The Tumour Initiation Project

Thesis submitted for the MSc by Research degree (September 2023)

Aneesh Aggarwal

Wolfson College

MRC Weatherall Institute of Molecular Medicine

Nuffield Department of Women's & Reproductive Health

With immense gratitude to my supervisors:

Prof Ahmed Ahmed & Dr Mara Artibani

Word Count: 26,942

Abstract

High-grade serous ovarian cancer (HGSOC) is the most lethal gynaecological malignancy – an imminently required screening tool may be developed through the study of tumour initiation. It is believed that mutagens from follicular fluid (FF) released during ovulations collaborate with components of retrograde menstruation to transform multipotent human fallopian tube (hFT) stem cells. In-house works recently illustrated that the WNT7A-FZD5 axis is fundamental to hFT stem cells *in vitro* as organoids. In this thesis, in hFT organoids, it was corroborated that FF reactive oxygen species (ROS), delivered as H_2O_2, are transduced intracellularly through NOX wherein they potently induce dsDNA breaks. Their lower toxicity compared to previous monolayer studies is likely insidious; since cells better escape death, they accrue DNA damage – sitting closer to the transformative 'Goldilocks Zone'. Curiously, FF transferrin was only genotoxic in collaboration with H_2O_2, perhaps through the former triggering an intracellular Fenton reaction. Similarly, FF Fe^{3+} was only toxic when paired with FF transferrin, suggesting that the latter shuttles in the former, perhaps driving lipid peroxidation (LPO). Fascinatingly, mutagens appeared to preferentially target already-proliferating stem cells. Surrogate Wnt and CHIR99021, synthetic factors, were shown to activate Wnt/β-Catenin signalling, whereas FF IGF2 and FF insulin did not. Regardless, these all promoted survival of stressed and unstressed organoids; corresponding pathway manipulators in FF likely enable ROS-damaged stem cells to expand *in vivo*, a carcinogenetic process. FF progesterone impressively abrogated this, inhibiting organoid growth ~30-fold more potently than monolayer studies predict, indicating therapeutic value in HGSOC prevention. FF estradiol also slightly antagonised organoid formation, and both hormones induced differentiation. Lastly, using aforementioned survival factors, media were successfully established to enable the recovery of organoids post-lentiviral genetic manipulation. Methods to purify mutant populations were then refined, and a highly cost-effective protocol for organoid clonal selection was developed, facilitating the next research stages.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

5-FU	5-Fluorouracil
A4	Androstenedione
aa	Amino Acid
AIG	Anchorage-Independent Growth
ALDH	Aldehyde Dehydrogenase
aNHEJ	Alternative Non-Homologous End Joining
AP	Apurinic
APC	Adenomatous Polyposis Coli
BER	Base-Excision Repair
BMP	Bone Morphogenetic Protein
BRCA	Breast Cancer Susceptibility Protein
BSA	Bovine Serum Albumin
Cas9	Caspase 9
CAT	Catalase
CBP	CREB-Binding Protein
CCOC	Clear Cell Ovarian Carcinoma
CFTR	Cystic Fibrosis Transmembrane Regulatory Gene
CNA	Copy Number Aberration
cNHEJ	Classical Non-Homologous End Joining
COMT	Catechol-O-Methyltransferase
COX2	Cyclo-Oxygenase 2
CRD	Cysteine-Rich Domain
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
ctDNA	Circulating Tumour DNA
CYP450	Cytochrome P450 Enzyme
DES	Diethylstilbestrol
DMT1	Divalent Metal Transporter 1
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco’s Phosphate Buffered Saline
DPI	Diphenyleneiodonium Chloride
DSB	Double-Stranded DNA Break
dsDNA	Double-Stranded DNA
DVL	Dishevelled
E2	Estradiol

ECD	Extracellular Domain
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFP	Enhanced Green Fluorescent Protein
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-to-Mesenchymal Transition
EnOC	Endometroid Ovarian Carcinoma
EOC	Epithelial Ovarian Cancer
ER	Estrogen Receptor
FA	Fanconi Anaemia
FACS	Fluorescence-Activated Cell Sorting
FAP	Familial Adenomatous Polyposis
FBS	Fetal Bovine Serum
FF	Follicular Fluid
FFA	Free-Fatty Acid
FGF-10	Fibroblast Growth Factor 10
FRT	Female Reproductive Tract
FSH	Follicle Stimulating Hormone
FTE	Fallopian Tube Epithelium
FZD	Frizzled Receptor
GPCR	G Protein-Coupled Receptor
GSH	Glutathione
GSR	Glutathione Reductase
GTP	Guanosine Triphosphate
HAA	Heterocyclic Aromatic Amines
Hb	Haemoglobin
hFT	Human Fallopian Tube
HGSOC	High Grade Serous Ovarian Cancer
HPV	Human Papillomavirus
HR	Homologous Recombination
HRMA	High Resolution Melt Analysis
HSC	Hematopoietic Stem Cell
hTERT	Human Telomerase Reverse Transcriptase
hTf	Holo-Transferrin
ICD	Intracellular Domain
IGF1R	Insulin-Like Growth Factor Receptor 1
IGF2	Insulin-Like Growth Factor 2
IgG	Immunoglobulin G

IHC	Immunohistochemistry
IHH	Indian Hedgehog
IL-6	Interleukin 6
IVF	*In Vitro* Fertilisation
KO	Knockout
LGSOC	Low Grade Serous Ovarian Cancer
LGR	Leucine-Rich G Protein-Coupled Receptor
LH	Luteinising Hormone
LPO	Lipid Peroxidation
LTR	Long Terminal Repeat
MaSC	Mammary Stem Cell
MDA	Malondialdehyde
miRNA	Micro RNA
MMR	Mismatch Repair
MOC	Mucinous Ovarian Carcinoma
MOI	Multiplicity of Infection
MPSC	Micropapillary Serous Borderline Tumour
NAC	N-Acetylcysteine
ncMMR	Non-Canonical Mismatch Repair
NEAA	Non-Essential Amino Acids
NER	Nucleotide Excision Repair
NGS	Next Generation Sequencing
NHEJ	Non-Homologous End Joining
NLS	Nuclear Localisation Signal
OEI	Ovarian Epithelial Inclusion
OFE	Organoid Forming Efficiency
OSE	Ovarian Serous Epithelium
P4	Progesterone
PAH	Polycyclic Aromatic Hydrocarbons
PanIN	Pancreatic Intraepithelial Neoplasia
PDAC	Pancreatic Ductal Adenocarcinoma
PGE2	Prostaglandin E2
PKA	Protein Kinase A
Porcn	Porcupine
PPV	Positive Predictive Value
PTCH	Patched
PUFA	Polyunsaturated Fatty Acids
RNA	Ribonucleic Acid

ROCK	Rho-Associated Protein Kinase
ROS	Reactive Oxygen Species
RSPO	R-Spondin
RTK	Receptor Tyrosine Kinase
SBT	Serous Borderline Tumour
SC	Serous Cystadenocarcinoma
SCOUT	Secretory Cell Outgrowth
sCRC	Serrated Colorectal Carcinoma
scRNA-seq	Single-Cell RNA-Sequencing
SFRP	Secreted Frizzled-Related Protein
sgRNA	Single Guide RNA
siRNA	Small Interfering RNA
SOD	Superoxide Dismutase
ssDNA	Single-Stranded DNA
STIC	Serous Tubal Intraepithelial Carcinoma
T-ALL	T-Cell Acute Lymphoblastic Leukaemia
Tf	Transferrin
TfR1	Transferrin Receptor 1
TGFβ	Transforming growth factor-β
UVA	Ultraviolet A
WNT	Wingless-related integration site protein
Wnt/PCP	Wnt/Planar Cell Polarity
WβC	Wnt/β-catenin

CHAPTER 1

Epidemiology and Subtypes of Ovarian Cancer

1.0 Epidemiological Overview

Worldwide, every two minutes a woman is diagnosed with ovarian cancer, the most lethal of the female-specific malignancies (Momenimovahed *et al.*, 2019). In 2020, an estimated 313,959 new cases of ovarian cancer were diagnosed, representing about 1.6% of all new cancer cases, with 207,252 deaths reported; 2.3% of all cancer-related deaths (Sung *et al.*, 2021). Predictive modelling of ovarian cancer's global burden projects an unfortunate trend, estimating 60% increase in deaths by 2040, primarily due to population aging and growth (Bray *et al.*, 2018).The disease primarily affects women aged 45 and above, with incidence sharply increasing after menopause (Reid *et al.*, 2017). Most of the disease burden lies with the most common variant, high-grade serous ovarian carcinoma (HGSOC) which is frequently diagnosed late at Stage 3 or 4, and is responsible for 70-80% of ovarian cancer deaths (Kurman and Shih, 2016). Late diagnosis is attributable, in part, to the non-specificity of presenting symptoms, such as bloating, bowel disturbance and dysuria.

Diagnostically, ovarian ultrasound is highly operator-dependent, and biomarker CA-125 has a sensitivity of just 50% for Stage I ovarian cancer, compared to 98% for Stage IV (Tiwari *et al.*, 2016). Disappointingly, a recent nationwide study, the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) deemed CA-125 as an ineffective for use in screening (Menon *et al.*, 2021). This finding comes as no surprise. Even in the context of only considering suspected cases of ovarian cancer, CA-125 offers a sensitivity of 77% and specificity of 94%, with positive predictive value (PPV) of 10% (Funston *et al.*, 2020). This is grossly inadequate; the low prevalence of ovarian cancer mandates a platform that offers sensitivity >75%, specificity >99.6% and a PPV of 10% critically in non-suspected cases (Bast *et al.*, 2019). Early diagnosis is critical; the 5-year survival for Stage I ovarian cancer is 93%, plummeting to 13% for Stage IV (CRUK, 2018). Predictive computer modelling has indicated that effective screening could yield a 43% reduction in ovarian cancer mortality, and save over £50,000 per year per patient (Havrilesky *et al.*, 2008).

Of grave concern, in the UK healthcare strains are prolonging time to investigation for patients with suspected ovarian cancer. From 2006 to 2016, this rose from 12 to 20 weeks on average (Stone, 2021). There is a dire need for improved screening tools.

1.1 Histological Subtypes of Ovarian Cancer

Ovarian cancer constitutes a diverse group of neoplasms derived from various cellular origins with unique mutagenic drivers. The categorisation of these tumours into Type I and Type II was first proposed by Kurman and Shih in 2008, and later refined (Kurman and Shih, 2010, 2016). This dualistic model is based on distinct clinical, morphological, molecular and genetic features, and the differentiating details of each category are outlined below (Figure 1A). Epithelial ovarian carcinomas (EOCs) account for 90% of all cases, and encompass the 5 most common ovarian cancer subtypes. Of these, high-grade serous carcinoma is Type II, and low-grade serous, endometroid, clear cell and mucinous carcinoma are Type I (Figure 1B). These can be easily delineated using four immunohistochemical markers (Figure 1C) – WT1, p53, Napsin A and PR (Köbel and Kang, 2022). More infrequent are germ cell tumours and sex cord-stromal tumours (malignancies of true ovarian origin), and rare histotypes including the Type I Brenner tumour (urothelial-like) and Type II carcinosarcoma (malignant mixed mesodermal tumour) (Kurman and Shih, 2016). To gain inspiration about the roots of HGSOC, it is prudent to look into the origin stories of Type I malignancies.

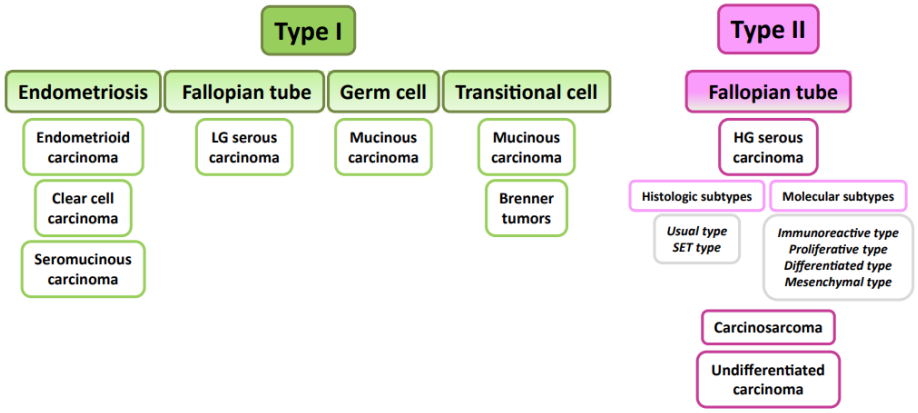
1.2 Endometroid Ovarian Carcinoma (EnOC)

In two seminal publications, John Sampson first proposed that during menstruation, retrograde blood flow occurs, delivering shed endometrial cells to local peritoneal tissues through the fallopian tubes or to distal sites through embolic spread via the uterine veins (Sampson, 1927, 1940). Here, they stick to the surfaces of organs, where they grow, thicken and bleed across each menstrual cycle. The proliferation of this ectopic tissue is intimately linked to 17β-Estradiol (E2). In endometriotic tissue,

A

	Feature	Type I Tumour	Type II Tumour
Onset	Stage	Frequently early	Usually advanced
	Tumour grade	Low grade	High grade
	Progression	Slow and indolent	Rapid and aggressive
	Origin	Precursor lesions	Distal fallopian tube
Genetic	TP53 mutation	Infrequent	Ubiquitous
	Somatic mutation burden	High	Low
	Homologous recombination	Rarely defective	Frequently defective
	Chromosomal instability	Low	High
Clinical	Early detection	Possible	Difficult
	Response to chemotherapy	Fair	Excellent (but recur)
	Actionable mutations	Sometimes present	Rare
	Overall clinical outcome	Good	Poor

B



C

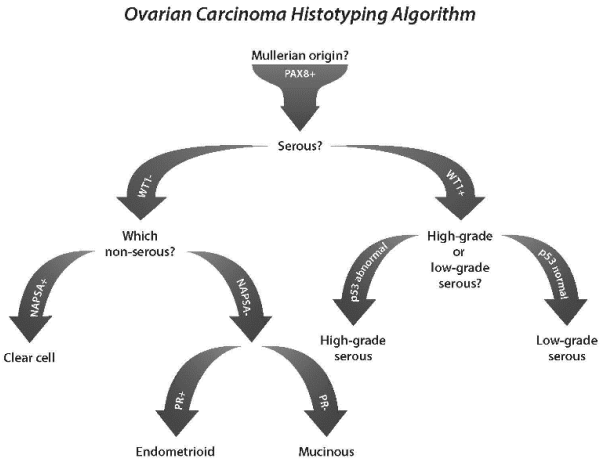


Figure 1. *Features of Type I and Type II ovarian cancer.* ***(A)*** *Type I cancers are less aggressive than Type II, and carry better prognoses.* ***(B)*** *The putative origin (middle row) of each ovarian cancer subtype (bottom row).* ***(C)*** *A panel of markers enabling distinction between subtypes. [(A) data and (B) figure taken from (Kurman and Shih, 2016); (C) figure taken from (Köbel and Kang, 2022)]*

aberrant DNA hypomethylation drives GATA6 production instead of GATA2, leading to ERβ, steroidogenic factor 1 (NR5A1) and aromatase (CYP19A1) synthesis (Chantalat *et al.*, 2020). NR5A1 drives steroidogenic acute regulatory protein (StAR) production, which imports cytosolic cholesterol into the mitochondrial, and aromatase catalyses androgen conversion to estrogens (Attar *et al.*, 2009). As such, endometriotic cells can synthesise E2 *de novo*; local concentrations are high. Although the precise role of ERβ compared to ERα is unclear, it is known when dimerised by E2 to stimulate a range of growth and survival pathways, facilitating ectopic colony formation (Chantalat *et al.*, 2020). While progesterone (P4) antagonises E2-mediated proliferation of endometriotic cells, GATA6 and E2-bound ERβ suppress progesterone receptor (PR) expression to robustly counteract this (Bulun, Wan and Matei, 2019).

These phenomena enable successful seeding of endometriotic tissue during retrograde menstruation into the ovary, in proximity to the ovarian surface epithelium (OSE). It is likely that ectopic tissue implants into open cortical inclusion cysts or corpus luteal cysts remnant post-ovulation (Varga *et al.*, 2022). It is important to note that endometriotic colonies are mostly stromal with scant epithelial cells, however they predispose to epithelial ovarian cancers (Bulun, Wan and Matei, 2019). It is probably these few cells, or perhaps metaplastic OSE cells lining the cyst, that transform. The stromal tissue lacing the cyst bleeds into the cavity, and the combination of free iron, haemoglobin (Hb) and aggregating inflammatory products confer genotoxic effects on neighbouring cells, which may be potentiated by catalysis of reactive oxygen species production through the Fenton reaction (Figure 2C). Moreover, a downstream target of ERβ is cyclo-oxygenase 2 (COX2), which generates prostaglandin E2 (PGE2). E2-bound ERβ and PGE2 further drive mutagenic inflammatory processes (Figure 2D) (Bulun, Wan and Matei, 2019). Additionally, a direct effect of high local E2 in forming quinone-based DNA adducts, as occurs in breast tissue, may cause double-stranded DNA (dsDNA) breaks, further driving tumour initiation (Cavalieri and Rogan, 2021). As a consequence of genotoxic mechanisms, classical Type I driver mutations (PIK3CA, KRAS and ARID1A) are frequently observed (Bulun, Wan and Matei, 2019).

1.3 Low Grade Serous Ovarian Carcinoma (LGSOC)

The current consensus model of low-grade serous ovarian cancer (LGSOC) onset proposes that the transforming cell of origin is from the distal fallopian tube epithelium (FTE), which invade ovarian stroma through endosalpingiosis to form ovarian epithelial inclusions (OEIs) (J. Li *et al.*, 2012). Expansion of OEIs to size >1cm leads to reclassification as serous cystadenoma (SC), which next progresses to a typical non-invasive serous borderline tumour (SBT) as it gains atypia and proliferative advantage. This advances to a non-invasive micropapillary serous borderline tumour (MPSC), and then to an invasive MPSC, better known as LGSOC (Vang, Shih and Kurman, 2009). Two gene expression profiling studies each with a 6-gene panel have robustly evidenced a FTE and not ovarian serous epithelial (OSE) origin for all stages of this slowly progressive model: OEI, SC, SBT and LGSOC (Qiu *et al.*, 2017; Wang *et al.*, 2019). Further validating this model, the advancement from SC to SBT is frequently observed, most LGSOCs have been firmly associated with SBT, early SBT and MPSC stromal invasions are histologically indistinguishable from LGSOC, and MPSCs invade stroma more readily than SBT (Smith Sehdev, Sehdev and Kurman, 2003; Longacre *et al.*, 2005; J. Li *et al.*, 2012).

In adopting a new residence in ovarian cysts, endosalpingiotic tissue is exposed to continuous oxidative stress. Cells in female reproductive tissues continually produce ROS, against which enzymatic oxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GSR) protect, alongside non-enzymatic antioxidants such as Vitamins C and E (Sharma and Agarwal, 2004; Sugino, 2006). At low levels, ROS stimulates growth, angiogenesis and steroidogenesis (Rizzo *et al.*, 2012). However, excessive ROS causes oxidative damage to the cellular environment – DNA, proteins and lipids (Yang *et al.*, 2021). Ovulation is an event of regular occurrence wherein reactive oxygen species are understood to have an indispensable role in follicle maturation and essential gene expression changes (Shkolnik *et al.*, 2011). Moreover, in the peri-ovulatory period, a sharp spike occurs owing to acute

inflammation driving follicle rupture, and the associated increased metabolic activity (Rizzo *et al.*, 2009). Further to regular acute stress, subacute inflammation from ovarian steroidogenesis during the luteal phase (Figure 2A), and chronic inflammation within the inclusion cyst that worsens with ageing (Figure 2B), contribute continuous ROS and dsDNA damage (Fujii, Iuchi and Okada, 2005; Huang *et al.*, 2019). As for other ovarian cancer subtypes, chronic estrogen exposure from the ovarian stroma may cause DNA damage and cell survival (Drapkin and Hecht, 2002), although in LGSOC it is noteworthy that ER+ status is overall protective. LGSOCs are 90% ER+ and 50% PR+, and as for EnOC, PR- status is linked to poorer outcomes (Cheasley *et al.*, 2022). Critically, Cohen *et al.* (2016) illustrated that gradual progression from normal tissue to SC to SBT to LGSOC may separately occur due to intrinsic stress. Each stage is accompanied by increasing intracellular ROS and lipid peroxidation (LPO) stress (Figure 2E), likely due to metabolic dysregulation. Along this pathway, cystic tubal epithelial cells acquire mutations in upstream MAPK regulators (*KRAS*, *BRAF* or *ERBB2*) conferring proliferation, as is classical for Type I tumours. In eventually transforming to LGSOC, SBT cells acquire multiple allelic imbalances (chromosomes 1p, 5q, 8p, 18q, 22q and Xp), leading to chromosomal instability, albeit not as severe as in HGSOC (J. Li *et al.*, 2012).

1.4 Mucinous Ovarian Carcinoma (MOC)

Roughly 80% of mucinous carcinomas affecting the ovary are metastatic, most frequently from the gastrointestinal tract. Distinguishing true origin has historically been difficult, and until recently, molecular and genetic studies have been unreliable due to misclassifications (Ledermann *et al.*, 2014). Out of the 20% that are true ovarian primaries, 80% are at Stage I. Analogous to LGSOC, MOC is understood to progress from mucinous cystadenoma to mucinous borderline tumour, with increasing occurrence of *KRAS* mutation across this trajectory (Babaier and Ghatage, 2020). The question remains as how mucinous tissue localises in an inclusion cyst to begin with. 5% of MOC cases develop alongside ovarian teratomas, indicating a germ-cell origin

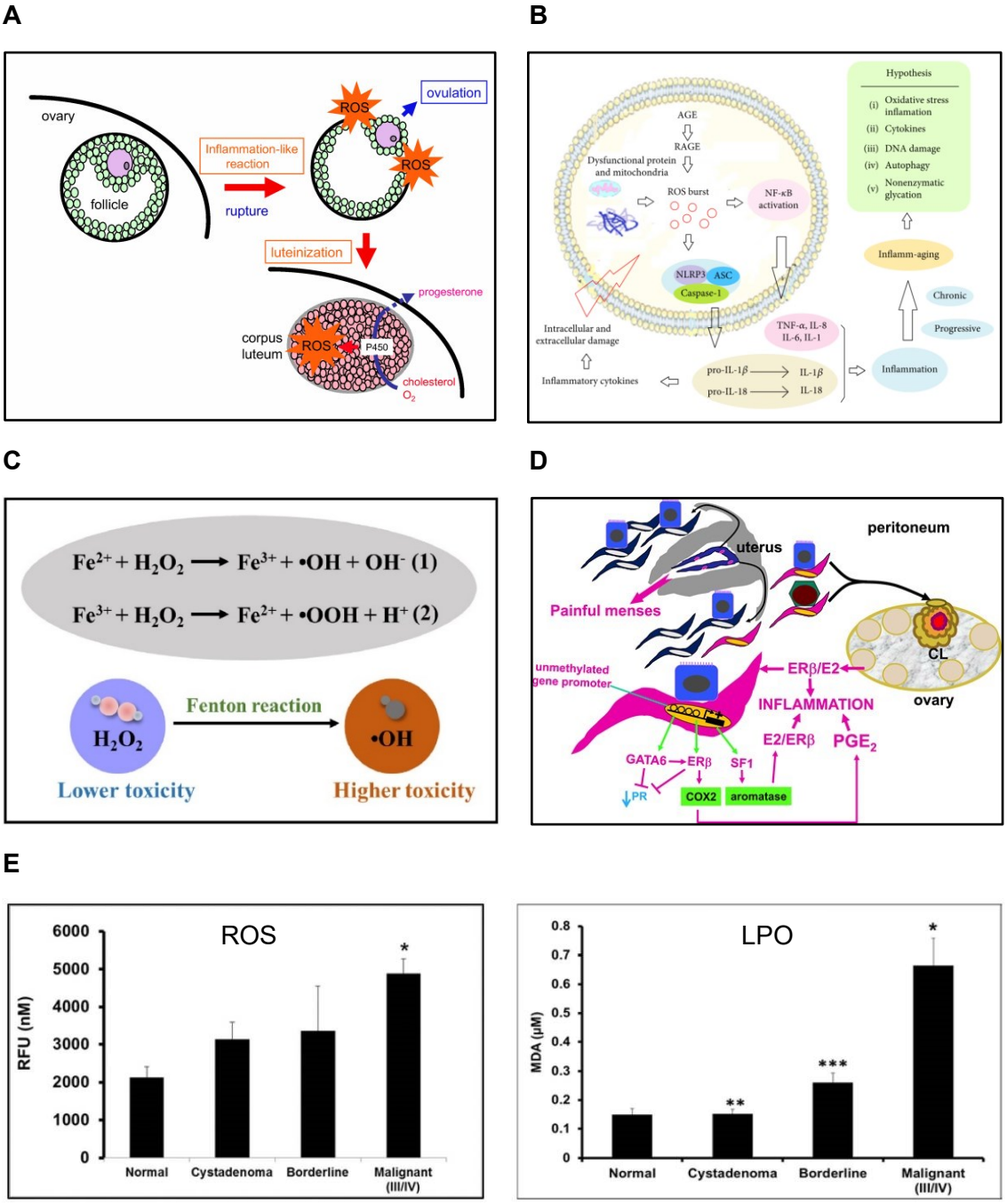


Figure 2. *The 5 major sources of mutagenic stress for Type I ovarian cancers.* ***(A)*** *Ovulation is inflammatory, generating a burst of ROS, and post-ovulation, progesterone synthesis via the mono-oxygenase reaction generates further ROS.* ***(B)*** *Metabolic ageing is expedited within inclusion cysts, increasing ROS.* ***(C)*** *In endometriosis, retrograde menstruation or haemorrhagic ovulation, free iron from blood can undergo a cyclical Fenton reaction with oxidants to generate free radicals.* ***(D)*** *Ectopic endometriotic stromal cells drive ERβ, COX2 and aromatase expression, leading to E2 and PGE2 upregulation, which drive inflammation.* ***(E)*** *As pre-malignant cells within the inclusion cyst progress towards cancer, they (Left) intrinsically produce more ROS which (Right) causes more LPO, generating increasing amounts of genotoxic aldehydes. [(A) taken from (Fujii, Iuchi and Okada, 2005); (B) taken from (Huang et al., 2019); (C) taken from (Meng et al., 2020); (D) taken from (Bulun, Wan and Matei, 2019); (E) taken from (Cohen et al., 2016)]*

(Elias *et al.*, 2018). Another possibility is that mucinous metaplasia of epithelial inclusion cysts occurs – with this said, it is exceedingly rare that such metaplasia is detected. Mucinous inclusion epithelium can be of endocervical, gastric or intestinal phenotype (Babaier and Ghatage, 2020). Exclusively for endocervical-like MOC, up to a half of cases are associated with endometriosis and exhibit frequent *ARID1A* mutation, similar to EnOC (Wu *et al.*, 2012). This suggests a role for retrograde menstruation-related fragment seeding for a subset of cases; gastrointestinal-like MOCs have no such association, which is fortunate – this would be difficult to explain. Separately, association between Brenner tumours and MOC has long been established. A study of rare mixed Brenner and mucinous tumours revealed a monoclonal origin between the components (Wang *et al.*, 2015). Walthard's nests are epithelial cell clusters in the distal fallopian tube connective tissue believed to have undergone transitional metaplasia. One elegant hypothesis is that they can undergo urothelial metaplasia to seed Brenner tumours, or mucinous metaplasia to see gastrointestinal-like MOC (Tafe *et al.*, 2016). Dependent on the MOC subtype, once ectopic or metaplastic involvement in an OEI has occurred, the mucinous epithelial cells are prone to the same physiological insults as in LGSOC or EnOC.

1.5 Clear Cell Ovarian Carcinoma (CCOC)

Clear cell carcinomas (CCC) are characterised by the enrichment of the cytoplasm with glycogen, creating a clear appearance. Aside from in the female reproductive tract and in the kidney, CCCs are extremely rare (Ji *et al.*, 2018). The histology and immunophenotypes of ovarian, uterine and cervical CCCs are near identical, suggesting a common origin, although subtle subtype-unique proteomic and morphological features do exist (Fata *et al.*, 2015; Ju *et al.*, 2018). Additional to EnOC, CCOC is the other major EOC subtype closely related to endometriosis; the endometriosis prevalence in CCOC is 35.9%, markedly higher than 19.0% for EnOC (Van Gorp *et al.*, 2004). Interestingly, ovarian carcinomas of endometriotic origin are not known to have site-specific pathognomonic mutations; for instance, *ARID1*,

PIK3CA, *CTTNB1* and *APOBEC* mutations exist in both EnOCs and CCOCs (Gamallo *et al.*, 1999; Wiegand *et al.*, 2010). The question therefore remains if endometriotic epithelial cells are truly serving as the progenitor to two independent carcinomas. It has been postulated that the heterogeneity of uterine epithelium in containing less-differentiated secretory cells and terminally-differentiated ciliated cells could explain this. Using a panel of 5 protein markers of differentiation to study an endometrial organoid model, it was found that CCOC shares features of the ciliated cell lineage, whereas EnOC is derived from secretory cells (Cochrane *et al.*, 2017). Along with generating oxidative stress through Fenton cycling, the haemorrhagic process in endometriotic cysts along with the provision of free-iron causes an aggressive inflammatory response mediated by IL-6 (Sanchez *et al.*, 2014). IL-6 has been evidenced to drive differentiation towards ciliated cells in the trachea, switch differentiation trajectories in the immune system, and contribute to differentiation in repair of muscle tissue following exercise (Chomarat *et al.*, 2000; Muñoz-Cánoves *et al.*, 2013; Tadokoro *et al.*, 2014). The leading model of CCOC therefore proposes that IL-6 high endometriotic cysts lead to the transformation of cells in the process of committing towards a differentiated ciliated fate, whereas IL-6 low endometriotic cysts predominantly comprise of less-differentiated secretory cells available for transformation (Cochrane *et al.*, 2017). The CCOC picture therein indicates that when studying HGSOC initiation, it will be essential to consider stemness influence, as well as the variety of mutagens and nature of the transforming cell.

CHAPTER 2

Current Perspectives on the Origin of HGSOC

2.0 HGSOC – An Origin in the Fallopian Tube?

Since even early HGSOC cases feature malignant ovarian lesions, it has historically been assumed that the disease originates from the ovarian serous epithelium (OSE). Fathalla (1971) was the first to recognise that incessant ovulation and ovarian cancer are closely linked, and it was initially suspected that 'tear and repair' of the OSE leads to the accumulation of DNA damage, mediating transformation. Moreover, the observation that post-ovulation, OSE tissue could invaginate to form a cortical inclusion cyst with an inflammatory environment and hormonal carcinogenetic drivers furthered belief in this hypothesis (Auersperg *et al.*, 2001; Fleszar *et al.*, 2018). At the turn of the millennium, increasing findings of dysplastic changes in fallopian tubes incidentally removed from patients at high-risk for developing ovarian cancer prompted the consideration that fallopian tube secretory epithelial cells are the origin of HGSOC (Piek *et al.*, 2001).

2.1 Shared Histology between HGSOC and the Fallopian Tube Epithelium

During embryological development, a single-celled squamous epithelial layer covers the inner cavity of the body, namely the coelomic epithelium. Being intensely metabolically active, and in generating underlying stroma through epithelial to mesenchymal transition (EMT), this coelomic lining is critical in abdominal organogenesis (Carmona *et al.*, 2013). Coelomic epithelium overlying the gonadal ridge, which gives rise to the ovary, specialises into flat-to-cuboidal OSE cells – the uncommitted mesothelium. By contrast, coelomic epithelium above the upper-lateral aspect of the gonadal ridge, which gives rise to the Müllerian ducts (that develop the fallopian tubes, uterus and endocervix) specialises into columnar cells – the Müllerian mesothelium (Auersperg *et al.*, 2001). The earliest clue for a tubal origin was that all serous ovarian carcinoma phenotypically resembles Müllerian mesothelium (Serov and Scully, 1973). However, the ease of metaplastic transformation of OSE towards

Müllerian phenotype and difficulty in explaining ectopia led to opposition of the tubal origin hypothesis (Feeley and Wells, 2001).

2.2 The *TP53* Mutation Signature Precedes HGSOC

During ovulation, the distal tubal epithelium, specifically the fimbrial projections that capture the egg, have exposure to high genotoxic stress from follicular fluid. An early report evidenced that mutant p53 signatures are frequently found in aggregations of secretory cells in patients with fallopian tube malignancy, serous tubal intraepithelial carcinoma (STIC), with preponderance towards the fimbriae (Lee *et al.*, 2007). These p53 signatures, along with STICs themselves, feature nuclear punctate staining for γH2AX, signifying double-stranded DNA (dsDNA) breaks in response to ongoing DNA damage, although STICs would uniquely display enhanced proliferative activity (MIB1/Ki67) along with genomic instability conferred by oncogene activation (Cyclin E). The importance of early *TP53* mutation has been further highlighted through studying heterozygous *BRCA1* or *BRCA2* mutant (*BRCA*+) patients, since they frequently have early serous carcinoma with frequent fimbrial mucosal lesions (Crum and Xian, 2010). Despite the enhanced risk of early ovarian cancer, many studies have not found a greater prevalence of p53 signatures in *BRCA*+ than in *BRCA*- patients (Lee *et al.*, 2007; Shaw *et al.*, 2009; Akahane *et al.*, 2022). Contrarily, other studies evidence that this trend does exist (Norquist *et al.*, 2010). Regardless of the controversy, it remains true that p53 signature is a relatively frequent finding in both *BRCA*+ and *BRCA*- patients. Furthermore, it has been shown that p53 signatures in heterozygous *BRCA*+ patients have enhanced proliferation, and that a subset of p53 signatures exist with loss of heterozygosity in *BRCA* that are endowed with carcinomatous features (Norquist *et al.*, 2010; Labidi-Galy *et al.*, 2017; Wu *et al.*, 2019). This collectively supports that *TP53* mutation in secretory cells typically precedes *BRCA* mutations, and that sequential allelic *BRCA* knockout on a *TP53* mutation background is permissive to the development of STICs, which can later seed in the ovary (Figure 3A). The concept of *TP53* as the fundamental driver mutation is robustly evidenced in that 97% of

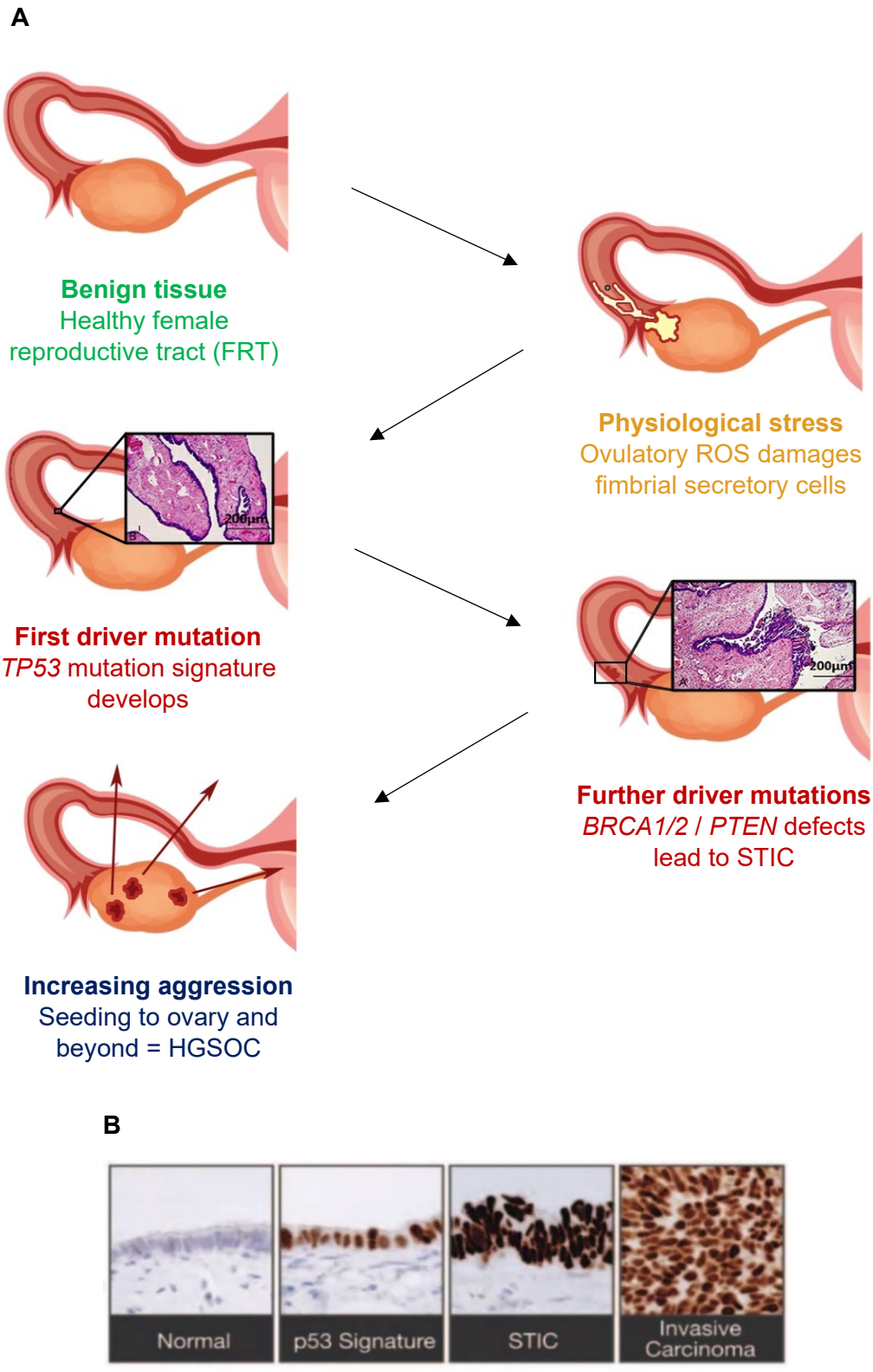


Figure 3. *The stepwise transformation from normal tubal epithelium to HGSOC.* ***(A)*** *Ovulatory ROS and retrograde menstruation (not shown) are genotoxic to fallopian tube epithelial cells, leading to local malignancy (STIC) that later spreads (HGSOC).* ***(B)*** *Immunostaining of mutant p53 shows progression of the p53 signature to cellular atypia and multilayering during the malignant stages. [(B) taken from (Karst and Drapkin, 2010)]*

carcinomas believed to originate from STICs have *TP53* mutations, with remaining cases showing p53 dysfunction (Ahmed *et al.*, 2010).

