clear cell carcinoma cell lines (RMG-1).[462] siRNA-mediated knockdown of *DKK3* in MCAS and RMG-1 cells increased cell proliferation, suggesting a tumour suppressor effect of DKK3. In addition, MCAS:si*DKK3* cells showed reduced protein expression for pro-apoptotic factors, including CASP3, -8 and -9, BAX, FAS/CD95, JNK and p53 and increased anti-apoptotic BCL2 protein expression, which plays in a role in mitochondrial outer membrane permeabilization (MOMP). However, overexpression of DKK3 in MCAS cells did not show any changes in the protein expression levels of all the apoptotic factors, suggesting that only a basal level of DKK3 is required for inducing apoptosis. Taken together, these results show that DKK3 acts as a tumour suppressor by promoting apoptosis in mucinous ovarian cancer *via* the mitochondria and the FAS death receptor path.[462]

Secreted Frizzled Related Proteins (SFRPs) are secreted WNT antagonistic proteins bearing homology to the cysteine rich domain (CRD) of FZD

receptors, capable of binding to extracellular WNT ligands to prevent activation of cellular WNT signalling. In the human genome, five SFRPs have been identified, namely SFRP1, -2, -4, -5 and FRZB (Frizzled related protein, also known as SFRP3). In EOC, epigenetic alterations of *SFRP1*, *-2*, *-4* and *-5* has been reported.[463-470] MSP analysis of a panel of ovarian carcinoma cell lines representing all four major histological subtypes and a human OSE cell line found CpG island (CGI) DNA methylation in the SFRP1 promoter sequence of some cell lines but not in the HOSE cell line (summarized in Table 5).[463]

Table 5: Summary of SFRP1 CpG Island (CGI) Methylation Status in Cells lines of Ovarian Carcinomas and OSE

Cell Line	Histology	CGI Methylation Status	Transcript Expression
ES-2	Clear Cell	-	++
HTOA	Serous	-	+
JHOS-2	Serous	-	++
KURAMOCHI	Undifferentiated	-	+/-
MCAS	Mucinous	+	-
OV-90	Serous	+/-	+/-
RMG-I	Clear Cell	+/-	-
RMUG-L	Mucinous	+	-
RMUG-S	Mucinous	-	+
RTSG	Poorly diff.	+	-
TOV21G	Clear Cell	-	++
TOV112D	Endometrioid	-	+/-
TYK-nu	Undifferentiated	+	-
HOSE	OSE	-	+

Transcription expression profile supports the MSP analysis, whereby ovarian carcinoma cell lines with DNA hypermethylation to be devoid of *SFRP1* expression, and *vice versa*. Treatment of MCAS mucinous ovarian carcinoma cell line with 5-Aza-C demethylating agent showed reversal of *SFRP1* CGI promoter methylation status, showing that SFRP1 may be epigenetically modulated in some EOC. Furthermore, MSP analysis of *SFRP1* promoter CGI

status in 17 primary ovarian tumour specimens and seven endometrial cysts showed 2/17 (12%) tumour specimens with hypermethylation, while the only one endometrial cyst showed trace levels of methylation. Separately, another study found SFRP1 hypermethylation in 12/36 (33%) of ovarian clear cell adenocarcinoma samples.[470] This further supports the notion of SFRP1 regulation *via* epigenetic silencing in EOC.[463] A subsequent epigenetic study on a larger cohort of ovarian cancer patient samples (126 malignant, 14 borderline and 75 benign) found CGI hypermethylation to be significantly increased during malignant progression for *SFRP1*, *-2* and *-5*.[464] Furthermore, patient samples with *SFRP1* hypermethylation showed higher relative risk towards recurrence and a worse prognosis, and *SFRP1* methylation status showed significant concordance between a cohort of paired tissue/serum patient samples, potentiating *SFRP1* hypermethylation as a prognostic marker.[464] Taken together, these suggest the notion of prevailing aberrant WNT signalling as ovarian cancer progresses towards malignancy. Separately, SFRP2 was shown to inhibit WNT7A/FZD5-mediated β-catenin/TCF transcriptional activity in SKOV-3 and HEY ovarian carcinoma cell lines, evident by significant reduction in TOPflash luciferase activity.[156] In another study, *SFRP4* transcripts were differentially expressed among A2780 ovarian carcinoma cells and two chemoresistant-derivatives of the line (Adriamycin-resistant A2780-ADR and cisplatin-resistant A2780-Cis).[465] *SFRP4* expression was also higher in an immortalized OSE (IOSE) line. Interestingly, immunohistochemical analysis of IOSE and parental A2780 cells after cisplatin-treatment showed that a majority of cells that survived were negative for SFRP4 staining. Ectopic SFRP4 overexpression in A2780-

ADR and A2780-Cis chemoresistant lines abolished cisplatin-chemoresistance as compared to the controls. Conversely, siRNA-mediated knockdown of *SFRP4* in A2780 parental cells showed elevated chemoresistance to cisplatin. Immunoblots showed an inverse correlation between SFRP4 and β-catenin protein expression levels,[465] a trend observed from tissue microarray (TMA) immunohistochemical (IHC) analysis of primary mucinous[465] and serous carcinomas.[466] In another separate study, ascitic samples from two patients with progressive chemoresistance collected at later timepoints during treatment displayed reduced SFRP4 protein expression as compared to the earlier timepoint, accompanied by decreased GSK-3β and increased in active β-catenin expression.[467] These results indicate that the WNT/β-catenin pathway mediates the chemoresistance effect induced by SFRP4. Furthermore, TMA-IHC analysis of 104 primary ovarian mucinous tumour samples showed significantly lower SFRP4 expression in mucinous adenocarcinomas as compared to the benign or borderline groups.[465] Similarly, another TMA-IHC analysis involving 721 patient samples displayed significantly reduced SFRP4 expression in EOC compared to benign tumours.[467] In addition, this trend was also observed in patient plasma samples using ELISA-based detection (Enzyme linked immunosorbent assay) of SFRP4. Of clinical relevance, patient samples lacking membrane SFRP4 expression was found to be associated with shorter overall survival.[467] Taken together, these results indicate that SFRP4 promotes chemosensitivity in EOC *via* the WNT/β-catenin pathway and that lost of SFRP4 is associated with disease progression towards malignancy and presents a worse prognosis.[465-467] Separately, *SFRP4* promoter

hypermethylation in ovarian clear cell adenocarcinoma tissues is rare, with a prevalence of ~10% (5/48).[470] In a follow-up study, introduction of SFRP4 to OVCAR-3 serous ovarian carcinoma cells with human recombinant SFRP4 (rSFRP4) reduced β-catenin/TCF/LEF transcription activity, as validated by TOPflash luciferase reporter assays.[468] Furthermore, SFRP4-treatment downregulated transcript levels of β-catenin target genes *CCND1*, *c-MYC* and *AXIN2*, but not of target genes of the β-catenin-independent (PCP and Ca^{2+}) pathways: *JNK*, *RHOA*, *RAC1* and *PRKCA*. Reintroduction of SFRP4 into OVCAR-3 cells impaired cell migration during wound healing assays, accompanied by increased integrin adhesion towards collagen and fibronectin. Furthermore, rSFRP4-treatment in OVCAR-3 cells increased protein expression of E-cadherin (CDH1), while decreasing expression of the mesenchymal intermediate filament vimentin (VIM) and the EMT-inducer TWIST1.[468] Interestingly, a near opposite trend was observed in the presence of WNT5A,[450] suggesting antagonism between WNT5A and SFRP4 in EOC.

SFRP5 hypermethylation prevalence during EOC disease progression was previously reported.[464] Another study reported a higher prevalence of *SFRP5* CGI promoter hypermethylation in ovarian clear cell adenocarcinoma (31/48, 65%) than in ovarian serous adenocarcinoma (2/15, 13%) while normal ovarian epithelium do not display *SFRP5* promoter hypermethylation.[470] A follow-up study showed a functional EOC phenotype in SFRP5.[469] MSP analysis of *SFRP5* showed strong hypermethylation in SKOV-3 and A2780 ovarian carcinoma cells, while OVCAR-3 and A2780/CP70 (cisplatin-resistant derivative of A2780 cells) cells were partially hypermethylated. Interestingly, treatment of SKOV-3 and A2780 cells with 5-Aza-C (DNA

methyltransferase inhibitor) restored *SFRP5* transcript expression, confirming epigenetic silencing *via* hypermethylation of *SFRP5*. This was also reported in several ovarian clear cell adenocarcinoma-derived cell lines (HAC-2, KK and RMG-II).[470] Exogenous overexpression of SFRP5 in SKOV-3 and OVCAR-3 cells reduced colony formation in soft agar and invasion in Matrigel. Additionally, enhanced chemosensitivity towards chemotherapeutic agents cisplatin and taxol was observed.[469] Furthermore, *SFRP5* re-expression abrogated tumourigenesis *in vivo* SCID mouse models. Conversely, the *vice-versa* trend was observed in A2780/CP70 cells after *SFRP5* shRNA-mediated knockdown (A2780/CP70:sh*SFRP5*).[469] TOPflash luciferase reporter assays showed reduced luciferase activity in SKOV-3 cells after exogenous SFRP5 overexpression. Furthermore, transcript expression of β-catenin target genes *CCND1* and *c-MYC* were also downregulated after SFRP5 re-expression in SKOV-3 and OVCAR-3 cells. Similarly, A2780/CP70:sh*SFRP5* cells showed nuclear β-catenin immunoreactivity and increased *CCND1* transcript expression. These would suggest that SFRP5 targets the β-catenin/TCF/LEF pathway. Of clinical relevance, majority of patients with *SFRP5* hypermethylation had poorer response to chemotherapy as compared to patients without *SFRP5* methylation. Furthermore, ovarian clear cell adenocarcinoma patients with *SFRP5* hypermethylation had shorter overall survival as compared to patients with unmethylated *SFRP5*.[470] This would suggest that epigenetic silencing of *SFPR5* enhances chemoresistance in EOC, resulting in poorer prognosis.

CHAPTER 1.4

Hypothesis and Aim

FZD7 transcript expression was previously reported to be up-regulated in SKOV-3 ovarian adenocarcinoma cells-derived spheroids.[243] Furthermore, previous studies carried out on primary neuronal and mammary epithelial cells showed that spheroids derived from these cells are enriched for the stem-like/ progenitor populations, evident by the ability of these spheroid-derived cells to differentiate into different lineages of the respective systems.[364,365] In addition, FZD7 was previously reported to regulate self-renewal of hESCs.[366] Taken together, these results suggest FZD7 to play in role in regulating self-renewal of stem-like/ progenitor populations in ovarian cancer to promote metastasis. To this end, the role of FZD7 in mediating spheroid formation was studied and it was hypothesized that abrogation of FZD7 in ovarian cancer cells will inhibit spheroid formation by promoting *anoikis*, apoptotic cell death induced by dissociating of anchorage-dependent adherent cells. As such, the aim of this study was to generate stable knockdowns against FZD7 in ovarian cancer cell lines with high FZD7 expression and to evaluate if silencing of FZD7 abrogates spheroid formation and induces *anoikis*.

CHAPTER 2

Materials and Methods

Cell Lines and cell culture

CH1 cells were kind gifts from Dr. Noriomi Matsumura (Kyoto University, Japan) while OV17R and OAW28 cells were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Dubelcco's Modified Eagle's Medium (DMEM) with high glucose (4500mg.L^{-1}) and sodium pyruvate, DMEM/ Ham's F-12 (1:1) media mix and fetal bovine serum (FBS) were from BioWest (Nuaillé, France). Supplements sodium pyruvate, bovine pancreatic insulin and hydrocortisone were all purchased from Sigma-Aldrich (St. Louis, MO). All cells were maintained in penicillin-streptomycin mix (Life Technologies-Gibco®, Grand Island, NY). See table below for culture conditions for each cell line.

	Media	FBS % (v/v)	Additives		
			Sodium Pyruvate	Insulin (μg.mL^{-1})	Hydrocortisone (μg.mL^{-1})
CH1	DMEM	10	1mM	-	-
OAWA28	DMEM	10	1mM	0.7	-
OV17R	DMEM/ Ham's F-12	5	1mM	10	0.1

RNA Isolation

RNA from cell material were isolated using TRIzol® reagent (Ambion®-Life Technologies, Grand Island, NY) under a modified protocol from that of the user guide. Briefly, cells were lysed in TRIzol® at an approximate volume: growth surface area ratio of 800μL:10cm^2 and mixed with 200μL of chloroform (amylene-stabilized, Sigma-Aldrich, St. Louis, MO) for every 1mL of TRIzol® used. After phase separation by centrifugation (12,000x g for

15min at 4°C), the RNA-enriched aqueous phase was further purified using RNeasy mini spin columns (QIAGEN, Germantown, MD), with the on-column DNase-treatment included prior to final elution.

Quantitative Real Time Polymerase Chain Reaction (qPCR)

An input of 500ng of total RNA of each individual samples were reverse transcribed into cDNA using pooled random hexamers and oligo-dT (Thymine) primers as according to user instructions of the RT^2 First Strand kit (SABiosciences-QIAGEN, Germantown, MD). qPCR reactions were prepared using the 2x SYBR Green RT^2 qPCR Master Mix (with ROX internal reference dye, SABiosciences-QIAGEN, Germantown, MD) with an input of ~5ng cDNA and mixed to a final reaction volume of 25μL per well. The same protocol was used for the human WNT Signalling Pathway Plus PCR Array (SABiosciences-QIAGEN, Germantown, MD). All qPCR reactions were ran on the 7900HT Real Time PCR System (Applied Biosystems/ Life Technologies, Grand Island, NY) under a 40-cycle standard run. Raw threshold cycle (C_T) values were determined with the SDS software (version 2.3). To calculate fold change differences of gene transcript expression, the raw C_T value of the gene of interest (GOI) was subtracted from the average C_T value of the house keeping genes (HKG) used in the array, namely beta-actin (ACTB), beta 2 microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1) and ribosomal protein, large 13A (RPL13A). This subtracted value becomes the ΔC_T, which is then compared between the control and the respective conditions to generate the $\Delta\Delta C_T$. Fold change is expressed as the

value from the negative exponent of $\Delta\Delta C_T$ to the base of two ($2^{-\Delta\Delta C_T}$). For calculation of percentage of transcript knockdown efficiency, the formula ($1-2^{-\Delta\Delta C_T}$) was used, with the overall value being expressed in percentage (%).