2.3 Genetic Changes Beyond *TP53* Mutation are Required to Initiate HGSOC

In pioneering work, Perets *et al.* (2013) constructed a murine cohort with sequential mutations targeted to the putative cells of origin, tubal secretory epithelial cells of the oviduct (the fallopian-tube equivalent), to understand the genetic drivers of HGSOC. PAX8 had previously been validated as a marker for tubal secretory epithelial cells, with absent expression in OSE and tubal ciliated epithelial cells (Bowen *et al.*, 2007). Using tetracycline-dependent *Pax8*-driven expression of Cre recombinase, adult murine cohorts of *Trp53*, *Brca1/2* and *Pten* knockout (KO) mice were generated, and oviducts harvested for study. This model revealed several critical findings: 1) *Trp53* & *Pten* KO initiated STIC, but not invasive HGSOC; 2) *Trp53* & *Brca2* KO slowly initiated invasive HGSOC; 3) *Trp53*, *Brca2* & *Pten* KO rapidly initiated invasive metastatic HGSOC, with genomic copy number alterations and pathway dysregulations consistent with TCGA study of human HGSOC; 4) *Trp53*, *Brca2* & *Pten* KO invasive metastatic HGSOC shared all immunohistochemical markers used in the clinical study of human HGSOC (The Cancer Genome Atlas Research Network, 2011; Perets *et al.*, 2013). Overall, this study gave substantial weight to a tubal origin for HGSOC. Although this was convincing, later evidence emerged that the human OSE contains rare expanses of epithelial (E-cadherin+) cells that are PAX8+ (Adler *et al.*, 2015). These cells were positive for calretinin and had flat-to-cuboidal morphology, verifying their origin as from the ovarian mesothelium rather than from endosalpingiosis. Critically, the finding that PAX8 increases during transformation *in vitro* warranted a re-investigation of the tissue of origin and stepwise mutagenesis.

2.4 Organoid Modelling Verified the Tissue of Origin and Key Genetic Changes

The inter-relation between stem cell biology and carcinogenesis was expedited by the advent of single-cell derived 3D organoids – small, multicellular, self-organising structures arising from stem cells cultured in an accurate *in vitro* recapitulation of their *in vivo* niche (Sato *et al.*, 2009). Epithelial organoids generate the diversity of organ-specific cell types and anatomical features of the tissue, thus their genetic manipulation can give insight into carcinogenetic processes (Hu *et al.*, 2020).

Lõhmussaar *et al.* (2020) established murine OSE and oviductal organoids, and interestingly found that both are Pax8+. In creating an organoid lirary of sequential knockouts (KOs) - *Trp53* KO, *Trp53* & *Brca1* KO, and *Trp53*, *Brca1* & *Pten/Nf1* KO – they identified several key differences: 1) *Trp53* KO alone in OSE organoids conferred vast chromosomal instability, uncharacteristic of HGSOC, whereas oviductal organoids required multiple KOs, conforming more closely to TCGA findings; 2) The mutant oviductal organoid library responded more to paclitaxel and niraparib than OSE, reflecting clinical response in HGSOC; 3) Triple KO oviductal organoids led to tumours when implanted orthotopically into bursae, unlike OSE organoids; 4) For subcutaneous tumour grafts, oviductal tumours grew larger and were more proliferative (Ki67-high) than OSE tumours (The Cancer Genome Atlas Research Network, 2011; Lõhmussaar *et al.*, 2020). This affirmed both the view that additional to *TP53* KO, mutations endowing proliferative advantage are likely necessary for transformation, and that the fallopian tube is the predominant tissue of origin in HGSOC.

These findings are consistent with previous works showing that Rb pathway dysregulation in *Trp53* mutants for both oviductal and OSE organoids evoke transformation, but that the former metastasise more readily, and have superior response to carboplatin and paclitaxel (Zhang *et al.*, 2019). Conservation of distinct transcriptional features based on the tissue of origin was shown, which aligns with

detailed proteomic and transcriptional studies to suggest that both human fallopian tube (hFT) and OSE-derived HGSOC subtypes exist – the latter being rarer and less responsive to conventional treatment, consistent with aforementioned pharmacological findings (Coscia *et al.*, 2016; Hao *et al.*, 2017). In summary, organoid models are proving to be an invaluable adjunct to mouse studies in studying mutational trajectories and cellular origins in HGSOC initiation (Psilopatis *et al.*, 2022).

2.5 HGSOC is Characterised by Dysregulation of Particular Signalling Pathways

Type II carcinomas are characterised by low somatic mutational burden but high copy number aberrations. The Cancer Genome Atlas Research Network (2011) undertook a detailed genomic study on a large HGSOC dataset, capturing mRNA expression, miRNA expression, DNA copy number, DNA promoter methylation, and whole-exome sequence changes. Frequent alterations were found through chromosomal aberration and mutations in 5 signalling cascades: Rb (67% of cases), PI3K/RAS (45%), Homologous Recombination (33%), NOTCH (22%) and FOXM1 (84%) pathways (Figure 4).

2.5a Rb Signalling in HGSOC

The Rb protein is a tumour suppressor that controls the G1/S checkpoint by inhibiting the activity of E2F-family transcription factors. In the absence of cellular stress, cytoplasmic complexes of Cyclin D-CDK4/6 and Cyclin E-CDK2 phosphorylate Rb. Hyperphosphorylated Rb releases E2F proteins, allowing the transcription of genes for S phase entry (López-Reig and López-Guerrero, 2020). In response to cellular stress, p53 is upregulated and activates its transcriptional target, the CDK inhibitor $p21^{CIP1}$. Alongside CDK inhibitors from other signals including the INK4 family of proteins (such as $p16^{INK4A}$) and $p27^{KIP1}$, $p21^{KIP1}$ blocks phosphorylation of Rb. Unphosphorylated Rb then binds to E2F, blocking G1/S progression in the context of cell damage. If, for instance, the cellular stress is DNA damage, ATM/ATR kinases

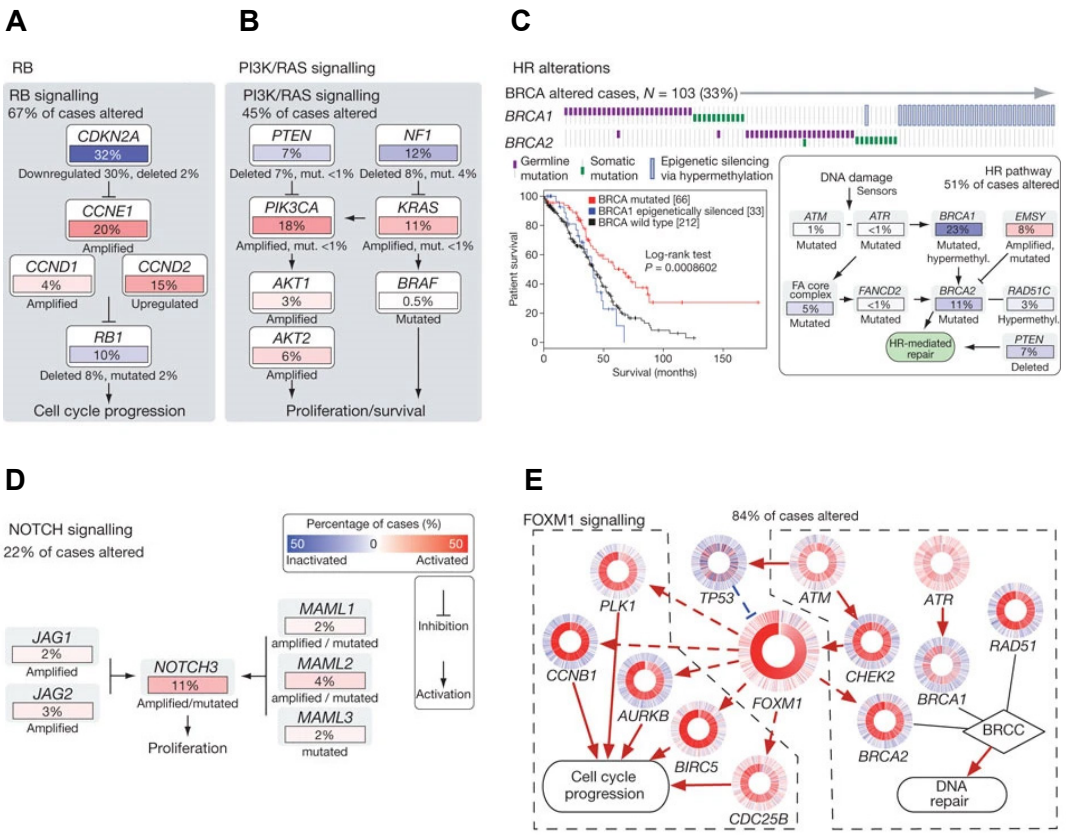


Figure 4. *The 5 pathways frequently dysregulated in HGSOC.* ***(A)*** *Aberrations in Rb signalling lead to constitutive E2F activity, resulting in G1/S checkpoint failure.* ***(B)*** *Activation of PI3K/RAS signalling leads to anti-apoptotic, pro-proliferative effects mediated through Akt.* ***(C)*** *Homologous recombination deficit prevents error-prone DNA repair, causing genomic instability.* ***(D)*** *Notch signalling is highly context-dependent but typically promotes stemness in hFT epithelium (Kessler et al., 2015).* ***(E)*** *FOXM1 upregulation is often downstream of p53/Rb; it promotes progression through G1/S, G2/M and mitosis. [Figure taken from (The Cancer Genome Atlas Research Network, 2011)]*

sense DNA breaks and activate p53, leading to cell-cycle arrest via the Rb pathway (Hamilton and Infante, 2016). Amongst these pathways, there is much complex cross-talk. Firstly, activation of PI3K/AKT/mTOR pathway by growth factors or in malignancy can lead to phosphorylation of Rb, leading to proliferation (Dong *et al.*, 2021). Secondly, activation of Ras/MAPK signalling drives expression of Cyclin D, so constitutive activity in cancer can bypass Rb control (Zhang and Liu, 2002). On the other hand, mechanisms exist to counteract this. For instance, ATM can directly modulate MDM2, the endogenous inhibitor of p53, to transform it into a *TP53* transcription factor. Additionally, MDM2 can directly upregulate Rb at the relevant cell-cycle stage during genotoxic stress (Hernandez-Monge *et al.*, 2021). With this

framework, it is easy to see why *TP53* mutation alone is insufficient to initiate HGSOC, and why subsequent Rb pathway mutations are frequent but not essential.

2.5b PI3K Activation in HGSOC

The PI3K/AKT/mTOR pathway is a multifaceted signalling cascade central to regulating cell growth, survival and metabolism. Activation occurs when growth factors bind and dimerise receptor tyrosine kinases (RTKs), or when ligands bind to G protein-coupled receptors (GPCRs). PI3Ks are the main downstream effectors; class IA PI3Ks (α, β & δ) relay RTK activity and class IB PI3K (γ) conveys GPCR signalling. Class IA PI3Ks have a regulatory subunit and a catalytic subunit, and it is the latter to which the α, β & δ isoforms correspond to: they are encoded by PIK3CA, PIK3CB and PIK3CD respectively (Rinne *et al.*, 2021). Of these, PIK3CA is the most often found mutated in epithelial breast and ovarian cancers (Levine *et al.*, 2005). PI3Ks phosphorylate PIP2 to PIP3, which recruits AKT to the cell membrane. mTORC2 and PIP3 then activate AKT by phosphorylation, which then exerts a variety of proliferative effects: cell growth, evasion of apoptosis, cell-cycle activation, and metabolic reprogramming. Eventually, PTEN dephosphorylates PIP3 back to PIP2 and shuts off the signal. Of note, once again there are profuse inter-relations between the pathways: 1) ATM/ATR can activate AKT directly (Fayard *et al.*, 2005); 2) DNA-PK, the only kinase involved in non-homologous end joining (NHEJ), can activate AKT in the context of DNA damage (Stronach *et al.*, 2011); 3) AKT activates MDM2, suppressing p53 in an unstressed environment; 4) When bound to GTP, Ras can directly activate PI3K (Castellano and Downward, 2011). Considering all of this, we can foresee the transformative power of aberrant activation of the PI3K/AKT/mTOR pathway, and understand how cells with disturbances in DNA repair and KRAS/BRAF pathways reap these benefits.

2.5c Homologous Recombination Deficit in HGSOC

Homologous Recombination (HR) is a critical cellular process to repair DNA double-stranded breaks (DSBs) that form in response to genotoxic agents. Its complex

orchestration is illustrated below (Figure 5). When genetic defects in HR occur, such as loss of BRCA1, cells become dependent on dangerous, non-templated DNA repair pathways to resolve DSBs. For instance, normally BRCA1 inhibits factor 53BP1, the co-ordinator of NHEJ, to promote HR (Bunting *et al.*, 2010). In BRCA1-deficient cells, factor 53BP1 blocks resection at DSBs thus inhibiting HR. Where multiple DSBs exist, NHEJ can adhere DSBs on different chromatids to generate chromosomal rearrangements and instability. Furthermore, in the case of uneven or 'ragged' DSBs, Artemis nuclease is recruited to trim bases, which leads to deletions and frameshifts (Savage and Harkin, 2015). Moreover, BRCA1 has a broad portfolio of involvements regulating genome stability: 1) It forms a complex with RAD51 and FANCD2 exclusively to repair DNA replication forks stalled at interstrand cross-links (Kim and D'Andrea, 2012); 2) It is required for ATM-mediated p53 activation to induce cell cycle arrest, and modulates p53 target genes (Fabbro *et al.*, 2004); 3) It serves as a transcriptional co-repressor for both ERα and c-Myc, throttling growth effects (Fan *et al.*, 2001; Li, Lee and Avraham, 2002). Interestingly, PTEN is understood to contribute to HR-mediated repair, and thus potentially plays a role in the regulation of genomic stability (Mansour *et al.*, 2018).

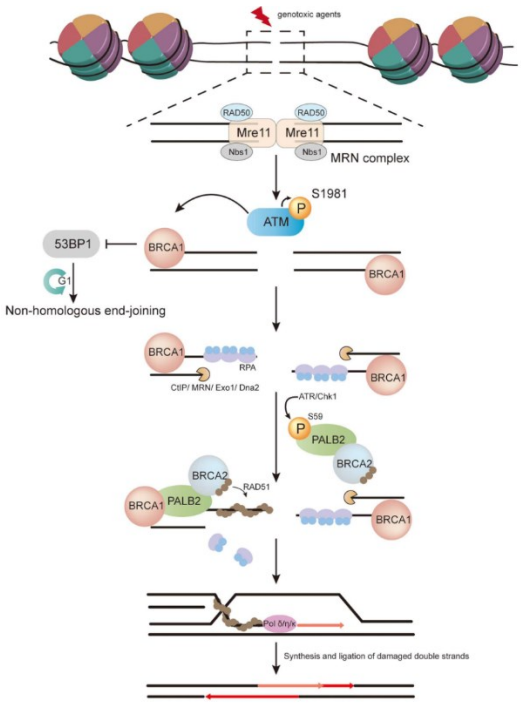


Figure 5. *Homologous recombination. The MRN complex, comprising MRE11, RAD50, and Nbs1, localizes to DSBs and recruits ATM. Upon autophosphorylation, ATM monomers activate H2AX, forming γH2AX which recruits BRCA1 (Bakkenist and Kastan, 2003). Simultaneously, CtIP, with MRN, initiates 5'-3' resection at DSBs, which is completed by Exo1, Dna2, and BLM (Sartori et al., 2007). ssDNA coated by RPA localise ATR/Chk1, which phosphorylates PALB2 (Syed & Tainer, 2018). Active PALB2, in conjunction with BRCA2, replaces RPA with RAD51. RAD51-ssDNA then forms a Holliday junction, engaging DNA polymerases δ/η/κ for strand elongation, culminating in junction resolution and DNA ligation. [Figure taken from (Wu et al., 2020)]*

With this all in mind, it comes as no surprise that 33% of HGSOC patients are *BRCA*-altered, that 51% have HR deficiency, and that 15% of cases have BRCA germline mutations. Curiously, the mode of *BRCA* defect appears to be significant. Epigenetically silenced and wild-type cases had similar 5-year survival rates at roughly 25%, whereas this was around 50% for mutant cases (Figure 4C) (The Cancer Genome Atlas Research Network, 2011). There are only highly speculative discussions as to why this is the case (Moschetta *et al.*, 2016). Another incompletely answered question is why hereditary *BRCA* mutation predisposes patients specifically to breast, ovarian, pancreatic and prostate cancer - the common link is that these tissues are estrogen responsive. However, a conflict occurs in that, for instance, *BRCA1*-mutant tumours are ERα negative (Savage and Harkin, 2015). Two concepts could collectively resolve this, for instance, in breast tissue. Firstly, estrogen can directly cause bulky DNA adducts and DSBs, and evidence points towards a unique role of BRCA1 in resolving these (Savage *et al.*, 2014). Secondly, despite BRCA1 repressing ERα transcriptional targets, BRCA1 upregulates ERα, which maintains luminal cells (Liu *et al.*, 2008; Gorski *et al.*, 2009). Thus, with BRCA1 loss-of-function, ERα+ luminal cells dedifferentiate to a ER- basal/stem-like state, and the cells have high genomic instability with particular vulnerability to local estrogenic metabolites. Hereditary *BRCA* mutations can therefore lead to basal-like breast cancers, which feature mutational trajectories and genomic scarring strikingly similar to HGSOC (Prat *et al.*, 2014).

2.5d Notch Pathway Dysregulation in HGSOC

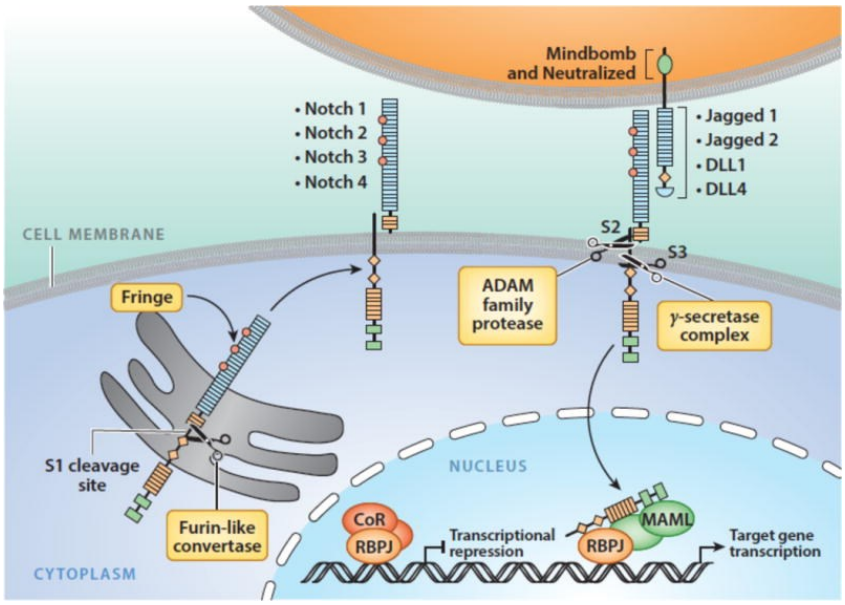
Through complex interactions with p53-related, immune, metabolic, apoptotic, angiogenic and proliferative cascades (including RAS/MAPK pathway), Notch signalling (Figure 6A) can dynamically behave in both an oncogenic and tumour suppressive manner, dependent on cancer type and exact cellular processes (Figure 6B) (Aster, Pear and Blacklow, 2017). The aforementioned ubiquity of *TP53* mutations in HGSOC indicates that the interaction between p53 and Notch is particularly important (Ahmed *et al.*, 2010). In breast tissue, Notch pathways inhibit expression of

pro-apoptotic p53 targets, and p53 inhibits the Notch transcription complex through interaction with MAML1 (Yun *et al.*, 2015). In T-ALL, Notch signalling drives Hes-mediated downmodulation of PTEN, activating PI3K/Akt and MDM2, inhibiting p53 (Dotto, 2009). *NOTCH* genes are oncogenic in these contexts. However, in early cervical cancer, a keratinocyte-dervied tumour, Notch inhibits the AP1 transcription factor complex, blocking activity of HPV E6, amplifying p53 activity (Talora *et al.*, 2002). In Ewing sarcoma, Notch utilises Hes/Herp transcriptional repressors to directly downregulate MDM2, activating p53 – this Notch signalling is disabled by the *EWS-FLI1* translocation (Ban *et al.*, 2008). In these settings, Notch is tumour suppressive. Indeed, these oncogenic and tumour suppressive pathways co-exist in many cancers, enabling Notch to switch roles dynamically.

However, study of non-canonical signalling, independent of the CSL transcription factor, may be particularly critical to processes underpinning tumour initiation. One such pathway includes the interplay between Wnt and Notch, sometimes referred to as ‘Wntch’ signalling. In combination, they play a role in embryonic induction, polarity in cell division, cell fate and cell growth (Jin *et al.*, 2009). For instance, both pathways are required for the maintenance of Lgr5+ intestinal stem cells; in the absence of one or the other, differentiation towards enterocyte, goblet cell, entero-endocrine or Paneth cell fate occurs (Yin *et al.*, 2014). In hFT organoids, Notch signalling is critical to stemness. Inhibiting γ-secretase with DBZ blocks Notch activity, and causes severe organoid infolding and an increase in ciliated markers, signifying differentiation. A global gene shift is observed, including downregulated stemness markers AXIN2, LGR6 and OLFM4 (Kessler *et al.*, 2015).

Interaction between the two pathways is incompletely understood but crossover is believed to occur at many levels: 1) In *Drosophila*, Notch activity represses Wingless (Wg) pathways, the counterpart of vertebrate Wnt-1, in the context of cell fate determination (Brennan *et al.*, 1999); 2) Notch receptor interacts directly with Wg, without producing NICD, as well as with Dishevelled, the co-ordinator of Wnt signalling

A



B

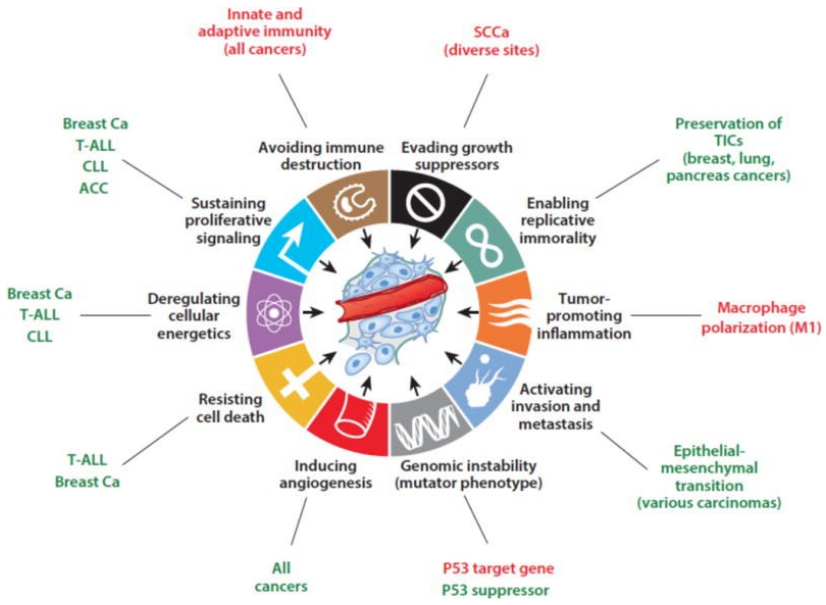


Figure 6. *Notch signalling and its roles in cancer.* ***(A)*** *NOTCH has four membrane-bound ligands from the Delta and Serrate families. The NOTCH receptors have an extracellular domain (NECD) that is connected to an inside domain (NICD) by a Ca^{2+}-stabilised bridge. On ligand binding, ADAM10/17 cuts off NECD, which then enters the signal-sending cell for recycling. This cleavage is the rate-limiting step, thought to rely on mechanical force caused by ubiquitination of the intracellular ligand tail by homologues of Mindbomb and Neuralized (Le Borgne et al., 2005). In the signal-receiving cell, γ-secretase then cleaves the NICD, which translocates to the nucleus. In complex with CSL and MAML, the canonical targets are upregulated: Myc, p21, Cyclin D and HES proteins (Widlund, 2012).* ***(B)*** *Notch signalling can be oncogenic (green) or tumour suppressive (red), depending on context. [(A) and (B) taken from (Aster, Pear and Blacklow, 2017)]*

(Axelrod *et al.*, 1996; Wesley, 1999); 3) Presenilin-1, part of the γ-secretase complex; 4) GSK3β targets β-catenin for destruction and downregulates Notch2 activity (Espinosa *et al.*, 2003); 5) NICD is a co-activator for LEF1, one of the β-catenin transcription factors (Ross and Kadesch, 2001); 6) β-catenin can upregulate Notch1 and NICD, as well as amplify the transcriptional activity of NICD (Jin *et al.*, 2009). Overall, with Notch and WβC signalling being intimately intertwined, their individual role in tissues is difficult to disentangle.

Insights may come from the fact that healthy tissues are often carpeted in somatic mutations (Lee-Six *et al.*, 2019). The recent finding that *NOTCH1/2/3* mutations in healthy hFT epithelial biopsies are more frequent than expected for gene size may imply that *NOTCH* mutation facilitates growth (Wietek *et al.*, Pre-Publication). This could serve as an intrinsic protection mechanism to outcompete more tumourigenic clones. Although this appears to conflict with the aforementioned role in hFT stemness, we have established Notch behaviour to be highly context-dependent, and further exploration is warranted (Kessler *et al.*, 2015).

2.5e FOXM1 Overexpression in HGSOC

FOXM1 is a multifunctional transcription factor overexpressed in all 32 TGCA-studied cancer types, with evidence of its genomic signature in 84% of HGSOC cases This is as expected, since *FOXM1* is an E2F1 target gene, and p53/Rb dysregulation is almost universal (Barger *et al.*, 2019). During HGSOC carcinogenesis, overexpression of several proliferative factors frequently occurs, including Gli1/2 (Shh pathway) (Teh *et al.*, 2002), c-Myc (Pan *et al.*, 2018), STAT3 (Mencalha *et al.*, 2012) and Twist-1 (Qian *et al.*, 2013). These all bind *FOXM1* promoter to induce gene expression, and FOXM1 can even bind its own promoter as part of a positive feedback loop. Interestingly, cellular stress upregulates FOXM1, which upregulates antioxidants such as SOD (Park *et al.*, 2009). SOD then cyclically promotes E2F1/Sp1-driven activation of FOXM1 (P.-M. Chen *et al.*, 2013). Thus, FOXM1 may particularly enable escape from apoptosis

caused by ovulatory ROS damaging naïve hFT cells (de Kok and van Maanen, 2000; Zona *et al.*, 2014).

FOXM1 is reputed for its low affinity to its recognition site, which confers its transcriptional promiscuity; it can interact with DNA either through *FOX* consensus sequences or in liaison with several transcriptional complexes (Littler *et al.*, 2010). Through diverse pathway-controlling gene targets, FOXM1 co-ordinates: 1) G1/S checkpoint progression (*SKP2* and *CKS1*); 2) G2/M checkpoint progression (*PLK1*, *CDC25B* and *CCNB1*); 3) Mitotic progression (*AURKB* and *KIF20A*); 4) Cellular migration and invasion (*KRT5*; *KRT7*; *ITGA1*; *ITGB2*; *LAMA*-family; and *FN1*). 5) Matrix remodelling (*MMP2* and *DLX1*). 6) Stemness (*CTNNB1*) (Liu, Barger and Karpf, 2021). 7) DNA repair – HR, NHEJ, short-patch base excision repair (BER), long-patch BER and Mismatch Repair (MMR) (through the respective target genes *NBS1*, *CSK1*, *EXO1*, *XRCC1* and *POLB*/*POLE*) (Zona *et al.*, 2014). In considering the various functions, it can be envisaged that the combination of HR deficiency and FOXM1 overexpression could lead to a proliferative, adhesive, ROS-resistant stem cell with genomic instability – a recipe specifically for HGSOC.

CHAPTER 3

The Cells of Origin of Cancer

3.0 The Cancer Cell of Origin – an Overview

In tumorigenesis, both loss of gatekeeper (cell-cycle control) and caretaker (DNA repair) functions are required in a single cell, bestowing proliferative advantage and genetic plasticity for survival. Tissue-resident multipotent stem cells already have self-renewal capacity, thus are one-step closer to transformation than non-stem cells. Confusingly, there are two types of multipotent stem cell. In high-turnover tissue, such as colorectal epithelium, homeostatic stem cells are prevalent and active for ongoing tissue regeneration (Barker *et al.*, 2007). In low-turnover tissue such as liver and pancreas, homeostatic cells are infrequent and dormant, however, unipotent cells re-gain potency as facultative stem cells upon tissue injury (Yanger and Stanger, 2011). Stem cell homeostasis may depend on a hierarchical model wherein master stem-cells can regenerate, but also give rise to less potent multipotent stem cells (Figure 7A). The alternative is a neutral competition model in which stem cells are intrinsically equipotent and some become dominant purely by chance (Figure 7B). It is likely that both models

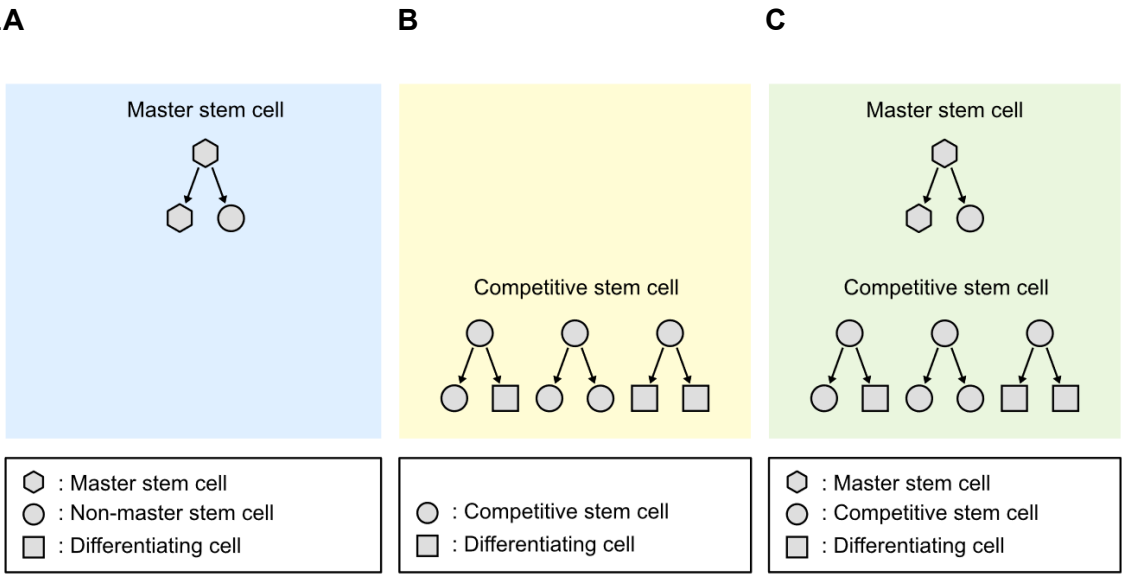


Figure 7. *Modelling of stem cell homeostasis.* ***(A)*** *Hierarchical Model: master stem cells may generate more committed stem cells, as well as true progenitors* ***(B)*** *Neutral Competition Model: by chance alone, some stem cells are lost (top right), some survive (top left), and some expand (top middle).* ***(C)*** *Hierarchical Neutral Competition Model: master stem cells always regenerate, but their more committed stem cell progeny randomly compete for dominance. (Figure taken from (Nakamuta, Yoshido and Naoki, 2022)*

co-exist (Figure 7C); master stem cells generate non-master stem cells, which neutrally compete (Nakamuta, Yoshido and Naoki, 2022). In the hFT, it is known that secretory cell outgrowths (SCOUTs) of >30 clonal cells with PAX2 downregulation precede the p53 signature, and are the earliest known pre-HGSOC lesion (Chen *et al.*, 2010). Whilst it is unclear how far up the putative stemness hierarchy hFT cells must be to form a SCOUT, it is likely to be a stratified stochastic risk. Thus, the hFT multipotent stem cells are the current best candidate for the HGSOC origin. However, in this chapter, exploration of the cells of origin of better-studied carcinomas explain why this must be said guardedly.

3.1 The Cell of Origin of Intestinal Adenocarcinoma

The intestinal epithelium comprises of crypts and villi; invaginations and protrusions across the surface. This epithelium is understood to be a high-turnover tissue; cells typically survive 3-5 days. In order for a stem cell to survive and repopulate the epithelium, it must have a considerably longer lifespan than this. After the discovery of the importance of Wnt/β-Catenin (WβC) signalling in maintenance of intestinal crypts, a search of Wnt-target genes found that Lgr5 was uniquely expressed in a limited number of cells at the site of deepest invagination of the crypt (Barker *et al.*, 2007; Flier *et al.*, 2007). To evidence these as the stem cells, lineage tracing was conducted. Two constructs were bred into individual mice: for the first, EGFP and tamoxifen-inducible Cre were expressed under *Lgr5* promoter, and for the second, a floxed STOP cassette was upstream of LacZ. With this setup, current Lgr5+ cells would report EGFP, and after tamoxifen treatment, current or former (differentiated) Lgr5+ cells would report LacZ. By day 60-post tamoxifen, in both the small intestine and colon, crypts were found to be entirely Lgr5 progeny with complete cellular heterogeneity (Figures 8A – 8C). This validated Lgr5+ cells as the stem cells in these intestinal epithelium (Barker *et al.*, 2007).

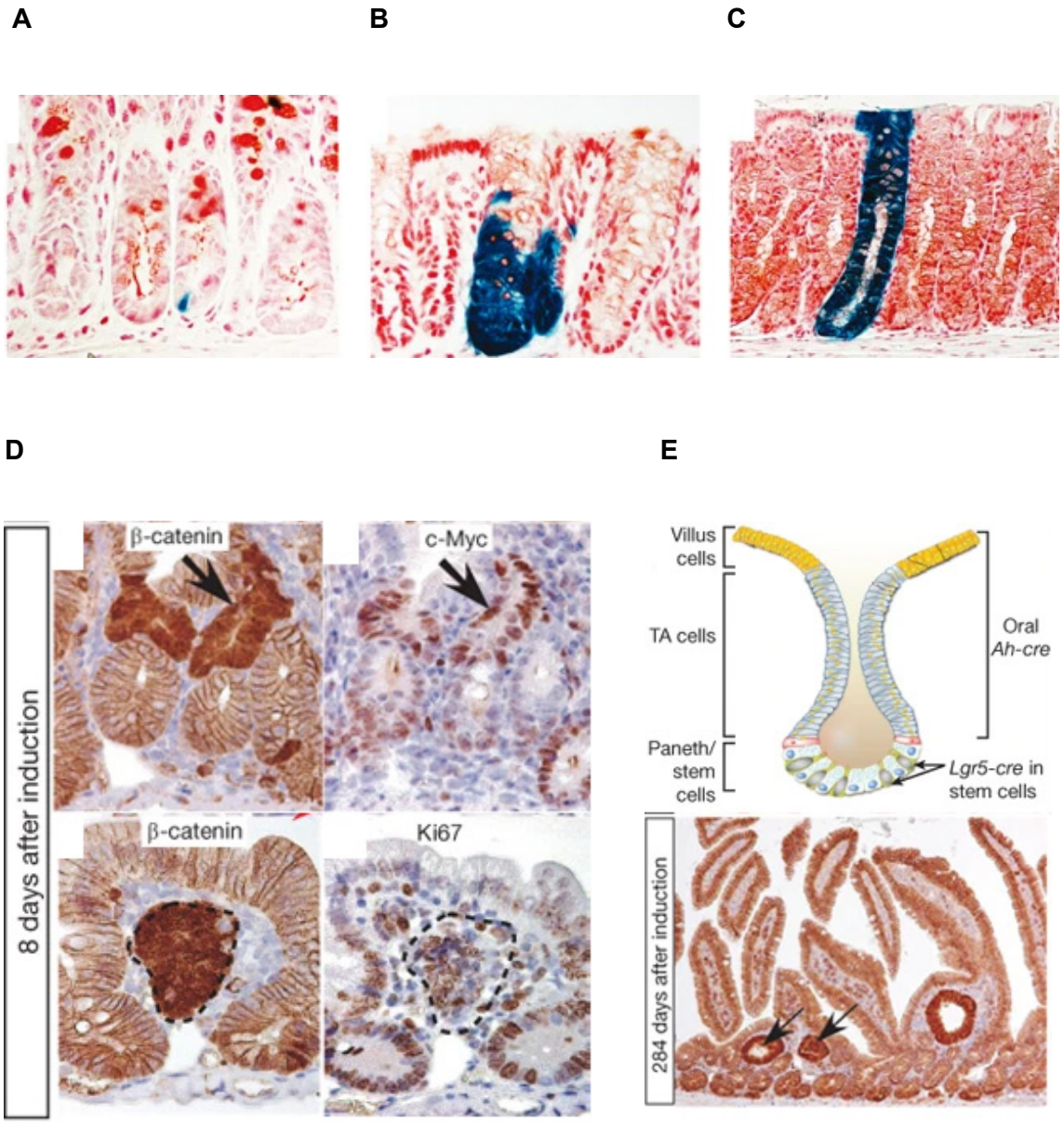


Figure 8. *The intestinal stem cell is the origin of intestinal adenocarcinoma.* ***(A-C)*** *Histology of the colonic crypts of a LacZ-reporter mouse for Lgr5 lineage tracing at (A) Day 1 (B) Day 5 (C) Day 60, showing that a single Lgr5+ cell repopulates the entire crypt, evidencing Lgr5 as the stem cell marker.* ***(D)*** *Targeting Apc knockout to Lgr5+ intestinal stem cells results in adenoma formation within as little as 8 days, with β-catenin overexpression in glands, overexpression of the Wnt-target genes, high proliferation (Ki67), and visible glandular atypia.* ***(E)*** *Targeting Apc knockout to transit amplifying (TA) non-stem cells results in no adenoma within 284 days; although β-catenin overexpression occurs, malignant or proliferative changes are not observed. (Figures (A-C) taken from (Barker et al., 2007); Figures (D) and (E) taken from (Barker et al., 2009)*

Within the small intestine, we now understand that at the crypt base, Paneth cells confer the niche for adjacent Lgr5+ stem cells through paracrine signalling. Further up, there is a transit-amplifying zone (TA) comprising the differentiating progeny of the stem cells, and tuft, goblet and enteroendocrine cells constitute the fully differentiated population (Huels and Sansom, 2015). In intestinal adenocarcinoma, the most frequent initiating mutation is biallelic knockout of *APC*, resulting in constitutive Wnt activity (Kinzler and Vogelstein, 1996). By breeding mice with the aforementioned *Lgr5*-driven construct with mice with floxed *Apc*, tamoxifen-induced biallelic knockout of *Apc* targeted to the intestinal stem cells was conducted. In both the small and large intestine, in as little as 2-3 weeks, multi-villous adenomas were found in a vast number of crypts (Figure 8D). Throughout the adenomas, Lgr5+ cells were found scattered throughout, c-Myc was uniformly present, and β-catenin was high (Barker *et al.*, 2009). Corroborating this, *Apc* deletions targeted to other intestinal stem cell markers, Lrig and Prominin1, also rapidly induce adenomas (Zhu *et al.*, 2009; Powell *et al.*, 2014).

By contrast, adenomatous formation is limited, but not absent, in non-stem populations. In place of the *Lgr5*-driven construct, *Ah-Cre* was used to express Cre in the presence of β-naphthoflavone. By administering the drug orally, β-naphthoflavone penetrance into crypts was low, so *Apc* knockout was primarily targeted at TA cells. Although β-catenin high foci were seen, after 30+ weeks very few progressed to adenomas (Figure 8E) (Barker *et al.*, 2009). Next, since Paneth cells confer the stemness niche, it is reasonable to suspect that their proliferation could be tied to that of Lgr5+ cells. However, *Apc* and *Kras* knockout in Paneth cells does not result in tumour formation (van Es *et al.*, 2019 - Supplementary Information, Figure S3C). Additionally, study of tuft cells shows that *Apc* loss alone does not induce tumorigenesis, but when inflammation is introduced, then progression to adenoma is occurs (Nakanishi *et al.*, 2013; Westphalen *et al.*, 2014; Huels and Sansom, 2015). In line with this, WβC hyperactivation alone could not initiate tumours in differentiated intestinal cells alone, however with added stress, NF-κB signalling led to transformation (Schwitalla *et al.*, 2013).

More generally, intestinal microenvironmental changes have been shown to enable transformation of non-stem cells (Davis *et al.*, 2015). It appears likely that additional stress or mutations separate to WβC-signalling are required to drive tumorigenesis in non-stem cells (Huels and Sansom, 2015). Likewise, enforced PPAR-δ signalling, the result of a high-fat diet, has been shown to increase intestinal adenoma formation atop *Apc* loss in non-stem cells, but not stem cells (Beyaz *et al.*, 2016 - Supplementary Information). This suggests that WβC signalling can be further amplified by stress in the former, but is already maximised in latter. Overall, the findings in colorectal cancer substantiate the stemness-stratified stochastic risk model.

3.2 The Cell of Origin of Basal-Like Breast Carcinoma

The breast epithelium comprises of two layers – inner luminal epithelial cells and outer myoepithelial cells, that branch to form terminal duct lobular units. The current understanding is that a bipotent mammary stem cell (MaSC) exists that can differentiate into luminal and basal progenitors. The luminal progenitor can subspecialise to ductal or alveolar luminal cells, and the basal progenitor forms myoepithelial tissue (Figure 9A) (Fu *et al.*, 2020). Studies using retroviral marking of mouse mammary cells provided the early knowledge that self-renewing single cells existed that could repopulate an entire mammary gland – MaSCs (Kordon and Smith, 1998). In order to isolate these rare cells, one team conducted immunolabelling to exclude well-characterised endothelial (CD31) and haematopoietic (CD45 and TER119) cells, and identified a CD24+/CD29^{high}/Sca1^{low} population capable of self-renewal, present in the basal but not luminal epithelium (Shackleton *et al.*, 2006; Stingl *et al.*, 2006). Within these cells, a Lgr5+/Tspan8^{high} subset readily produced the most complex glandular structures imputable to their unique ability to produce all basal lineages. Moreover, this population was found near the nipple, the oldest portion of the ductal tree, supporting an embryonic origin, whereas Lgr5+/TSpan8^{low} cells were found in distal branches and had reduced repopulating capacity; thus Tspan8 was considered the putative embryonic MaSC marker (Fu *et al.*, 2017).

As the picture of hierarchical differentiation began to establish for mammary gland epithelium, it was suspected that breast cancer subtypes would correspond to similar-behaving cellular subtypes: 1) ER- *BRCA*-mutation related basal-like cancers were conceptualised to originate from *BRCA1* silencing causing differentiation arrest in MaSCs; 2) ERBB2+ cancer was thought to result from 17q12 (ERBB2) amplification causing hyperproliferation of MaSCs; 3) Proliferative gain in early progenitor cells was believed to give rise to ER+/- Luminal-B cancer; 4) Tumorigenic change in committed progenitors was suspected to result in Luminal-A cancer (Melchor and Benítez, 2008). Although highly intuitive, research findings have not supported this model. One study isolated three populations: $CD24^{low}$/Sca1− basal progenitor cells (containing MaSCs), $CD24^{high}$/Sca1−/ER− luminal progenitor cells, and $CD24^{high}$/Sca1+/ER+ luminal differentiated cells. Excision of floxed *Brca1* exons on a heterozygous *TP53* background was targeted to luminal progenitors (*Blg-Cre*) and basal cells (*K14-Cre*) (Molyneux *et al.*, 2010). Interestingly, targeting biallelic *Brca1* knockout to luminal progenitors produced tumours with histology that closely phenocopies basal-like breast cancer (Rakha, Reis-Filho and Ellis, 2008). By contrast, targeting basal progenitors and therefore MaSCs produced cancerous histology resembling malignant adenomyoepithelioma – a rare tumour with features indicating a true stem cell origin (Molyneux *et al.*, 2010).

As discussed previously, BRCA1 upregulates ERα to maintain luminal cells - thus, a mechanism is clear for how *BRCA1* knockout leads to their de-differentiation to the early ER- progenitor state (Figure 9B), which carries basal-like features (Liu *et al.*, 2008; Gorski *et al.*, 2009). Furthermore, the ease of de-differentiation in this tissue has been observed in organoid models, wherein YAP/TAZ expression has been shown to revert differentiated mammary gland cells into MaSCs (Panciera *et al.*, 2016). Critically, the unexpected finding that the cell of origin in basal-like breast carcinoma is putatively not the MaSC conveys that we cannot assume the HGSOC origin based on phenotypic features of cell subtypes.

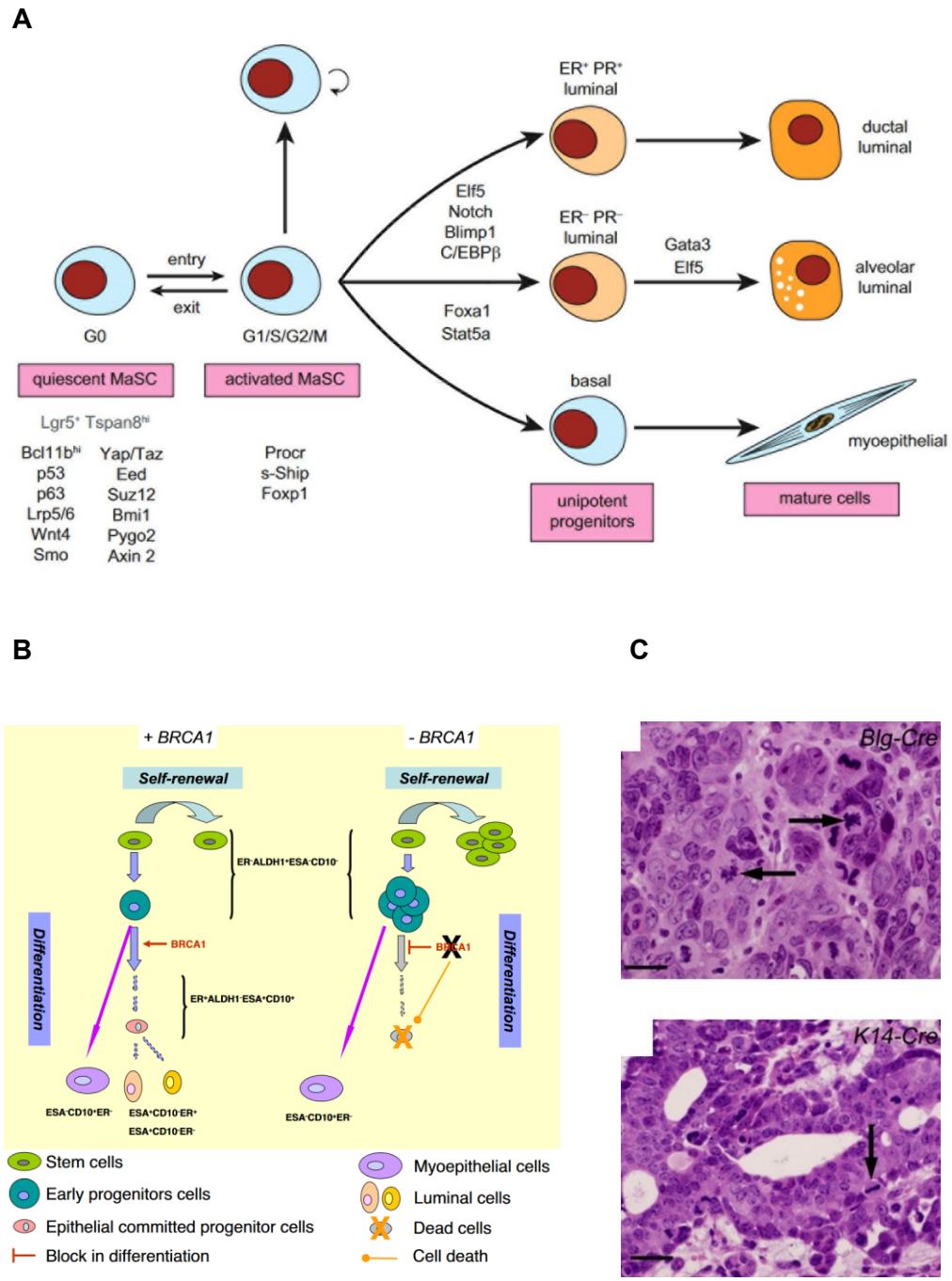


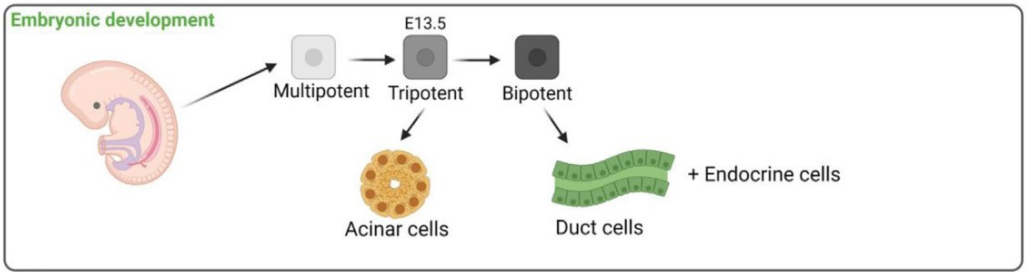
Figure 9. *The malleable fate of breast cells interplays with cancer subtype specification.* ***(A)*** *Lgr5+/Tspan8+ cells mark embryological mammary stem cells, which respond to several molecular cues to eventually determine luminal ductal (ER+/PR+), luminal alveolar (ER-/PR-) or myoepithelial fate.* ***(B)*** *BRCA1 is essential for the differentiation of early ER-/PR- progenitors to ER+ luminal cells; with BRCA1 knockout, this process fails.* ***(C)*** *BRCA1 exon deletion in ER- luminal cells under the Blg promoter leads to non-glandular basal-like breast cancer (top), whereas targeting basal cells under the K14 promoter results in malignant adenomyoepthelioma, which is visibly glandular (bottom). [(A) taken from (Fu et al., 2020); (B) taken from (Liu et al., 2008); (C) taken from (Molyneux et al., 2010)]*

3.3 The Cells of Origin of Pancreatic Ductal Adenocarcinoma

Over the past 50 years, progress in pancreatic cancer survival is the worst out of the non-rare malignancies (CRUK, 2013). The majority of cases (>90%) are pancreatic ductal adenocarcinoma (PDAC), the source of which is the exocrine pancreas (Mergo *et al.*, 1997). To find the cell of origin, an understanding of the embryonic development of the acinar, ductal and endocrine system is required. The first stage of pancreatogenesis involves the formation of the pancreatic bud, a duodenal expansion. The multipotent progenitor cells are positive for embryonic marker PDX1, acinar marker PTFA1 and ductal marker SOX9. These differentiate to two populations of tripotent cells with distinguishing markers – PTF1A+ tip cells and SOX9+ trunk cells (Jennings *et al.*, 2015). These eventually commit, with the tip cells forming acinar cells, and bipotent trunk cells forming ductal and endocrine cells (Figure 10A) (Backx *et al.*, 2022).

Similar to the liver, the pancreas has a low cell turnover, with activation of progenitors observed only during tissue injury (Bonner-Weir and Sharma, 2002; Zhou and Melton, 2018; Campana *et al.*, 2021). Early studies evidenced this progenitor population as originating from the ductal epithelium; after partial pancreatectomy in mice, Pdx1 re-appeared in most mature duct cells, regenerating endocrine islets (Bonner-Weir *et al.*, 2004). The adoption of a transient stem cell-like state qualifies this population as facultative stem cells (Zipori, 2004). Ablation of both acinar and endocrine cells, but sparing ductal cells, again showed that Sox9+ ductal cells regained Pdx1 expression, and could regenerate both missing lineages (Criscimanna *et al.*, 2011). More recent works evidence the importance of the WβC pathway in regenerative response; pancreatic damage caused by duct ligation is observed to upregulate Axin2 and Lgr5, and ductal organoids were found to express these alongside Pdx1, unlike normal ductal epithelium (Huch *et al.*, 2013). Moreover, only Sox9+ ductal cells could be established as long-lasting organoids from single cells (> 5 months), whereas Ptf1a+ acinar cells could only form organoids in clusters, with short-

A



B

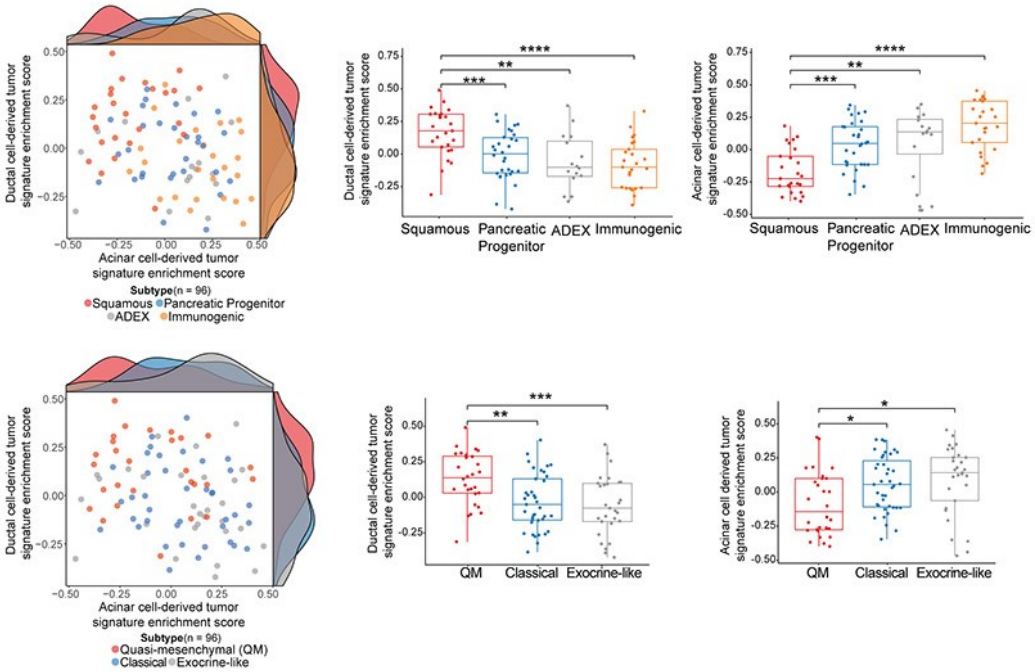


Figure 10. *The cells of origin of pancreatic ductal adenocarcinoma subtypes are fully differentiated.* ***(A)*** *In pancreatic embryonic development, and in resurgence after tissue injury, PDX1+ multipotent progenitors differentiate to tripotent cells, which form PTFA1+ tip cells that generate acini, as well as bipotent progenitors that form SOX9+ duct cells.* ***(B)*** *Targeting acinar cells gives rise to five subtypes of PDAC, whereas targeting ductal cells leads to two subtypes. [(Figure (A) taken from (Backx et al., 2022); Figure (B) taken from (Flowers et al., 2021)]*

term survival noted (3-4 passages) (Huch *et al.*, 2013). This strongly evidences that facultative pancreatic progenitors with stem-like features arise from ductal epithelium.

However, it is well documented that with stress, acinar cells can transdifferentiate to ductal cells through Nestin-positive intermediates, and other native cell types can readily intercalate with these metaplastic cells (Means *et al.*, 2005;

Blaine *et al.*, 2010). Accordingly, both acinar and ductal epithelial cells have been interrogated as the possible origin of PDAC. A seminal study to answer this question established an accurate inducible-carcinogenetic murine model. Using *Ptf1a* or *Sox9* with tamoxifen-dependent *Cre* to respectively target acinar or ductal cells, *Kras* oncogene was constitutively expressed along with null, monoallelic or biallelic *Trp53* knockout to create a tumorigenicity gradient. (Flowers *et al.*, 2021). The insightful findings were: 1) Oncogenic *Kras* activation alone can initiate PDAC in acinar cells, whereas subsequent *Trp53* mutation was required in ductal cells. 2) Early acinar-to-ductal metaplasia and pancreatic intraepithelial neoplasia (PanIN) lesions were observed in the acinar model, whereas this was not observed within the ductal model. 3) Distinct transcriptional signatures are observed for acinar-derived PDACs (upregulating extracellular matrix and cell adhesion pathways) and ductal-derived PDACs (enriching ribosome function and glycolysis genes). Overall, the acinar signature corresponded to the predominant classical subtype of PDAC (further subclassified as progenitor, ADEX, immunogenic, and exocrine-like), whereas the ductal signature aligned with the rarer basal-like subtype (the squamous and quasi-mesenchymal subclasses) (Figure 10B). The PanIN-independence and unique biomarking of duct-derived PDAC has been robustly corroborated by a further study (Ferreira *et al.*, 2017).

However, another team dispute that acinar cells transform more readily than ductal cells; when inducing *Kras* activation and *Trp53* homozygous knockout, ductal PDACs arose sooner with more aggression than acinar PDACs (Lee *et al.*, 2019). The context of *Trp53* knockout could be highly relevant – on this background, *Kras* oncogenesis is sufficient for immediate ductal cell transformation, whereas metaplastic change is required prior to acinar cell transformation. This accentuates the importance of interrogating mutations in the full context of concomitant genomic changes and cellular stresses.

Thus, when considering the origin of HGSOC, although stem cells are the likely suspect, there are three caveats: 1) Superadded physiological stress may facilitate the transformation of non-stem cells, as seen in colorectal cancer; 2) The possibilities of lineage specification changes and de-differentiation with *BRCA1* mutation opens all fallopian tube epithelial cells as possible candidates, as learned from basal-like breast cancer; 3) Targeting different fallopian tube epithelial cells may lead to different subtypes of HGSOC/LGSOC, as for PDAC.

CHAPTER 4

Maintenance of Stem Cells *In Vitro*

4.0 Immediate Study of *ex vivo* Samples has Limitations

Accurate *in vitro* study of tumour initiation requires a suitable culture system to grow stem cells, or the use of fresh tissues. A single-cell transcriptomic study of the cellular heterogeneity of the human fallopian tube (hFT) epithelium revealed that both cryopreservation and establishment of monolayer culture altered the single cell transcriptomes; changes with overnight culture included induction of otherwise rare genes (*CD44*), loss of hormone receptors (*ESR1*), loss of stemness markers (*LGR5* and *RSPO1*), loss of tissue-specific markers (*OVGP1*) and loss of ciliary organisation (Hu *et al.*, 2020). Furthermore, single-cell analysis of hFTs has revealed that, additional to secretory and ciliated epithelial cells, a diversity of cell types comprise the epithelium and stroma (Ulrich *et al.*, 2022). The hFT mesenchymal tissue (fibroblasts, myofibroblasts, smooth muscle), vascular components (pericytes, blood endothelial cells, lymphatic endothelial cells) and immune cells (B cells, T/NK cells, mast cells and macrophages) are all believed to have intimate roles in HGSOC tumour initiation, with contributions pre- and post- metastasis being likely (Charbonneau *et al.*, 2013; Touboul *et al.*, 2014; Motohara *et al.*, 2019). Only when studying fresh tissue are both cellular architecture and cellular heterogeneity fully preserved. However, obtaining early specimens in HGSOC is rare, and the sample can only be studied at a single time-point (Labidi-Galy *et al.*, 2017). Additionally, aside from flash-treatment with chemicals, hormones and putative mutagens, followed by immediate assays, it is difficult to interrogate the transformative behaviour of the tissue. Thus, fresh tissues are insufficient for stepwise genomic study of HGSOC tumorigenesis.

4.1 Stem Cells are Rapidly Lost in Primary Cultures

The simplest *in vitro* system is primary cell culture, the growth of freshly obtained cells harvested from organs. However, long-term culture of primary epithelial tissue has historically caused rapid loss of stem cells, leading to apoptosis (Grossmann *et al.*, 2003). This was largely attributable to the loss of anchorage to extracellular matrix (ECM) proteins through integrins (Sträter *et al.*, 1996). Use of ROCK inhibitor Y-

27632 in primary cultures has been evidenced to substantially reduce the scale of this detachment associated death (Koyanagi *et al.*, 2008). In 2D hFT epithelial cultures optimised to maintain stem cells, treatment with Y-27632 increased stem cell/colony survival from 3-7 days to 5 weeks, and prevented a change from epithelial-like to fibroblast-like morphology (Alsaadi, 2021). Additionally, activation of growth factor pathways such as IGF1R or EGF, acting through PI3K/mTOR, can confer further resistance to anoikis (Wilson *et al.*, 2019). In establishing primary cultures in this way, one advantage is that early passages contain microenvironmental components with intercellular signalling. However, one key disadvantage, separate to aforementioned transcriptomic changes in 2D, is that wider factors interplay limiting the feasibility of indefinite stem cell maintenance.

4.2 Feeder-Layer Cultures Maintain Stem Cells but Contain Undefined Factors

To maintain multipotent stem cells *in vitro*, it is now understood that the provision of stemness niche factors is required. Prior to this knowledge, it was found that human epidermal keratinocytes could be established as single-cell derived clones when plated in 2D cultures on top of fibroblasts (Rheinwald and Green, 1975). In this protocol, embryonic mouse fibroblasts, 3T3 cells, were plated and lethally irradiated to arrest growth pre-confluence. The enrichment of the growth medium by the 3T3 cells promoted proliferation of human keratinocyte stem cells for 20-50 generations. Feeder layer culture systems have been used to expand keratinocyte cells such as oral mucosal epithelial cells for treatment of ocular surface disorders (Nakamura *et al.*, 2004), as well as limbal epithelial cells (Sugiyama *et al.*, 2008) and iris/retinal pigmented epithelial cells (Johnen *et al.*, 2011). They were additionally used to grow embryonic and induced pluripotent stem cells for differentiation to selected lineages, before feeder/serum-free systems were popularised (Llames *et al.*, 2015). Importantly, combined use of irradiated fibroblast-feeder systems and ROCK inhibition with Y-27632 have been observed to maintain indefinite proliferation with a stem-like state in prostate, breast, liver and lung epithelia, non-keratinocyte cells (Liu *et al.*, 2012;

Suprynowicz *et al.*, 2012). However, the key disadvantages of feeder-layer cultures include the absence of well-defined culture components and, particularly in xenogenic co-cultures, exposure to foreign pathogens, alongside lack of known feeder systems for many multipotent stem cell types (Amit *et al.*, 2004). With modern technologies in stem-cell maintenance, feeder-layer cultures are growing redundant.

4.3 Stem Cell Immortalisation is Feasible but Dysregulates Critical Pathways

The finite lifespan of primary cells in culture can be overcome through immortalisation; a means of transforming primary cells to acquire indefinite proliferative capacity. This can be achieved by multiple approaches, including: 1) Incorporating telomerase or telomerase reverse transcriptase (hTERT) that collectively stabilise chromosomal ends (Tsai *et al.*, 2010); 2) Inactivation of p53 and Rb protein through introduction of E6/E7 Human Papillomavirus, E1A/E1B adenoviral oncogenes or SV40 (Shay, Pereira-Smith and Wright, 1991; White, 2006); 3) Provision of oncogenes *HRAS*/*KRAS* to bypass cell-cycle arrest (Liu *et al.*, 2004). For human mesenchymal stem cells, immortalisation has been an effective way to retain stem-like features, and stem cells from the apical papilla (SCAPs) have been immortalised for use in endodontic treatment (Tsai *et al.*, 2010; Cheng *et al.*, 2023). Moreover, immortalisation of colonic epithelial cells has been possible such that cells express the key epithelial stemness marker LGR5, remain capable of multilineage colonic differentiation, and can form organotypic cultures (Roig *et al.*, 2010). It should be noted that in most immortalised tissue cultures, the true endogenous stem cells will be lost. Additionally, it is well recognised that immortalised versus primary cells are known to have atypical cellular behaviours, reduced cellular heterogeneity, genomic and genetic aberrations, and have limited relevance for *in vivo* work (Richter *et al.*, 2021). An underlying consideration of deal-breaking importance is that all discussed mechanisms of immortalisation interfere with *TP53*/p53; use of this model would eliminate our ability to effectively study the mutation that ubiquitously initiates HGSOC (Ahmed *et al.*, 2010).

4.4 Organoids are an Advanced Culture System for Stem Cells

In the late 2000s, with the intestinal stem cells in the basal area of crypts identified as being marked by Lgr5, the Clevers Laboratory sought to recapitulate the stemness niche *in vitro*, in an effort to maintain these cells (Sato *et al.*, 2009). *In vivo*, niche-conferring Paneth cells secrete Wnt3a and several proliferative signals in a paracrine manner to stem cells, and Lgr5 maintains the receptors for Wnts in the cell membrane (Sato, van Es, *et al.*, 2011). It was therefore reasoned that promoting WβC signalling, providing a consortium of growth factors, and polymerisation within extracellular matrix (ECM) should allow stem cells to be cultured.

However, the finding that intestinal stem cells spontaneously developed into ‘mini-guts’ or ‘organoids’ was unforeseen, and their physiologically accuracy has opened up another era of research. For instance, a forskolin-induced swelling assay on patient-derived intestinal organoids is licensed as a method to evidence with 100% accuracy the efficacy of channel-modulating cystic fibrosis drugs (ivacaftor and lumacaftor) in patients with non-classical (non-ΔF508) *CFTR* mutations (Boj *et al.*, 2017). Moreover, intestinal organoids are able to act as ‘molecular band-aids’ to seal intestinal epithelial lesions, and the first in-human organoid transplantation trial is underway to treat intractable ulcerative colitis (Tokyo Medical and Dental University), with highly promising initial results (Watanabe *et al.*, 2022; Yui, 2022).

It therefore comes as no surprise that organoid research to accurately model cancer initiation, progression and treatment is well underway for a wide array of tissue types. With this said, organoid culture is challenging to optimise. First and foremost, to understand its essence, a deep dive into WβC signalling is required.

CHAPTER 5

Wnt Signalling and Organoid Culture

5.0 Wnt/β-catenin Signalling – an Overview

The Wnt/β-catenin signalling pathway is an evolutionarily conserved pathway that regulates important aspects of cell fate determination, cell migration, cell polarity, neural patterning, and organogenesis during embryonic development. Its dysregulation has been implicated in various human diseases – in particular, cancer. In 1982, Nusse and Varmus found that proviral insertion of the mouse mammary tumour virus at the locus of *Wnt1* (formerly known as *Int1*) led to tumorigenesis, characterising its involvement as a proto-oncogene in mice. Within the next decade, the downstream molecule β-catenin was discovered independently by multiple groups, owing to its functional pleiotropy. It was first shown to form the cell adhesion complex with E-cadherin as well as α-catenin and γ-catenin (plakoglobin) (Ozawa, Baribault and Kemler, 1989). Shortly after this, its role as a transcriptional co-activator in *Drosophila* and *Xenopus* developmental biology was found (McCrea, Turck and Gumbiner, 1991). It had previously been shown that Wingless in *Drosophila* had a role in regulating the anterior-to-posterior axis within the embryonic parasegments (Nüsslein-Volhard and Wieschaus, 1980). This later transpired to be homologous to Wnt1, and its corresponding role in invertebrate and vertebrate axis patterning was soon described (Rijsewijk *et al.*, 1987; McMahon and Moon, 1989). In 1996, Xenopus microinjection studies of LEF-1 and Tcf-3 showed that they can form complexes with β-catenin, necessary for the transcription of Wnt/β-catenin target genes (Behrens *et al.*, 1996; Molenaar *et al.*, 1996). Over the past two decades, the importance of the WβC axis in stem cell biology has become increasingly apparent.

5.1 Production and Secretion of Wnt Ligands

The Wnt ligands are a 19-member family of highly hydrophobic, cysteine-rich secreted glycoproteins. Historically, this made them difficult to purify and their crystalline structure elusive, especially as their primary sequences differed from typical protein folds. However, their structure was clarified using Xenopus Wnt8 bound to mouse Frizzled-8 (Fz8) (Janda *et al.*, 2012). Wnts usually contain 350 residues

resembling a ‘hand’, with an ‘index finger’ and ‘thumb’ to interact with targets. Post-translational modification is key for their maturation; Wnt3a, for instance, is palmitoylated at its conserved cysteine (C77), adding to hydrophobicity. Mutating at this site (C77A) leads to loss of function. Additionally, Wnt3a is modified at a conserved serine (Ser209) with monounsaturated palmitoleic acid by the acylating enzyme, Porcupine (Porcn) (Kadowaki *et al.*, 1996; Zhai, Chaturvedi and Cumberledge, 2004; Takada *et al.*, 2006). Mutation of Ser209 therefore hinders acylation, release of Wnt3a from the endoplasmic reticulum, and secretion (Takada *et al.*, 2006). After Porcn has modified Wnt, transporters such as Wntless, Evi or Sprinter chaperone it to the plasma membrane (Bartscherer *et al.*, 2006; Bänziger *et al.*, 2006; Goodman *et al.*, 2006; Coombs *et al.*, 2010). Polarised vesicular release mechanism co-exist; apical Wnt release is typically acylation-independent, whereas basolateral secretion is acylation-independent (Chen *et al.*, 2016; Takada *et al.*, 2017). Their extreme hydrophobicity may enable direct, exosome-free partition into neighbouring membranes, amplifying local concentrations for efficient paracrine signalling (Willert *et al.*, 2003).