Spheroid Formation and Anoikis Assay

Ovarian carcinoma cell lines cultured on adherent plastic (Delta-treated surface, Thermo Scientific-Nunc, Grand Island, NY) were trypsin-dissociated (Nacalai Tesque, Kyoto, Japan) between 70-90% confluency and strained with a 40μm-porosity strainer (Greiner Bio-one, Frickenhausen, Germany) to acquire a suspension of single cells. Single-cells suspension were seeded onto neutrally charged and hydrophilic flat-bottomed 96-well ultra-low attachment surface (ULAS, Corning, Tewksbury, MA) at a density of 200 cells/ well and left in culture for a week to determine spheroid formation capabilities. ULAS coating prevents ionic interactions between cells and the surface, disrupting anchorage-dependent adhesion in the process, thereby allowing the evaluation of anchorage-independent growth capabilities. Protocol for *anoikis* assay is the same as that mentioned in the spheroid formation assay, with the exception that the entire cell suspension was harvested after various timepoints (between 24-168hrs) for either Annexin V flow cytometry cell sorting or immunoblotting.

Fluorescence-activated Cell Sorting (FACS) Flow Cytometry

Briefly, adherent cells were trypsin-dissociated and re-suspended in FBS-supplemented media. $1x10^5$ cells were counted and washed twice with 1x PBS (phosphate-buffered saline). For suspension cultures, the entire cell suspension

was trypsin-dissociated (for dissociating spheroids) prior to resuspension, counting and PBS wash as before. PBS-washed cells were subsequently rinsed in 1x Annexin V Binding Buffer (AVBB) and stained in 100μL of AVBB-based staining solution, which consists of pacific blue-conjugated Annexin V (Life Technologies-Molecular Probes®, Carlsbad, CA) at 5μL per $1x10^5$ cells and propidium iodide (Sigma-Aldrich, St. Louis, MO) at $2\mu g.mL^{-1}$. Cells were incubated for 15 min at room temperature, washed once in AVBB before suspension in 1x PBS (200μL/ 1x 10^5 cells). Stained cells were strained through 70μm porosity cell strainer (Greiner Bio-one, Frickenhausen, Germany) prior to FACS analysis. FACS was performed on the LSRII flow cytometer (Becton-Dickinson™, San Jose, CA) and data analysed with the FACSDiva software (Becton-Dickinson™, San Jose, CA).

shRNA and shRNA^{miR}-mediated Knockdown

Ribonucleic acid interference (RNAi) *via* short hairpin RNA (shRNA) was used to generate stable clones of cells with the knockdown for the genes of interest. Lentiviral particle transduction was used to generate stable knockdowns for *FZD7* and *WLS*. For *FZD7*, the MISSION® shRNA system (designed by The RNAi Consortium [TRC], Cambridge, MA; distributed by Sigma-Aldrich, St. Louis, MO) was used. The sh*FZD7* lentiviral plasmids were purchased from Sigma-Aldrich (St. Louis, MO). Briefly, each lentiviral plasmid vector (pLKO.1-Puro) contains various 21-base sense and antisense short hairpin sequence verified to target specific regions of *FZD7* (see table below for sequence). The microRNA (miRNA) miR-155-based sh*WLS* plasmids (pMSCV-Blst: sh*WLS*^{miR155}) were kind gifts from Dr. YU Jia from

Prof. David VIRSHUP's lab (Duke-NUS Graduate Medical School, Singapore). Briefly, two 21-base *WLS* coding sequence were separately cloned into the stem loop region of the miR155 precursor expression cassette, flanked by synthetic introns of the pSM155 miRNA vector.[471] Of note, the mature miR155 sequence has been replaced by the WLS coding sequence, effectively eliminating any miR155-specific off-target effects. The advantage of this system is the utilization of the precursor sequence of miR155 into naturally processing the sh*WLS*^{miR155}-cassette, thereby improving the knockdown efficiency. The entire sh*WLS*-miRNA expression cassette was subsequently cloned into the pMSCV-Blst vector to allow Blasticidin-based antibiotic selection for stable clones. When selecting for stable clones, CH1 and OV17R cells were cultured in conditions as previously mentioned above, with the addition of either puromycin (Life Technologies-Gibco®, Carlsbad, CA) or blasticidin (Sigma-Aldrich, St. Louis, MO) for selection antibiotics at concentrations stated below:

	Puromycin (μg.mL^{-1})	Blasticidin (μg.mL^{-1})
CH1	1.0	4.0
OAW28	4.0	N.A.
OV17R	4.0	20.0

pLKO.1-puro: sh*FZD7* (sense strand in blue, anti-sense strand in red)

	Sequence	**From – To**	**Region**
778	CCGGCAACGGCCTGATGTACTTTAACT CGAGTTAAAGTACATCAGGCCGTTGTT TTG	0778 – 0798	CDS
1867	CCGGGGGCCTGTTTCTGTAACTTTCCT CGAGGAAAGTTACAGAAACAGGCCCT TTTTG	1867 – 1887	CDS
2030	CCGGCGGCGATGTGAATCGTCAAAGC TCGAGCTTTGACGATTCACATCGCCGT TTTTG	2030 – 2050	3'UTR

pMSCV-Blst: sh*WLS*^{miR155}

	Sequence	**From – To**	**Region**
155-1	ACCAAGAAGCTGTGCATTGTT	0333 – 0353	Exon 01
155-5	TGGACATTGCCTTCAAGCTAA	0649 – 0669	Exon 02

Selection antibiotics-resistant single colonies were individually picked after culture (~2 months) and expanded. Knockdown efficiency of individual clones were validated *via* transcript expression profiling relative to the respective controls.

Western Immunoblotting

Whole cell lysates were prepared using radio-immunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louis, MO) supplemented with phosphatase inhibitor cocktail set II and protease inhibitor cocktail set I (EMD Millipore, Billerica, MA). Lysate supernatant were collected after 30min lysis on ice and centrifugation at 9000x *g* (10 min at 4^{o}C). Bicinchoninic acid (BCA) protein assay kit (Thermo Scientific-Pierce™, Rockford, IL) was used to quantitate the lysates as per instructions stated on the user guide. Lysates were resolved on SDS-PAG (sodium dodecyl sulphate polyacrylamide) gel with varying polyacrylamide concentrations and transferred onto Immobilon®-FL polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA), before blocking with the Odyssey® blocking buffer (LI-COR, Lincoln, NE) for 1hr at room temperature. Membranes were subsequently stained with the primary antibodies of interest and incubated overnight at 4^{o}C and washed vigorously in 1x TBST (tris-buffered saline supplemented with 0.1% (v/v) tween-20) thereafter. Membranes were then probed with IRDye® secondary antibodies for an hour at room temperature and washed with 1x TBST as before. Blots were analyzed on the Odyssey® CLx infrared imaging system (LI-COR, Lincoln, NE).

Immunofluorescence staining and Confocal Imaging

Cells were cultured on glass coverslips (Marienfeld-Superior, Lauda-Königshofen, Germany) and fixed in 4% paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA) for 10min and permeabilized in 0.05% TRITON® X-100 (Sigma-Aldrich, St. Louis, MO). All reagents were prepared in 1x PBS and carried out room temperature unless specified. PFA-fixed cells were probed with respective primary antibodies of interest for 1hr and fluorescently labeled with Alexa Fluor® antibodies (Life Technologies-Molecular Probes®, Carlsbad, CA) for 1hr. For visualization of F-actin filaments, cells were stained with tetramethylrhodamine B isothiocyanate (TRITC) conjugated-Phalloidin at 50μg.mL^{-1} for 30min (Sigma-Aldrich, St. Louis, MO). Cells were counterstained with VECTASHIELD® mounting medium with 4',6-diamidino-2-phenylindole (DAPI, Vector-Laboratories, Burlingame, CA) and imaged using the Nikon A1^{+} confocal microscope (Nikon Instruments, Tokyo, Japan).

CHAPTER 3

Results

Stem-A molecular subtype teratocarcinoma and ovarian carcinoma cell lines generally have higher FZD7 transcript expression

Based on the *FZD7* transcript expression profiling of the library of ovarian carcinoma cell lines available from our collection, it was observed that cell lines with the highest *FZD7* expression fall into the Stem-A molecular subtype, including the teratocarcinoma cell lines PA1 and CH1, and several ovarian carcinoma cell lines COV318, OV17R, OVCAR-3, OAW28, JHOS-4, and TOV-112D all having over 2-fold change expression over PEO1 (Figure 1A), supporting earlier reports of FZD7's role in the maintenance of the stemness phenotype. However, it should also be noted that some Stem-A-classified ovarian carcinoma cell lines (OVK18, IGROV-1 and OAW42) displayed very low *FZD7* transcript expression. Furthermore, when *FZD7* transcript expression profile is grouped between Stem-A and non Stem-A cell lines, the Stem-A group shows significantly (p=0.0011) higher expression as compared against the non Stem-A group (Figure 1B).

Knockdown of FZD7 in spheroid-forming ovarian cancer cells disrupts spheroid-formation

Previously, *FZD7* transcript expression was shown to be up-regulated in spheroids formed by SKOV-3 ovarian adenocarcinoma cells,[243] suggesting that FZD7 (presumably through the WNT signalling pathway) plays a role in mediating spheroid formation. To test this further, a human teratocarcinoma cell line of ovarian origin (CH1) was chosen for subsequent studies for two

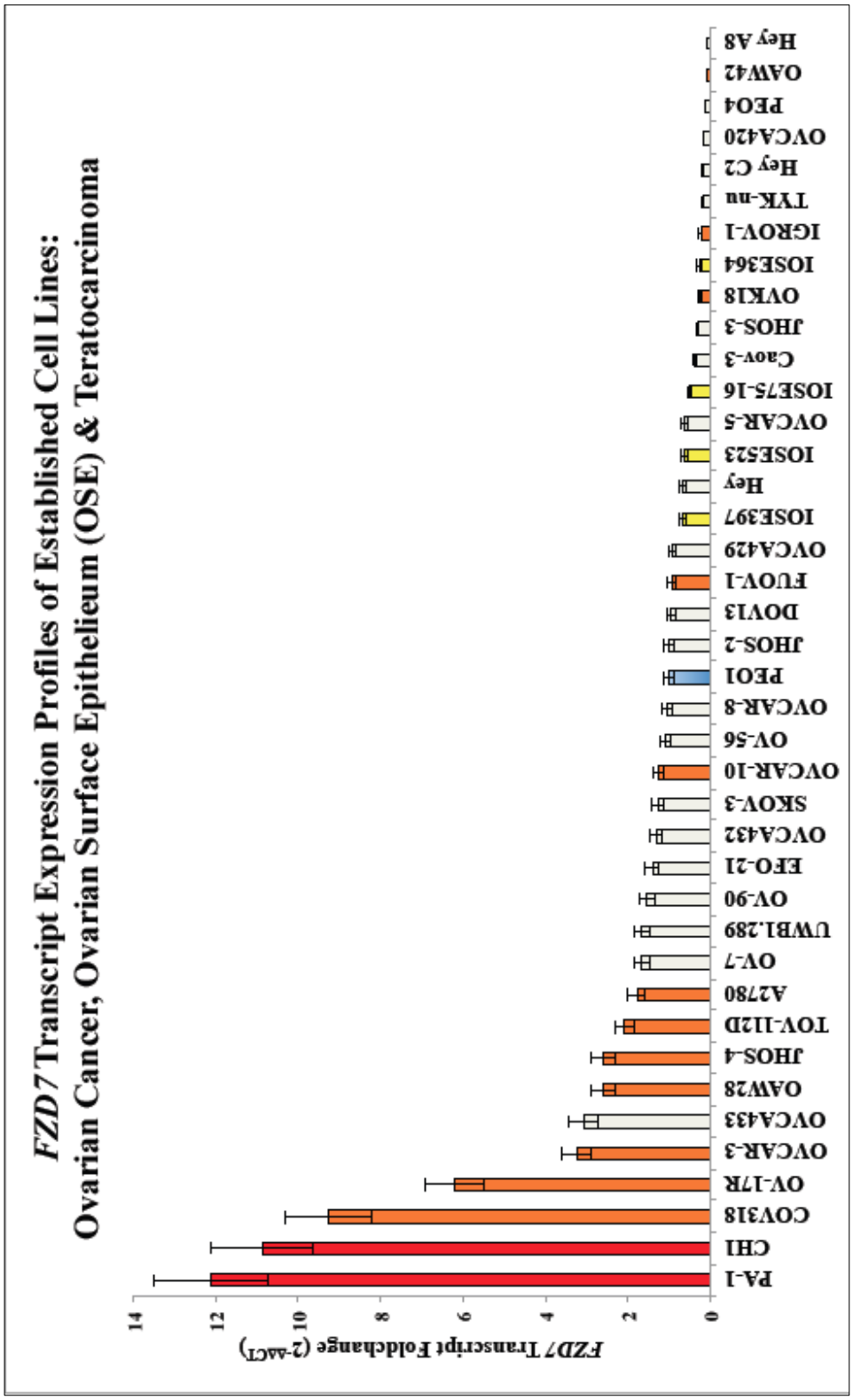


Figure 1A: *FZD7* transcript expression profile from a library of ovarian carcinoma cell lines. $\Delta\Delta C_T$ values were obtained by normalizing the expression profile against that of PEO1, an epithelial ovarian adenocarcinoma cell line[472] (marked in blue). Error bars represent the standard error (SE) of experimental duplicates. Teratocarcinoma cell lines are shaded in red, Stem-A molecular subtype cells are shaded in orange[31] while immortalized ovarian surface epithelium (IOSE) cell lines are shaded in yellow.