5.2 WNT Ligands Interact with FZD Receptors

The 19 mammalian WNTs can bind to 10 FZDs, which comprise of 7 transmembrane α-helices, and despite strictly being G protein-coupled receptors (GPCRs) this is not their main modality (Fredriksson *et al.*, 2003; Huang and Klein, 2004). They contain an extracellular N-terminus containing a 120-residue cysteine-rich domain (CRD) for Wnt binding and Fzd dimerisation, and the intracellular C-terminus has a KTXXXW motif vital for canonical signalling (Umbhauer *et al.*, 2000; Dann *et al.*, 2001). First, in XWnt-Fz8, the acylation of XWnt8 Ser187 at the ‘thumb’ domain tip, presumably the equivalent of palmitoleic acid binding to Ser209 on Wnt3a, was shown to fully engage a groove in the CRD domain. Second, a disulphide bridge (Cys315-Cys325) in the XWnt8 ‘index finger’ interacts with a hydrophobic recess between a-helices in the CRD (Janda *et al.*, 2012). These CRD contact sites are highly conserved and essential, but not sufficient, to activate canonical signalling. Canonical signalling

requires WNT to dimerize FZDs with LRP5/6 co-receptors, and different Wnts engage these co-receptors through different binding sites (Bourhis *et al.*, 2010). Recently, complex structural modelling showed this to be mediated by a Wnt N-terminal loop and C-terminal NC-linker that typically liaise with E1/E2 domains of LRP5/6 (Figure 11) (Tsutsumi *et al.*, 2023). With other Wnts (such as Wnt3a) instead binding E3/E4 domains, this explains why LRP6 antibodies variably potentiate or inhibit canonical signalling in different Wnts (Gong *et al.*, 2010). However, when studying WNT-FZD interactions, it must be considered that some WNTs can bind multiple FZDs, some

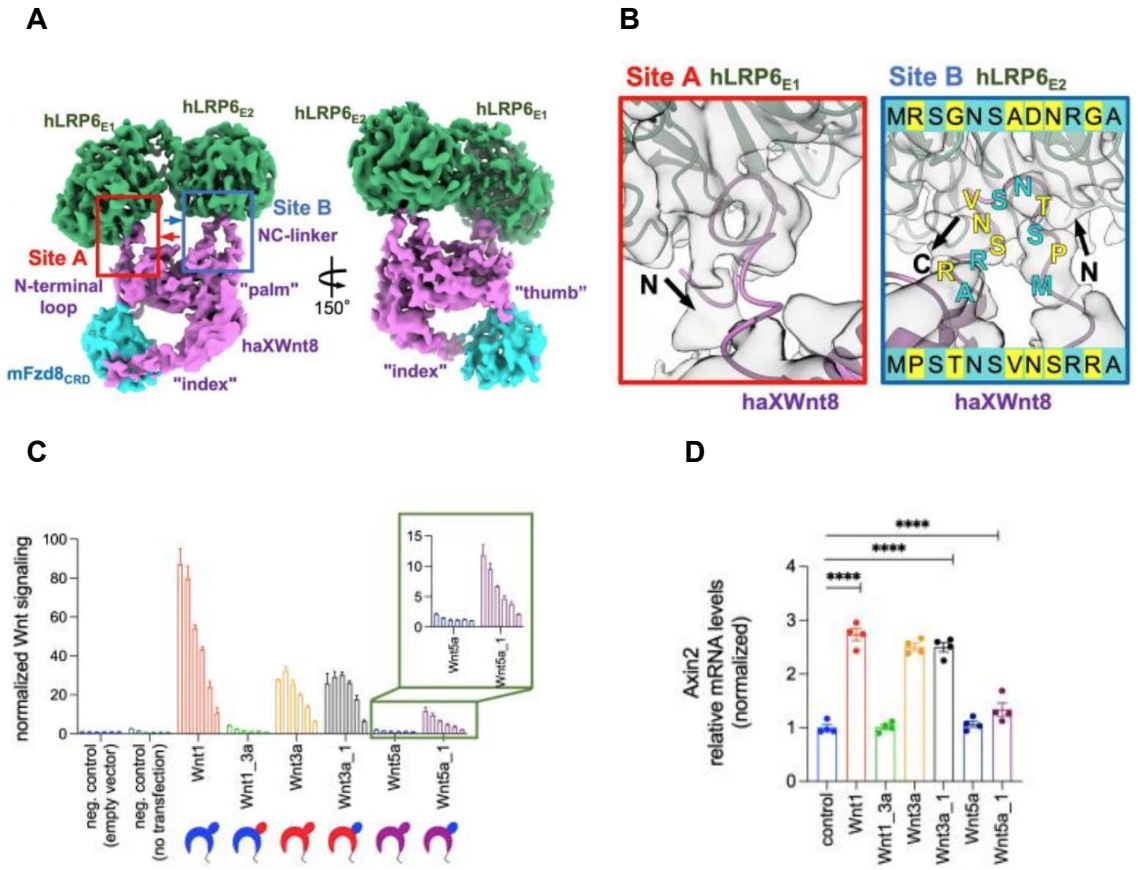


Figure 11. *To activate canonical WβC signalling Wnt NC-linkers must engage LRP5/6 co-receptors.* ***(A)*** *An engineered complex of high affinity Xenopus Wnt8 (haXWnt8), mammalian FZD8 CRD domain ($mFZD8_{CRD}$) and human LRP6 E1/E2 domains ($hLRP6_{E1E2}$); the haXWnt8 NC-linker is required to cause vast conformational change in the hLRP6 E2 β-propellor.* ***(B)*** *Close-up of sites on the haXWnt8 N-terminal loop and C-terminal loop (NC-linker) that respectively bind the E1 and E2 funnels in the $hLRP6_{E1E2}$ β-propellors.* ***(C-D)*** *Grafting the NC-linker into Wnt5a (Wnt5a_1) bestows canonical WβC signalling ability as shown by (C) TOPbrite assay and (D) Wnt target gene Axin 2 upregulation. [Figures taken from (Tsutsumi et al., 2023)]*

FZDs can bind multiple WNTs, redundancy is observed in WNT-FZD signalling, and several non-canonical WNT-FZD pathways exist (Chen and Struhl, 1999; Medina, Reintsch and Steinbeisser, 2000; Carmon and Loose, 2010). Moreover, although FZDs primarily interact with WNTs, they can also bind several other ligands – pathway activators such as R-spondins (RSPOs) and Norrin, or inhibitors such as Secreted FZD-Related Proteins (SFRPs) (Nam *et al.*, 2006; Bhat *et al.*, 2007; Chang *et al.*, 2015). Understanding individual roles in this complex cascade therefore requires care.

5.3 LGR5/6 and RSPOs Maintain Membrane FZDs

A discussion on WβC signalling and the stemness niche is incomplete without studying LGR5/6, the markers of many adult stem cells (Leushacke and Barker, 2012). LGR4/5/6 protein are orphan 7TM leucine-rich repeat-containing GPCRs, that along with their homologues LGR4/6, serve as receptors for R-spondins (RSPOs). Without this interaction, membrane FZDs degrade and Wnt signalling invariably fails. In mammals, there are 4 RSPOs (RSPO1-4) which share 40-60% sequence similarities, and substantial structural features (Nam *et al.*, 2006). The first Rspo domain is a hydrophobic N-terminal signal-peptide that enables secretion and promotes local effects (Jin and Yoon, 2012). The second domain comprises of two cysteine-rich furin like repeats involved in Lgr5 receptor binding. The Lgr5 ECD forms a horseshoe-like structure with 17 leucine-rich repeats (LRR), out of which Rspo1 binds to the concavity across LRR3-9 (de Lau *et al.*, 2014); The β-hairpin of the Furin-2 repeat of RSPO1 forms a phenylalanine clamp around Ala190 of Lgr5, which is indispensable to Wnt-signalling. Further interactions occur between two Rspo1 Furin-1 residues and Lgr5, boosting signalling, but are dispensable (Peng, de Lau, Forneris, *et al.*, 2013). The binding interface is conserved across Rspo1-4/Lgr4-6 in multiple species, highlighting its pivotal nature (Wang *et al.*, 2013).

Stabilised Rspo/Lgr(5/6) complexes degrade membrane E3 ubiquitin ligases Rnf43/Znrf3, to prevent their degradation of FZDs. Structural studies have clarified how

this occurs. In a trimeric complex of Rnf43 ECD and Lgr5/RSPO, it was shown that Furin 1 and Furin 2 RSPO1 domains bridge Lgr5 to Rnf43 (P.-H. Chen *et al.*, 2013). Similar interactions were illustrated between Rspo1/Znrf3, as well as Rspo2/Rnf43-Znrf3 (Peng, de Lau, Madoori, *et al.*, 2013; Zebisch *et al.*, 2013). The trimer of Rspo/Lgr/E3-ligase is understood to drive auto-ubiquitination and clearance of Rnf43/Znrf3 from the plasma membrane, sustaining Fzd receptors to enable Wnt-signalling (Hao *et al.*, 2012; Hao, Jiang and Cong, 2016). In the absence of RSPO1, the de-ubiquitination protein USP42 stabilises RNF43/ZNRF3 at the membrane, ensuring little to no FZD activity (Giebel *et al.*, 2021).

Revisiting intestinal crypts briefly, interestingly, while Paneth cells supply Wnt3a and essential stemness signals in a locally concentrated paracrine manner, it is stromal cells that supply Rspo3 to overcome the absolute barrier to WβC signalling – maintenance of Fzds (Sato *et al.*, 2011; Kabiri *et al.*, 2014). A model therefore forms: the intestinal epithelial stem cell most embedded in the underlying ‘niche-establishing’ stroma that interacts most intimately with a neighbouring ‘niche-restricting’ Paneth cell is most likely to clonally dominate crypt regeneration.

5.4 Disassembly of the β-catenin Destruction Complex

When the canonical Wnt pathway is ‘Off’, AXIN1 behaves as a flexible cytosolic scaffold for CK1α, GSK3β and β-catenin to form the β-catenin destruction complex (Figure 12A). Firstly, co-recruitment of GSK3β and β-catenin to AXIN1 is reciprocally productive; GSK3β phosphorylates AXIN1, ensuring its cytosolic stability, and GSK3β gains 20,000 fold greater efficacy in phosphorylating β-catenin (Yamamoto *et al.*, 1999; Dajani *et al.*, 2003). This is attributed to proximity effects alone; GSK3β does not gain catalytic activity from binding to the scaffold (Stamos and Weis, 2013). Similarly, when CK1α is recruited to a distal site on AXIN1, it is better able to phosphorylate β-catenin

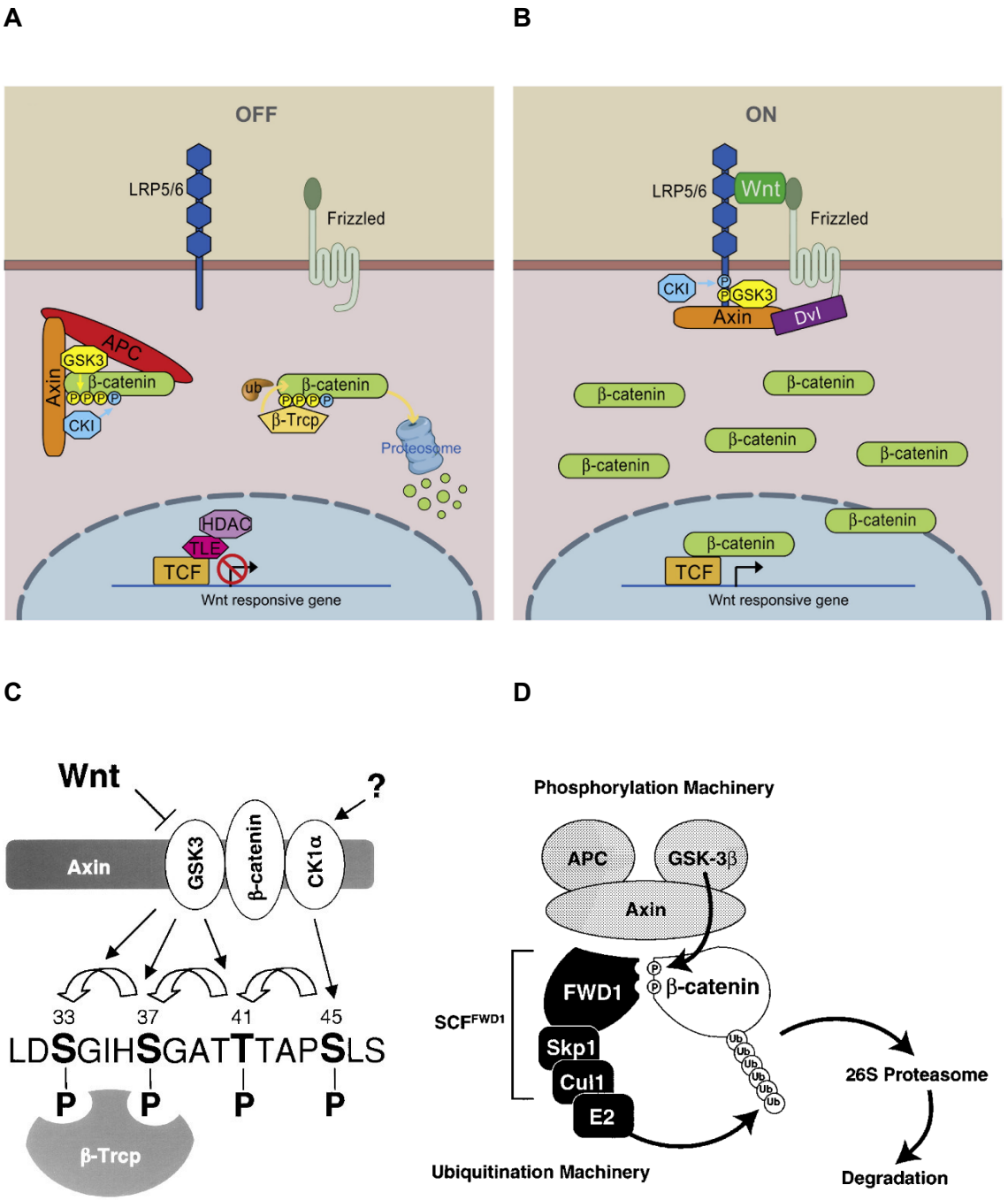


Figure 12. *Canonical WβC signalling.* ***(A)*** *Without Wnt, a cytoplasmic complex of AXIN, CK1α, GSK3β and APC forms, which targets β-catenin for destruction.* ***(B)*** *With Wnt ligand, a complex with Fzd and LRP5/6 recruits components of the β-catenin destruction complex to the membrane, resulting in its disassembly – β-catenin is free to translocate to the nucleus and co-activate TCF.* ***(C)*** *Within the β-catenin destruction complex, CK1α and GSK3β surround β-catenin, creating an N-terminus 'phospho'-degron tail that recruits β-TrCP.* ***(D)*** *Axin, β-TrCP (FWD1) and β-catenin collectively recruit Skp1/Cul1/E2 ubiquitin-conjugating enzymes, appending a multi-ubiquitin tail to β-catenin that is recognised by the 26S proteasome for degradation. [(A) and (B) taken from (MacDonald, Tamai and He, 2009); (C) taken from (Liu et al., 2002); (D) taken from (Kitagawa et al., 1999)]*

due to proximity effects rather than increase in catalytic ability (Sobrado *et al.*, 2005). At a proximal site, the RGS domain of AXIN1 recruits APC (Fagotto *et al.*, 1999). This must occur in order for GSK3β and CK1α to phosphorylate APC, and it is the phosphorylated APC domain that competes with AXIN1 for binding to β-catenin (Ikeda *et al.*, 2000; Ha *et al.*, 2004). The β-catenin destruction complex is then complete – first, β-catenin is phosphorylated by CK1α at Ser45, then GSK3β phosphorylates Thr41, Ser37 and Ser33 (Figure 12C) (Liu *et al.*, 2002).

This phospho-'degron' motif is a docking site for the F-box E3 ubiquitin ligase β-TrCP (FWD1) that recruits the Skp1/Cul1/E2 apparatus, which multi-ubiquitinates β-catenin, targeting it for proteasomal degradation, shutting off the WβC signal (Figure 12D) (Kitagawa *et al.*, 1999). Throughout this process, APC can be viewed as the master co-ordinator. It has roles in recruiting β-catenin, and maintaining the β-catenin phospho-'degron' by protecting it from surrounding phosphatases (Ha *et al.*, 2004; Su *et al.*, 2008). Additionally, a distal site of AXIN1 can recruit PP2A and PP1, which dephosphorylate AXIN1 and are conventionally thought to negatively regulate Wnt signalling (Luo *et al.*, 2007). It is thought, however, that in the right context, PP2A may serve to dephosphorylate APC, liberating phosphorylated β-catenin for destruction (Xing *et al.*, 2003). APC may therefore liaise with other proteins to co-ordinate cyclical AXIN1 and β-catenin turnover, creating scope for a responsive signalling pathway.

When the canonical Wnt pathway is switched 'on' by WNT-FZD-LRP5/6 signalling complex formation, Dishevelled (DVL) is recruited to the LRP5/6 intracellular domain (ICD), which oligomerises multiple signalling complexes (MacDonald, Tamai and He, 2009). This facilitates the loading of AXIN1-GSK3β complexes (Zeng *et al.*, 2008). The LRP5/6 ICD contains 5 P-P-S[/T]-P-x-S[/T] (proline/serine/threonine) repeats – primarily GSK3β, alongside other proline-directed kinases including Protein Kinase A (PKA), PFTK1 and GRK5/6, phosphorylates the first serine residues in these sites (Niehrs and Shen, 2010). This primes the non-proline directed kinase CK1α to phosphorylate the x-S serines and various N-terminal sites (Zeng *et al.*, 2005). Tightly-

bound cytosolic membrane complexes with AXIN1, CK1α and GSK3β thus form, preventing the assembly of β-catenin destruction complexes (Figure 12B). Unphosphorylated β-catenin is then cytosolically available to exert nuclear effects.

5.5 β-catenin Transcriptional Targeting

β-catenin has some sequence homology to Importin-β/Beta-karyopherin/Transportin, suggesting it directly enters the nucleus (Fagotto, Glück and Gumbiner, 1998). Non-hydrolysable GTP analogues, RAPGEF5 and its Rap1a/b GTPases appear to aid this process (Griffin *et al.*, 2018). Moreover, the sparing of APC recruitment to the membrane in WβC signalling, its high affinity to β-catenin, and its functions in cytoplasmic-nuclear shuttling validate it as a β-catenin nuclear chaperone (Henderson, 2000). Thus, utilising several strategies, nuclear translocation is rapid.

Being core to the WβC axis, β-catenin has a ‘two-fold handicap’; it cannot bind DNA and lacks transactivation potential (Söderholm and Cantù, 2021). It primarily exerts its effects through binding TCF/LEF, but also interacts with other transcription factors including FOXO, Pit1, Prop, HIF1α and SOX family proteins (Kioussi *et al.*, 2002; Essers *et al.*, 2005; Olson *et al.*, 2006; Kaidi, Williams and Paraskeva, 2007; Kormish, Sinner and Zorn, 2010). There are four TCF/LEF proteins – 3 activators, TCF7 (=TCF1), LEF (=TCF1a), TCF7L2 (=TCF4), and 1 repressor TCF7L1 (TCF3) – although alternative splicing and promoter variation create a spectrum of isoforms (Hoppler and Kavanagh, 2007). TCF/LEFs have a conserved HMD box and basic tail forming the DNA binding domain. A second, small DNA binding domain, the ‘C clamp’, depends on four cysteine residues for its function (Atcha *et al.*, 2007).

Without β-catenin, TCF/LEFs inhibit transcription with the assistance of co-repressors, in particular Groucho/TLEs (Figure 12A) (Cavallo *et al.*, 1998). The central domain harbouring these co-repressors is directly outcompeted with β-catenin abundance, switching gene repression to activation (Figure 12B) (Daniels and Weis,

2005; Arce, Pate and Waterman, 2009). β-catenin then recruits Creb-binding protein (CBP) and p300 to the Wnt-response element, acetylating histone subunits H3 and H4 to drive transcription of Wnt-target genes (Hecht *et al.*, 2000; Kioussi *et al.*, 2002). The list of Wnt target genes is extensive, however they can be clustered into cellular functions (Table 1) (Nusse, 2023). From this non-exhaustive list, it is clear how hyperactivation of WβC signalling alone, as for colorectal cancer, advances several of the hallmarks of cancer (Hanahan and Weinberg, 2011; Lecarpentier *et al.*, 2019).

Processes	***Targets***	***Functions***	***References***
Cell growth & proliferation	c-Myc; Cyclin D; FGF18/9/20; Met; VEGF; Telomerase;	Proliferation; cell cycle progression; cell growth; wound healing; angiogenesis; immortalisation	(Duronio and Xiong, 2013; Farooq *et al.*, 2021)
Cell adhesion & migration	CD44; uPAR; MMP2/7/9	Cell-cell interactions; proteolysis; remodelling of extracellular matrix	(Sneath and Mangham, 1998; Zhai *et al.*, 2022)
Signal transduction	PPARδ; EGF receptor; c-jun/fra-1	Lipid metabolism; cell survival; AP-1 transcription factor-related pathways	(Gazon *et al.*, 2018; Liu *et al.*, 2018)
Tissue development and maturation	BMP4; Runx2; Islet1; Tbx3	Bone/cartilage development; osteoblast differentiation; heart/nervous system development; mesoderm formation	(Lozano-Velasco *et al.*, 2022)
Tissue immune response	IL8; CTLA4	Inflammation; negative regulation of T-cell activation	(Fousek, Horn and Palena, 2021)
Pluripotency	Nanog; Oct4; Sox2	Activation of embryonic stemness modules and stem cell self-renewal	(Takahashi and Yamanaka, 2006)
Epithelial-to-Mesenchymal Transition	Snail; Twist	Downregulate E-cadherin; upregulate fibronectin	(Hu *et al.*, 2020)
Promote WβC signalling	Wnt3a; LGR5; DLL1; Jagged	Wnt ligand; Fzd receptor maintenance; Notch ligands	(Takam Kamga *et al.*, 2020)
Repress WβC signalling	DKK1; AXIN2; SFRP2; SOX9	LRP5/6 inhibitor; β-catenin destruction complex; Wnt competitor	(Topol *et al.*, 2009)

Table 1. *Wnt target gene-encoded proteins. Activation of the canonical Wnt module provides cells advantages for growth, invasion, microenvironment optimisation, and self-renewal. [Full database of Wnt target genes compiled by (Nusse, 2023)]*

5.6 Non-Canonical Wnt signalling

Despite inevitable cross-talk between pathways, non-canonical Wnt signalling is defined as being independent of β-catenin. There are at least 9 well categorised non-canonical pathways, but two main branches exist – the Wnt/Planar Cell Polarity (Wnt/PCP) and the Wnt/Ca^{2+} pathways (Semenov *et al.*, 2007; Komiya and Habas, 2008). Whilst Wnts and Fzds are traditionally categorised into 'canonical' and 'non-canonical', this is often unhelpful as context-dependent variability means classifications disagree (Xiao *et al.*, 2017; Chae and Bothwell, 2018). With this said, there is consensus that certain Wnts (Wnt4, Wnt5a and Wnt11) and certain Fzds (Fzd3 and Fzd6) are typically non-canonical (Xiao *et al.*, 2017).

Although apical-basal polarity is well characterised in epithelial cells, the Wnt/PCP pathway regulates the orthogonal axis, controlling directionality across the epithelial surface itself. For instance in vertebrates, it orientates stereocilia in the inner ear epithelium, hair follicle organisation, patterning in gastrulation, and cell migration of neural crest cells (De Calisto *et al.*, 2005; Matsui *et al.*, 2005; Wang and Nathans, 2007). It relies on a non-canonical Wnt-ligand binding to Fzd3/6 independent of Lrp5/6, with downstream GTPase activity (Figure 13 - Left). With Wnt/PCP target genes co-ordinating invasion and migration, overactivation drives EMT and poor outcomes in several malignancies – for instance, in pancreatic cancer (aberrant Ror2 induction) and ovarian cancer (Ror1 overexpression) (Zhang *et al.*, 2014; Carbone *et al.*, 2018; Chen *et al.*, 2021).

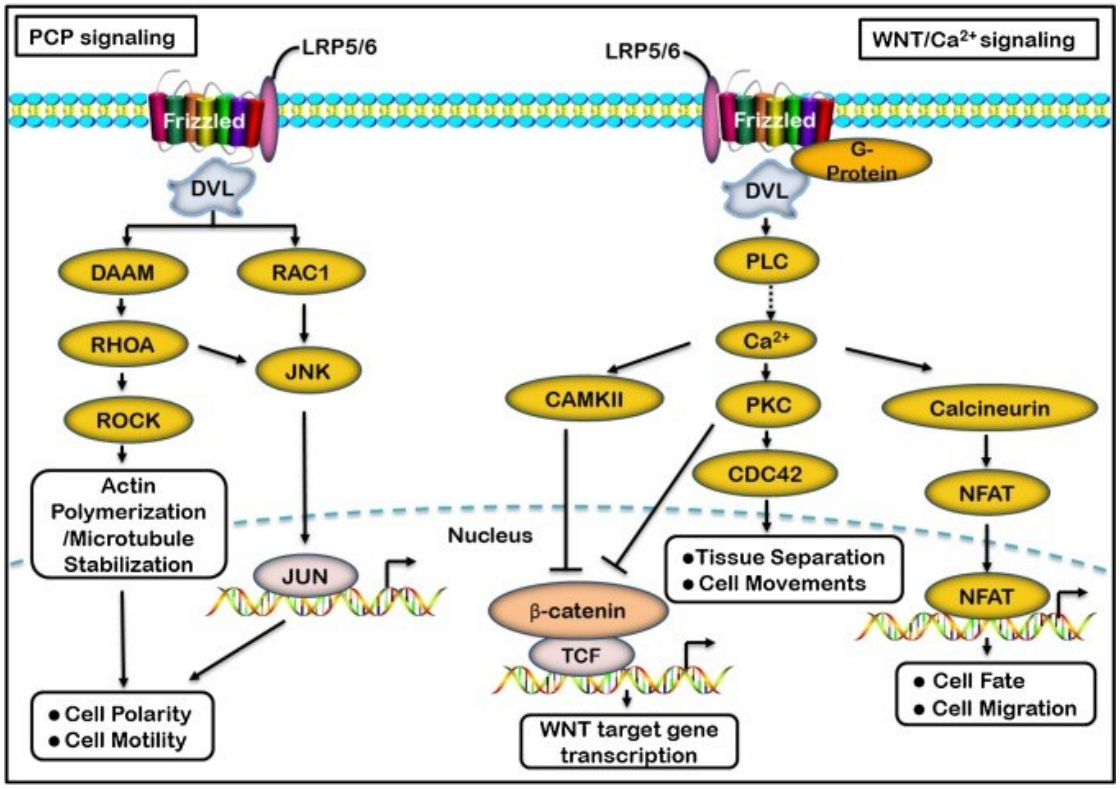


Figure 13. *An overview of non-canonical Wnt/PCP and Wnt/Ca^{2+} signalling. [Figure taken from (Xiao et al., 2017)]*

(Wnt/PCP Signalling - Left). *First, a Wnt5a-type ligand binds to the Fzd(3/6) receptor, and utilises the Ror2 or Ryk co-receptors in place of Lrp5/6, the vitality of which to canonical signalling was recognised earlier (Lu et al., 2004; Nishita et al., 2006). Two pathways then follow. Firstly, the Ror2/Ryk signal activates Dvl, which recruits a scaffold protein, Daam1. The N-terminus of Daam1 creates a complex with RhoA-GDP and a Rho guanine exchange factor, WGEF (Habas, Kato and He, 2001; Tanegashima, Zhao and Dawid, 2008). This generates a functioning GTPase which activates Rho-associated Kinase (ROCK) and myosin, leading to cytoskeletal remodelling and microtubule stabilisation (Komiya and Habas, 2008). Secondly, DVL DEP domain recruits RAC1 GTPase, activating JNK and the multi-modular AP1 complex, regulating cell polarity, motility and morphogenesis (Xiao et al., 2017).*

(Wnt/Ca^{2+} Signalling - Right). *Wnt(5a) binding to Fzd(3/6)-Ror1/2 enables the G protein complex to recruit DVL, which co-activates PLC. This then cleaves PIP2 to DAG and IP3, the latter of which drives Ca^{2+} release from the ER (Xiao et al., 2017). It is also thought that a Dvl-Axin-GSK3β complex may form at the Fzd intracellular domain, phosphorylating Ror1/2 to directly activate PLC independent of GPCR activity (De, 2011). Although recruitment of BC destruction components would imply synergy of canonical and non-canonical pathways, they in fact antagonise each other; intracellular Ca^{2+} activates Calcineurin, CamKII and PKC, the latter two of which inhibit nuclear β-catenin/TCF function (Gwak et al., 2006; Flentke et al., 2014).*

The Wnt/Ca^{2+} pathway was first discovered when it was found that injection of XWnt5a (but not XWnt-8) mRNA into zebrafish embryos induces calcium transients in the germinal disc (Slusarski *et al.*, 1997). Inhibitor studies later related this to Fzd G protein-coupled receptor (GPCR) activity (Slusarski, Corces and Moon, 1997). This pathway culminates in the synthesis of Calcineurin, CamKII and PKC, which collectively activate a variety of transcription factors (Figure 13 - Right). Calcineurin induces NFAT movement to the nucleus, controlling cell fate and migration (Mancini and Toker, 2009). PKC and CamKII activate NF-κB, driving inflammatory damage, proliferation, apoptosis evasion, and invasion/EMT (Jridi *et al.*, 2021). PKC, with RhoA and CDC42, activates CREB, causing similar downstream effects (Slater *et al.*, 2001). Again, pathway dysregulation is tied to poor outcomes; in colon cancer, CamKIIβ and NFAT1/2/3/4 expression signatures increase at advanced stages, and correspond strongly to reduced survival (Sarabia-Sánchez *et al.*, 2023 - Supplementary Figure 3).

5.7 Factors to Establish Murine Intestinal Organoids

To grow murine intestinal organoids from single epithelial cells *in vitro*, a cocktail of factors are required to fulfil the mesenchymal and Paneth cell roles, with attention to WβC signalling (Sato *et al.*, 2009). *In vivo* models had evidenced Rspo1 to induce crypt cell proliferation on injection into mice (Kim *et al.*, 2005). The importance of Wnt proteins (Wnt3a) *in vivo* was understood due to the anti-proliferative effects of overexpressing the endogenous Lrp6 co-receptor inhibitor, Dkk1 (Pinto *et al.*, 2003). Epidermal Growth Factor (EGF) is also essential, evidenced by knockout of its receptor causing intestinal epithelial defects, absent crypt development and aberrant maturation (Duh *et al.*, 2000). This is ascribable to the downstream effect of EGFR-family heterodimers in activating PI3K-AKT, MAPK and JAK-STAT pathways, encouraging proliferation, survival and stemness (Figure 14A) (Abud, Chan and Jardé, 2021). Noggin was shown *in vivo* to be a patterning mesenchymal-to-crypt signal inhibiting BMP4 action through Type I BMP receptors, which otherwise drives differentiation through R-Smads (1/5/8) and Co-Smad (Smad4) (Figure 14B) (Qi *et al.*, 2017).

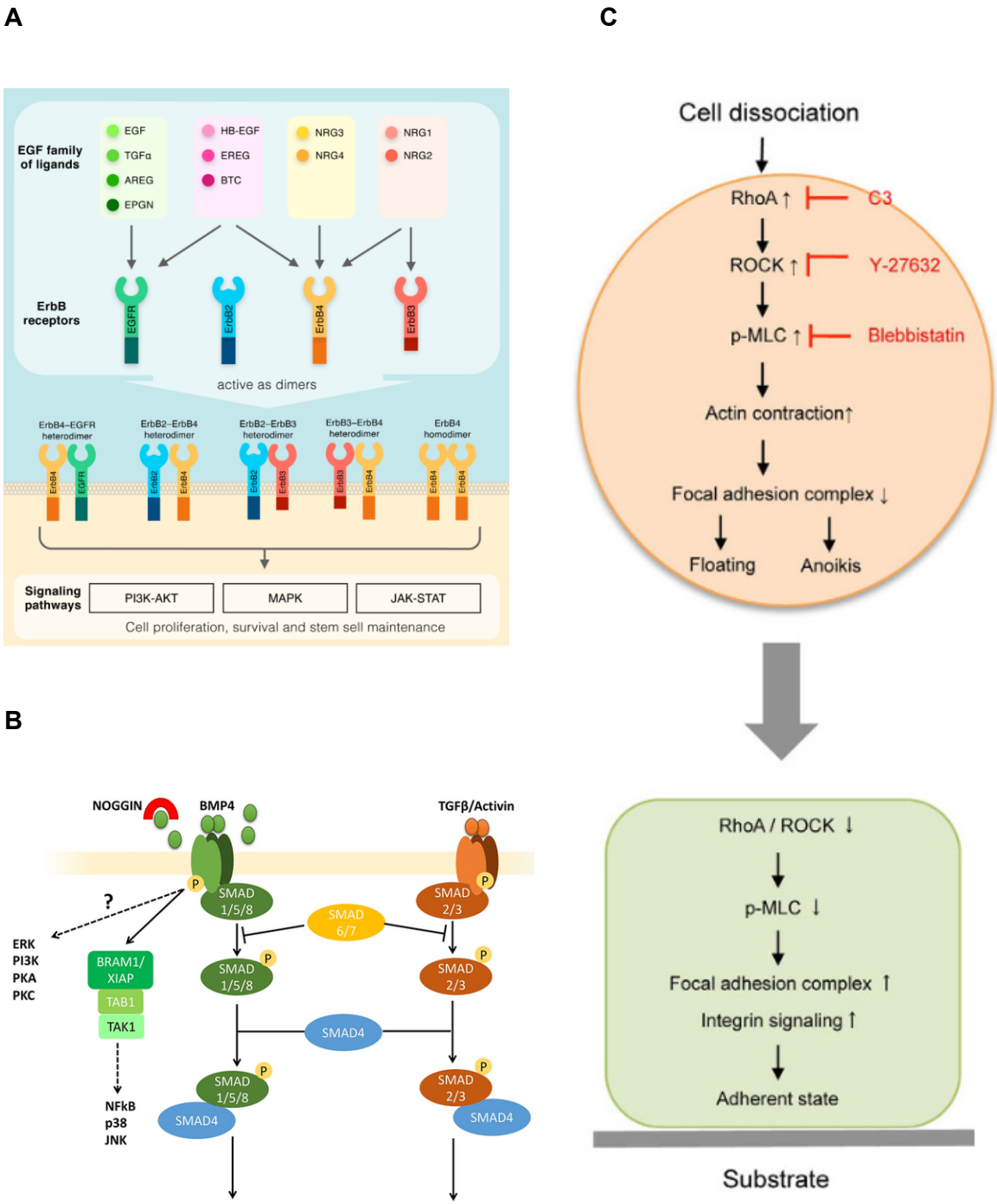


Figure 14. *Mechanisms of essential organoid factors.* ***(A)*** *Members of the EGF family of ligands dimerise corresponding receptors, activating signalling pathways that confer growth advantages.* ***(B)*** *The two canonical TGFβ pathways include BMP-SMAD1/5/8 and Activin/Nodal-SMAD2/3 signalling, which drive differentiation – Noggin inhibits the former, and A83.01 the latter.* ***(C)*** *Cell dissociation increases RhoA/ROCK/p-MLC, driving actin contraction, which suppresses the focal adhesion complex and integrin signalling – ROCK inhibitor Y-27632 opposes this. [(A) taken from (Abud, Chan and Jardé, 2021); (B) taken from (Roberts et al., 2022); (C) taken from (Okumura et al., 2016)].*

Moreover, the Rho Kinase (ROCK) inhibitor Y-27632 antagonises the RhoA/ROCK/p-MLC-mediated cell death, anoikis, that occurs in intestinal epithelial cells lacking cell-cell interactions (Figure 14C) (Hofmann *et al.*, 2007; Okumura *et al.*, 2016). These form the major constituents of organoid media. Lastly, Laminins α1/α2 are enriched at the crypt base, with Laminins α3/α4 enriched in the villous region (Sasaki *et al.*, 2002; Antfolk and Jensen, 2020); cells were thus polymerised in laminin-rich ECM, prior to addition of medium.

5.8 Factors to Establish Human Intestinal Organoids

Although more physiologically relevant, human intestinal organoids are more difficult to establish, and require further factors (Sato, Stange, *et al.*, 2011). p38 MAPK causes ligand-mediated degradation of EGFR, and drives secretory cell differentiation, so p38 inhibitor SB202190 is required for long-term culture (Frey *et al.*, 2006). Moreover, although Noggin inhibits BMP4-Smad1/5/8 pathways, TGFβ/Activin/Nodal signalling through SMAD2/3 also blocks differentiation; this pathway is suitably repressed with SB431542 or the more potent A83.01 (Sato, Stange, *et al.*, 2011). Other less essential signals are often manipulated in intestinal organoid growth: 1) Valproic acid or Jagged-1, Notch activators, maintain the stemness state, and are beneficial for early phase single-cell cultures (Grabinger *et al.*, 2014); 2) Nicotinamide inhibits sirtuins, which regulate senescence and differentiation, but its value in cultures is debated (Denu, 2005; Fujii *et al.*, 2015); 3) PGE2 activates components of canonical Wnt-signalling and protects against anoikis, as well as causing organoid swelling through luminal anion secretion (Buchanan and DuBois, 2006; Fujii *et al.*, 2016); 4) IL-22 activates JAK/STAT3 signalling, which potentiates Notch and Wnt pathways, and can be used in replacement for EGF (Lindemans *et al.*, 2015); 5) GSK3β inhibition with CHIR99021 disassembles the β-catenin destruction complex, directly activating WβC signalling, however its promiscuity means it likely causes broad transcriptional changes (Yin *et al.*, 2014; Beurel, Grieco and Jope, 2015). The collective effect of many of these factors is illustrated below (Figure 15) (Holmberg *et al.*, 2017).