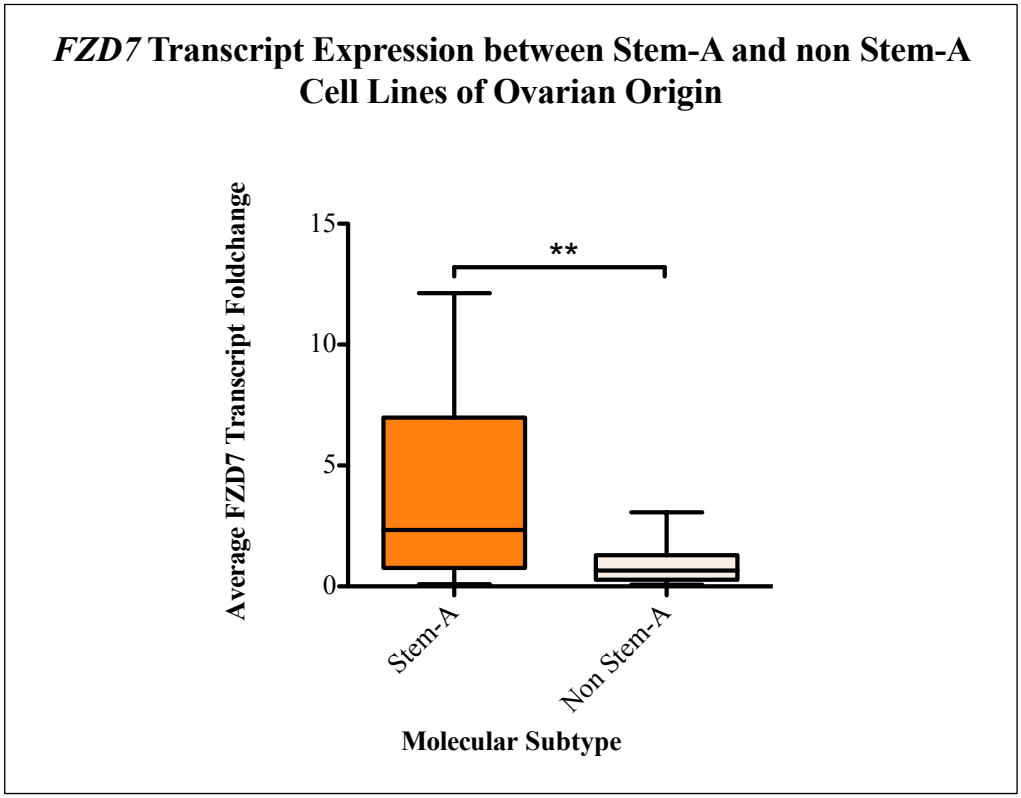


Figure 1B: Box plot comparing the relative *FZD7* transcript expression fold change between Stem-A and non Stem-A cell lines. Unpaired T-test (two-tailed, unequal variance) was performed to determine statistical significance. Double asterisks indicate *p*–value <0.005.

reasons: i) CH1 has one of the highest FZD7 transcript expression levels in our library of ovarian cancer cell lines, and ii) CH1 cells grow spheroids at a relatively fast rate due to the short doubling time and thus provide a model whereby the role of FZD7 in mediating spheroid formation can be quickly validated. In this study, *FZD7* transcript expression from CH1-derived spheroids showed ~1.6 fold increase relative to CH1 cells cultured in adherent conditions (Figure 2A), supporting initial observations made in SKOV-3 derived spheroids.[243] As such, lentiviral-transduction based techniques were used to introduce constructs carrying short hairpin RNA (shRNA) sequences into parental CH1 cells and single colonies were validated after puromycin-selection. Of the various sh*FZD7* constructs used, two showed the best knockdown of ~51% (sh*FZD7*: 778-6, termed CH1:*778-6* from here on) and

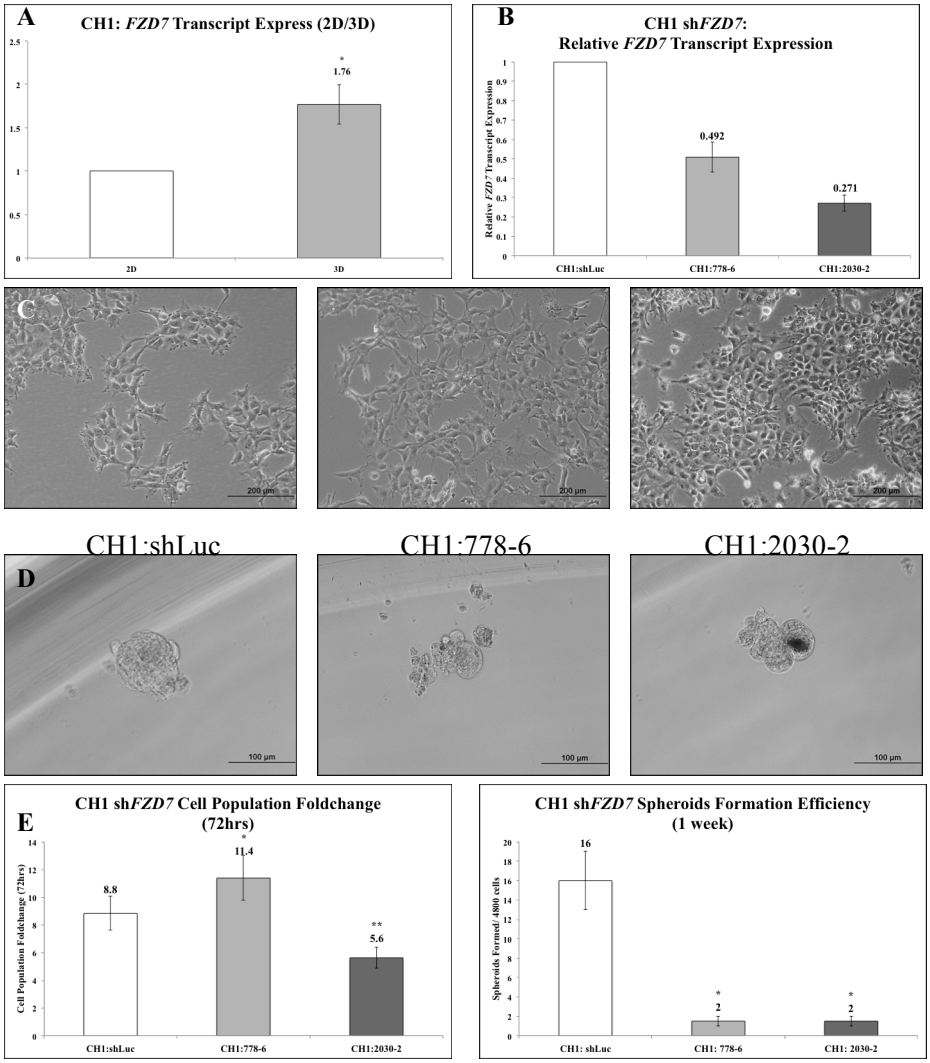


Figure 2: Characterization of CH1 s*hFZD7* cells after lentiviral transduction. A) *FZD7* expression is upregulated in CH1 and SKOV-3-derived spheroids. B) Bar chart depicting the knockdown levels of two CH1 sh*FZD7* clones (778-6 and 2030-2) relative to the luciferase control. C) Phase contrast images of CH1 sh*FZD7* cells during adherent culture at 10x magnification and D) suspension culture at 20x magnification. E) Reduced cell doubling rates and F) abrogation of spheroid formation of CH1 cells after sh*FZD7* knockdown. Single asterisks (*) represent statistical significance with p-value <0.05 while double asterisks (**) statistical significance with *p*-value <0.005. Student's T-test were performed (two-tailed, equal variance).

~73% (sh*FZD7*: 2030-2, termed CH1:*2030-2* from here on, Figure 2B) respectively. As a control, a construct of the same backbone targeting the *luciferase* gene (shLuc, termed CH1:shLuc) was used.

Following expansion of these stable clones, CH1:2030-2 cells were observed to display a more compact epithelial-like morphology as compared to the mesenchymal morphology of CH1:shLuc luciferase control cells (Figure 2C).

In addition, CH1:2030-2 cells showed reduced proliferation (Figure 2E). However, these differences were not observed in CH1:778-6 *FZD7* knockdown cells. CH1:shLuc, CH1:*778-6* and CH1:*2030-2* cells were subsequently cultured in suspension for 1 week to test for spheroid formation efficiency (Figures 2D & F). After which, CH1:shLuc cells on average displayed a spheroid formation efficiency of 300 cells/spheroid (1 spheroid-forming cell for every 300 viable cells). In contrast, after FZD7 knockdown, both CH1:778-6 and CH1:2030-2 cells has a spheroid formation efficiency of ~2400 cells/spheroid, clearly indicating that FZD7 plays in role in mediating spheroid formation in CH1 cells.

FZD7 knockdown in CH1 cells promotes anoikis via the extrinsic pathway

Anoikis is defined as a form of apoptotic cell death induced by abrogating cell-substrate adhesion. To investigate whether the suppression of spheroid formation in CH1 sh*FZD7* was caused by *anoikis*, annexin V was used to detect the presence of phosphatidylserine (PS) phospholipid in CH1 sh*FZD7* cells that were cultured in suspension. In normal viable cells, PS is located in the cytoplasmic cell membrane. However, during early and intermediate stages of apoptosis, PS is translocated to the outer surface of the plasma membrane, which can be readily detected by the Ca^{2+}-dependent annexin V phospholipid-binding protein.[473-475] Using fluorescently-conjugated annexin

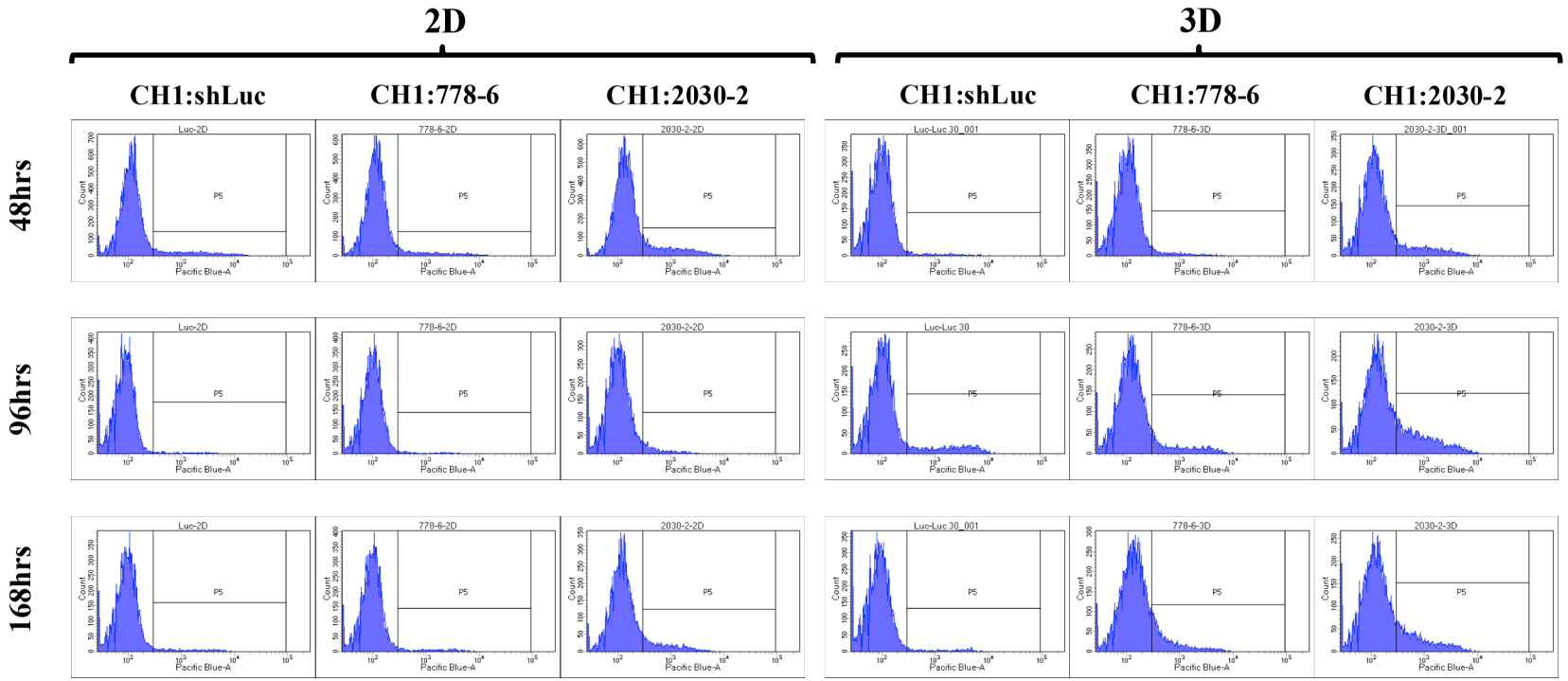


Figure 3A: FACS analysis of the anoikis assay of CH1 cells after FZD7 knockdown (sh*FZD7*). Cells were grown as either adherent (2D) or non-adherent/ suspension (3D) cultures over three timepoints (48, 96 and 168 hrs) and stained with the apoptotic marker Annexin V and analysed *via* flow cytometry.

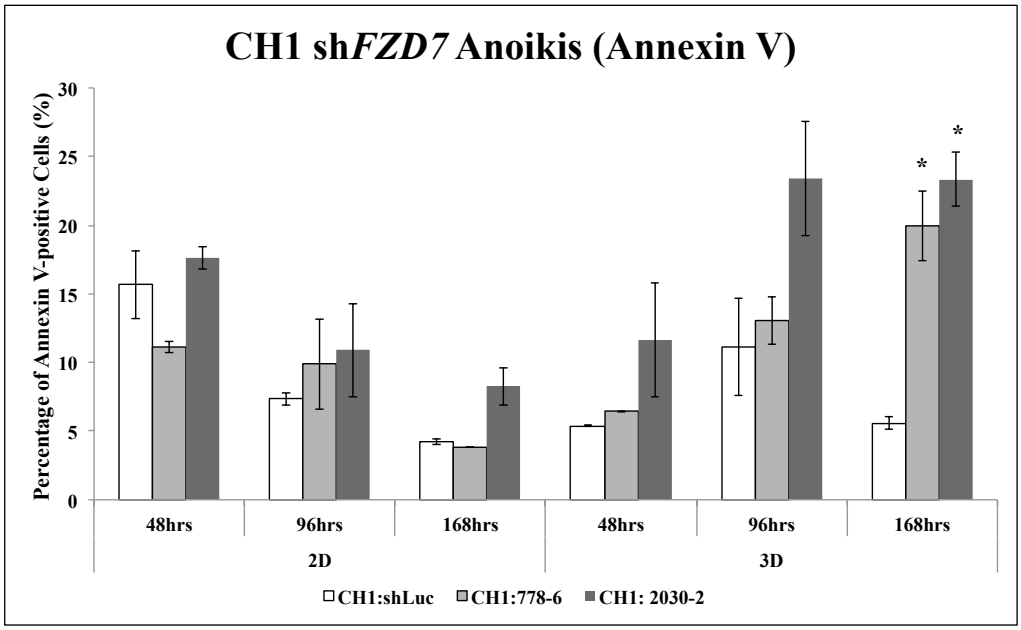


Figure 3B: Summary of the *anoikis* assay of CH1 sh*FZD7* cells over the indicated timepoints. Student's T-test (two-tailed, equal variance) was performed to determine statistical significance, with single asterisk (*) representing statistical significance (*p*-value < 0.05).

V, FACS *via* flow cytometry can be used to quantitate the population of annexin V-positive cells.[474] In this study, CH1 sh*FZD7* cells were cultured in suspension as before and cells at the various time-points (48hrs/2days, 96hrs/4days and 168hrs/1week) were harvested and stained for annexin V-conjugated with the Pacific Blue fluorescent probes and analyzed *via* FACS (Figures 3A & B)

As shown, annexin V-positive cells increased in suspension culture over time. In addition, pro-apoptotic-related markers were investigated in whole cell lysates harvested from the same experiments, including: Caspase-3 (CASP3) and Poly (ADP-Ribose) Polymerase (PARP) and the anti-apoptotic Myeloid Cell Leukemia 1 (MCL-1) (Figure 3C). In this study, cleaved CASP3 was detected early in CH1 suspension cultures (48hrs) for both shLuc control and sh*FZD7* knockdowns, corresponding to the cleavage of PARP, further increasing in intensity at the 96hr time-point. However, cleavage activity of CASP3 and PARP were observed to be higher after *FZD7* knockdown. Of note, the band intensity of the cleaved CASP3 bands (p19 and p17) were both higher after sh*FZD7*-mediated knockdown in suspension culture. The activation of CASP3-PARP cascade indicates that FZD7 promotes *anoikis* in CH1 cells *via* the extrinsic pathway. Furthermore, Caspase-9 (CASP9) was also probed for but did not show any differences, indicating that FZD7 did not mediate the intrinsic pathway of apoptosis (data not shown). Interestingly, down-regulation of anti-apoptotic Mcl-1 in CH1 sh*FZD7* cells (in both adherent/2D and suspension/3D cultures) suggests that FZD7 mediates the extrinsic apoptotic pathway through regulation of Mcl-1. As Mcl-1 is part of the anti-apoptotic Bcl-2 family, Bcl-2 was also probed for but was not detected (data not shown).

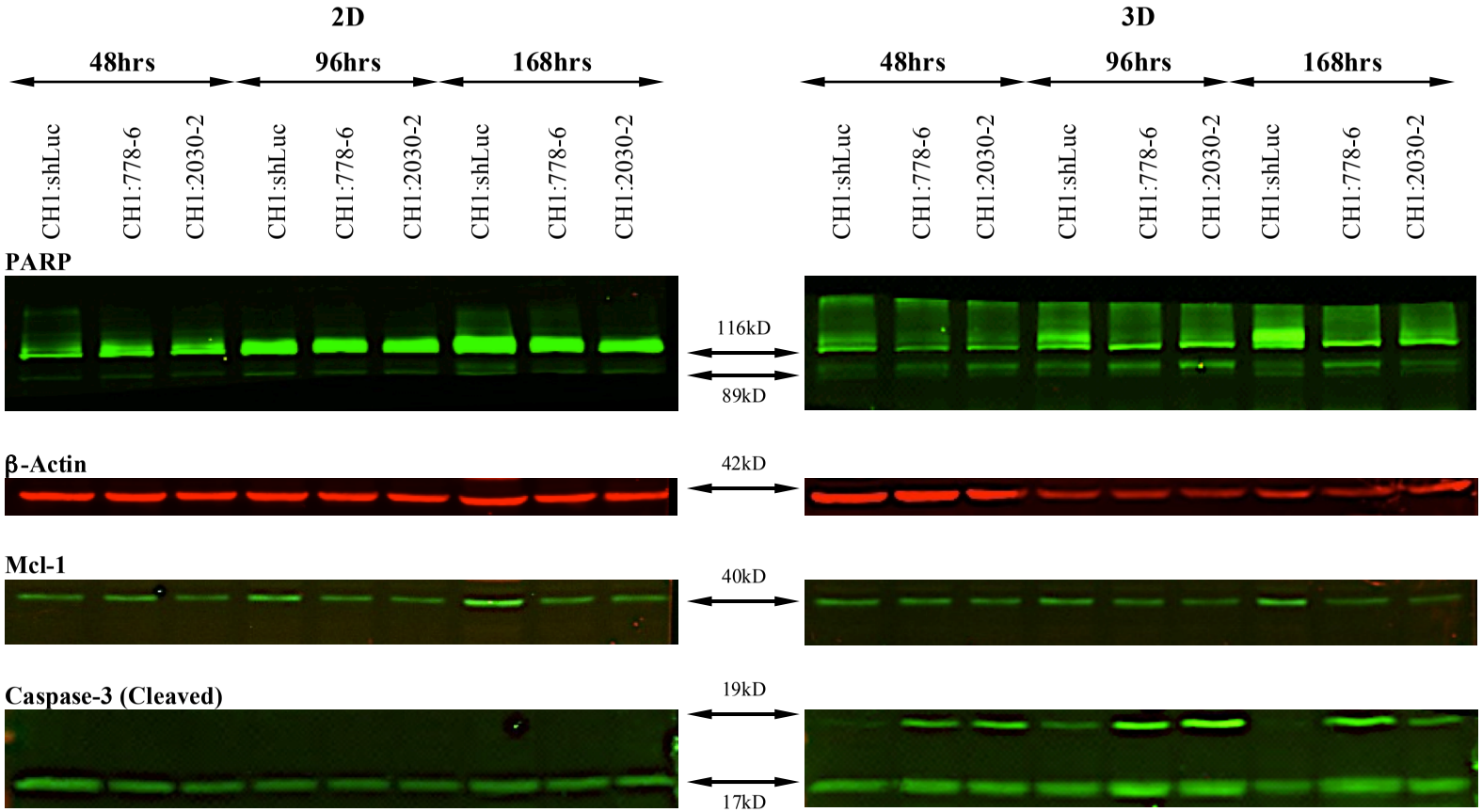


Figure 3C: Immunoblots of CH1 sh*FZD7* cells cultured in both adherent (2D) and non-adherent (3D) conditions over three timepoints (48, 96 & 168hrs) and probed with the following antibodies: PARP, Mcl-1 and cleaved Caspase-3 (Casp-3). β-actin was used as loading control.

FZD7 knockdown in OV17R ovarian adenocarcinoma cells promoted anoikis

As the results presented thus far are based on CH1, a teratocarcinoma cell line of ovarian origin, these data were validated in an ovarian cancer cell line instead. Using the same sh*FZD7* constructs used for CH1 cells (sh*FZD7*:778 and sh*FZD7*:2030), stable knockdowns of *FZD7* were established in OV17R, a stem-A classified ovarian adenocarcinoma cell line with high *FZD7* transcript expression (Figure 1A). From the stable clones established, three were selected, one from the sh*FZD7*:778 construct and two clones from the sh*FZD7*:2030 construct (2030-1/clone 1 and 2030-3/clone 3), with transcript

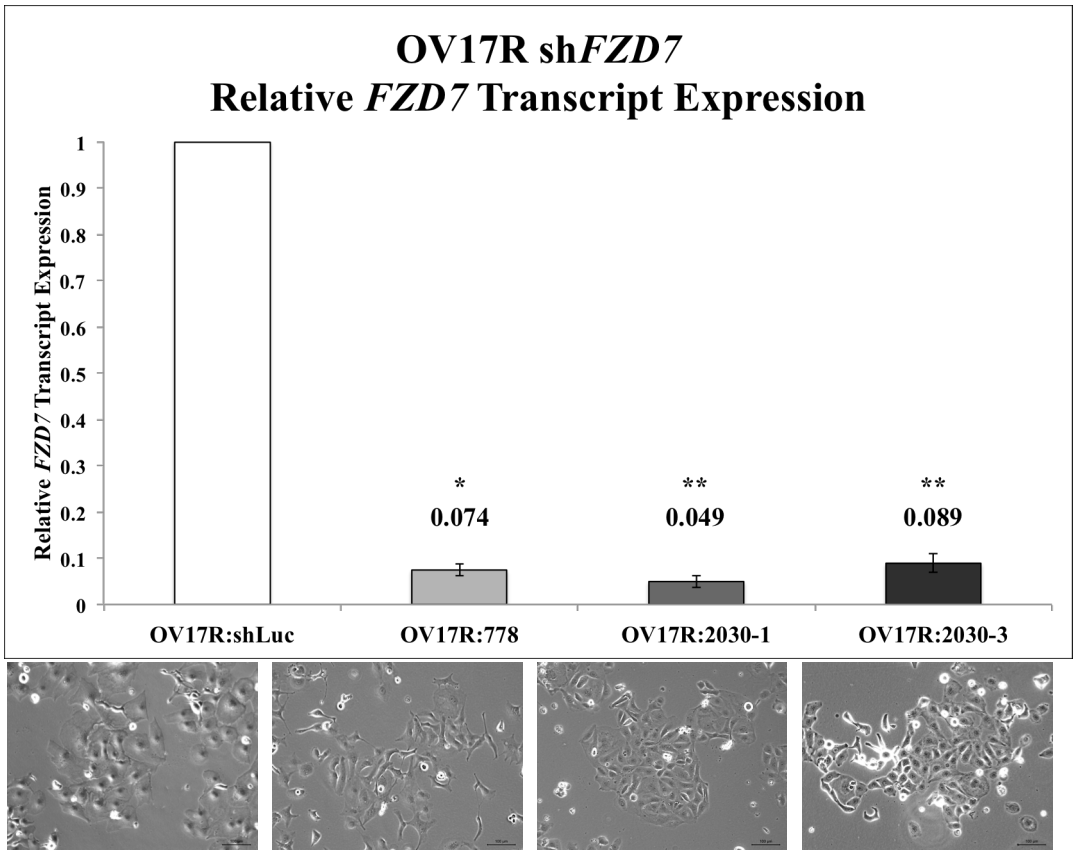


Figure 4A: *FZD7* knockdown in OV17R cells. Bar chart illustrating the relative *FZD7* transcript expression levels after sh*FZD7*-mediated knockdown. Student's T-test (two-tailed, equal variance) was used to determine statistical significance. Single asterisk (*) indicates statistical significance with p-value <0.05 while double asterisks (**) indicate statistical significance with p-value <0.005. Numbers below asterisks represent the average *FZD7* transcript expression remaining after sh*FZD7* knockdown, relative to the shLuc control. Phase contrast images below show the morphology of OV17R cells after *FZD7* knockdown. Images were captured at 10x magnification.

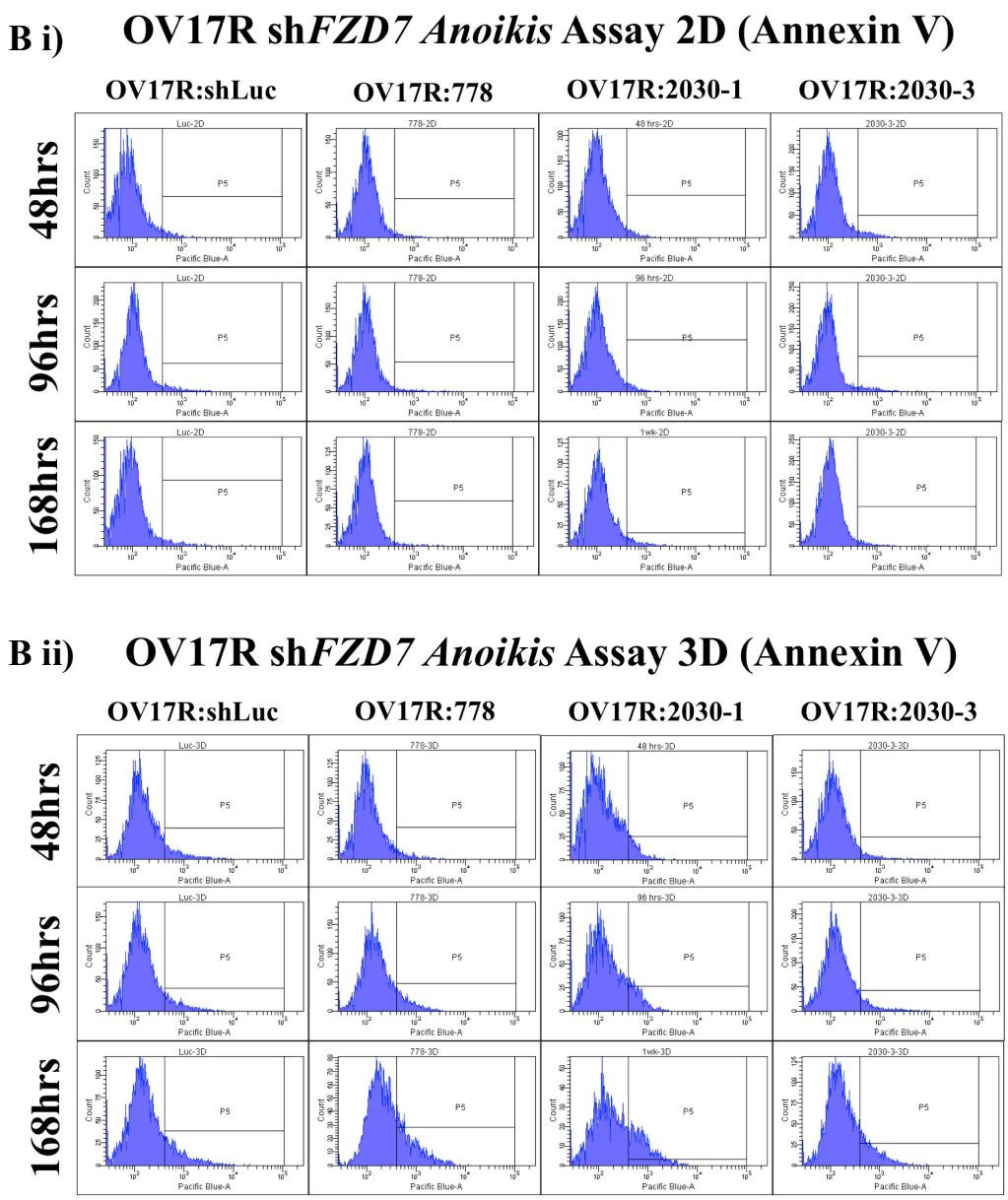


Figure 4B: FACS analysis of the *anoikis* assay of OV17R cells after *FZD7* knockdown (OV17R sh*FZD7*).
B i) Histograms of Annexin V-positive cells derived from the respective OV17R cells in adherent (2D) cultures over the three timepoints (48, 96 and 168 hrs). B ii) Histograms of Annexin V-positive cells derived from the respective OV17R cells in non-adherent / suspension (3D) cultures over the three timepoints (48, 96 and 168 hrs) ran in parallel with those described in (B i)