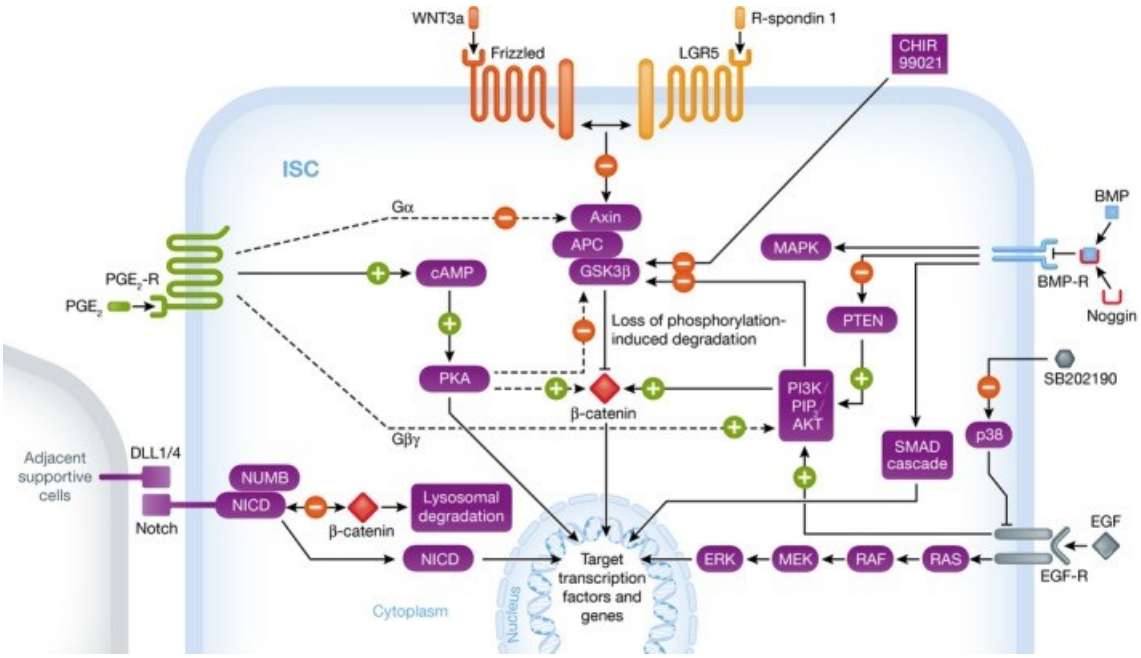


Figure 15. *Mechanisms of several less essential organoid factors. Through diverse routes, the influence of SB202190, DLL1/4, PGE2 and CHIR99021 on nuclear β-catenin and growth signalling are shown. [Figure taken from (Holmberg et al., 2017)]*

5.9 The WNT7A-FZD5 axis is Critical to hFT Organoid Culture

Single cell transcriptomic study of hFT epithelial cells (CD45- EpCAM+) revealed that WNT7A is the only expressed WNT (Alsaadi *et al.*, 2022). This is consistent with the role of Wnt7a in co-ordinating specification of the oviduct and the uterus in embryonic development, and exclusively localising to these epithelia in the adult female reproductive tract (Miller and Sassoon, 1998). Transcriptomics identifies the native FZD receptors found in the hFT epithelium as FZD3/5/6/10, however as aforementioned, FZD3/6 participate are classically associated with non-canonical Wnt signalling. Utilisation of antibodies targeting the FZD5 CRD domain (IgG-2921 and IgG-2919) decreased organoid formation by 80-90% and suppressed the Wnt-activity (7TGC) signal by >60%, similar to the observed effect of blocking Wnt-release with Porcupine inhibitor LGK-974, whereas targeting FZD3/6/10 had no such impact (Alsaadi *et al.*, 2022). Although anti-FZD5 antibodies cross-react with the closely related FZD8, this FZD is absent in the hFT epithelium. Overall, there is strong

indication that the WNT7A-FZD5 axis is the primary driver of stemness maintenance in hFT epithelium, thus promotion of this axis should drive organoid growth.

In hFT organoids WNT7A+ secretory cells, themselves Wnt-inactive, were usually adjacent to WNT7A- Wnt-active cells. The former likely are niche-conferring cells providing paracrine sustenance of the latter, putative stem cells (Figure 16A). However, infrequent WNT7A+ Wnt-active cells were found (Alsaadi, 2021); although these likely reflect differentiating stem cells, an autocrine stemness model, which has precedent in hair follicles is possible (Figure 16B) (Lim *et al.*, 2016).

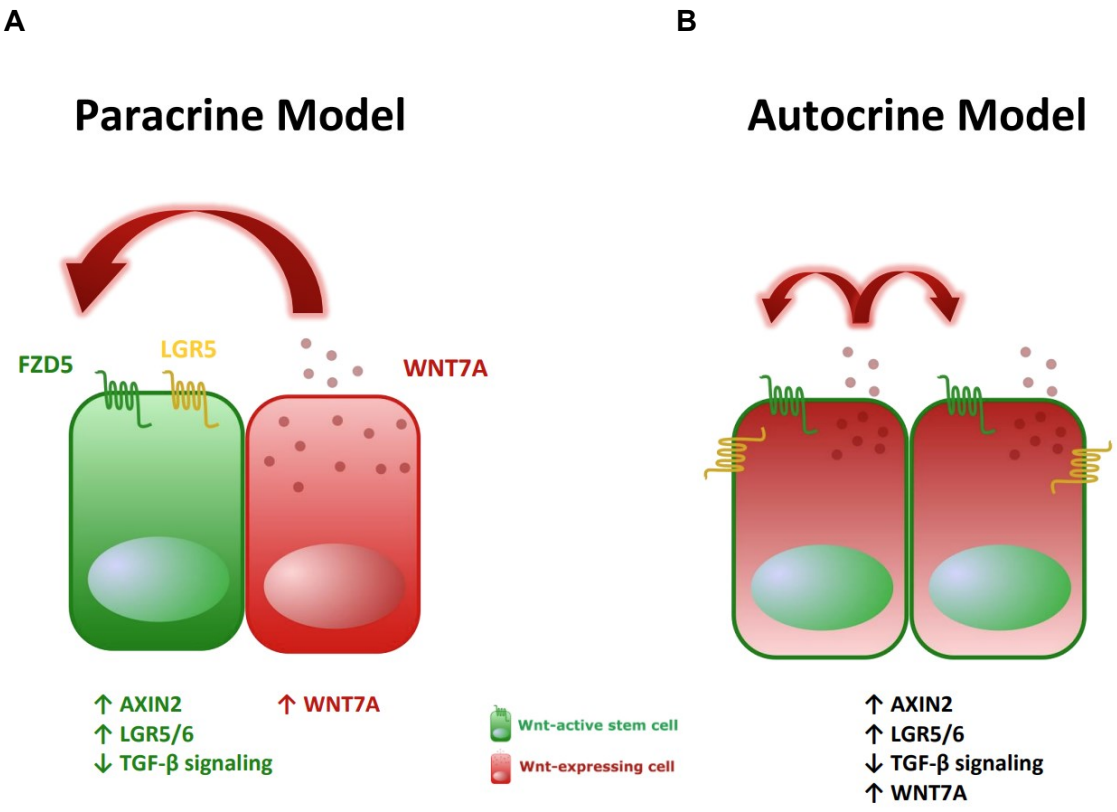


Figure 16. *Possible models for the maintenance of fallopian tube epithelial stem cells.* ***(A)*** *In the paracrine model, the most likely case, short-range release or intermembrane transfer of WNT7A by a Wnt-expressing cell generates high local concentrations for FZD5 signalling in an adjacent stem cell, which therefore becomes Wnt-active.* ***(B)*** *In the autocrine model, which cannot be excluded, the Wnt expressing cell and Wnt-active cell are the same – self-sustenance. [Figure taken from (Alsaadi, 2021)]*

CHAPTER 6

Disease Modelling

6.0 Advantages and Disadvantages of 2D and 3D Cultures

To accurately study a disease, the culture of participating cells, the creation of a relevant growth environment, and introduction of the causative factors are required. Considering this, 3D hFT organoid culture offers a plethora advantages compared to 2D culture for the study of ovarian cancer initiation (Table 2) (Jensen and Teng, 2020). On the other hand, 3D organotypic cultures have variable viability, are missing microenvironmental components, lack interorgan communication, and are unable to re-capitulate complicated hormonal fluxes, in comparison to *in vivo* animal models (Kim, Koo and Knoblich, 2020). However, the cost, complexity and difficulty in stepwise control of mutagenesis severely limits the animal model.

Feature	***2D Monolayer***	***3D Organoids***	***References***
hFT cell morphology	Flattered, elongated, fibroblastic shape	Columnar cells, accurate to the tissue	(Lawrenson *et al.*, 2013; Alsaadi, 2021)
hFT cellular heterogeneity	Culture dominated by proliferative 'cell cycle' cells	Full portfolio of cell-cycle, EMT, differentiated, KRT17 cluster and ciliated cells are maintained, in relevant ratio	(Hu *et al.*, 2020; Alsaadi, 2021)
hFT stem cells	Rapidly lost	Maintained; organoids are intrinsically derived from them	(Kessler *et al.*, 2015; Alsaadi, 2021)
hFT transcriptome	Immediate aberrant changes	Unusual changes are far more infrequent	(Hu *et al.*, 2020; Smirnov, Melino and Candi, 2023)
Cell-cell and cell-ECM interactions	Greatly reduced	Prolific; frequent cell-cell junctions, and communication through ion gradients and paracrine signals	(Sato, van Es, *et al.*, 2011; Soares *et al.*, 2012)
Drug responses	Misleading, with low IC_{50}	More accurate; co-ordinated multicellular responses and architecture confer improved drug resistance	(Lõhmussaar *et al.*, 2020; Yang *et al.*, Pre-Publication)
Culture conditions	Excessive oxygen and medium exposure	More physiologically representative access	(Kapałczyńska *et al.*, 2018)

Table 2. *3D organoids carry many advantages in comparison to 2D monolayer cultures. The pharmacological, cellular and contextual inaccuracies of 2D models limit their utility in particular in cancer research.*

We also understand that mouse oviduct (mOV) organoids grow equipotently in a Wnt-independent manner, whereas hFT organoids are Wnt-dependent (Alsaadi, 2021). Since our suspicion is that, *in vivo*, mutagenic stress from follicular fluid (FF) drives these already gatekeeper-free stem cells to transform, the different stemness physiology means using mOV stem cells to model tumorigenesis is inherently flawed. Moreover, *in vivo*, mouse oviducts are distally shielded from FF stress and growth factors by their encapsulating ovarian bursae (Hosotani *et al.*, 2021). Thus, mOv stem cells establish under different environmental exposures to hFT stem cells.

The differing physiology/environment partly discredit the leading models of ovarian tumorigenesis which have, to date, targeted mOV stem cells (Perets *et al.*, 2013; Lõhmussaar *et al.*, 2020). The value of organoids in ovarian cancer research is exhibited by the fact that only 27% of drugs efficacious in animal models translated to clinical sensitivity in Phase II clinical trials, whereas personalised treatment could be established in 88% of patients in an organoid-based high-throughput screening study (Asghar *et al.*, 2015; Witte *et al.*, 2020). Consensual with this, the FDA recently lifted the requirement for animal testing of new drugs prior to human clinical trials – a landmark change (Wadman, 2023).

6.1 Genetic Manipulation Alone is Insufficient in Modelling Tumour Initiation

Gene editing studies to model stepwise progression towards cancer in organoids are common, as we observed for ovarian cancer (Lõhmussaar *et al.*, 2020). Whilst targeting the putative cell of origin is possible in these models, to date, these works have not incorporated endogenous physiological stress to contextualise the effect of selected genetic changes. For instance, the Clevers laboratory found that in the context of *TP53*, *PTEN*, *SMAD4* and *NF1* knockouts in human cholangiocyte organoids, a background of *BAP1* tumour suppressor mutation was essential for transformation to cholangiocarcinoma on xenotransplantation (Artegiani *et al.*, 2019). However, all risk factors for this malignancy, such as primary sclerosing cholangitis,

liver fluke infestation, and ductal bile stasis, lead to chronic inflammation, with cytokine-driven activation of effector cells causing oxidative DNA damage (Braconi and Patel, 2010). Without long-term inflammatory stress in the model, it is difficult to conclude that *BAP1* is the 'release switch' for cancer onset; perhaps this role *in vivo* belongs to a different mutation or epigenetic change that confers severe genetic vulnerability to inflammatory stress.

Another group investigated serrated colorectal cancer (sCRC), a CRC subtype with particularly poor prognosis and unique genetic trajectory. A library of colorectal organoids was established, with background $Braf^{V600E}$ mutation, and then with sequential biallelic deletions in *TgfBr2* (*ΔT*), *Rnf43/Znrf3* (*ΔRZ*), *Cdkn2a* – encoding $p16^{Ink4a}$ (*ΔI*), and *MLH1* (*ΔM*). Since $Braf^{V600E}$, *ΔRZ* and *ΔM* respectively confer constitutive EGFR activity, WβC signalling and microsatellite instability (MSI), use of EGFR inhibitors, withdrawal of Wnt activators (Rspo-2 or Wnt3a) and treatment with chemotherapeutic 5-fluorouracil (5-FU) were correspondingly used to select for mutant organoids (Lannagan *et al.*, 2019). However, *in vivo*, mutant-selection in the large bowel is co-ordinated alongside at least 5 differently-acting classes of faecal mutagens; pyrolytic foodstuffs cause polycyclic, nitrogenous DNA adducts, whereas bile acids and fecapentenes drive epigenetic changes (Schut and Snyderwine, 1999; de la Monte and Tong, 2009; Ewa and Danuta, 2017). Although the sCRC study conducts orthotopic transplantation to evidence greater tumorigenicity with increased somatic mutation burden, the team do not interrogate the nature of mutagenic stress or multi-omic changes post-transplantation (Lannagan *et al.*, 2019). Thus, the works do not capture which mutations are driven by or cause vulnerability to individual mutagens.

6.2 Combining Endogenous Stress with Genetic Manipulation Improves Modelling

The co-study of genetic changes and cellular stress has been highly insightful for understanding the development of a variety of diseases. For instance, in a murine model of pancreatic ductal adenocarcinoma (PDAC), deletion of *Tigar*, which encodes

an antioxidant protein, was explored on a background of *Kras* mutation, with/without *Trp53* mutation or deletion (Cheung *et al.*, 2020). *Tigar* deletion drove an increase in intracellular ROS, which delayed PanIN progression, and could be rescued with antioxidant NAC treatment. However, *Tigar* deletion was also shown to amplify metastatic spread, in particular to the lung, through pro-migratory Erk signalling in relation to increased mitochondrial ROS production. This corroborates similar studies where *Tigar* deletion slows the onset of *Apc*-null intestinal adenomas, or loss of antioxidant Nrf2 retards PanIN progression, but without improvement in overall survival (DeNicola *et al.*, 2011; Cheung *et al.*, 2013).

Separately, the study of alcohol intake alongside genetic manipulation has given profound insight into haematopoietic stem cell (HSC) attrition (Garaycoechea *et al.*, 2018). Normally, the dangerous ethanol metabolite acetaldehyde is safely converted to acetate by Aldh2. In mice, it was shown that deletion of *Aldh2*, and not Fanconi anaemia (FA) crosslink-repair gene *Fancd2*, drives HR repair in HSCs. While Aldh2 is therefore the main protectant against ethanol-related toxicity, subsequent knockout of HR and FA crosslink-repair proteins showed that these collaboratively repair acetaldehyde-damaged DNA. Similarly, FA and NHEJ (Ku70) repair were shown to mediate genomic stability. Fascinatingly, *Trp53* deletion additional to Fancd2 and Aldh2 KO were shown to rescue HSCs from toxicity, unexpectedly without expense of genome stability. Critically, this contextualises works conducted without endogenous stressors, which suggest p53 downregulates the FA DNA repair pathway (Jaber *et al.*, 2016). Garaycoechea *et al.* (2018) therefore evidence p53 to be a gatekeeper rather than caretaker in alcohol-related HSC attrition. Thus, *Trp53* mutation likely protects against alcohol-related myelodysplasia, but predisposes to myeloproliferative cancers.

6.3 Stress has Rarely Been Studied Alongside Genetic Changes in Organoids

Although murine studies are suitable to study initiation of stem cell disease with natural sources of stress, organoid models are a cheaper alternative for which stepwise

genomic characterisation is easily possible. Currently, there is little in the literature about interrogating tumorigenesis in this manner.

The exception is a very recent paper, wherein varied physiological conditions with genetic manipulation of hepatocyte organoids gave detailed insights into the aetiology of hepatic steatosis, a precursor condition for hepatocellular carcinoma (Hendriks *et al.*, 2023). First, patient-derived healthy hepatocytes were modified by prime editing to have graded predisposition to steatosis, with heterozygous or homozygous *PNPLA3 I148M* mutation, and by CRISPR/Cas9 for biallelic *PNPLA3* KO. In unmodified medium, *PNPLA3* deficiency led to some spontaneous steatosis, with 8% of biallelic KO cells affected. However, in free fatty acid (FFA)-enriched medium, cells assimilated vastly more triglycerides with increasing *PNPLA3*-deficiency; 75% greater deposition was found for homozygous *PNPLA3 IL148M* compared to naïve cells. Here, both genetic and environmental factors initiated the disease. Second, CRISPR/Cas9 was used to create *APOB*-/- and *MTTP*-/- organoids to model familial hypobetalipoproteinaemia and abetalipoproteinaemia, which predispose to hepatic steatosis. For these, *de novo* lipogenesis with impaired fat secretion was revealed as the disease mechanism, rather than assimilation of exogenous FFAs. Thirdly, bulk RNA-sequencing on drug-treated naïve and modified organoids identified mechanisms of both drug action and side effects – valuable knowledge that enables accurate and holistic patient management.

Relating this to ovarian tumorigenesis, it is believed that the endogenous mutagens typically include ROS, iron and hormones (such as estradiol), mostly from follicular fluid. Accordingly, distinct transcriptional signatures are already associated with oxidative stress, iron-related ferroptosis and long-term estradiol treatment in relevant tissues (Han *et al.*, 2008; Turco *et al.*, 2017; Yu *et al.*, 2021). The carcinogenic potential of these mutagens may be context-specific; for instance, HR deficiency may promote stemness and proliferation, with concomitant susceptibility to bulky oestrogenic adducts as in basal-like breast carcinoma (Liu *et al.*, 2008; Gorski *et al.*,

2009; Savage *et al.*, 2014). They may also be cell-specific, for instance, targeting more chromatin-open stem cells. Moreover, as seen with PDAC *Tigar* mutation, it could be that ROS does not cause hFT STICs, but promotes EMT to enable metastatic invasion (Cheung *et al.*, 2020). Another angle is that our view of *TP53* mutation as always a harmful culprit in HGSOC could even be misled; perhaps its occurrence prevents tubal atrophy in a subset of patients with high ovulatory ROS, with elevated HGSOC risk being merely a consequence (Garaycoechea *et al.*, 2018). Thus, genetic manipulation of hFT organoids with exposure to environmental chemicals could give deep insights into the cause, nature and treatment of HGSOC.

CHAPTER 7

Aims and Objectives

7.0 Aim 1: Characterise hFT Stem Cells.

a) Expand hFT organoids transduced with the 7TGC Wnt-reporter construct.
b) Refine methods to quantify Wnt-activity using 7TGC-transduced organoids.
c) Explore which physiological factors promote the WβC stemness axis.
d) Develop methods to optimise organoid culture to maximise growth.
e) Enhance 3D media to create ‘recovery solutions’ for stressed organoids.

7.1 Aim 2: Study Components of Follicular Fluid and their Role in Tumorigenesis.

a) Explore how endogenous mutagens cause DNA damage and cell death.
b) Consider the combinatorial effects of endogenous mutagens.
c) Study the factors enabling physiological rescue of hFT organoids from H_2O_2.
d) Investigate if stem cells are particularly vulnerable to endogenous mutagens.
e) Explore the roles of local hormones in hFT organoid behaviour.

7.2 Aim 3: Establish a Protocol for Genetic Manipulation of hFT Organoids.

a) Assess the lentiviral construct design and suitability for genetic modification.
b) Attempt rescue of hFT organoids immediately post-transduction.
c) Establish long-term cultures for genetically manipulated hFT organoids.
d) Optimise methods to purify mutant hFT organoid populations.
e) Develop a clonal selection protocol to isolate organoids of interest.

CHAPTER 8

Methods and Materials

8.0 Human Clinical Samples

Human fallopian tubes (hFTs) were obtained from patients undergoing hysterectomy with bilateral salpingo-oophorectomy for a benign ovarian mass, or debulking surgery for a gynaecological malignancy. hFTs from benign cases were presumed to be wild-type, and those from malignant cases were presumed to harbour regions of cellular atypia. Surgery was conducted at the Department of Gynaecological Oncology, Churchill Hospital, Oxford University Hospitals NHS Foundation Trust, United Kingdom. Patients recruited were adults with capacity who provided written informed consent. After harvesting, specimens were collected within 2 hours and processed immediately to maximise viability. Tissue handling complied with the Human Tissue Act 2004 and Data Protection Act 2018. Most cases were recruited within the Gynaecological Oncology Targeted Therapy Study 01 (GO-Target-01; Research Ethics Approval #11-SC-0014; Berkshire NRES Committee), and the remaining cases as part of the Oxford Centre for Histopathology Research (OCHRe) and the Oxford Radcliffe Biobank (ORB) (IRAS ID 262470; Research Ethics Committee Reference 19/SC/0173).

In this thesis, hFTs used to establish organoids for the purpose of studying response to chemical insult or hormones, or for genetic manipulation leading to carcinogenesis were derived from a single patient confirmed to have an ovarian mass but non-malignant disease. In this patient, critical target regions in exons of *TP53*, *BRCA* and *PTEN* were confirmed to be wild-type through Sanger sequencing by May Sallam. In one experiment, organoids derived from the hFT of a patient with HGSOC were found to respond unusually to physiological damage (Figure 26E), and were discounted for further study. The hFTs used to establish Wnt-reporter organoids (Figure 18) with the intention of studying stemness, growth signalling and stem cell injury were derived from a patient with endometrial carcinoma with confirmed benign tubal histology on retained sections. Lentiviral synthesis and transduction of the 7TGC construct was performed previously by Abdulkhaliq Alsaadi. Beyond these detailed, no

further hFTs, tissues or cell types were used to generate organoids for any experimental purpose.

8.1 Tissue Dissection and Processing

The hFTs were washed three times with cold Dulbecco's Phosphate Buffered Saline (DPBS) to remove excess red blood cells, and the supernatant was discarded. The hFTs were then transferred to a 60mm TC-treated cell culture dish. Using sterile scalpels, the tissue was macerated, focusing on the fimbrial end. Within 5 minutes, 5ml of pre-warmed 'Digestion Medium' (R0, 0.5mg/ml DNase I, 100units/ml Collagenase Type I) was applied. Enzymes, stored at -20°C, were added to R0 immediately before use. Tissue was further macerated until fragments were <1mm^3. Afterwards, Y-27632 (ROCK inhibitor) was added at 10μM, and the mixture was transferred to a gentleMACS C tube. This was then connected to a gentleMACS Dissociator with a pre-set high-tempo dissociation programme for 1 hour at 37°C. The cell suspension was diluted to 15ml in R0 and filtered through a MACS Smartstrainer (70μm). R0 was used to clear blockages. The filtrate was centrifuged at 300g for 5 minutes. Supernatant was discarded, and ACK red blood cell lysis buffer was added for 3 minutes. The buffer was diluted, pelleted, and then washed in DPBS. A sample was mixed with Trypan Blue and counted in a Nexelcom cytometer; typical yield was 1 million cells.

8.2 Organoid Culture

8.2a Establishment Directly from hFTs

Two hours before dissection, a 6-well plate was pre-warmed to 37^{o}C in an incubator. An aliquot of Cultrex™ ECM was thawed on ice, and two sets of sterile tip boxes were placed in a -20^{o}C freezer. After extracting single cells from hFTs, they were transferred to a 1.5ml Eppendorf Safe-Lock Tube and centrifuged at 700g for 5 minutes, forming a visible pellet. The supernatant was removed without disturbing the cells. Using the 20-200uL tips from the freezer, 100μL of Cultrex™ ECM per 1 million

cells was added and mixed. Using the chilled 2-30µL tips, 20µL domes were plated in the pre-warmed 6-well plate. The plate was quickly inverted and incubated at 37°C for 45 minutes to allow Cultrex™ ECM gelation. The plate was gently shaken to confirm rigid dome formation, then overlaid with 2ml of hFT 3D organoid medium containing ROCK inhibitor that was optimised in previous works (Alsaadi, 2021). Pre-warming the plate promoted rapid Cultrex™ ECM polymerization, ensuring dome adhesion, and chilled tips prevented premature gelation during dome placement.

8.2b Variations of hFT Organoid Medium

Previous works established an effective 3D medium to successfully grow hFT organoids (Alsaadi, 2021). Within a base medium of Advanced Dulbecco's Modified Eagle Medium/F12 (DMEM/F12), the following are added: HEPES buffer, 12mM; Penicillin/Streptomycin, 1%; Nicotinamide, 1mM; N-acetylcysteine, 1mM; A83-01, 5µM; Forskolin, 10µM; B27 Supplement, 2%; EGF Recombinant Human Protein, 100ng/ml; N2 Supplement, 1%; Human R-Spondin-1 (RSPO1), 500ng/ml; Human FGF-10, 100ng/ml; Human Noggin, 100ng/ml. This can be refrigerated at 4°C for use within 4 weeks. Moreover, when establishing organoids from primary tissues, passaging organoids as single cells, culturing organoids post CRISPR/Cas9 genetic manipulation, or promoting the growth of struggling organoids, Y-27632 dihydrochloride, 10µM, is additionally added, immediately prior to using medium. Later, this thesis shows that to culture precious, struggling organoids, further addition of both Wnt Surrogate-Fc Fusion Protein, 10pM, and Human Insulin, 1µg/ml, is highly effective. Separately, for the occasional maintenance of hFT stem cells for up to 2 weeks as 2D monolayers, the medium comprised of DMEM/F12, Fetal Bovine Serum (FBS), 5%, and HEPES, Penicillin/Streptomycin, EGF and Y-27632 all at the aforementioned concentrations (Kessler *et al.*, 2015).

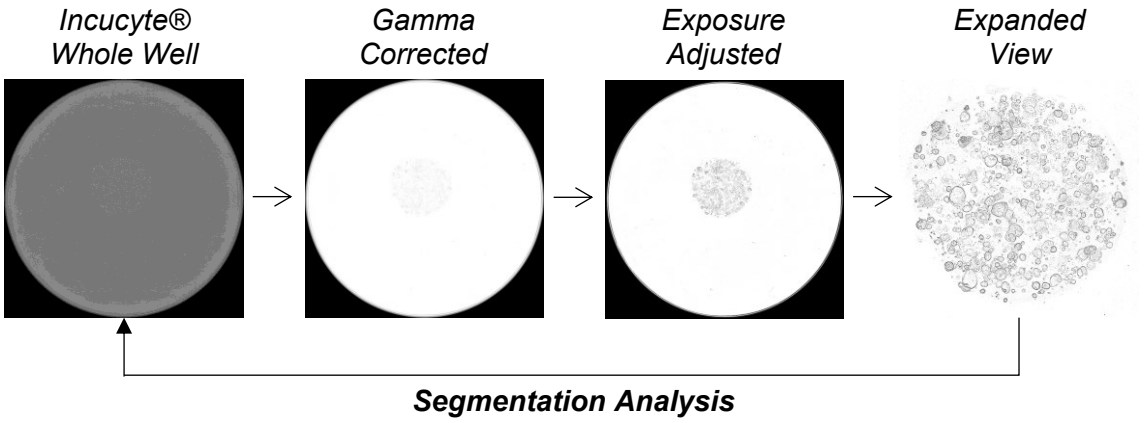
8.2c Maintenance of Organoid Culture

After establishment hFT organoids usually reached confluence within 5-7 days, necessitating fresh 3D medium every 3-4 days. For harvesting, medium was discarded and Dispase (1Unit/ml) vigorously pipetted into the Cultrex™ ECM domes for disruption. Enhancing enzyme-matrix protein contact improved digestion. After 15 minutes, further pipetting with a p1000 tip flush to the plastic of the well sheared the organoids. The suspension was collected in a 15ml Falcon™ tube, and after centrifugation at 600g for 5 minutes, supernatant was carefully removed with a p1000 tip. For routine passage, cells were resuspended in DPBS, further sheared to oligocellular clumps, and transferred to a 1.5ml Eppendorf Safe-Lock Tube. Then, they were re-seeded in Cultrex™ ECM (1:5 radio). For controlled seeding for studies, cells were resuspended in 500µL Trypsin (0.05%) for 5 minutes at 37^{o}C in a water bath. Following this, Trypsin was neutralised with 500uL R10 (RPMI 1640, 10% FBS, 1% Penicillin/Streptomycin), and organoids sheared to single cells. After pelleting/washing in DPBS, cells were counted as described previously, cells were seeded in domes at a density of 200,000/100µL Cultrex™ ECM. Separately, for 2D expansion, confluent wells were rinsed with DPBS, overlayed with Trypsin and neutralised with R10 as before, and then re-seeded in 2D medium at 1:3 ratio.

8.3 Organoid Growth Assays

Experiments were set up such that single 20µL domes each containing 40,000 single cells were each placed in individual wells, subject to individual chemical conditions at t=0. Imaging was performed with the Sartorius Incucyte® at Day 4-8, depending on growth rate of the control well(s). Initially, Incucyte® ‘Whole Well’ scans were taken and deconvoluted in ImageJ/Fiji by gamma correction and exposure adjustment, to distinguish true organoids from artefacts (Figure 17A). With these visuals, a segmentation analysis algorithm was optimised with the in-built Incucyte® software using band-pass filters for object size, density and eccentricity. For object

A



B

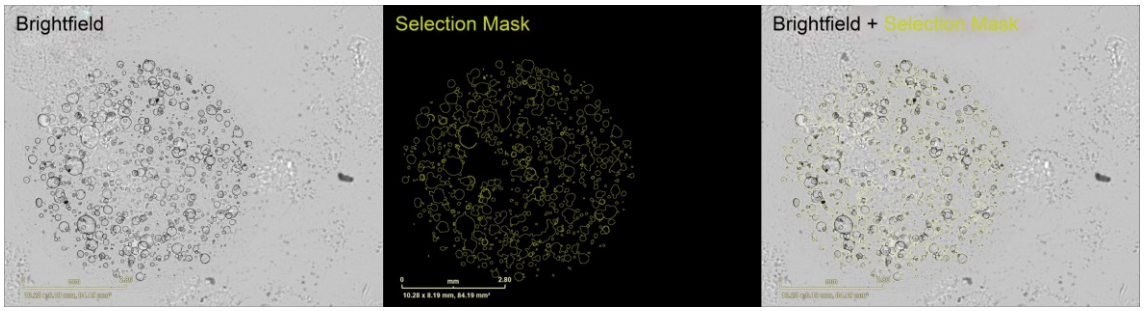


Figure 17. *Incucyte® methods to study organoid count, mean area and confluence.* ***(A)*** *With the Incucyte 'Whole Well' module, organoids are hardly visible – image processing enables accurate segmentation analysis.* ***(B)*** *With the organoid module, segmentation can immediately be performed.*

masks, hole-filling was avoided, to better account for overlapping organoids at marginally different focal heights. This enabled accurate, standardised measurement of organoid count, mean area and confluence. Later, access to the Incucyte® 'Organoid' scan and analysis toolkits became available, a new segmentation algorithm was defined, and this became the primary method to quantify growth (Figure 17B).

8.4 Fixation of Organoids as Single Cells

For flow cytometric analysis, or cell-by-cell study otherwise, a method to fully dissociate and fix organoid cells was optimised. Organoids were first harvested from domes and trypsinised to single-cells as previously described. The Eppendorf

ThermoMixer was then pre-warmed to 37°C and set to 700 oscillations/minute. Meanwhile, single-cells were pelleted in a 1.5ml Eppendorf Safe-Lock Tube at 700g for 5 minutes, and medium fully aspirated. Working quickly, 100uL of pre-prepared methanol-free Paraformaldehyde, diluted in DPBS to 4%, was added with a p200 pipette, with 40-cycles of rapid pipetting to generate a single cell suspension. The tube was immediately transferred to the active Eppendorf ThermoMixer for 15 minutes, for fixation in motion. Once retrieved, 400µL of DPBS containing 0.4M Glycine was vigorously pipetted into the PFA. This yielded the benefits of fluid diluting the PFA, glycine blocking the unreacted aldehyde groups, and cell disruption preventing crosslinking of clumped cells. Since cells shrink during fixation, pelleting at 800g for 5 minutes was then required. Two further washes in DPBS/glycine were conducted, and cells were pelleted and stored at 4°C in a refrigerator for study within 2 weeks.

8.5 Immunofluorescence Staining

8.5a Fixed Cells

Each tube of fixed cells in DPBS/glycine were simultaneously blocked and permeabilised by re-suspension in 100uL of pre-prepared Block/Perm Buffer (DPBS, 10% FBS, 3% Bovine Serum Albumin (BSA), 0.2% Triton X-100) for 1 hour at room temperature. After this, cells were incubated with 75uL of a non-bovine primary antibody (anti-γH2AX or anti-PAX8, both at 1:250) dissolved in Block/Perm Buffer, and refrigerated at 4°C in an opaque box for 24 hours. Cells were then incubated with 75uL of a fluorophore-labelled secondary antibody (at 1:250) dissolved in Block/Perm Buffer, at room temperature for 45 minutes in a dark drawer. Cells were counterstained with 100µL of DAPI (at 1:100) in DPBS for 15 minutes, to identify fixed cells. Finally, the suspension was made up to 250µL with DPBS, and transferred to a single well of a round-bottomed 96-well plate.

8.5b Live Cells

Cells which had been formerly transduced with the 7TGC reporter construct, a fluorophore variant of the TOPFlash assay, did not require immunolabelling. They were trypsined to single cells as described, then re-suspended in 250µL of Fluorescence-Activated Cell Sorting (FACS) Buffer (Ca^{2+}/Mg^{2+}-free DPBS, 10% FBS, 1% BSA, 2mM EDTA, 0.5% Sodium Azide), and transferred to a round-bottomed 96-well plate as before. Counterstaining with DAPI was not necessary, as the transduced construct ensured strong, short-lived constitutive fluorescence (mCherry) that identified live cells.

To study cell death, first, pre-prepared 10x Annexin V Binding Buffer (DiH_2O, 0.1M pH 7.4 HEPES, 1.4M NaCl, 25mM CaCl) was diluted to 1x in DiH_2O. Annexin V-FITC Apoptosis Staining / Detection Kit (AbCam) and DAPI counterstain were dissolved (both at 1:100) in this solution, to label cells undergoing early apoptosis and already dead cells respectively. Cells were then trypsinised as described, and resuspended in 250uL of the complete solution. They were then plated as above and stored in a dark drawer for 15 minutes prior to analysis.

8.6 Confocal Microscopy

hFT single cells from 7TGC-transduced Wnt-reporter organoids were seeded at a low density of 20,000 cells/100µL Cultrex™ ECM as 5µL domes on pre-warmed glass-bottomed 8-well chamber slides (Thistle Scientific) or 4-well chamber dishes (Cellvis). Domes were overlayed with 3D organoid medium with separate treatment conditions, which was replaced every 48 hours. After 7-14 days of growth, organoids z-stack images were captured using the Leica Thunder Widefield Microscope or a Zeiss LSM980 Inverted Confocal Microscope, and processed in ImageJ/Fiji.

8.7 Lentivirus Synthesis and Transduction

Lentiviral vectors were used to deliver Cas9, selection colours, and dual single guided RNA cassettes consisting of hU6-sgRNA1 and hH1-sgRNA2. Each lentivirus enabled the targeted KO of a critical gene, and with each transduction, a unique fluorescent marker was activated. Synthesis and transduction steps were performed by May Sallam, and recovery and purification steps were optimised or planned within this thesis, as later detailed in the results sections.

8.8 Fluorescence-Activated Cell Sorting

Organoids were trypsinised to single cells as described, and re-suspended in 1ml of Organoid FACS Buffer (50% FACS Buffer + 50% 3D organoid medium + 10μM Y-27632), with DAPI counterstaining (1:100) to exclude dead cells (frequently auto-fluorescent). FACS was performed using the BD FACSymphony™ S6 6-way Cell Sorter and analysed with BD FACSDiva™ Software (Version 9.4). Cells were sorted into 1ml of 3D organoid medium with 10μM Y-27632 in a 1.5ml Eppendorf Safe-Lock Tube, immediately seeded densely in 3D domes (1,000,000 cells/100uL Cultrex™ ECM), and grown in 3D organoid recovery medium.