knockdown efficiency between 90-95% (Figure 4A). Two clones from the sh*FZD7*:2030 construct were selected as both clones show different morphologies (Figure 4A, phase contrast images). Next, OV17R sh*FZD7* cells were cultured in suspension conditions identical to that of CH1 sh*FZD7* cells

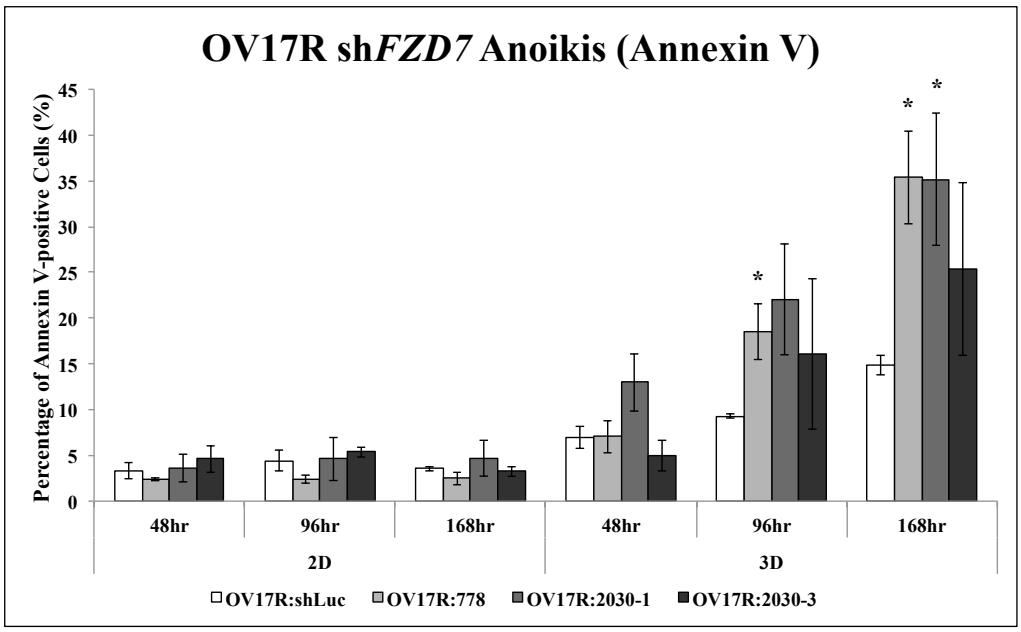


Figure 4C: Summary of the *anoikis* assay of OV17R sh*FZD7* cells over the indicated timepoints. Student's T-test (two-tailed, equal variance) was performed to determine statistical significance, with single asterisk (*) representing statistical significance (*p*-value < 0.05).

and Annexin V-positive cells were quantitated *via* FACS flow cytometry analysis as before (Figure 4B/C). Based on the FACS analysis carried out, OV17R:778 and OV17R:2030-1 cells showed significant increment of annexin V-positive cells after 96 and 168 hours respectively when in suspension culture (OV17R:778_{96hrs} *p*-value= 0.040; OV17R:778_{168hrs} *p*-value= 0.017; OV17R:2030-1_{168hrs} *p*-value= 0.050). However, immunoblotting of lysates harvested after 48 and 96 hours of suspension culture did not show the similar trend as compared to that of CH1 sh*FZD7* cells (Figure 4D), with only a slight increment of the cleaved-PARP band (89kD) observed at 48hrs of OV17R:2030-1 and that total Mcl-1 expression was downregulated in OV17R:2030-1 cells after 96hrs suspension culture. Cleaved CASP3 antibody detected only the 17kD cleaved band but not the 19kD band. This may indicate that the *anoikis* induced by FZD7 in OV17R cells could possibly undergo a different mechanism as compared to CH1 cells.

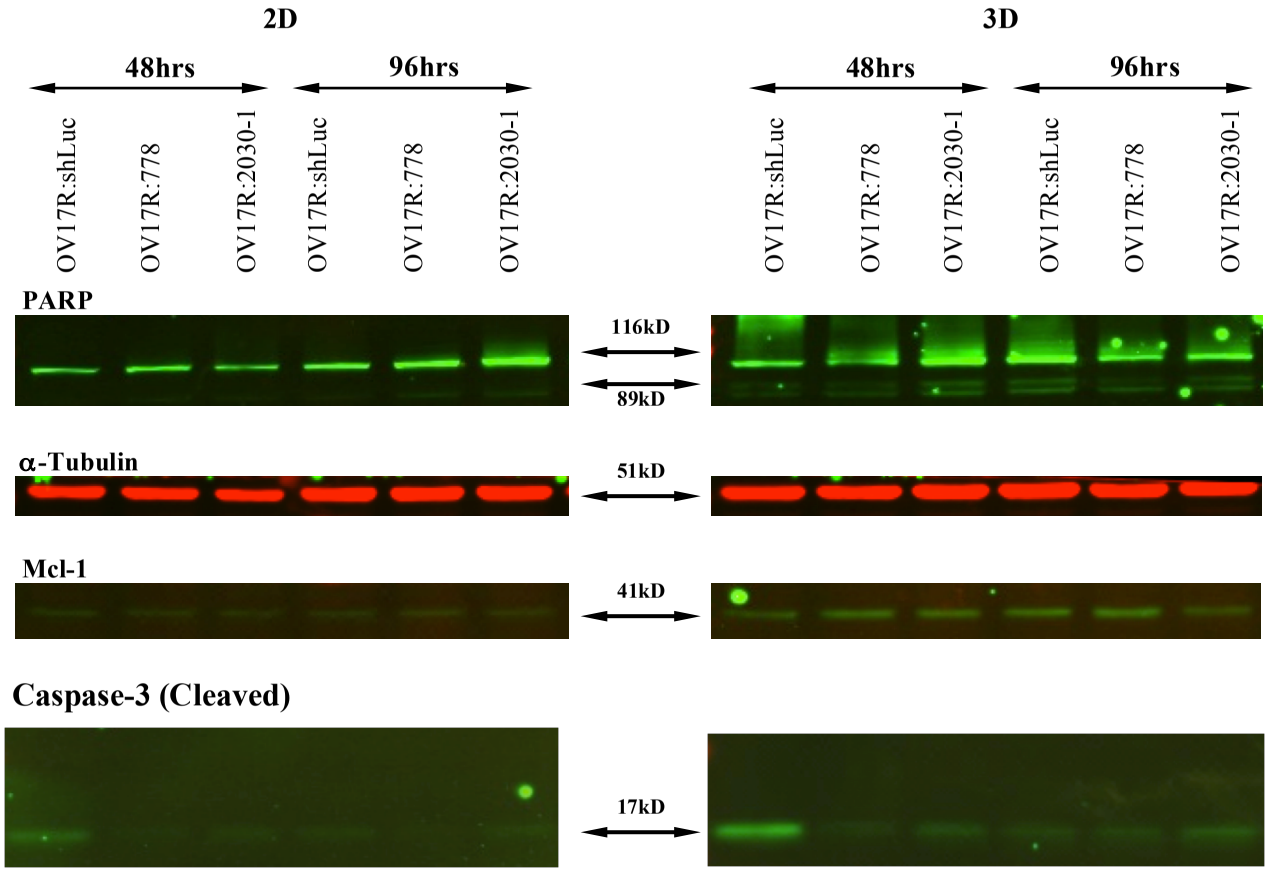


Figure 4D: Immunoblotting of OV17R shFZD7 cells after culture in either adherent (2D) or suspension (3D) conditions over 48 and 96hr timepoints probed against PARP, total Mcl-1 and cleaved Caspase-3 (Casp-3).
α-tubulin was used as loading control.

Knockdown of FZD7 induces colony compaction and induced cytoskeletal reorganization in both CH1 and OV17R cells

As the WNT/PCP-pathway is known to induce cytoskeletal reorganization and morphological transformations, it would seem plausible that the compaction of colonies formed in CH1 and OV17R sh*FZD7* cells may be due to the PCP-pathway. To this end, confocal imaging was used to observe the distribution of actin filaments *via* phalloidin staining of F-actin in CH1 and OV17R cells after *FZD7* knockdown (Figure 5A).

In CH1 sh*FZD7* cells, colonies formed appeared more compact in clone CH1:2030-2, as noted previously. Of interest, Z-stacked images revealed CH1:shLuc control cells to display a denser F-actin network as compared to CH1:2030-2 cells (sidebar of Figure 5A II). However, there was no observable difference seen between CH1:shLuc control and CH1:778 cells.

In OV17R sh*FZD7* cells, the effect of cytoskeletal reorganization was more visible, with a drastic change in F-actin distribution observed in OV17R:2030-1 and 3 clones. One striking feature observed was a seemingly random layout of F-actin after *FZD7* knockdown, as compared to a more orderly distribution in OV17R:shLuc control cells, whereby F-actin was observed to be mainly distributed around the periphery of each cell. Furthermore, there was a clear demarcation between F-actin and cell-cell junctions (represented by β-catenin, a marker of adherent junctions) in control cells, whereas β-catenin staining co-localized with F-actin in OV17R sh*FZD7* cells, reinforcing the observation that loss of *FZD7* disrupted cytoskeletal organization.

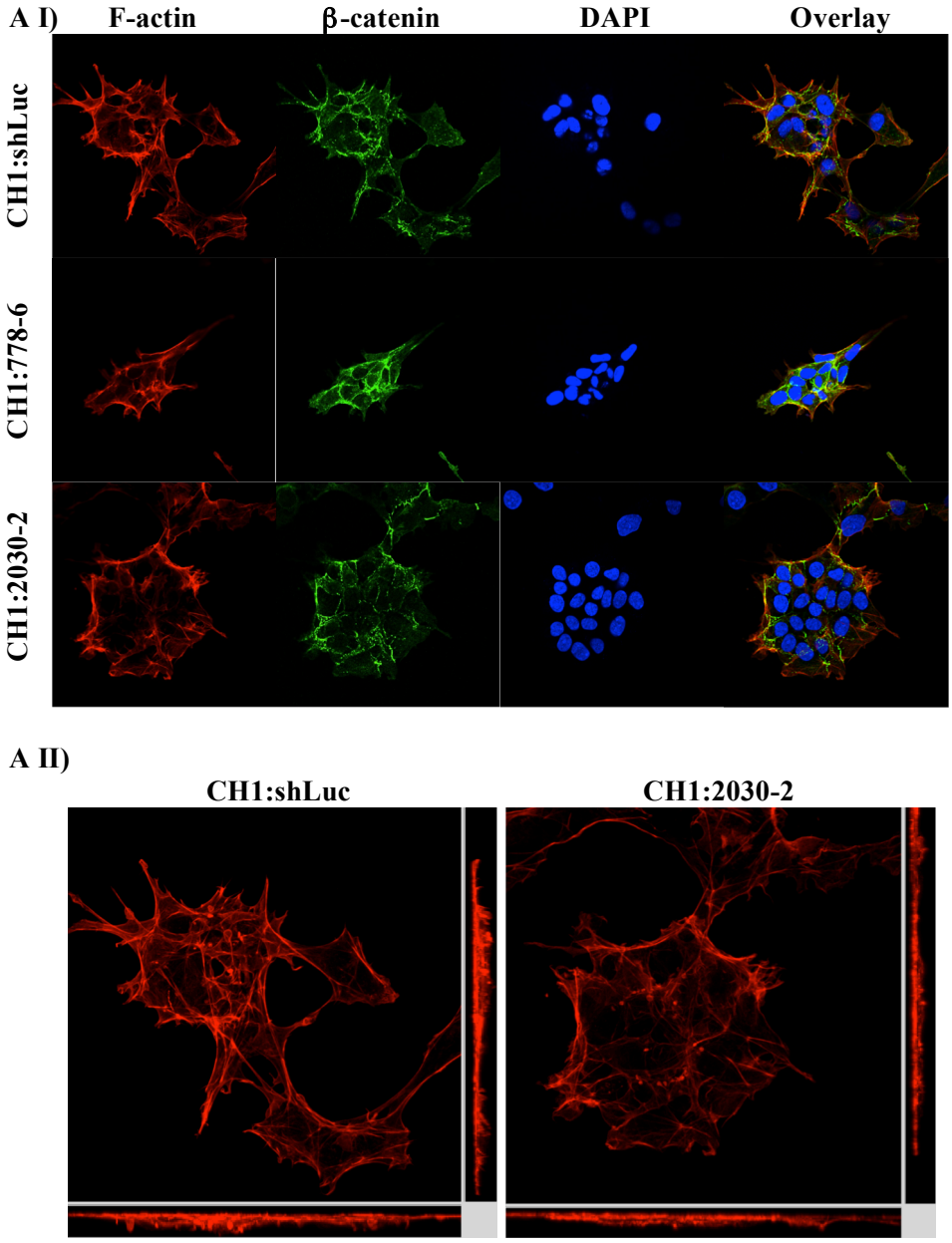


Figure 5A: Confocal images of CH1 sh*FZD7* cells stained for F-actin (red), β-catenin (green) and DAPI nuclear stain (blue). AI) Images of CH1:shLuc and CH1 sh*FZD7* cells. A II) Enlarged Z-stacked images of CH1:shLuc and CH1:2030-2 cells stained against phalloidin (F-actin) showing greater detail on the distribution of F-actin. Notice the side bar indicating the stacked y-axis view, displaying the overall thickness of the cells. Of interest, CH1:shLuc cells show more depth in F-actin, as noted by the change in thickness of F-actin distribution and less intense phalloidin staining after *FZD7* knockdown. All images were taken at 60x magnification.

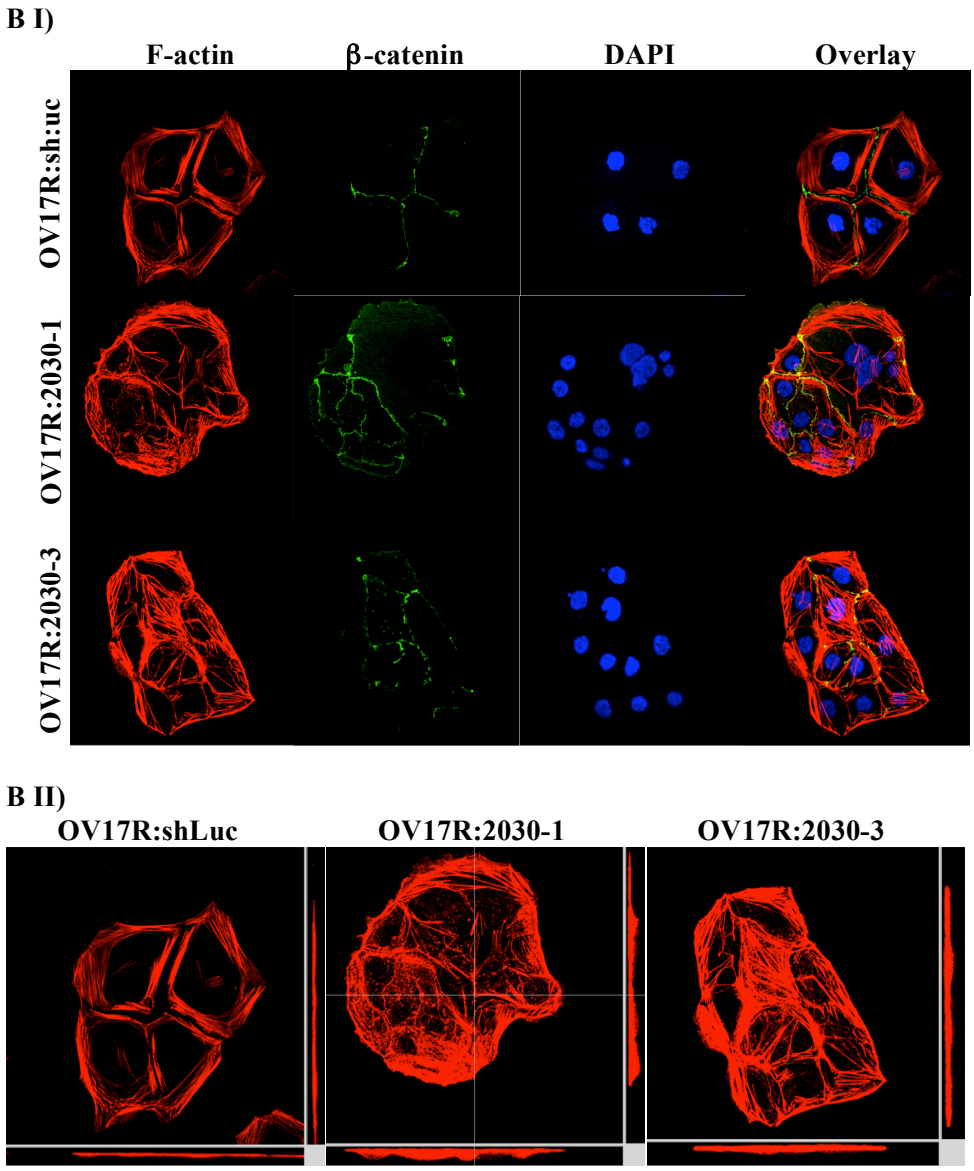


Figure 5B: Confocal images of OV17R sh*FZD7* cells stained for Phalloidin (F-actin, red), β-catenin (green) and DAPI nuclear stain (blue). Notice the distribution of F-actin and compaction of nuclear distance after *FZD7* knockdown. B II) Enlarged Z-stacked images of OV17R:shLuc and OV17R:2030-1 and -3 cells stained against phalloidin (F-actin) showing greater detail on the distribution of F-actin. Notice the side bar indicating the stacked y-axis view, displaying the overall thickness of the cells. In contrast to what was seen in CH1 sh*FZD7* cells, OV17R sh*FZD7* cells show more depth in F-actin, as noted by the change in thickness of F-actin distribution and less intense phalloidin staining after *FZD7* knockdown. Images were taken at 60x magnification.

Knockdown of FZD7 abrogated Phosphorylation of DVL2/3 proteins

Dishevelled proteins have been previously reported to mediate the PCP-pathway (refer to section 1.2 of Introduction). Furthermore, FZD10 was previously reported to activate the PCP-pathway by regulating Dvl2/3 phosphorylation in synovial sarcoma.[253] As such, it would be interesting to see if *FZD7* knockdown has any effect on the PCP-pathway through Dvl2/3 phosphorylation. Immunoblots from total cell lysates of CH1 sh*FZD7* and OV17R sh*FZD7* cells probed against Dvl2 and Dvl3 antibodies showed Dvl2/3 dephosphorylation in OV17R sh*FZD7* cells but not in CH1 sh*FZD7* cells. In particular, OV17R:778 cells expressed much lower total Dvl2/3 proteins, evident by the less intense bands observed (Figure 6). As such, these results indicate FZD7 to have a role in the phosphorylation of Dvl2/3 proteins in OV17R ovarian adenocarcinoma cells but not in CH1 ovarian teratocarcinoma cells.

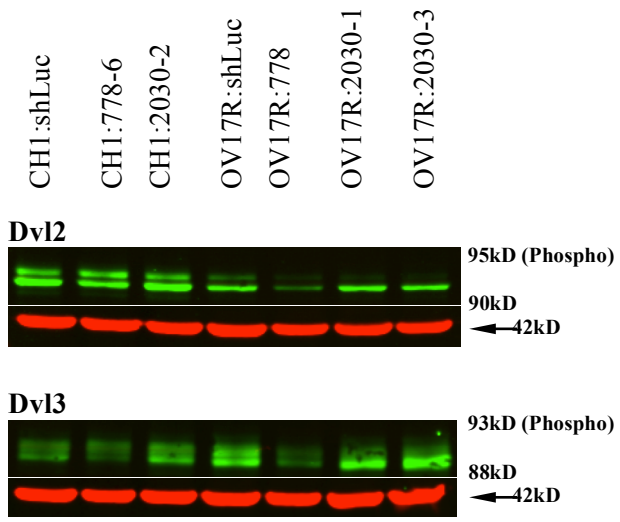


Figure 6: Immunoblots of CH1 sh*FZD7* and OV17R sh*FZD7* cells probed against Dvl2 (95/90kD) and Dvl3 (93/88kD) proteins. The top bands (green) of each blot represents the phosphorylated form while the lower green band shows the dephosphorylated band. β-actin (51kD, red) was used as loading control.

Cyclin D2 (CCND2) is up-regulated in OV17R shFZD7 cells

To better understand the WNT-related genes regulated by FZD7 in OV17R cells, a commercially available qPCR array on human WNT signalling pathway was used to analyse the transcript expression profile of 84 WNT pathway related genes between OV17R shLuc control and OV17R sh*FZD7* cells. cDNA reverse transcribed from each of the conditions (control and 3 knockdown clones) were ran on the qPCR array in triplicates, and compared with the transcript expression profile of the shLuc control. Based on the selection criteria that all three of the sh*FZD7* clones must show statistical significance (Student's T-test: two tailed, equal variance) for the particular genes analyzed, two genes were found to pass this criteria, namely *Cyclin D2* (*CCND2*) and *WNT5B*. Both genes were up-regulated, with an average fold change of 1664 for *CCND2* (*p*-value range: 0.005-0.02) and 8.1 for *WNT5B* (*p*-value range: 0.01-0.04). Immunoblot of the respective OV17R sh*FZD7* total cell lysates were probed against a CCND2 antibody and does indeed show CCND2 up-regulated expression in all three of the OV17R sh*FZD7* clones (Figure 7). Interestingly, OV17R exogenously expressing a *FZD7* cDNA construct also shows a slight up-regulation in CCND2. The same CCND2 antibody was also probed against CH1 sh*FZD7* total cell lysates. Of the four clones probed against, only one showed a slight increment of CCND2 expression (CH1:1735-6), while a very faint band from the other three clones was observed, showing a very slight increment relative to the shLuc control. However, the increment of CCND2 expression in CH1 sh*FZD7* cells is almost negligible compared to what was observed in OV17R sh*FZD7* cells.

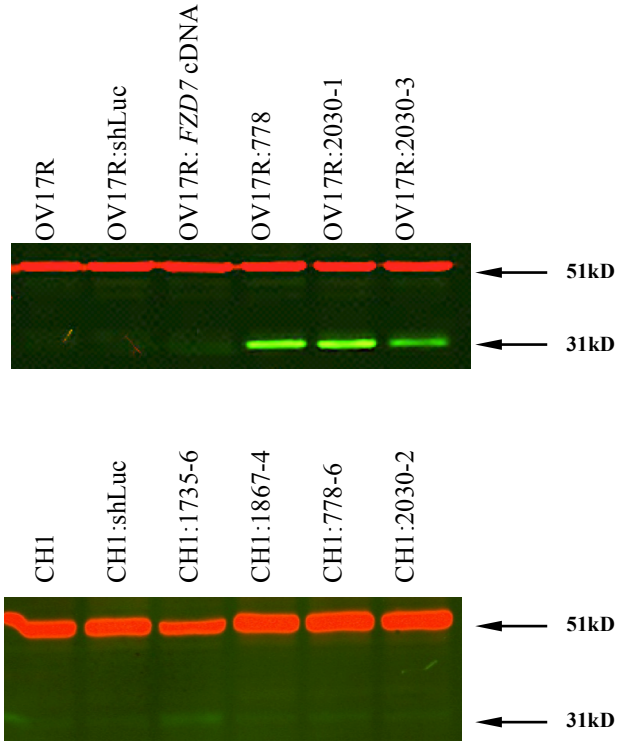


Figure 7: Immunoblot of OV17R and CH1 sh*FZD7* total cell lysates probed against a CCND2 mAb (green, 31kD). α-tubulin (red, 51kD) was used as a loading control.

WLS, a WNT-transporter molecule is up-regulated in suspension cultures of ovarian teratocarcinoma and cancer cells

In order to validate the sh*FZD7* data, it was sought to target another WNT-related component to determine if the same pro-*anoikis* phenotype could be observed. WLS was eventually chosen as a target to disrupt. WLS is a seven-transmembrane molecule responsible for transporting newly synthesized WNT ligands from the rough ER to the cell surface for secretion (refer to section 1.2 of 'Introduction). As such, by targeting WLS to inhibit WNT secretion, thereby 'starving' the cells from WNT ligands to inhibit WNT signalling, similar in effect to the disruption of the receptor (FZD7) level. In an initial screen for WLS expression, several cell lines of human ovarian origin that readily form spheroids (PA1, CH1 and the HEY-series: HEY, HEY A8 and

HEY C2) were screened for WLS expression. Interestingly, spheroids formed from these cells when cultured in suspension showed up-regulated WLS expression relative to their respective adherent cultures (Figure 8A). This novel finding would suggest that WNT signalling activity may be elevated in suspension or spheroid cultures.

WLS knockdown in PA1 cells reduced rate of cell growth

Two sh*WLS* constructs containing precursor elements of microRNA-155 (miR-155) within the backbone vector were used to generate stable knockdowns of WLS in PA1, CH1 and OV17R cells. Essentially, the encoding of an artificial intronic miR-155 sequence flanking the sh*WLS* sequence improves knockdown efficiency by allowing miR-155 to lead the sh*WLS* sequence to target the *WLS* mRNA for degradation. After lentiviral transduction of viral particles carrying the respective sh*WLS*$^{\text{miR-155}}$ constructs, stable clones were selected and knockdown validated. Transcript expression profiling shows knockdown efficiency between 75-80% relative to the empty vector controls (Figure 8B) and immunoblots of total cell lysates probed against a WLS mouse monoclonal antibody (mAb) showed reduced protein levels (Figure 8C), consistent with the level of transcripts detected. As an initial characterization, the cell growth rates were determined over a 72hrs period and PA1 cells carrying the sh*WLS*$^{\text{miR-155}}$-5 construct (PA1:sh*WLS*-5) showed significant reduction in growth rates (Figure 8D).