8.9 Appendix 1: Catalogue Numbers & Equipment

Reagent	*Source*	*Catalogue №*
Cell Culture Supplies and Media		
DPBS, no calcium, no magnesium	ThemoFisher	#14190094
RPMI 1640 (+ L-Glutamine)	ThermoFisher	#21875091
Advanced DMEM/F-12	ThermoFisher	#12634010
Penicillin-Streptomycin (10,000U/ml)	ThermoFisher	#15140122
Corning® tissue-culture treated culture dishes	Sigma-Aldrich	#CLS430196
Fetal Bovine Serum, qualified, heat inactivated, E	ThermoFisher	#10500064
Cultrex Basement Membrane Extract, Type 2, Pathclear	Bio-Techne	#3532-010-02
Corning Matrigel Basement Membrane Matrix, Phenol Red-Free, LDEV-Free	Scientific Laboratory Supplies	#356237
Poly-L-lysine solution (0.1% w/v in H_2O)	Sigma-Aldrich	#P8920
Dispase in Hanks' Balanced Salt Solution (5 U/ml)	Stemcell Technologies	#07913
Trypsin-EDTA (0.5%), no phenol red	ThermoFisher	#15400054

Reagent	*Source*	*Catalogue №*
Enzymes and Inhibitors		
DNAse I	Stemcell Technologies	#100-0683
Collagenase Type I, powder	Life Technologies	#17018029
Y-27632 dihydrochloride	Bio-Techne	#1254/10
TheraPEAK™ ACK Lysing Buffer	Lonza	#BP10-548E

Reagent	*Source*	*Catalogue №*
Chemicals and Buffers		
HEPES (1M)	ThermoFisher	#15630056
Nicotinamide	Sigma-Aldrich	#N0636-100G
A83.01	Stemcell Technologies	#72022
Forskolin (FSK)	Bio-Techne	#1099/10
B-27 Supplement	ThermoFisher	#12587010
Sodium Azide (99%)	Sigma-Aldrich	#199931
Sodium Chloride (NaCl)	Sigma-Aldrich	#S9888
Calcium Chloride ($CaCl_2$)	Scientific Laboratory Supplies	#C4901
EDTA Solution (0.5mM)	Sigma-Aldrich	#E8008
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	#D8418
Sodium Butyrate	Sigma-Aldrich	#B5887
Sucrose	Sigma-Aldrich	#S0389
Opti-MEM™ Reduced Serum Medium	ThermoFisher	#51985026
Polybrene Reagent	Millipore UK	#TR-1003-G
Nutlin-3a	Bio-Techne	#6075
Diphenyleneiodonium chloride	MedChemExpress	#HY-100965
Ferric Citrate	MedChemExpress	#HY-N1428C
Estradiol	MedChemExpress	#HY-B0141
DMT1 Blocker 2	MedChemExpress	#HY-126302
Progesterone	MedChemExpress	#HY-N0437
Entacapone	MedChemExpress	#HY-14280
Hydrogen Peroxide (H_2O_2) Solution (30% w/w in H_2O, contains stabilizer)	Sigma-Aldrich	#H1009
Human Holo-Transferrin Protein, CF	Bio-Techne	# 2914-HT
CHIR99021	Bio-Techne	#4423/10
IWP-2	Bio-Techne	#3533/10

Reagent	*Source*	*Catalogue №*
Growth Factors and Supplements		
Epidermal Growth Factor (EGF), human, recombinant	ThermoFisher	#PHG0311
N-2 Supplement	ThermoFisher	#17502048
Human RSPO1	Peprotech	#120-38-100
Fibroblast Growth Factor-10 (FGF-10), human, recombinant	Peprotech	#100-26
Noggin, human, recombinant	Peprotech	#120-10C
WNT Surrogate-Fc Fusion Protein	ImmunoPrecise Antibodies	#N001-05
Insulin, Human Recombinant	Sigma-Aldrich	#91077C

Reagent/Tool	*Source*	*Catalogue №*
Cell Analysis Tools		
Trypan Blue Solution, 0.4%	Life Technologies	#15250061
Nexcelom™ Cellometer® Disposable Counting Chambers (PD100 Slides)	ThermoFisher	#11522186
Cellometer® Auto T4 Bright Field Cell Counter	Sigma-Aldrich	#Z692441
MACS® SmartStrainers (70 µm)	Miltenyi Biotec	#130-110-916
MycoAlert Mycoplasma Detection Kit	Lonza	#LT07-118

Reagent	*Source*	*Catalogue №*
Fixing Reagents		
Albumin, Bovine Serum, Fraction V, Fatty Acid/Nuclease/Protease-Free	Sigma-Aldrich	#126609
Glycine, ReagentPlus	Scientific Laboratory Supplies	#G7126
Pierce 16% Formaldehyde (w/v)	ThermoFisher	#28908
Triton™ X-100	Sigma-Aldrich	#X100

Antibody/Stain	*Organism*	*Dilution*	*Source*	*Catalogue №*
Antibodies and Stains				
Phospho-Histone H2A.X (Ser139) (20E3) (=γH2AX)	Rb mAb	1:250	Cell Signaling	#9718
PAX8	Rb pAb	1:250	Proteintech®	#10336-1-AP
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)	Goat	1:250	AbCam	#AB150077
Goat Anti-Rabbit IgG H&L (Alexa Fluo®) 647) preadsorbed	Goat	1:250	AbCam	#AB150083
Annexin V-FITC Apoptosis Staining / Detection Kit	-	1:100	AbCam	#AB14085
DAPI (100ng/ml)	-	1:100	Sigma-Aldrich	#D9542

Article	*Source*	*Catalogue/Unit №*
Laboratory Equipment		
Swann-Morton Surgical Scalpels	MediSupplies	#PMC0099
gentleMACS C Tubes	Miltenyi Biotec	#130-093-237
gentleMACS™ Octo Dissociator with Heaters	Miltenyi Biotec	#130-096-427
Eppendorf ThermoMixer® C	ThermoFisher	#5382000031
Mr. Frosty™ Freezing Container	ThermoFisher	#5100-0001
8 well chamber slide, TC treated	ThistleScientific	#IB-80826
4-Chamber 35mm glass bottom dish with 20 mm microwell, #0 cover glass	Cellvis	#D35C4-20-0-N
Leica DM18 Thunder Widefield Microscope	Leica Microsystems	-
Zeiss LSM980 Inverted Confocal Microscope with Airyscan 2 and NIR Detector	Zeiss	-
Attune™ NxT Flow Cytometer	ThermoFisher	-
BD FACSymphony™ S6 Cell Sorter	BD Biosciences	-
GloMax® 20/20 Luminometer	Promega	#E5311
Incucyte® S3 Live-Cell Analysis Instrument	Sartorius	-

Reagent	*Source*	*Catalogue №*
Genetic Engineering and Transfection Reagents		
MEM Complete Medium, with NEAA, 1 mM sodium pyruvate, 2mM L-Glut, and 10% FBS	Sigma-Aldrich	#SLM-244
Transfer Plasmid (02_pLentiCRISPR_dual_sgRNA_p53 eGFP)	In House	-
Packaging Plasmid (psPAX2)	Addgene	#12260
Envelope Plasmid (pMD2.G)	Addgene	#12259

Programme	*Source*	*Version*
Software and Data Analysis Tools		
(Fiji Is Just) ImageJ	Fiji/ImageJ	2.9.0/1.54b
IncuCyte® Graphical User Interface	Sartorius	2020B
BD FASDiva™ Software	BD Biosciences	v9.4
Prism	GraphPad	9.0.0 (121)

CHAPTER 9

Results Section 1:
Stemness and Growth

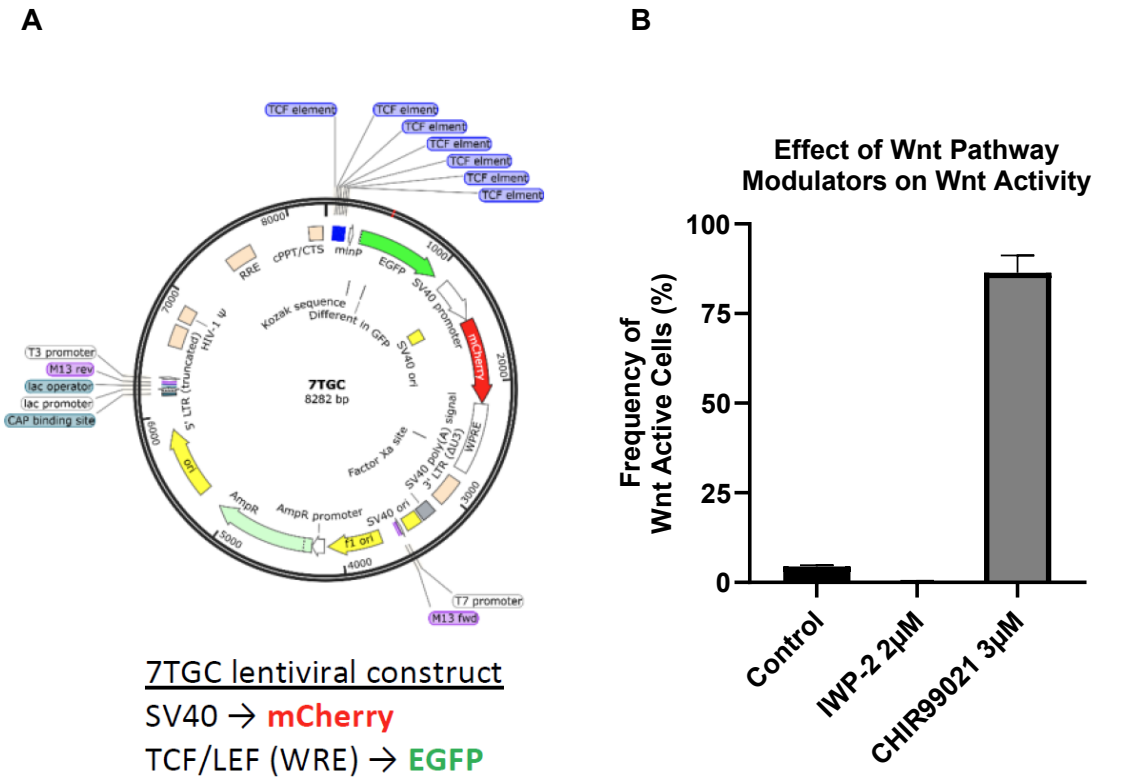
9.0 Re-Validating the Wnt-Reporter Organoids

This chapter relies on the integrity of previously-transduced hFT organoids to reputably report Wnt-activity, thus their chemical responses were re-tested. The criticality of WβC signalling to the sustenance of hFT stem cells is epitomised by previous works showing that withdrawal of RSPO1 blocks organoid formation, with only extremely poor rescue possible. The 7TGC-transduced Wnt-reporter organoids contain a 7xTCF-EGFP element (Figure 18A), thus reflect functioning nuclear β-catenin levels. First, it was confirmed that Wnt inhibition (IWP-2) and β-catenin stabilisation (CHIR99021) could modulate Wnt-activity as expected (Figure 18B), which was then observed directly by confocal microscopy (Figures 18C & 18D). Together with previous validation experiments and the finding that hFT organoids establish from Wnt-active and not Wnt-inactive cells, we can robustly infer that a subset of Wnt-active cells are the multipotent hFT stem cells. Being the putative HGSOC origin, the first step is to further interrogate what regulates their abundance, by modulating stemness.

9.1 Directly and Indirectly Supporting the WNT7A-FZD5 Axis

9.1a Surrogate Wnt and CHIR99021

Promoting this stemness axis is important, as it may elucidate stem cell markers to enable targeted genetic studies. Previously, FZD5 antagonism was shown to potently inhibit Wnt-activity. Logically, FZD5 activation should do the reverse. However, there are a few challenges. Firstly, failure to couple FZD5 to the Lrp5/6 co-receptor may direct non-canonical rather than canonical signalling. (Cavodeassi *et al.*, 2005; Slater *et al.*, 2013). Secondly, high hydrophobicity of Wnts makes solubilisation difficult, meaning commercially available Wnt7a sources are largely functionless (Tüysüz *et al.*, 2017; Alsaadi, 2021). This has led to the advent of hydrophilic ‘Surrogate Wnt’ agonists. In line with suspicion, the specific mediator of this axis, Surrogate Wnt, was able to robustly promote WβC-activity in approximately 60% of cells, compared to >80% for CHIR99021 (Figures 19A and 19B). Since Surrogate Wnt



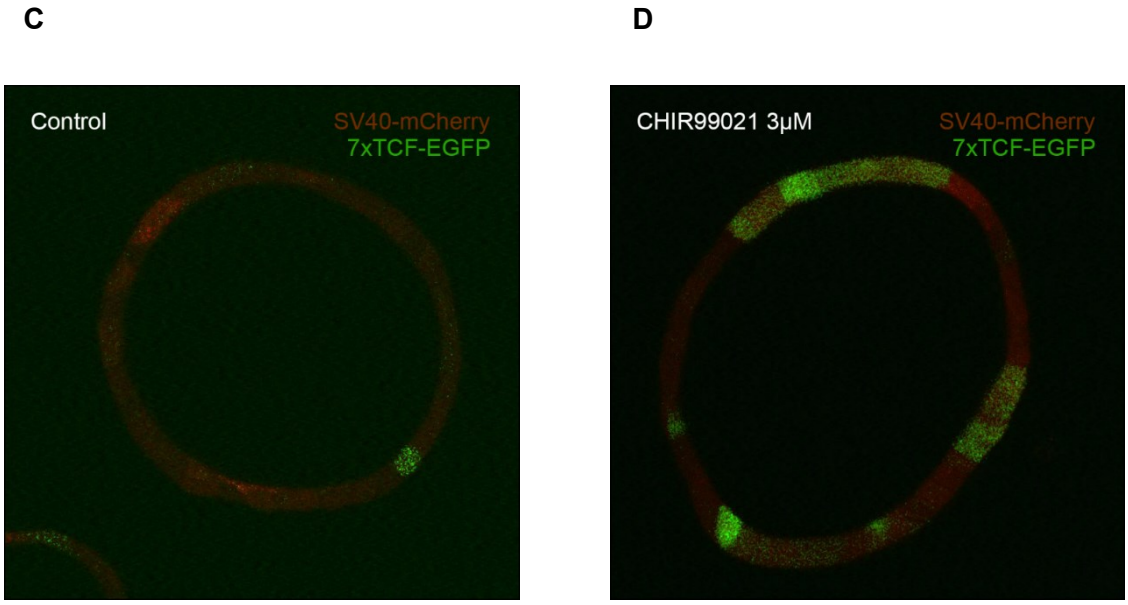


Figure 18. *Wnt-reporter organoids behave as anticipated.* ***(A)*** *The 7TGC construct drives mCherry synthesis constitutively in all cells under SV40 promoter, and 7xTCF elements drive EGFP production, additionally labelling Wnt-active cells.* ***(B)*** *Around 4% of cells are Wnt-active (EGFP+), repressed to 0% by IWP-2 (Porcupine inhibitor), and boosted to >80% by CHIR99021.* ***(C)*** *Confocal microscopy evidences rare Wnt-active cells (2-3 per organoid) in untreated conditions.* ***(D)*** *GSK3β inhibition causes many cells to discretely become Wnt-active – microscopy rules out drug-induced autofluorescence.*

requires the FZD5 receptor which most cells possess, whereas CHIR99021 disassembles the β-catenin destruction complex in every cell regardless of receptors, this follows expectations (Alsaadi, 2021). However, it was observed that Wnt-activation alone did not correspond linearly to organoid growth. For instance, 10pM Surrogate Wnt increased Wnt-activity only 1.4-fold, whereas 500pM increased it 12-fold, however, the former increased organoid growth by 1.8-fold, and the latter by 2-fold - a less impressive difference (Figure 20B).

To reconcile this non-linearity of WβC-activity and growth, we return to the study of colorectal cancer, and APC. Study of patients with Familial Adenomatous Polyposis (FAP) with germline *APC* mutations evidence that the second allelic hit only leads to transformation if a small degree of APC activity is retained (Lamlum *et al.*, 1999). Whilst counterintuitive, it is thought that optimal clonal proliferation requires a degree of β-catenin destruction and nuclear export; the 'just-right' model of Wnt signalling (Albuquerque *et al.*, 2002). Supporting this, overexpression of β-catenin is known to induce apoptosis co-ordinated by Bcl-x(L) (Kim *et al.*, 2000). It is similarly noteworthy that over-treatment with certain Surrogate Wnts begins to restrict growth of mouse intestinal organoids (Chen *et al.*, 2020).

In addition to explaining the responses to Surrogate Wnt concentrations, the 'just-right' model may explain why the complete uncoupling of the β-catenin destruction complex through CHIR99021, despite potently promoting Wnt-activity, was sometimes found to be toxic (Figure 19F). Furthermore, previous laboratory works have recommended that truncating β-catenin to eliminate the 45aa phospho-'degron' tail could be an effective way of studying stemness (Alsaadi, 2021). However, deletion of β-catenin Serine 45 alone in human colonocytes has been shown to retain susceptibility to Wnt treatment, *APC* loss and GSK3β activation (Parker, Rudeen and Neufeld, 2020). The findings thus far suggest that this 'just-right' model may be more suitable for the study of growth and transformation of stem cells.

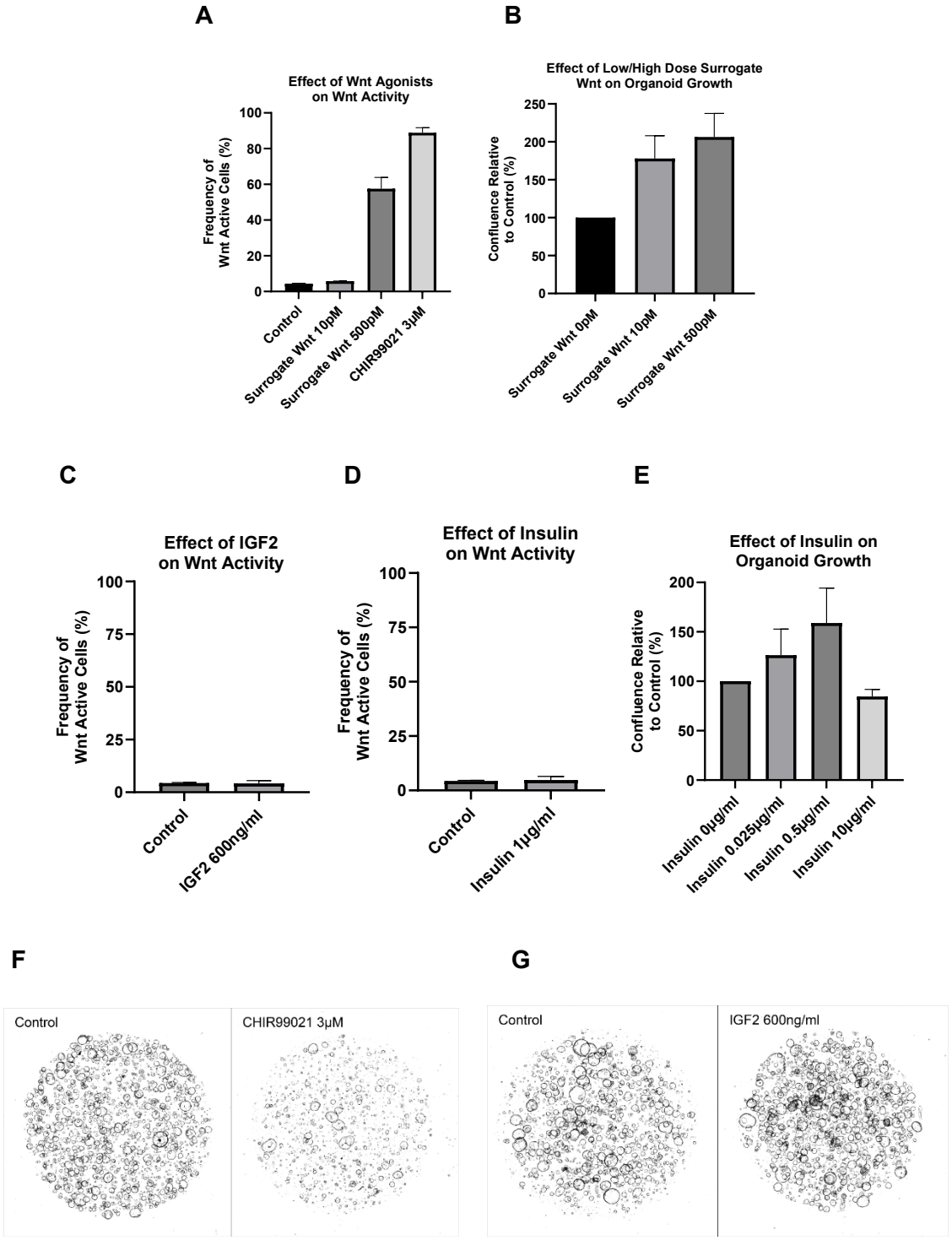


Figure 19. *Wnt-activity does not equal growth.* ***(A)*** *FZD5-LRP5/6 coupling with sufficient Surrogate Wnt or CHIR99021 potently promote Wnt-activity. (B) Although only Surrogate Wnt 10pM only marginally promotes Wnt-activity, it supports organoid growth by 80% of the 500pM dose.* ***(C & D)*** *IGF2 and Insulin do not promote Wnt-activity.* ***(E)*** *Doses of insulin ~0.5μg/ml effectively promote organoid growth.* ***(F)*** *Despite potent Wnt pathway activation, CHIR99021 was occasionally toxic, usually when added to fresh medium.* ***(G)*** *IGF2 visibly promotes organoid growth.*

9.1b Insulin-Like Growth Factor 2 (IGF2) and Insulin

Next, physiologically relevant factors were sought that could promote organoid growth and stemness. Particular interest was taken to the follicular fluid (FF)-enriched Insulin-Like Growth Factor 2 (IGF2), since its FF concentration is 600-1000ng/ml, around 10x greater than the typical upper effective dose (Choi *et al.*, 2006). Moreover, direct treatment of immortalised hFT epithelial cells evidences that: 1) IGFBP2/6-bound IGF2 and PAPP-A are released in FF during ovulation; 2) PAPP-A is recruited onto fimbrial epithelial glycosaminoglycans and cleaves IGFBP2/6-IGF2 to liberate IGF2 in the membrane vicinity 3) IGF2 signals through IGF1R to exert intracellular effects. Thus, AKT/mTOR drives AIG (overcoming anoikis) and AKT/OCT4/NANOG boosts stemness (clonogenicity) (Hsu *et al.*, 2019). Accordingly, it was first confirmed that IGF2 could promote organoid growth (Figures 19G and 32D). On a similar note, an alternative ligand of IGF-1R, insulin, which binds with a lower affinity than the IGFs, is also found in the FF at relevant concentrations, from ~700-2800ng/ml (Diamond *et al.*, 1985). Although insulin had variable effects in promoting organoid confluence (Figure 20E), it was observed that it encouraged organoids to establish sooner.

To further explain these effects, it was considered if IGF2 and insulin are capable of promoting the WβC axis. As the central regulator of IGF2 activity in the hFT, AKT is known to interact with WβC signalling: 1) It impedes the nuclear translocation of FOXO3a which competes with β-catenin for binding to TCF (Liu *et al.*, 2015); 2) It phosphorylates and thus partly inhibits GSK3β (Hermida, Dinesh Kumar and Leslie, 2017); 3) It phosphorylates β-catenin to directly promote transcriptional activity (Fang *et al.*, 2007). Furthermore, provisional single cell RNA-seq data on hFT Wnt-active cells evidences DPP4 as a marker in the largest cluster (Alsaadi, 2021). Since DPP4 destroys incretins, boosting insulin and thus blood glucose, this may suggest that ovulatory insulin serves to feed metabolically-active hFT stem cells. Despite this, the vastly high FF dose of IGF2 and the FF dose of insulin could not firmly activate WβC signalling through Akt or otherwise (Figures 19C and 19D); it is thus thought that Akt-mediated effects in the hFT are largely independent of β-catenin. For insulin, it is

possible that it may drive membrane trafficking of GLUT4 as the mechanism for increasing the import of glucose as metabolic fuel – this is observed to occur *in vivo* and *in vitro* in the closely related endometrial epithelium (Long *et al.*, 2021). Moreover, the knowledge that DPP4 can cleave many factors beyond incretins to locally constitute the stemness niche, along with the miniscule size of the hFT epithelium, points to a local rather than wide-ranging action.

9.2 Developing an Organoid Recovery Medium

The combinatorial effect of these various organoid growth-promoting chemicals was next investigated, with the goal to cost-effectively create a recovery medium. On repeat studies, it was observed found that the doses of 1µg/ml insulin (not seen in Figure 20C, but shown robustly in Figure 32B) and 10pM of Surrogate Wnt (Figure 20B) independently promoted organoid formation. When combined, the anecdotal advantages of insulin with earlier formation of organoids was retained, with final confluence similar to that with Surrogate Wnt alone (Figure 20D versus Figure 20B). Moreover, in struggling cultures, treatment with insulin appeared to spare them from regions of necrosis, observed on multiple occasions.

Although IGF2 to some degree encouraged organoid growth at FF physiological range (Figure 20E), it co-operated poorly with Surrogate Wnt, leading to reduced organoid growth relative to the control (Figure 20F). The former combination was therefore utilised as a recovery medium. Very surprisingly, in a set of cultures in which old medium had been used, wherein hFT epithelial cells had survived in ECM to Day 7-12 but failed to establish organoids, addition of 10pM Surrogate Wnt and 1µg/ml Insulin led to organoids blossoming within 24 hours. Despite Surrogate Wnt being used in doses 7-fold lower than the half-maximal TOPFlash response based on supplier studies (Figure 20G) (ThermoFisher, 2020), and without clear mechanistic basis to using insulin, this observation cemented faith in the efficacy of the duo.

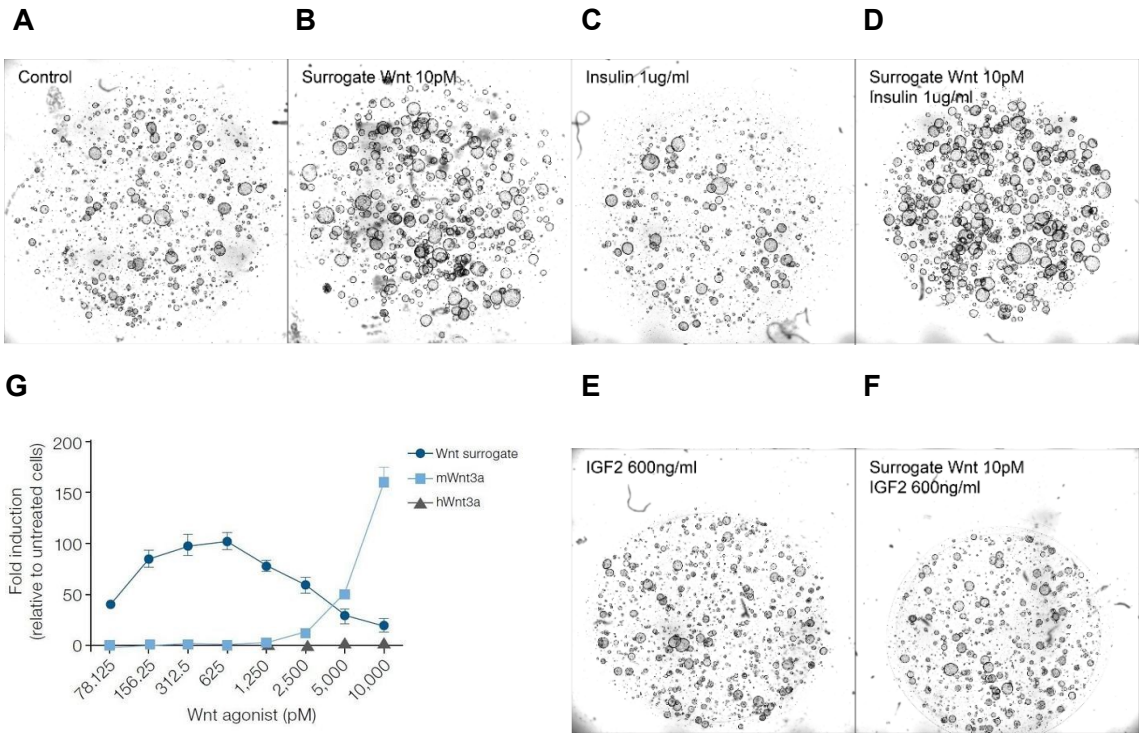


Figure 20. *Surrogate Wnt and insulin collaborate to support organoid growth.* ***(A)*** *Organoids grown in normal 3D organoid medium.* ***(B)*** *Extremely low dose Surrogate Wnt (10pM) potently increased growth.* ***(C)*** *Insulin (1µg/ml) alone had a marginal negative effect on growth in this batch, although organoids grew sooner, and Figure 34B illustrates its consistent benefits.* ***(D)*** *The combination of Surrogate Wnt and insulin provide the greatest increase in organoid size and number, with no necrotic regions.* ***(E)*** *High dose IGF2 alone provides a slight growth advantage* ***(F)*** *Surrogate Wnt and IGF2 do not synergise well.* ***(G)*** *It is conceivable that 10pM has a slight effect in promoting Wnt activity (y-axis). [(G) taken from (ThermoFisher, 2020)]*

9.3 Estrogenic Influences on Stemness and Growth

9.3a Follicular Fluid Contains Exceedingly High Levels of E2 and P4

At the point of ovulation, the follicular fluid is deeply enriched in both E2 and P4; E2 is the most concentrated FF molecule at 400-600ng/ml, 2900-fold higher than in the serum, and P4 is more variable (5-15µg/ml) but usually 800-fold higher than serum levels (Nagy *et al.*, 2019; Hsu *et al.*, 2022). Moreover, the quantity of follicular fluid released at ovulation is high, at ~2.7ml, leaving minimal scope for dilution prior to embathing of the hFT fimbrial epithelium (Simonetti, Veeck and Jones, 1985). No other tissue is exposed to such extreme bursts of these hormones, yet breast and even

prostate cancer are driven by lower circulating E2(/P4) levels (Omoto and Iwase, 2015). This warrants prompt investigation of their influence in ovarian tumorigenesis.

9.3b E2 Blocks WNT7A-Release yet can Exert a Separate Mitogenic Effect

The exact role of E2 in the hFT is uncertain. Firstly, it is known that estrogens downregulate WNT7A release in the anterior FRT, as observed in the Ishikawa cell line of endometrial adenocarcinoma, and recently evidenced in the hFT epithelium (Wagner and Lehmann, 2006; Alsaadi *et al.*, 2022). Moreover, treating mice with a highly potent agonist of ERα and ERβ, diethylstilbestrol (DES) leads to posteriorisation of the uterus, phenocopying *Wnt7a* deletion (Miller, Degenhardt and Sassoon, 1998; Sassoon, 1999). However, gestational DES treatment leads to enlarged uteri and induces mitogenic figures independent of Wnt7a status, although Wnt7a initially protects against E2-induced apoptosis (Carta and Sassoon, 2004). E2 may then later permit cell death, returning the hFT/uterus to the *status quo* post-ovulation. There is considerable evidence that the mitogenic effect is dependent on stromal estrogen receptors (Cooke *et al.*, 1997). It is notable that DES treatment and *Wnt7a* KO also disrupt Wnt5a patterning (Carta and Sassoon, 2004). Likewise, in a Wnt5a mutant heterograft model, DES-mediated Wnt7a suppression was abolished, but with normal epithelial proliferation (Figure 21A) (Mericskay, Kitajewski and Sassoon, 2004). With this data, the first step prior to hormonal study is to compile a corroborated model (Figure 21C).

9.3c Unifying the Likely Estrogenic Picture in the hFT

Recent laboratory works show that in hFT organoids, E2 alone suppresses WNT7A, but with failure of organoid proliferation, despite epithelial ERα presence (Figure 21B) (Alsaadi, 2021). Integrating this finding with historic murine endometrial study delivers the following complex picture: 1) WNT7A localises WNT5A to the stroma 2) WNT5A in turn inhibits epithelial WNT7A, keeping balance 3) E2-ERα directly inhibits WNT7A production/release 4) However, E2-ERα predominantly co-operates with stromal WNT5A-producing cells to inhibit epithelial WNT7A 5) E2-ERα may signal

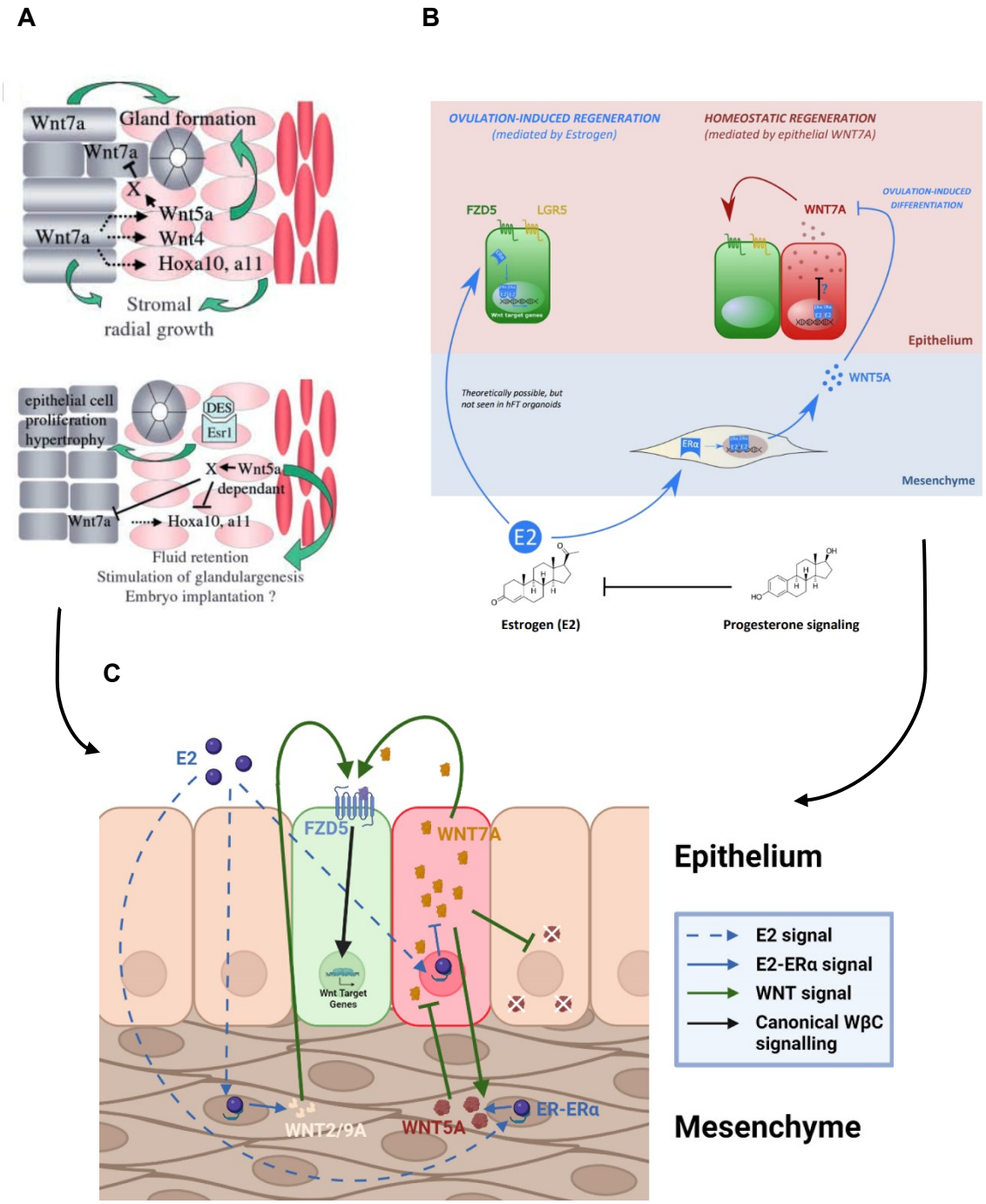


Figure 21. *The interplay between WNT7A, WNT5A and E2 in the posterior FRT.* ***(A)*** *In the endometrium, Wnt7a upregulates Wnt5a, which in turn downregulates Wnt7a possibly through an unknown factor. Estrogens (DES) act through mesenchymal ERα to promote epithelial hyperplasia.* ***(B)*** *In hFT epithelial organoids E2 suppresses WNT7A even without WNT5A, but is unable to hijack Wnt elements.* ***(C)*** *With hFT mesenchymal Wnts now known to be WNT2 and WNT9, an integrated model forms; E2-ERα can directly repress WNT7A release in the epithelium, and stimulate WNT5A and WNT2/9A release in the mesenchyme. WNT5A assists epithelial WNT7A repression, and WNT2/9A activate canonical signalling, possibly through FZD5, to cause epithelial hyperplasia. [(A) taken from (Mericskay, Kitajewski and Sassoon, 2004); (B) taken from (Alsaadi, 2021); (C) Created with BioRender]*

through an alternative stromal signal to induce epithelial proliferation. The latest single cell transcriptomic data of mesenchymal hFT cells suggests that this role may be fulfilled by WNT2 or possibly WNT9A producing cells (Alsaadi *et al.*, 2022). Moreover, RSPO3 appears to be the endogenous FZD stabiliser, and even this could be the estrogenic target. This proposed model is illustrated in Figure 21C.