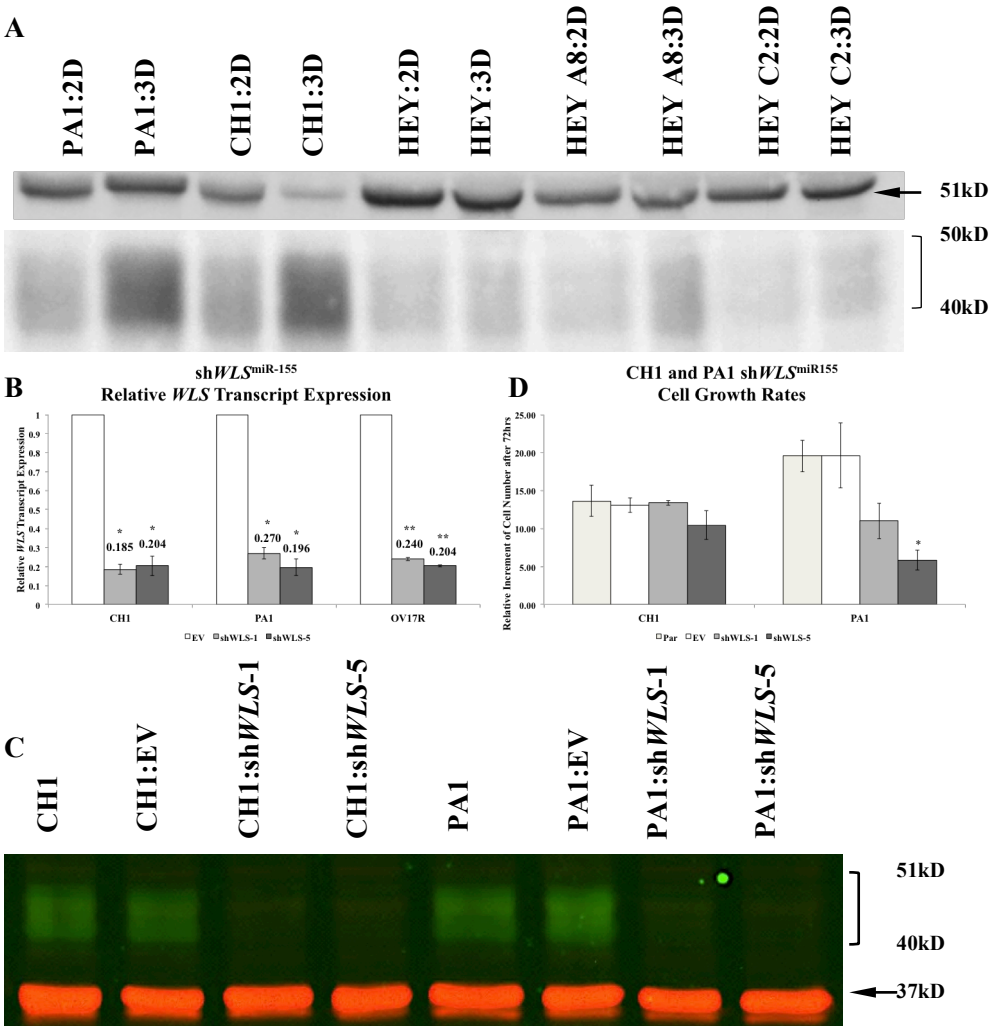


Figure 8: A) Immunoblot of various human cell lines of ovarian origin cultured in adherent (2D) or suspension (3D) conditions and probed against WLS (YJ5) mAb (between 40-50kD). β-tubulin was used as the loading control (51kD). B) Transcript expression of *WLS* after sh*WLS*$^{miR-155}$ mediated knockdown for both CH1 and PA1 cells. C) Immunoblot of total cell lysates of CH1 and PA1 cells after sh*WLS*$^{miR-155}$-mediated *WLS* knockdown. Total cell lysates were probed against the WLS (YJ5) mAb. GAPDH (37kD, red) was used as a loading control. D) Characterization of cell growth rates of CH1 and PA1 cells after *WLS*-knockdown. Cells were seeded overnight and manually counted 24hrs post-seeding and taken as T_0. Cells were trypsin-dissociated and counted as before after another 72hrs of culture. Bar chart represents the average rate of increment (T_{72}/T_0) of experimental triplicates and asterisk (*) indicates statistical significance (Student's T-test: two-tailed equal variance).

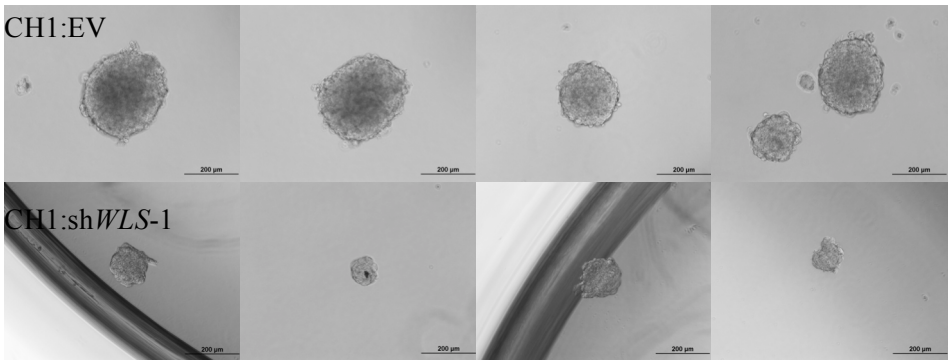
PA1 parental and PA1 empty vector control cells (PA1:EV) showed ~20x increment in cell numbers over the 72hrs period, whereas PA1:sh*WLS*-5 cells only increased by ~6x (*p*-value=0.03). Although PA1:sh*WLS*-1 cells showed reduced growth rates as well (11x increment), no statistical significance was found. CH1 cells stably expressing the sh*WLS*$^{miR\text{-}155}$-5 construct (CH1:sh*WLS*-5) also showed reduced cell growth rates (10.5x increment as compared to ~13x increment of parental and empty vector control cells). However, statistical significance was not achieved.

Inhibition of WLS slowed down rate of spheroid formation in CH1 cells

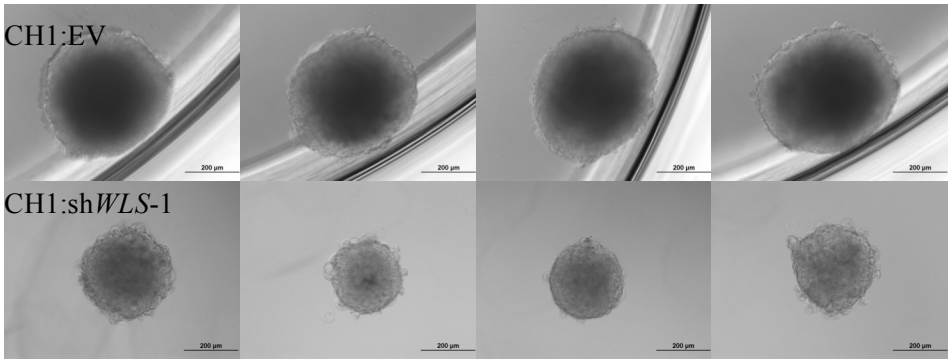
The ability for spheroid formation after sh*WLS*$^{miR\text{-}155}$-mediated knockdown was tested in CH1 cells. By the end of the first week, CH1:EV cells formed spheroids, averaging 263 ±61μm in diameter (Figure 9A/B). CH1:sh*WLS*$^{miR\text{-}155}$ cells formed spheroid-like aggregates averaging 101 ±31μm in diameter. However, after a two-week culture period, CH1:sh*WLS*$^{miR\text{-}155}$ cells formed spheroids similar in size to that of CH1:EV spheroids formed during the first week, averaging 286 ±35μm in diameter. CH1:EV spheroids continued growing during the second week, averaging diamaters of 444 ±6μm.

A)

1 week



2 weeks



B)

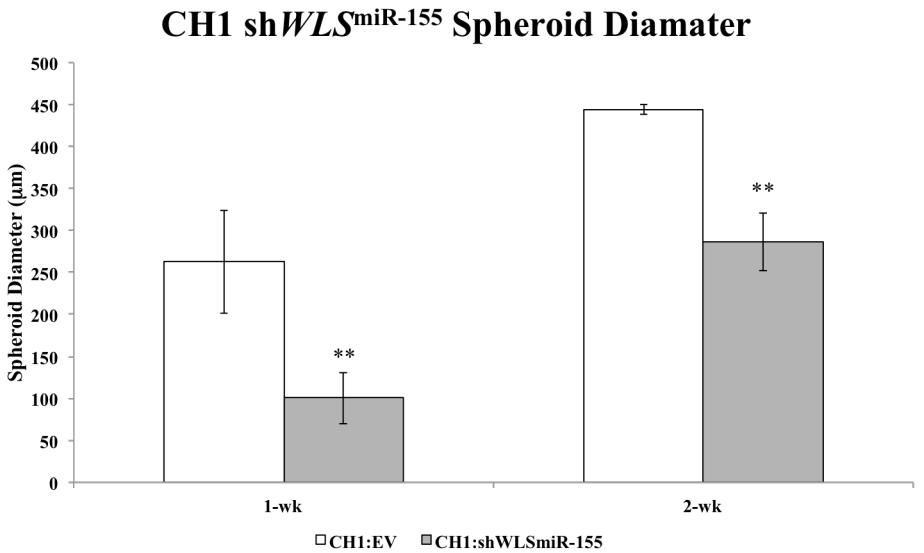


Figure 9: Effects of *WLS* knockdown on spheroid formation in CH1. A) Phase contrast images of spheroids formed by CH1:EV and CH1:sh*WLS*-1 cells after 1 week (top panel) and two weeks (lower panel). Images taken at 10x magnification. B) Bar charts summarizing the average diameters of spheroids formed over the two timepoints after sh*WLS*-mediated knockdown in CH1. Double asterisks represent statistical significance with p-value <0.005 (Student's T-test: two-tailed, equal variance).

shWLS$^{miR\text{-}155}$*-mediated Knockdown did not significantly induce anoikis*

Following the initial characterization, PA1:sh*WLS*$^{miR\text{-}155}$ cells was cultured in suspension to test for *anoikis* (Figure 10A). The general finding was that PA1 cells underwent *anoikis* over a period of 96hrs. However, no significant difference was obeserved between PA1:sh*WLS*-1/5 and PA1:EV control cells. Similarly, OV17R sh*WLS*$^{miR\text{-}155}$ cells also did not show statistically significant increment in annexin V-positivity, even though OV17R:sh*WLS*-5 cells showed relatively higher percentage of annexin V-positivity (Figure 10B). As such, no definitive conlusion could be made from this set of data.

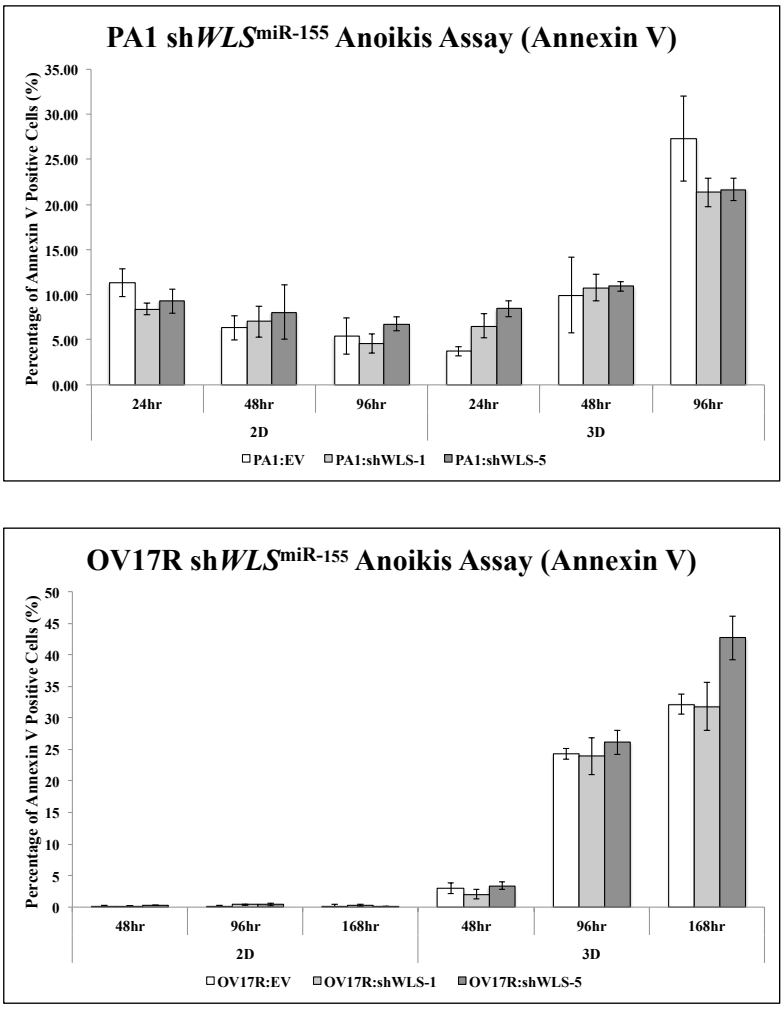


Figure 10: Summary of the *anoikis* assay of CH1 and OV17R sh*WLS*$^{miR\text{-}155}$ cells over the indicated timepoints.

CHAPTER 4

Discussion

One of the ways in which ovarian cancer cells metastasize is through the shedding from the primary tumour site into the patient ascites, eventually forming secondary tumourigenic growths along the mesothelial lining of the peritoneal cavity. As such, a better understanding of the mechanisms involved in mediating this process is of great importance towards management of disease progression. Essentially, ovarian cancer cells that have shed must possess resistance towards the *anoikis* process: programmed cell death of anchorage-dependent cells when 'displaced' in a non-anchored environment.

Previously, spheroids (three-dimensional spherical aggregates) formed *in vitro* by SKOV-3 ovarian adenocarcinoma cells were shown to express elevated transcript levels of the WNT receptor *FZD7*, indicating the involvement of WNT signalling pathway.[243] In this study, *FZD7* was shown to mediate *anoikis*-resistance, whereby stable knockdown of *FZD7 via* RNA-silencing techniques promoted *anoikis* in both CH1 ovarian teratocarcinoma and OV17R ovarian adenocarcinoma cells, resulting in the abrogation of spheroid formation in CH1 cells. Though this has not been reported in ovarian cancer, a similar finding has been reported in Wilm's tumour cells,[384] whereby anti-FZD7 antibody treatment *in vitro* induced cell death within the $FZD7^{+}$ population of Wilm's tumour cells, which are the stem-like/ progenitor pool of cells with spheroids-forming potential. As such, their study indicates that FZD7 is a potential therapeutic target used to eliminate the stem-like pool of Wilm's tumour cells marked by the expression of FZD7. The current study

described in this manuscript supports the notion of FZD7 as a therapeutic target in ovarian cancer, whereby CH1 sh*FZD7* cells cultured in suspension display increased levels of cleaved-CASP3 and PARP activity, which is known to be part of the extrinsic pathway of the apoptotic mechanism. In addition, Mcl-1, a Bcl-2 family anti-apoptotic/ pro-survival gene was down-regulated after *FZD7* knockdown, further reinforcing the finding that CH1 sh*FZD7* cells in suspension display higher proportion of annexin V-positivity, indicative of apoptotic activity/ *anoikis*.

Activation of WNT signalling has been reported to inhibit apoptosis, whereby the induction of WNT activity *via* Wnt3a-conditioned media rescued NIH3T3 cells from undergoing apoptosis in serum-starved conditions.[476] Furthermore, apoptosis-resistance derivatives of parental prostate cells showed enhanced TCF/LEF promoter activity, accompanied by elevated levels of nuclear and cytoplasmic β-catenin.[477] Conversely, antisense β-catenin treatment of mouse embryonic liver explant cultures increased apoptosis, as evaluated by TUNEL-staining,[478] while the inhibition of β-catenin-mediated Wnt activity *via* either Wnt1 mAb treatment or siRNA-silencing induced apoptosis in both rodent and cancer cells.[99,479] In addition, overexpression of the WNT antagonist DKK1 has also been reported to promote apoptosis.[480-482]

Caspase3 (CASP3) is a cysteine aspartase that acts as an executor caspase during the induction of apoptosis. It exists as a 32kD zymogen that is activated by a two-stage mechanism, whereby it is first cleaved into a 19/12kD active intermediate (p19/p12) at Asp175 by other initiator caspases. Two p19/p12

intermediates form a heteromeric complex and undergoes autocatalytic cleavage at Asp28, resulting in the final p17 form.[483] Interestingly, it has been shown that the p19 form can exist in the presence of cytochrome c (CYCS),[484] which is released during loss of mitochondrial outer membrane permeabilization (MOMP, reviewed in Tait and Green, 2013).[485] While basal CASP3 activity was detected in the adherent/2D cultures of CH1 cells regardless of FZD7 levels, the suspension/3D cultures do however show more cleaved-CASP3 after sh*FZD7*-mediated knockdown (evident by the more intense p19 and p17 bands relative to the control), which would support the role of FZD7 in mediating in *anoikis*. While the presence of the p19 form after sh*FZD7*-mediated knockdown in CH1 cells during suspension culture conditions would seem to suggest that the *anoikis* undergone is of intrinsic nature, it should be highlighted that duration of MOMP is usually within the order of minutes, which would argue against the timepoints (48-168hrs) when the p19 bands were detected. Furthermore, CASP9 activity (initiator caspase responsible for activating CASP3 in the intrinsic pathway) was not detected in this *anoikis* assay. Interestingly, β-catenin has been reported to be cleaved by CASP3 *in vitro* during *anoikis* in mammary epithelial cells,[486] indicating an inhibitory role of CASP3 on β-catenin-mediated WNT signalling. Furthermore, RNA-inhibition of β-catenin (*CTNNB1*) in multiple myeloma enhanced cleavage (activation) of CASP3[487] while si*CTNNB1* abrogated cisplatin-resistance in A2780 ovarian carcinoma cells, promoted apoptosis *in vitro* and suppressed tumourigenic growths *in vivo*.[488] In a similar trend, gene silencing of the WNT receptor *FZD7* in the current study promoted *anoikis via* the CASP3/PARP extrinsic pathway.

In this study, Mcl-1 was also down-regulated in CH1 cells after sh*FZD7*-mediated knockdown. Of relevance, Myc, a target gene of the WNT/β-catenin pathway, was previously reported to directly regulate Mcl-1 expression.[489,490] Furthermore, Mcl-1 expression correlated with GSK-3β inactivity (active WNT/β-catenin signalling) in multiple cancer cell lines, including ovarian cancer cells.[491] The down-regulation of Mcl-1 after *FZD7* knockdown in CH1 cells from the current study supports this observation, whereby reduced FZD7 expression is accompanied by reduced Mcl-1 expression.

Previously, cDNA microarray screening found *FZD10* expression to be up-regulated in synovial sarcoma,[492] siRNA-mediated silencing of which abrogated cell growth.[492] FZD10 was subsequently shown to regulate cytoskeletal reorganization through Dvl2/3 phosphorylation to activate the non-canonical Rac1-JNK pathway, whereby silencing of FZD10 ablated Rac1 activity.[253] Interestingly, the endogenous expression of FZD10 in a panel of synovial sarcoma cell lines directly correlated with the phosphorylation status of Dvl2/3 proteins.[253] Furthermore, the activation of Rac1 by Dvl2/3 phosphorylation enhanced *anoikis*-resistant anchorage-independent growths.[253] More recently, si*FZD7*-mediated knockdown in CH1, PA1 and OV17R cells reduced phosphorylated DVL2 (DVL2 pSer143) co-localization with γ-tubulin in the centrosome.[243] In addition, RhoA activity was suppressed and Rac1 activity increased after *FZD7*-silencing.[243] In the current study, Dvl2/3 proteins were shown to be dephosphorylated after sh*FZD7*-mediated knockdown in OV17R cells but not in CH1 cells. Interestingly, total proteins

for both Dvl2 and Dvl3 were down-regulated in OV17R:778 cells. The cause of this is unclear, though it is noted that CH1:778 cells do show down-regulated total Dvl3 proteins as well, suggesting that the CDS region targeted by the sh*FZD7*:778 construct may result in a non-functional truncated protein that plays a role mediating Dvl protein expression. Of more relevance, it would be of interest to determine if Dvl2/3 proteins do indeed play a role in mediating *anoikis* of ovarian cancer in the current model.

Cyclin D2 (CCND2) is one of the three human members of D–type cyclins responsible for initiating the G1→S transition during cell cycle progression by activating various cyclin dependent kinases (Cdk), including Cdk4/6.[493] CCND2 expression may be regulated by the WNT target gene c-Myc,[392,494,495] NF-κB[496-499] or PITX2,[431,500] which is an effector and regulator of WNT/β-Catenin signalling (refer to section 1.3 of 'Introduction'). CCND2 has been reported to be overexpressed in chronic lymphocytic leukemia[499] and significantly down-regulated in adherent cultures of prostate cancer stem cells.[501] In ovarian cancer, aberrant methylation of CCND2 promoter leads to down-regulated CCND2 expression and also poorer disease-free survival.[502] In murine ovarian granulosa cells, follicle-stimulating hormone (Fsh) up-regulates Ccnd2 expression to drive cell growth, ablation of Ccnd2 in mice resulted in hypoplasia.[503] In the current study, the mechanism by which how CCND2 is up-regulated after sh*FZD7*-mediated knockdown in OV17R cells is unclear. As CCND2 can be regulated by c-MYC, a WNT target gene, it was expected that the knockdown of FZD7 would reduce WNT activity and thus reduce expression of WNT target genes. Also, cyclin dependent kinase

inhibitor 1B (CDNKN1B/ $p27^{Kip1}$) inhibits cyclin D-Cdk4 complex formation (reviewed in Chu, Hengst and Slingerland, 2008).[504] Interestingly, c-MYC has been known to negatively regulate $p27^{Kip1}$ expression, as such, there may be a correlation between CCND2 and $p27^{Kip1}$ expression in OV17R sh*FZD7* cells. More needs to be done to verify if c-MYC is indeed down-regulated in the *FZD7* knockdown model to prove this. The involvement of PITX2 in regulating CCND2 seems unlikely in OV17R cells as that pathway requires DVL2 activity, which is clearly de-phosphorylated after sh*FZD7* knockdown in OV17R cells. Interestingly, activation of WNT/β-Catenin signalling by use of a GSK-3β inhibitor mediated cisplatin resistance by blocking nuclear translocation of NF-κB.[505] It is tempting to speculate that the knockdown of FZD7 disrupts WNT/β-Catenin activity, thus allowing NF-κB nuclear translocation to drive CCND2 expression. As such, aside from c-MYC, it would be of interest to see if NF-κB is involved in regulating CCND2 after sh*FZD7*-mediated knockdown as well.

WLS acts as a cargo transporter molecule for WNT ligands, carrying them from the rough ER to the cell surface for receptor binding. As such, targeting of WLS seemed an attractive alternate strategy to verify the role of WNT during spheroid formation. Interestingly, WLS up-regulated protein expression was observed in spheroids formed by various ovarian teratocarcinoma and adenocarcinoma cell lines, indicating active WNT transportation in spheroids, presumably for secretion and activation of the pathway/s involved. Knockdown of WLS slowed down the rate of spheroid formation in CH1 cells but did not fully abrogate spheroid formation. Interestingly, the eventual size

of spheroids in CH1:sh*WLS* cells matched that of the empty vector control cells, at an approximate one-week lag period. Furthermore, WLS-mediated knockdown did not show significant increment in *anoikis* activity in PA1 and OV17R cells, which supports the relative slower growth rates of CH1 sh*WLS* cells: inhibition of WLS slows suspension culture growth but not induce *anoikis* as opposed to what was observed in FZD7 knockdown model. It may be that decreased WLS activity dampened certain elements of cell cycle. Of some relevance, conditional deletion of *Wls* in mice models impaired hair follicular development, resulting in hair cycle arrest in the telogen or early anagen phase, accompanied by reduced proliferation.[506] Similarly, *Wls* loss of function (LOF) mice models show impaired hair growth and stunted body size in another study.[507] Interestingly, cyclin D1 (Ccnd1, a WNT/β-catenin target gene) was enriched in the telogen bulge of hair follicles but not in highly proliferative Ki-67^{Hi} anagen cells, suggesting that a pool of hair follicular stem cells in telogen phase with high Ccnd1 expression differentiates into transit amplifying cells to mediate anagen phase growths.[508] Taken together, it may be that the suppression of Wls in these cells suppressed Ccnd1 expression, thus arresting these cells in the telogen phase. In a similar way, inhibition of WLS in ovarian cancer may have the same effect during spheroid formation, whereby stem-like/ progenitor cells are unable to proliferate as normally would due to inhibition of WNT activity, thus a slower rate of growth was observed. The finding of elevated WLS expression in spheroids formed would seem to support this hypothesis, whereby increased WLS activity may indicate a highly proliferative environment, inhibition of which disrupts proliferative stimulants (such as CCND1) from being transcribed to drive proliferation.

Conclusion and Future Directions

In conclusion, the data from the current study shows FZD7 to play a role in protecting ovarian teratocarcinoma and adenocarcinoma cells from undergoing *anoikis*. Moreover, the cells with reduced FZD7 expression undergo cytoskeletal reorganization, as evident by the distribution of F-actin, presumably through the mediation of Dvl2/3 adaptor protein.

While the findings of this study may be preliminary, it would be of interest to validate the role that Mcl-1 plays in CH1 sh*FZD7* cells. One way of testing this would be to either overexpress Mcl-1 in CH1 sh*FZD7* cells to test if Mcl-1 overexpression will rescue CH1 sh*FZD7* cells from *anoikis*. Conversely, Mcl-1 may also be targeted for gene silencing in CH1 sh*FZD7* cells (generating double knockdowns) to determine if abrogation of Mcl-1 expression will further enhance *anoikis*. Other genes that may be targeted include CASP3 and PARP. The abolishment of *anoikis* by targeting these genes after sh*FZD7*-mediated knockdown will clearly show the dependence of the executor caspase (CASP3) in mediating *anoikis*. While the results of this study is of interest, one must acknowledge that this study did not include a FZD7 rescue model, which would undoubtedly make the role of FZD7 in inducing *anoikis* more convincing. As such, it is proposed to generate constructs that will enable stable FZD7 expression to facilitate the rescue study. Similarly, it will be of great interest to determine if the re-introduction of FZD7 would restore DVL2/3 phosphorylation. Conversely, the phenotypes of the double knockdowns of either FZD7/DVL2 or FZD7/DVL3 will help further determine if these proteins do indeed contribute towards *anoikis*.

Also, it will be of interest to see if β-catenin is cleaved by CASP3 after FZD7 knockdown, which can be easily carried out with probing immunoblots of whole cell lysates against a β-catenin antibody. In addition, it was not clearly shown whether β-catenin was involved in mediating the sh*FZD7* phenotype. The role of β-catenin could be tested by treating spheroid-forming cells with β-catenin-specific inhibitors, such as inhibitors of β-catenin responsive transcription (iCRTs).[509]

It has been previously reported that transient *FZD7* knockdown disrupted cell cycle progression, evident by increased G_0/G_1 subpopulations.[243] As such, cell proliferation after sh*FZD7*-mediated knockdown could be better characterized by analyzing the cell cycle profiles as well.

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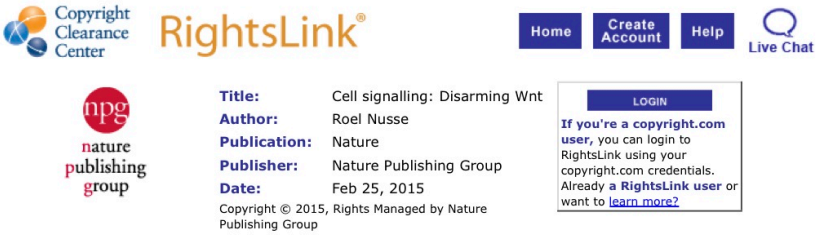

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