9.3d In Vitro E2 treatment Counter-Intuitively Drives Differentiation

Additional to the above models, E2 is recognised to hijack WβC signalling at many levels (Figure 22A) (Wang *et al.*, 2010). It can block the endogenous Lrp5/6 repressor Dkk1, contribute to the destabilisation of the β-catenin destruction complex through indirect GSK3β inhibition, and can liaise with Ezh2, ERα and β-catenin to transactivate E2 and Wnt-target genes (Shi *et al.*, 2007; Zhang *et al.*, 2008; Pinzone *et al.*, 2009; Varea *et al.*, 2009). ER target genes include Wnt4, Wnt5a and Wnt7a, and E2 is understood to downregulate SFRP2 which acts as a soluble competitor for Wnt ligands (Das *et al.*, 2000; Wang *et al.*, 2010). Despite this, *in vitro* it has been shown that E2 treatment of early passage hFT organoids (p1 or p2) at 27ng/ml, vastly diluted from FF concentrations, was sufficient to repress WNT7A release (Figure 22B). After 2 weeks of treatment, organoids began to show crippling invaginations and necrotic cores (Figure 22C). It was reasoned that this should represent rapidly differentiating organoids, in which a near-total switch from secretory (PAX8+/KRT7+) to ciliated (TUBB4+/CAPS+/FOXJ1+) cells would be expected (Hu *et al.*, 2020).

Limitation of samples during planned E2 experiments restricted access to early passage organoids in this thesis, and it is known that ERα is frequently diminished in long-term culture. Utilising passage 8 hFT organoids, it was robustly shown however that treatment with E2 did reduce PAX8 positivity in a dose-dependent manner (Figure 22D). This differs from a study on monolayer cultured FT epithelial cells treated with 50ng/ml E2 for 14 days that found no changes in PAX8/TUBB4 staining – the disagreement may arise from the sub-physiological doses used by the group. This is

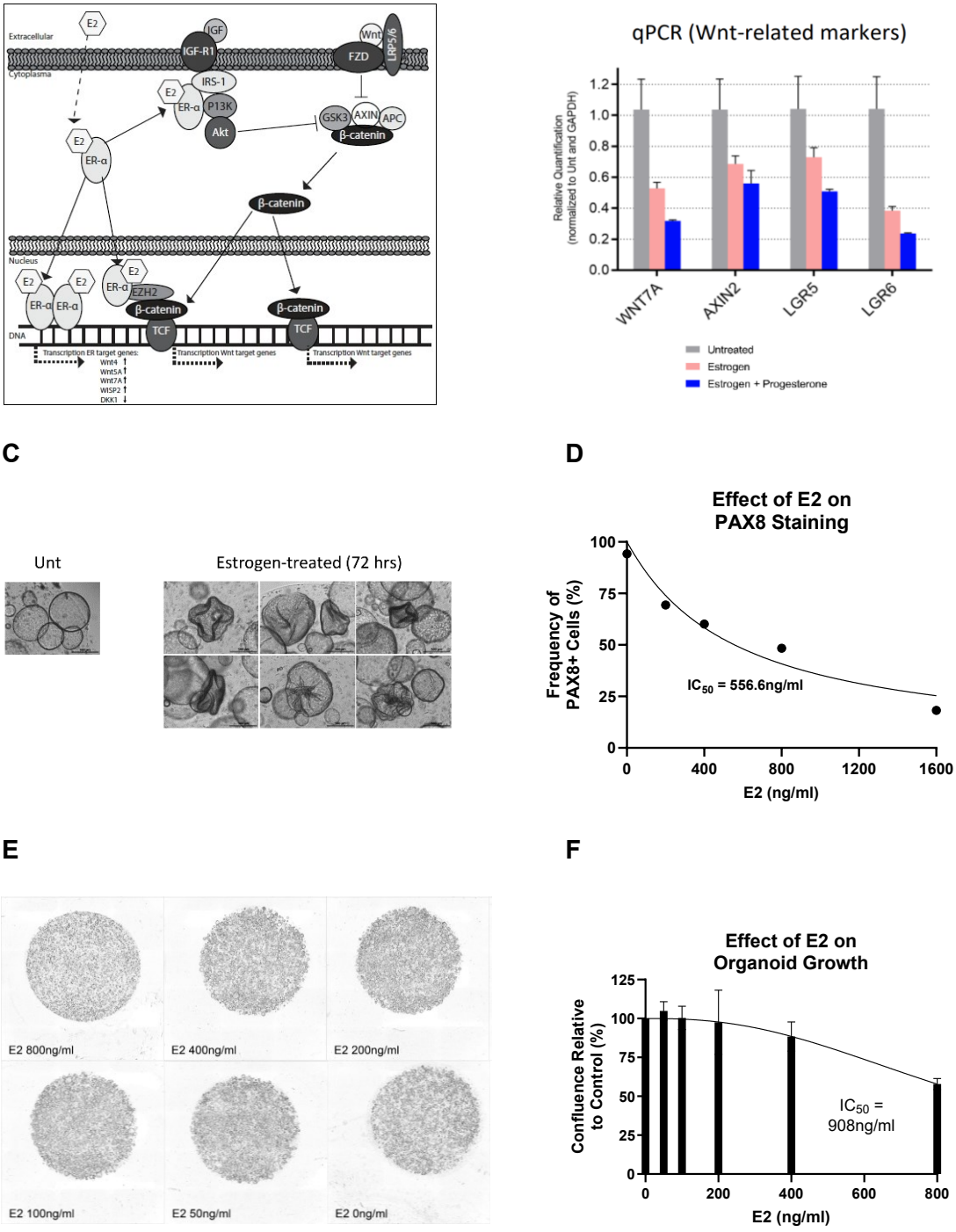


Figure 22. *E2 has a differentiating effect in hFT organoids.* ***(A)*** *E2 in theory can hijack WβC signalling at many levels.* ***(B)*** *qPCR illustrates significant downregulation of WNT7A and Wnt target genes with E2/P4* ***(C)*** *Treatment with E2 of early passage organoids causes crippling infolding, a differentiated phenotype.* ***(D)*** *The secretory cell marker PAX8 is greatly reduced by FF E2 doses, indicating widespread differentiation (IC_{50} CI 95% = 278.3 – 1033ng/ml).* ***(E & F)*** *At high physiological levels (~400ng/ml), organoid formation is inhibited (IC_{50} CI 95% = 765.8 – 1301ng/ml). [(A) taken from (Wang et al., 2010); (B) and (C) taken from (Alsaadi, 2021)]*

re-enforced by the inconsistent finding that the team noticed an increased fibrillary appearance, particularly with E2 doses of 100ng/ml (Chang, Ding and Chu, 2019).

Next, it was found that one-day treatment with FF-level diluents of E2, recapitulating ovulation, only attenuated organoid formation at high physiological range (Figure 22E). This may reflect that the brief timescale of ovulatory stress diminishes the effect, although this disagrees with previous observations (Carta and Sassoon, 2004). It may be that in late passage, partial ERα loss in WNT7A-producing cells occurred, limiting direct WNT7A suppression. Interpatient heterogeneity perhaps due to age or medication differences could instead be responsible; FT epithelial cell cultures from different patients were found to variably express ERα (Chang, Ding and Chu, 2019). In subsequent works, detailed experiments will be performed early on acquisition of a hFT from a pre-menopausal patient with benign disease.

9.4 *In Vitro* P4 Treatment Drives Differentiation as Expected

P4 is the hormonal counterpart to E2, largely serving as an inhibitor of WβC signalling. It is able to rapidly and directly induce DKK1 and FOXO1, leading to a suppression of TOPFlash Wnt-activity signal (Tulac *et al.*, 2006; Wang *et al.*, 2009). P4 is also able to directly counteract the WβC-promoting effects of E2; in the uterus it antagonises the E2-PI3K pathway that drives GSK3B activation, and it upregulates a portfolio of WβC pathway inhibitors (Chen *et al.*, 2005; van der Horst *et al.*, 2012). P4 may directly downregulate uterine Indian Hedgehog (IHH) – Patched (PTCH) signalling, which is a master regulator of WβC signalling, although this is disputed across other tissue types (Day and Yang, 2008; Wang *et al.*, 2010).

On the notion that E2 drives WNT7A suppression with failure of WNT signalling-hijack in hFT organoids, and on the basis that P4 drives DKK1/FOXO1 induction, *in vitro* one would not expect either to promote stemness, as was observed (Figure 23A). Consistent with this, loss of secretory marker PAX8 was observed in a dose-dependent

manner (Figure 23B), and previously, E2 +/- P4 treatment of fresh hFT organoids reduced Wnt-signalling markers LGR5, LGR6 and AXIN2 (Figure 22B) (Alsaadi, 2021). By stark contrast, in 2D hFT epithelial cell cultures, low-dose E2 (50ng/ml) or and, even more so, FF-dose P4 (5µg/ml), markedly increased these same Wnt-signalling markers, as well as stemness markers (SSEA3, SSEA4, ALDH1 and OLFM1) and Notch signalling markers (Hes1) (Chang, Ding and Chu, 2019). A partial explanation for this aberrance is that this group cultured their cells without ROCK inhibition, resulting in

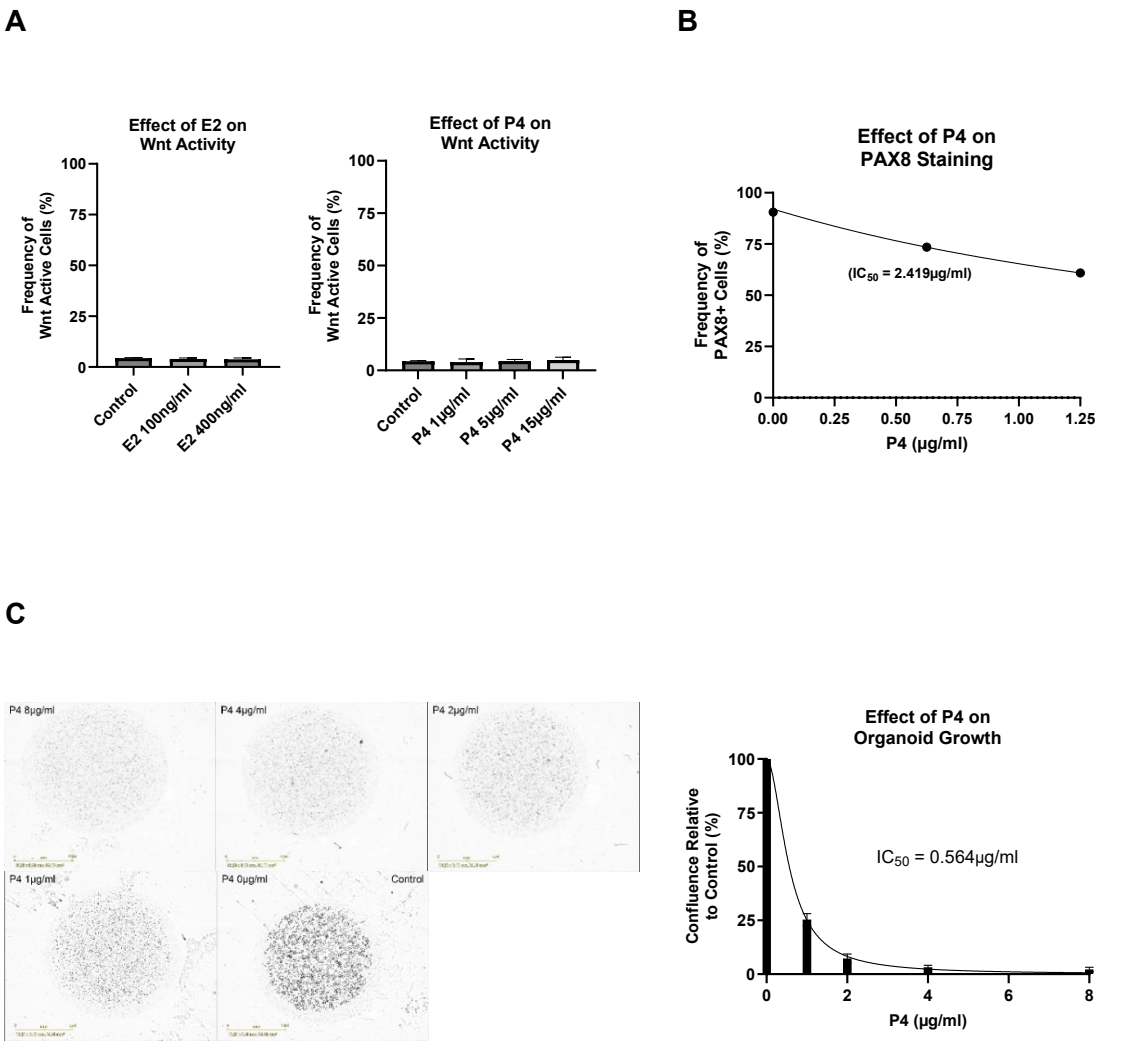


Figure 23. *P4 is powerfully able to impede organoid growth.* ***(A)*** *Neither E2 nor P4 promoted not inhibited Wnt-activity.* ***(B)*** *P4 likely induces differentiation, but less aggressively than for E2 (CI 95% incalculable).* ***(C)*** *The IC_{50} of 0.564µg/ml (CI 95% = 0.470 – 0.648µg/ml) is ~30 fold lower than expected from 2D models.*

complete loss of true stem cells within days. Notably, even overnight 2D hFT culture is associated with nontrivial transcriptional derangement (Hu *et al.*, 2020; Alsaadi, 2021).

With opposition of stemness in organoids, it was therefore expected that P4 would suppress organoid formation better than growth in 2D, however the extent to which this occurred was unprecedented (Figure 23C); in 2D, the LC_{50} is around 12-fold higher if PR-A+ and 30-fold higher if PR-A- (Wu *et al.*, 2017). This finding is of critical clinical interest, since this group found in 2D that brief (24h) treatment with supraphysiological P4 (30μg/ml) could selectively kill *TP53* suppressed (FT194) or dominant-negatively mutated (FT282-V cells). Cytokine analysis evidenced this to be necroptosis through TNF-α/RIPK1/RIPK3/MLKL signalling, leaving cellular ghosts. Even at this vast dose, in 2D only 50% killing occurred in each case (Wu *et al.*, 2017).

With far greater P4 potency in hFT organoids, it is anticipated that P4 at FF physiological levels (5-15μg/ml) may *in vivo*: 1) Entirely wipe the pre-malignant *TP53* mutant cells; 2) Eradicate excess stem cells, reducing the number available for transformation; 3) Throttle proliferation in all surviving stem cells. Supporting the prospect of such potent anti-tumorigenic effects of P4, 3-year usage of the contraceptive injection medroxyprogesterone acetate leads to a staggering 83% lifetime risk-reduction for developing epithelial ovarian cancer (Wilailak *et al.*, 2012).

Next, it was considered if the potent effects of P4 on organoid formation could be mitigated through physiological activation of non-Wnt pathways (IGF2) (Figure 24A) or through downstream activation of WβC signalling (CHIR99021) (Figure 24B). Although rescue was evident for the former two, P4 toxicity remains magnitudes greater than in 2D; the prospect of an anti-tumorigenic role remains. Finally, it was reasoned that if there is any slight degree of Wnt-hijack with E2, this could bypass potential P4-DKK1 effects – however, no rescue was observed, quashing this notion *in vitro* (Figure 24C). Overall, with an understand of FF factors that promote and inhibit stem cell proliferation, we can next explore this context of cellular stress.

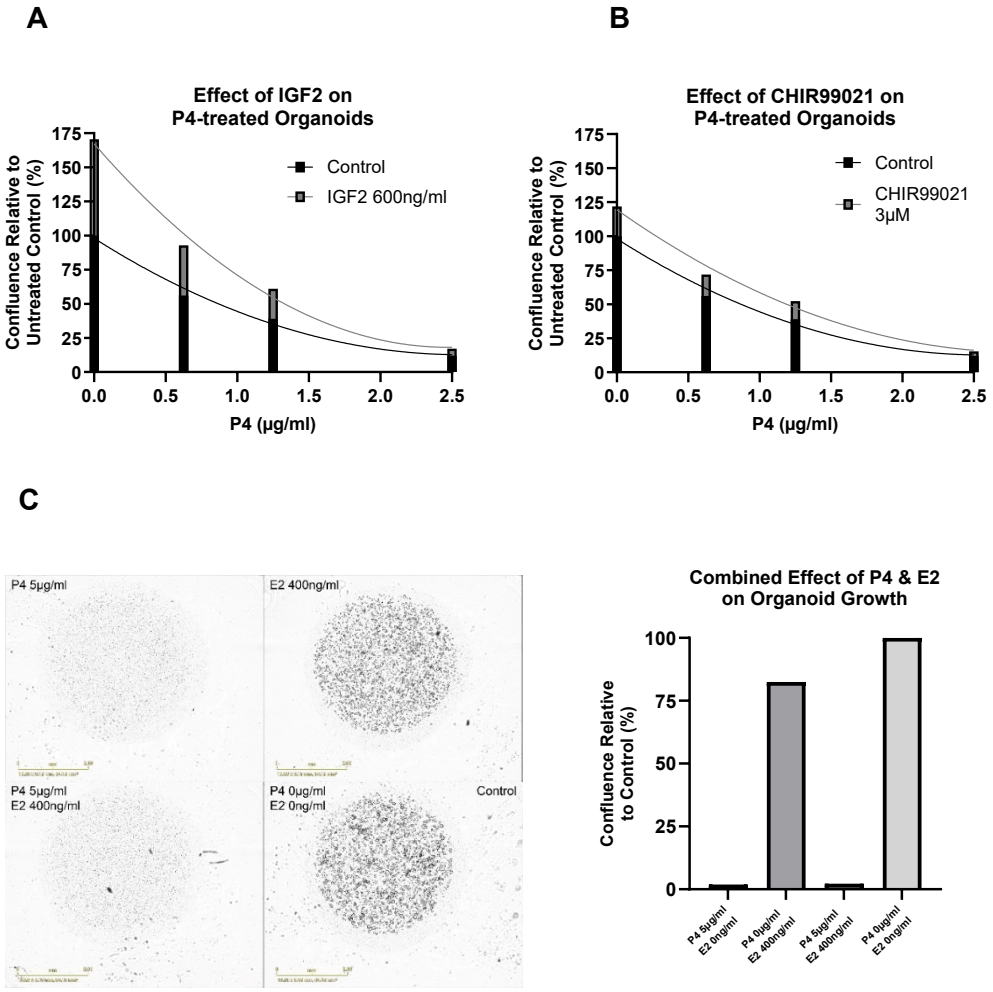


Figure 24. *The lethality of P4 can be partially mitigated by survival signals.* ***(A)*** *IGF2 provides ~70% increase in growth against P4, however at even half of low FF P4 concentrations (2.5µg/ml), near-complete toxicity is observed.* ***(B)*** *CHIR99021 provides a more modest survival advantage (~20%), again insufficient to overpower P4.* ***(C)*** *As anticipated, E2 is unable to rescue P4 effects.*

CHAPTER 10

Results Section 2:
Endogenous Mutagens

10.0 Mutagens Cause DNA Adducts and thus DNA Damage

Dependent on human behaviour, genetics, or mandatory function, every organ system is subjected to dangerous chemicals that drive malignant transformation. Damage to the hFT DNA likely falls best into this third category; ovulation and menstruation are required for life-bearing, and the damage to the hFT incurred by their counter-current flows in the hFT is just an unfortunate consequence. Inflammatory processes can directly generate reactive oxygen species (ROS), such as the hydroxyl radical (•OH), or reactive nitrogen species (RNS) that directly oxidise DNA (Cadet and Wagner, 2013). While most unstable chemicals react with cyclic or exocyclic nitrogen atoms of the DNA bases, carcinogens have unique signatures. The vulnerability of

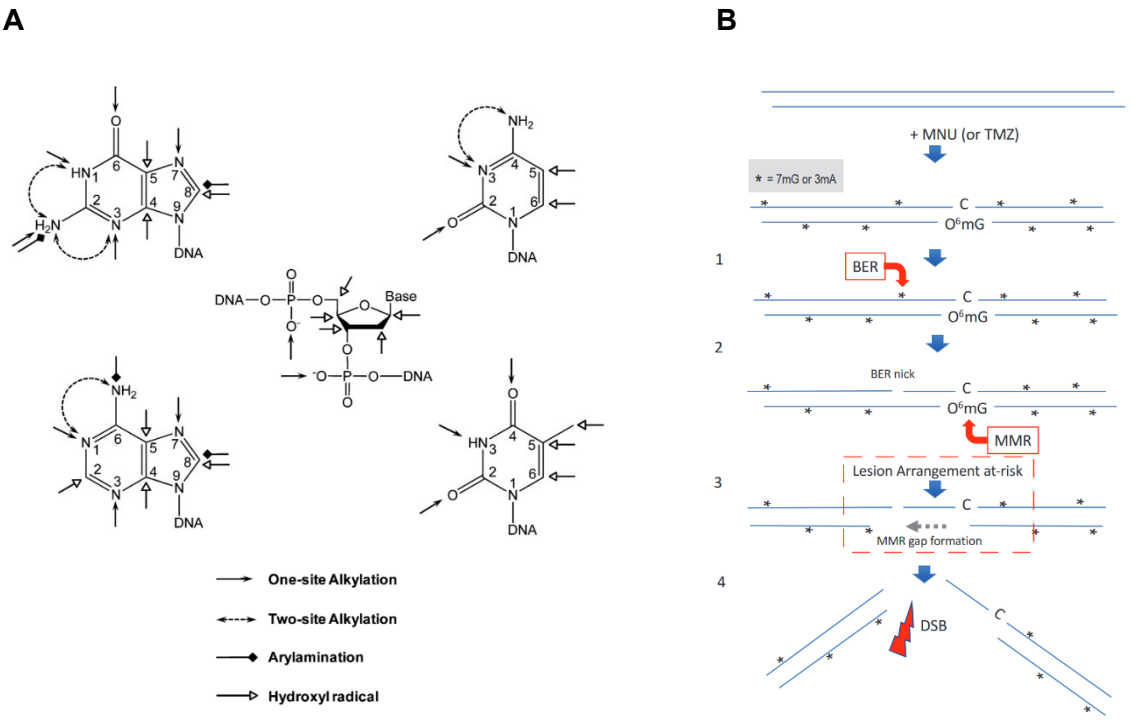


Figure 25. *Mutagens cause DNA adducts which frequently resolve into dsDNA breaks.* ***(A)*** *Alkylating agents such as polycyclic aromatic hydrocarbons (PAH), arylating agents such as heterocyclic aromatic amines (HAA), and oxidative agents (ROS), generate specific adducts on the four DNA bases – guanine (top left), cytosine (top right), adenine (bottom left), and thymine (bottom right).* ***(B)*** *DNA adducts, such alkylations caused by methylnitrosurea (MNU), are repaired by base excision/pruning – if these occur in proximity on adjacent strands, typically <500 nucleotides apart, DNA fractures, forming double stranded breaks (DSBs). [(A) taken from (Liu and Wang, 2015); (B) taken from (Fuchs et al., 2021)]*

base sites tailored to the nature of carcinogen is shown in Figure 25A (Liu and Wang, 2015). DNA adducts lead to DNA breaks in a number of ways. For instance, N7 guanine or N3 adenine adducts resolve to form apurinic (AP) sites, and during Base Excision Repair, transient single-stranded (ssDNA) breaks result. When this meets, for instance base pruning to repair a different lesion (such as an O6 guanine adduct) on the complementary strand, this leads to a DNA fracture – a double-stranded (dsDNA) break (Figure 25B) (Fuchs *et al.*, 2021).

10.1 Follicular Fluid ROS is therefore Potently Genotoxic

ROS has critical roles in folliculogenesis, follicle maturation and normal ovarian function, and is released in the inflammatory process of ovulation (Borowiecka *et al.*, 2012). Additional to forming oxidative adducts, ROS attacks membrane lipids and polyunsaturated fatty acids (PUFA), generating a family of aldehydes that form bulkier DNA adducts (Gentile *et al.*, 2017; Yun *et al.*, 2020). Its diverse routes to DNA damage mean we expect it to carry potent toxicity. Interestingly, patients have historically been found to have either high ROS within their FF (H_2O_2 equivalent: 400±25μM) or low ROS (H_2O_2 equivalent: 56±4μM) (Huang *et al.*, 2015).

It was reasoned that in hFT organoids, while the p53 response to dsDNA breaks is active, stem cells are shielded from extracellular chemicals and receive supportive signalling from their neighbours. In triplicate, the LC_{50} was found to be ~200μM (Figure 26A), and the imaging shows that in 3D, all physiological H_2O_2 levels (0-500μM) are strictly sub-lethal and have capability to transform (Figure 26A); the dot product of viability and γH2Ax induction is likely to represent transformative potential. These results differ from the equivalent monolayer studies in literature, wherein hFT epithelial cells had an LC_{50} of ~80μM (Huang, Chu and Chu, 2015). Moreover, this group showed that postovulatory fimbrial scrapings had potent (71%) γH2Ax induction, and that in an immortalised hFT epithelial line (FE25), γH2Ax was more readily induced at low H_2O_2 concentrations (Figure 26C) (Huang, Chu and Chu, 2015). As such, this

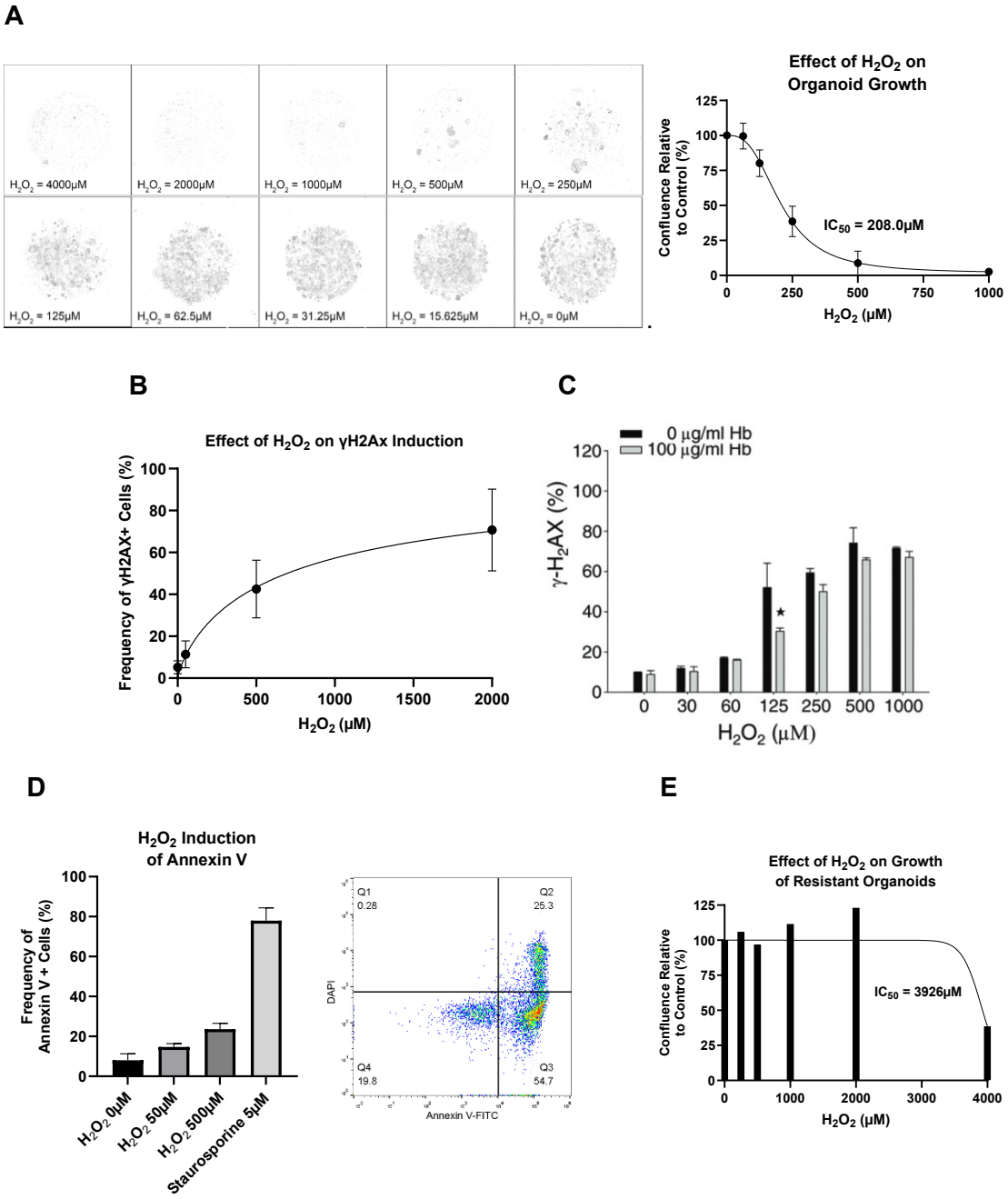


Figure 26. *H_2O_2 is less potent at inducing DNA damage in organoids compared to 2D models.* ***(A)*** *Rare organoids survive in concentrations as high as 1000μM, and the IC_{50} for organoid formation is 208μM (IC_{50} CI 95% = 176.2 – 251.0μM), greater than ~80μM observed in 2D.* ***(B and C)*** *H_2O_2 has roughly a 5-fold higher EC_{50} for causing dsDNA breaks in (B) hFT organoids (EC50 = 696.1μM, CI 95% = 426.8 – 1137μM) compared to (C) immortalised hFT cell line FE25 in 2D (left, black bars)* ***(D)*** *Annexin V induction at 24h increases with H_2O_2 concentration (left), but less than in FE25 2D cultures. As organoid processing caused some cell shearing, Q2 was unreliable, so the Q3:Q4 ratio (fraction of live cells Annexin V+) was used (Right).* ***(E)*** *Organoids established from one patient with suspected benign pathology were vastly resistant to oxidative stress, warranting study. [(C) taken from (Huang et al., 2016)]*

team proposed that other factors must greatly attenuate ROS to sub-lethal levels, to enable DNA damage to steadily accrue in surviving cells. However, the increased resilience in hFT organoids suggests that the 'Goldilocks' transformative H_2O_2 concentration is likely to be far higher than considered by the existing 2D models, negating the need for such attenuation. Compared to 2D, the milder induction of apoptosis in organoids as shown by Annexin V staining corroborates this further (Figure 26D).

Curiously, organoids from one patient, initially thought to be wild-type, were incidentally found to be resistant to toxicity at as high as 2000μM of H_2O_2, supraphysiological levels (Figure 26E). It is critical to next distinguish if this is due to a tumour suppressor gene mutation (e.g. *TP53*) or normal interpatient heterogeneity, as it may identify if genetic changes confer vulnerability to ROS-high FF, or if some patients are innately high-risk.

Next, it was considered how H_2O_2 causes dsDNA damage. In the 2D model, H_2O_2 was found to be transduced via NOX to generate intracellular ROS, as evidenced by NOX inhibition with Diphenyleneiodonium Chloride (DPI). This ROS is stabilised by Superoxide Dismutase (SOD) to form intracellular H_2O_2. Although H_2O_2 import is possible through aquaporins, inhibition studies evidenced this effect to be insignificant (Huang *et al.*, 2016). Given the stark transcriptional differences in 3D versus 2D, it was important to confirm this mechanism in hFT organoids (Hu *et al.*, 2020). Indeed, DPI was able to mostly rescue organoids from DNA damage caused by supraphysiological H_2O_2 (Figure 27A), as found in monolayer (Huang *et al.*, 2016). The slight residual γH2Ax induction could be due to: 1) Oxidation of membrane lipids leading to aldehyde-DNA adducts; 2) Some degree of aquaporin import of H_2O_2; 3) Partial NOX agonism by DPI itself; 4) Insufficient duration of pre-treatment with DPI (although a need to limit cell duration without media restricted this). Unfortunately the aggressive toxicity of DPI beyond 24h voids any prospects of therapeutic potential (Figures 27B and 27C).

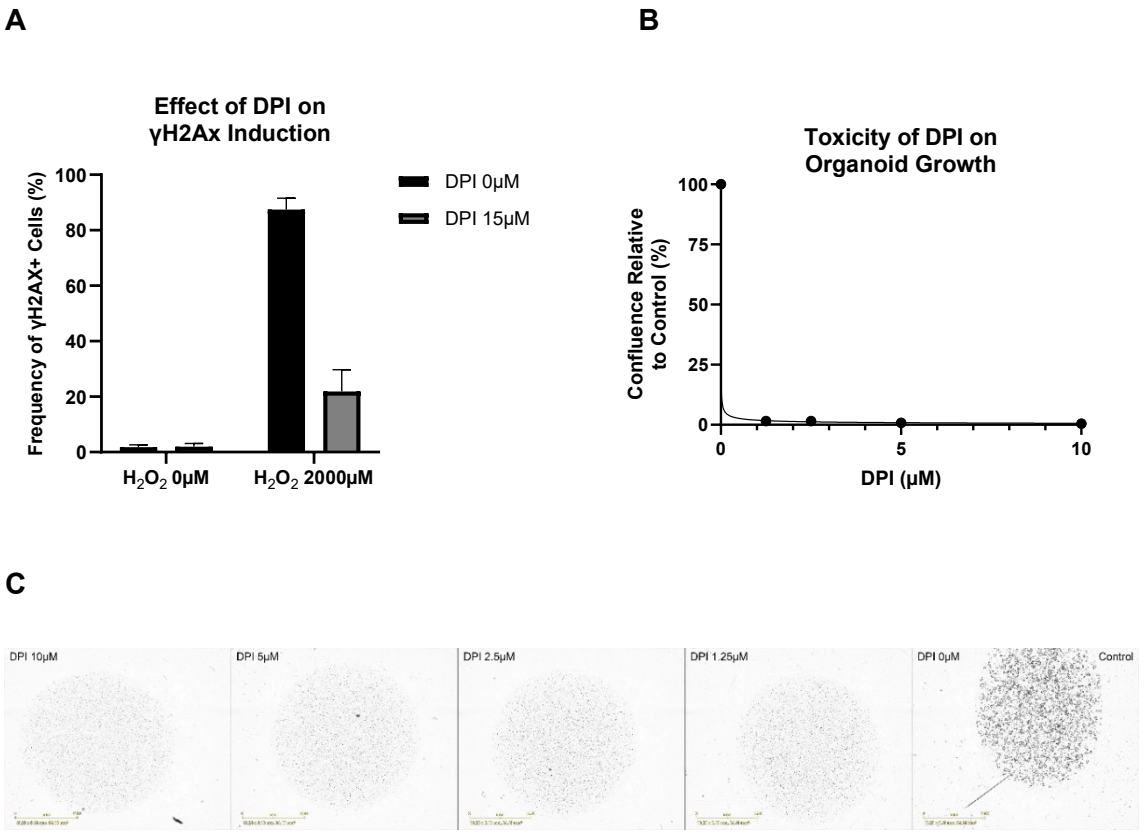


Figure 27. *Diphenyleneiodonium (DPI) protects against H_2O_2-induced DNA damage.* ***(A)*** *Even with supraphysiological concentrations of H_2O_2 (2000μM), 15 minutes of pre-treatment of organoids with the NOX inhibitor DPI abrogates DNA damage induction.* ***(B & C)*** *DPI has a typical EC_{50} of 1-3μM – even at the lowest bound, near-total toxicity to organoid formation was observed, so it is likely to be clinically unsafe.*

10.2 Iron and Transferrin are Mutagenic in the Correct Contexts

The hFT epithelium is exposed to iron, transferrin and haemoglobin (Hb) from three sources – ovulation (enriched when haemorrhagic), retrograde menstruation and endometriosis (Hsu *et al.*, 2022). The function of these is context-dependent. For instance, when Fe^{2+} is chelated by Hb, it may aggressively catalyse the Fenton reaction (Figure 2C), with Hb accepting the damage in place of the hFT (Widmer *et al.*, 2010). Although this sounds positive, ferryl-Hb might calibrate the sub-lethal genotoxic state (Figure 26C) (Huang *et al.*, 2016). On the other hand, if Fe^{3+} is bound to the plasma iron carrier, transferrin (Tf), this holo-transferrin (hTf) can bring Fe^{3+} intracellularly by binding the Tf receptor (TfR1), reduction to Fe^{2+} (STEAP), and import through DMT1

(Figure 28A) (Rockfield *et al.*, 2017). Separately, free Fe^{3+} can be reduced by DCYTB and directly imported by DMT1 (McKie *et al.*, 2001). These both generate the intracellular Fe^{2+} pool, juxtaposing the Fenton reaction to DNA, causing damage.

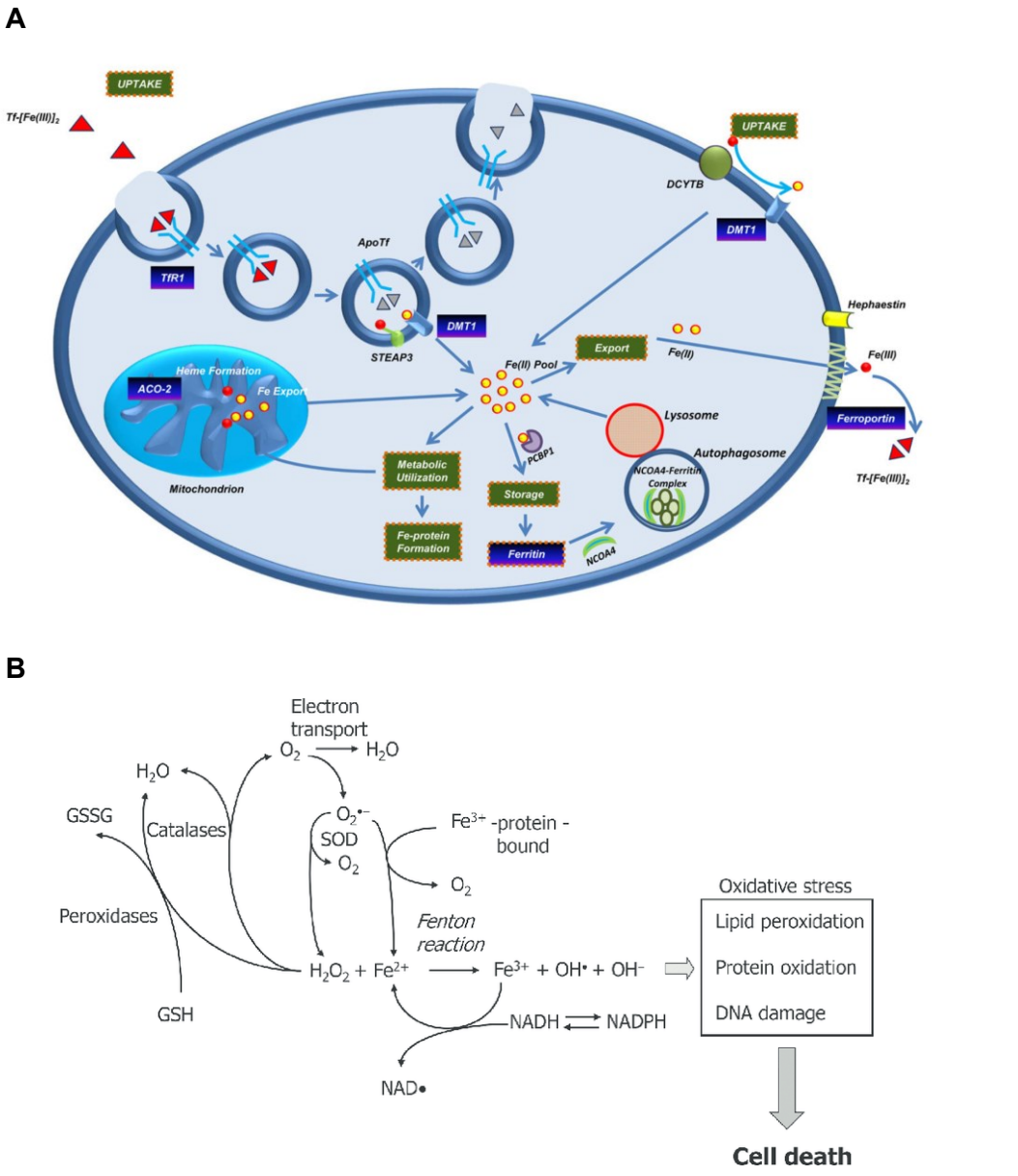


Figure 28. *Iron import can occur directly or with transferrin, catalysing free radical production.* ***(A)*** *Fe^{3+}-bound transferrin (holo-transferrin) binds to its receptor TfR1, causing endocytosis, reduction to Fe^{2+}, and import by DMT1. Alternatively free Fe^{3+} can be reduced at the membrane surface and directly imported by DMT1.* ***(A)*** *Antioxidant processes enacted by SOD, peroxidases and catalases create H_2O_2 as an intermediate. Fe^{2+} competes to react with H_2O_2, generating hydroxyl radicals (•OH) that can directly or indirectly attack DNA. [(A) taken from (Rama and Rodríguez, 2016); (B) taken from (Rockfield et al., 2017)]*

It is therefore of no surprise that iron metabolism is of significance in malignancy; in breast cancer, a 16-gene iron regulatory panel is an effective prognostic tool, and similar signatures have recently been identified in endometriosis/related ovarian cancers (Miller *et al.*, 2011; Wang *et al.*, 2023).

The role of free Fe^{3+} in HGSOC initiation, in the absence of H_2O_2, has been studied in 2D culture. With chronic treatment with Fe^{3+} levels 250-fold lower than FF levels, at 250nM, a vast increase in RNA/DNA base damage by oxidative adduct 8-OHdG has previously been found in a hFT secretory cell line (FT194), with mild increase in γH2Ax, and interestingly an increase β-catenin (Rockfield, Kee and Nanjundan, 2019). In this thesis, to understand if this translates to an effect due to free Fe^{3+} released due to ovulation, hFT organoids were instead treated one-time with high-dose ferric citrate. Contrasting the 2D findings, even at 320-fold higher levels, there was no obvious suppression or stimulation of organoid formation (Figure 29A), and no induction of γH2AX was seen (Figure 29B). In part, the immortalisation in FT194 (large T antigen) likely contributes to the dsDNA damage, and it is likely that chronic low-dose treatment will be required to observe tangible effects on WβC signalling (Rockfield, Kee and Nanjundan, 2019).

For hTf, it is expected that facilitated Fe^{2+} import should amplify dsDNA damage in both 2D and 3D. In 2D cultures for primary hFT epithelial cells, doses <10% of FF levels have been shown to induce γH2AX at up to 200% of the control, and elevated intracellular ROS was observed, salvageable by knockdown of TfR1 (Shigeta *et al.*, 2016). Interestingly, for hFT organoids, even FF doses as vast as 4mg/ml did not impair organoid formation (Figure 29C) and no obvious γH2AX induction was observed (Figure 29D). One possibility that no effects were seen for high hTf (and Fe^{3+}) in organoids is that cells in 2D are far more proliferative, with unlimited access to oxygen – they may generate the intracellular substrate (H_2O_2) for Fe^{2+} for the Fenton reaction.

To investigate the mechanistic basis of Fe^{3+} and hTf further, first, blockage of DMT1 was attempted with DMT1 Blocker 2 (DMT1B2). Owing to its toxicity, a dose-response curve was established, and it was investigated how physiological Fe^{3+} / hTf modulate this. Curiously, externalising Fe^{3+} had a protective effect, yet externalising hTf

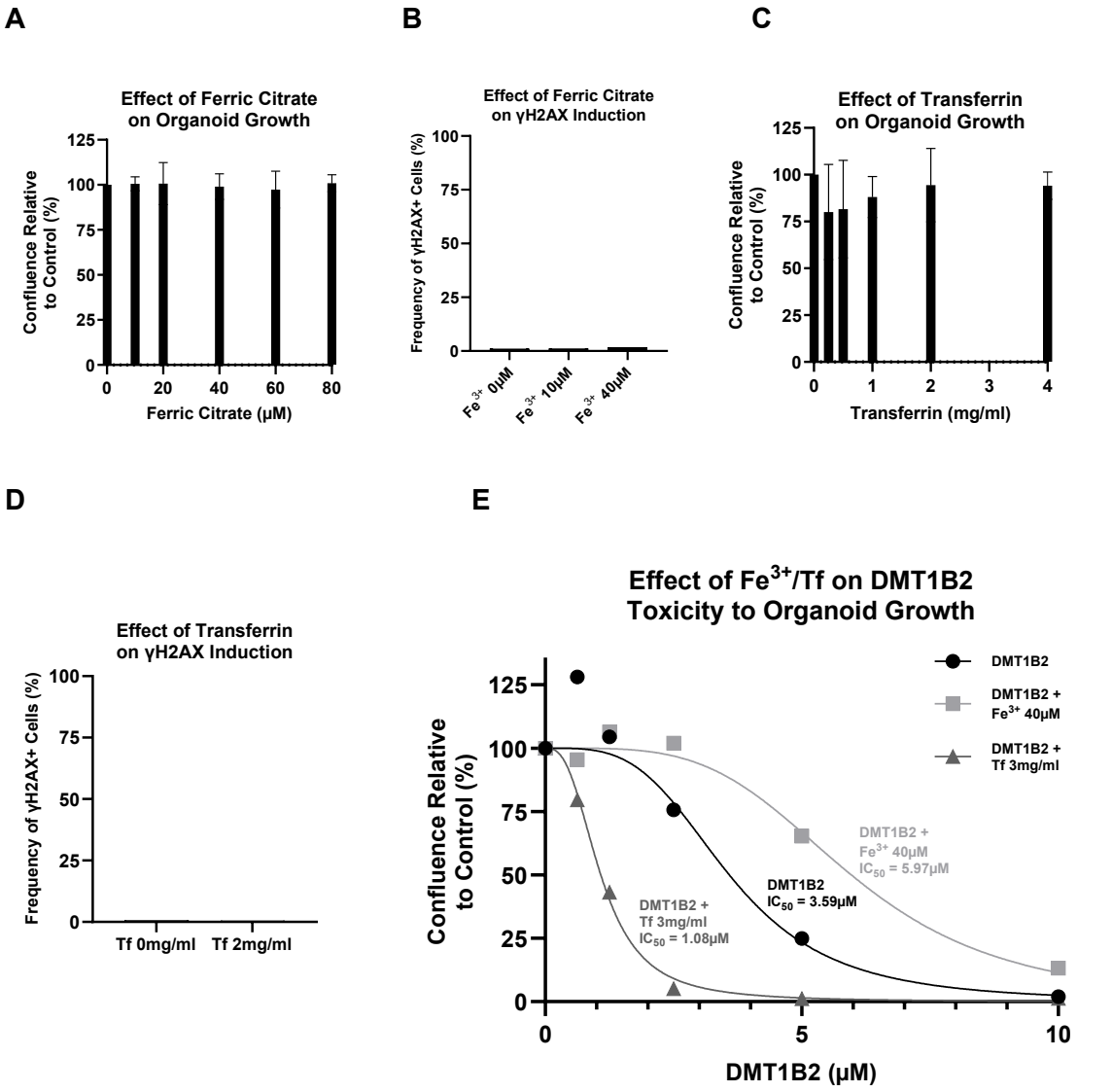


Figure 29. *Iron and transferrin are not toxic alone at physiological ranges.* ***(A & B)*** *Fe^{3+} at FF concentration (~40μM) (A) does not impact organoid growth, and (B) does not induce γH2AX.* ***(C & D)*** *Similarly, at FF levels of Tf (4mg/ml) (A) organoid growth is unaffected and (B) γH2Ax is not induced.* ***(E)*** *When divalent ion import is blocked with DMT1B2, Tf potently increases toxicity (IC_{50} 1.08μM; CI 95% = 0.98 – 1.19μM), whereas Fe^{3+} potently reduces toxicity (IC_{50} CI 95% = 5.20 – 6.96μM), compared to the control (IC_{50} CI 95% = 2.30 – 5.77μM).*

appeared potently toxic (Figure 29E). An explanation for the former could be that dying cells toxified by DMT1B2 had high intracellular H_2O_2, and externalising Fe^{3+} allowed neutralisation of extracellular oxidants. In the case of the latter, DMT1B2 blockade may mean that endosomes form with heavily concentrated free Fe^{2+} (unloaded from hTf-TfR1 by STEAP), that oxidises membrane lipids, generating genotoxic aldehyde adducts. Use of Ferristatin II, which degrades TfR1, or anti-TfR1 antibodies may be more suitable to study hTf externalisation (Byrne *et al.*, 2013; Candelaria *et al.*, 2021).

Next it was considered how Fe^{3+} and hTf would synergise with H_2O_2, since their toxic effects are inter-related (Figure 28B). In a novel finding, hTF was observed to significantly potentiate γH2AX induction by H_2O_2 in a dose-dependent manner (Figure 30A), at physiological FF levels, with the associated toxicity increase (Figure 31B). This, combined with the absence of toxicity of hTf alone (Figures 29C and 29D), corroborates a redefined mechanism of tumorigenicity: 1) hTf co-ordinates Fe^{2+} import, but this is safe cytosolically at low levels without any substrate; 2) NOX transduces H_2O_2 intracellularly, providing the substrate; 3) These undergo a Fenton reaction to aggressively generate oxidative DNA adducts.

The concept of endosomally concentrated Fe^{2+} alone causing toxicity, and the concept of hTf serving as an Fe^{2+} messenger for genotoxic collaboration with H_2O_2, inspired an idea. Perhaps the excessive hTf and Fe^{3+} in FF enable the cyclical import of vast quantities of Fe^{2+}, conferring toxicity alone. In endometriotic tissue, bleeding generates free iron, which Tf scavenges for import, resulting in an iron-high Tf-low state (Kobayashi *et al.*, 2009). For an unclear reason since there is not necessarily ovarian bleeding, FF from endometriosis patients also contains an iron-high Tf-low imbalance (Li *et al.*, 2020). It was reasoned that, in the non-overload state, the 52.5nM of Tf is plenty to import the 29.5μM Fe^{2+}, and that in iron-overload (endometriosis), the 42.5nM is still ample to import the 40.1μM Fe^{2+}. It was hypothesised that in combination, iron toxicity would now be observed, but greater for iron-overload – in another novel finding, this was exactly what occurred (Figure 30C).

Although it may be tempting to dismiss this mechanism as more relevant to endometriosis-linked endometroid and clear cell ovarian carcinomas, these are associated with endometriotic cortical inclusion cysts, and less subjected to FF toxicity. Meanwhile, iron overload in hereditary haemochromatosis has been associated with HGSOC, and mucosal iron deposits are frequently found in the hFTs of women diagnosed with advanced HGSOC (Gannon *et al.*, 2011; Seidman, 2013; Rockfield, Kee and Nanjundan, 2019). Regardless, the value of studying the collaborative effects of mutagens is apparent.

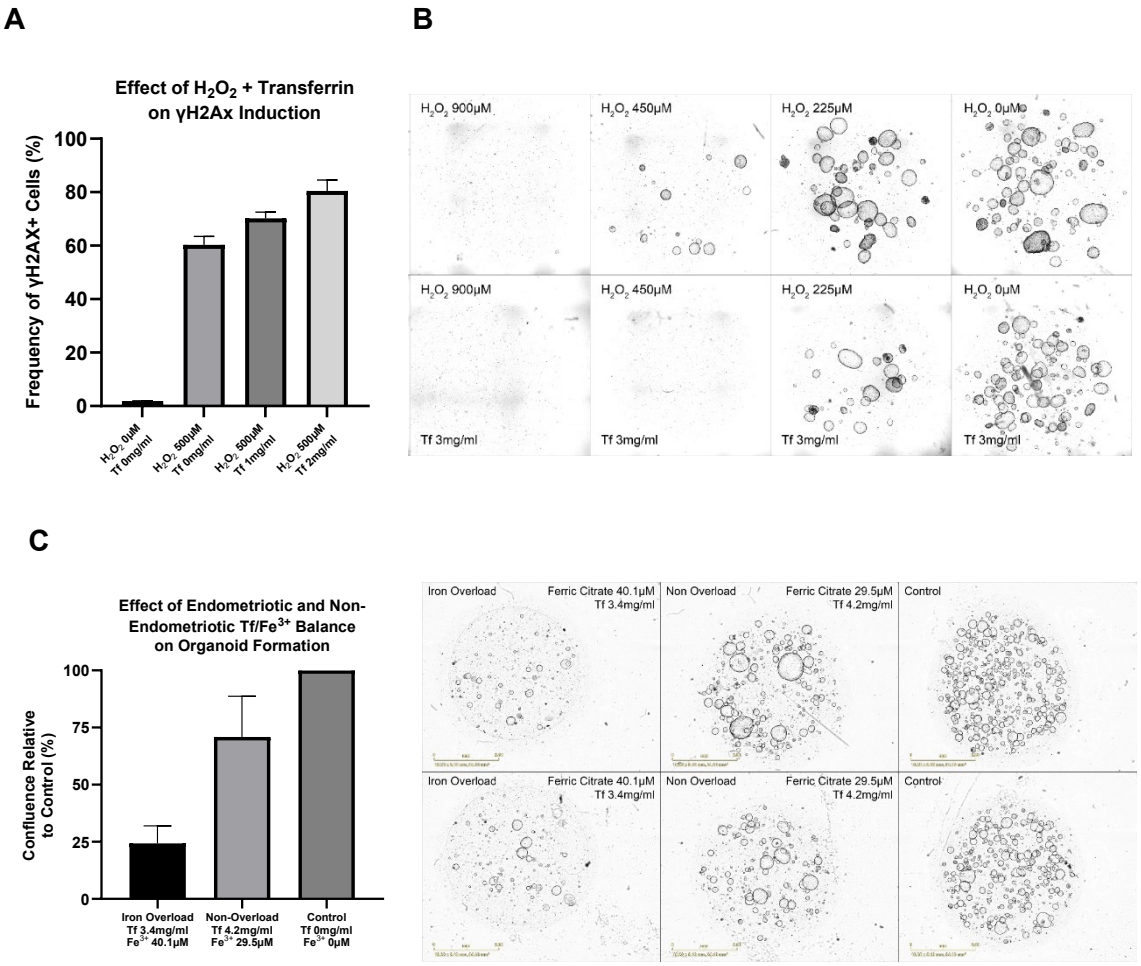


Figure 30. *Iron-related mutagens are only toxic to organoids in the correct context.* ***(A)*** *Tf is unable to induce γH2AX alone, but can strongly potentiate induction by H_2O_2 (p=0.0010, Two-Way ANOVA between H_2O_2 500μM ± Tf 2mg/ml).* ***(B)*** *Tf heightens the toxicity of H_2O_2.* ***(C)*** *Endometriotic patients have iron overloaded FF, with high Fe^{3+} and low Tf, which appears more toxic than the normal balance (p = 0.2355). Note that individually, neither Fe^{3+} nor Tf were toxic (Figures 29A – 29D).*

10.3 Estradiol is not Genotoxic to Naïve hFT Organoids

In the past, estrogens have been viewed as carcinogenic due to proliferative effects through hormonal signalling rather than direct mutagenic effect, for instance in breast cancer (Feigelson and Henderson, 1996). However, in ovarian breast, prostate and thyroid cancers, as well as Hodgkins lymphoma, depurinating estrogenic adducts are common. These are consequent to E2; through Phase I enzyme CYP1B1 or CYP1A/3A, 4-OH-(E1/E2) or 2-OH-(E1/E2) are generated, which if not extrahepatically conjugated by Phase II enzyme COMT, are further oxidised to quinone (E1/E2)-3,4-Q or (E1/E2)-2,3-Q. This can depurinate (N7-)guanine and (N3-)adenine, if not neutralised by conjugation by GSH (Figure 31A) (Cavalieri and Rogan, 2017). Thus E2 can create bulky DNA adducts and drain antioxidant reserves. In a monolayer model of genetically stable, immortalised mammary epithelial cells (MCF10A), knockdown of BRCA1 led to E2 / 2-OH-E2 / 4-OH-E2-driven surges in dsDNA breaks (γH2AX), p53 response (53BP1), stalled replication forks (pS4/8-RPA), and genomic instability (metaphase aberrations) (Savage *et al.*, 2014). Although these findings verify that BRCA1 is vital to repair bulky DNA adducts and maintain genome integrity, curiously, slight induction of these responses were visible in the albeit immortalised control at serum-territory concentrations, 1800-fold below FF levels.

It was hypothesised that in hFT organoids, delivering an overwhelmingly large E2 dosage would cause dsDNA damage, however, remarkably they appeared safe (Figure 31B). To see if functional loss of safe metabolism could contribute to transformation, hFT cells were pre-treated with the COMT inhibitor entacapone, but again no γH2AX induction occurred, and organoid formation remained minimally impaired in physiological E2 range (Figure 31C). The presence of superior DNA repair / HR machinery would not reconcile these findings; instead, perhaps hFT cells possess a safer metabolic pipeline to recycle E2. It will be fascinating to subsequently see if *BRCA1* KO organoids allow E2 to function as a genotoxin.

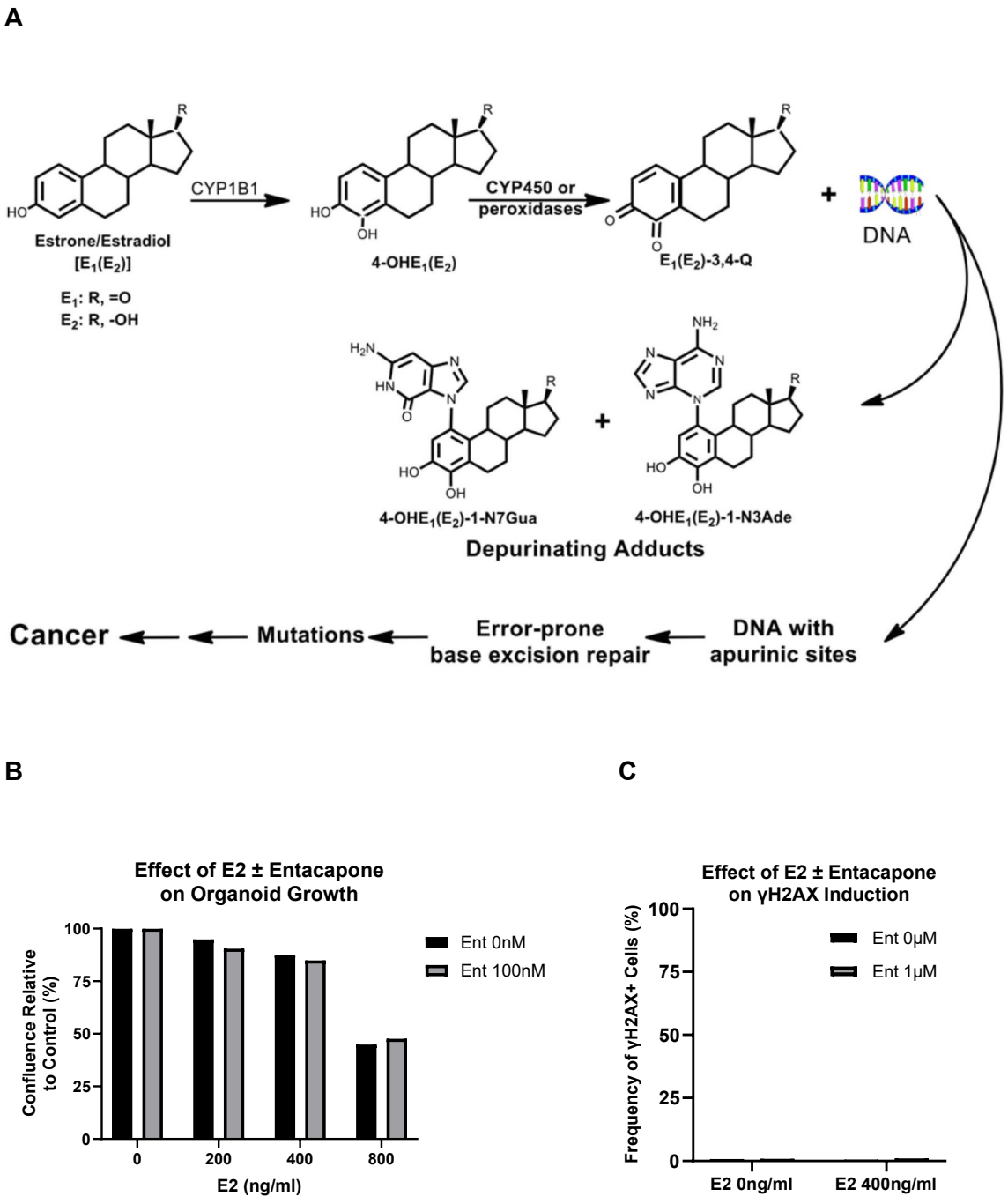


Figure 31. *Although estrogens can cause DNA adducts, this was not observed in hFT organoids.* ***(A)*** *Metabolism of estrogens by cytochrome P450 enzymes can lead to the formation of quinone metabolites that primarily form depurinating DNA adducts – note that this pathway can be safely subverted by COMT and GSH.* ***(B)*** *Even with COMT inhibition with entacapone, vastly high FF E2 concentrations did not increase the toxicity of E2 to organoid growth.* ***(C)*** *With or without high-dose COMT inhibition, E2 could not induce γH2Ax breaks. [(A) taken from (Cavalieri and Rogan, 2017)]*

10.4 Rescue from Genotoxic Stress is Possible Through Multiple Pathways

Whilst Hb may have the capability to reduce ROS to sub-lethal levels, this relies on a decrease in DNA damage per surviving cell (Huang *et al.*, 2016). A more sinister prospect would be if FF factors provide escape from cell death. First, it is understood through siRNA studies and Annexin V assays that activation of WNT(-1)/β-catenin/TCF signalling opposes apoptosis by inhibiting cytochrome c release from mitochondria (Chen *et al.*, 2001). In combination with aforementioned pro-proliferative effects, it was reasoned that Surrogate Wnt and CHIR99021 should protect against H_2O_2 stress in organoids - this was reputably found (Figures 32A & 32B). *In vivo*, activation of these pathways could be executed through Wnts embedded in microvesicles or endosome-derived exosomes. The latter has been robustly evidenced in a human colon cancer cell line (Gross *et al.*, 2012). Moreover, equine FF is know to contain 25 miRNAs that most notably regulate 88 Wnt genes, and porcine FF exosomes contain miR-31-5p which silences *SFRP4* mRNA (da Silveira *et al.*, 2012; Gross *et al.*, 2012). Additionally, FF RSPO2 concentrations are ~1ng/ml, which correlates to typical ED_{50} values (Kadim, Al-Wasiti and Qader, 2017; BioTechne, 2023). It is likely therefore that hFT epithelial cells are further protected against H_2O_2 by these agents *in vivo*.

Additionally, IGF1R/AKT signalling appears to have direct anti-apoptotic functions. Firstly, IGF1R-derived AKT inhibits GSK3β, preventing TIP60-dependent acetylation of K120 of p53. This post-translational modification is essential for it to drive apoptosis upon DNA damage (Duan and Maki, 2016). Insulin can likely also exert these effects, and in porcine follicular granulosa cells has been shown at FF concentrations (~1μg/ml) to downregulate Bim mRNA and its greatest splice variant BimEL – a Bcl2-family apoptotic protein (Han *et al.*, 2019). Thus, it was reasoned that, similar to WβC activation, anti-apoptotic and proliferative pathways activated by IGF2 and insulin should promote survival against stress. As anticipated, both conferred recognisable protection against H_2O_2 toxicity (Figures 32C – 32E).

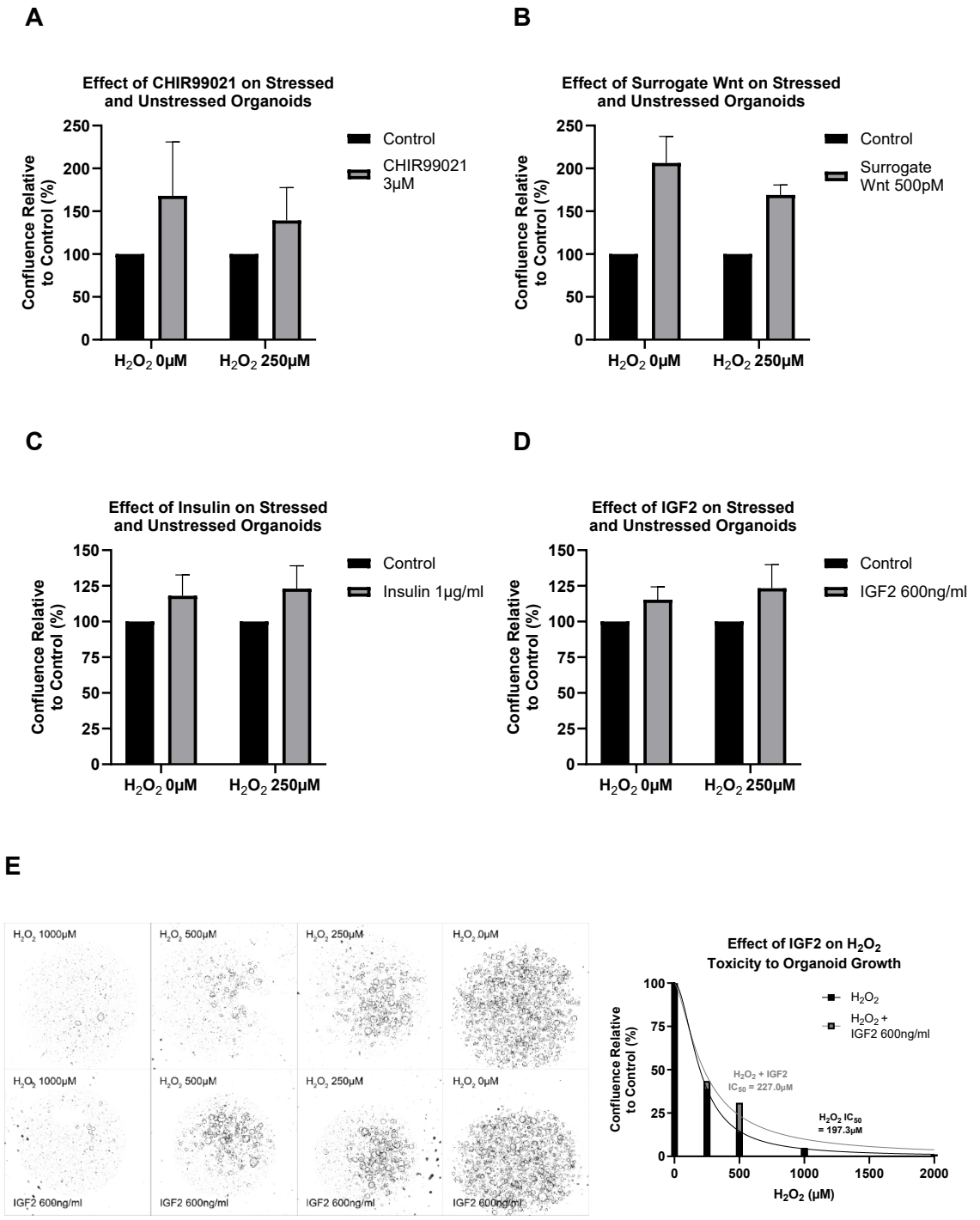


Figure 32. *Using WβC activators or insulin-like factors promotes organoid growth with/without oxidative stress.* ***(A)*** *CHIR99021 is capable of greatly increasing confluence, but its effect is variable, as seen in Figure 19F (p=0.0357, Two-Way ANOVA).* ***(B)*** *Surrogate Wnt at 500pM is the most reliable and potent growth-promoting agent in this figure (p<0.0001).* ***(C)*** *Insulin appears to have a marginal but consistent effect in supporting both stressed and unstressed organoids (p=0.0116).* ***(D)*** *IGF2 has a slightly lesser effect than insulin (p=0.0073).* ***(E)*** *The growth-promoting effect of even the weakest agent (IGF2) is evident from Incucyte imaging.*

In fact, considering the number of powerful survival factors in FF, the greatly elevated IC_{50} in hFT organoids (Figure 26A) versus monolayer, and the likely advantages of mesenchymal-to-epithelial signalling now raises the question if cells would readily survive high ROS (~500μM) *in vivo* (Huang *et al.*, 2015). If so, then ROS-reduction by Hb is protective, not dangerous, contrary to Huang *et al.* (2016). There is also some evidence that insulin, through PI3K and ERK signalling, can also suppress excess ROS from concentrating within cells, thus may synergise to this effect with Hb (Kang *et al.*, 2003). The overall effect could be found by obtaining post-ovulation fimbrial scrapings as in previous works, but from multiple patients, and co-staining for dsDNA breaks (γH2AX) alongside early apoptosis markers (Annexin V) (Huang, Chu and Chu, 2015).

10.5 Stem Cells may be Particularly Vulnerable to DNA Damage

In eukaryotes, DNA is complexed with histone proteins as chromatin in units called nucleosomes, enabling compaction, protection against damage, and gene regulation. In euchromatin, the transcriptionally active regions of the genome, the nucleosomes are loosely spaced, whereas in heterochromatin, which is transcriptionally inactive, tight nucleosome packing and repressive histone modifications are present (Alberts *et al.*, 2002). The predominant argument to which sites are vulnerable is not if heterochromatin is exposed at the nuclear periphery, or if it has superior steric shielding from oxidative adducts, but instead that base-excision repair (BER) apparatus is preferentially targeted to euchromatin at the nuclear core (Amouroux *et al.*, 2010; Yoshihara *et al.*, 2014; van Steensel and Belmont, 2017; Poetsch, 2020). Validating this, knockout of *OGG1*, encoding the enzyme that excises the 8-oxoG adduct in BER, results in loss of preponderance of 8-oxoG adducts to the nuclear periphery (Akatsuka *et al.*, 2006). Moreover, it has been demonstrated that as cells increasingly commit from pluripotency to multipotency to fully differentiated cells, the fraction of euchromatin decreases, and heterochromatin increasingly localises to the nuclear periphery (Ugarte *et al.*, 2015).

Considering this, it was hypothesised that multipotent stem cells in hFT organoids would be more vulnerable to dsDNA breaks than the remaining cells since: 1) There is more euchromatin, which is less guarded from mutagens; 2) The euchromatin is more accessible at the nuclear periphery compared to differentiated cells; 3) The euchromatin rapidly undergoes BER, creating ssDNA breaks which, if in proximity, can fracture DNA; 4) These are the only dividing cells, and adducts can interfere with DNA replication machinery. To test this, Wnt-reporter organoids were subjected to oxidative stress, and γH2AX induction was compared in multipotent stem cell-enriched Wnt-active cells, and multipotent stem cell-absent Wnt-inactive cells. Crucially, Wnt-active cells had greater γH2AX activity than Wnt-inactive cells when organoids were expanding (Figure 33A), although the abrogation of this at confluence (Figure 33B) wherein organoids stopped growing suggests that adduct interference with replication might be the main mechanism. However, it is important not to conflate increased γH2AX with increased dsDNA breaks here, since multipotent stem cells have enhanced DNA damage response, of which γH2AX is a component (Vitale *et al.*, 2017). This finding may instead be indicating superior protection.

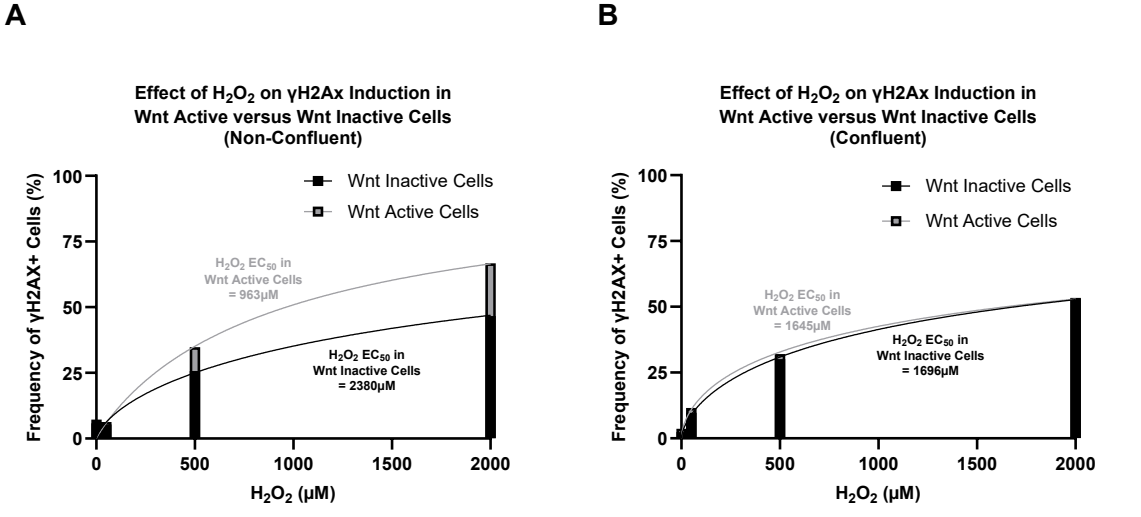


Figure 33. *Proliferating stem cells appear particularly vulnerable to oxidative stress.* ***(A)*** *At sub-confluence, Wnt-active cells, presumably rapidly dividing, had ostensibly higher γH2AX induction with H_2O_2-treatment than Wnt-inactive cells.* ***(B)*** *At confluence, wherein growth stopped, there was no difference in γH2AX induction by H_2O_2 between Wnt-active and Wnt-inactive cells.*

CHAPTER 11

Results Section 3:
Genetic Manipulation

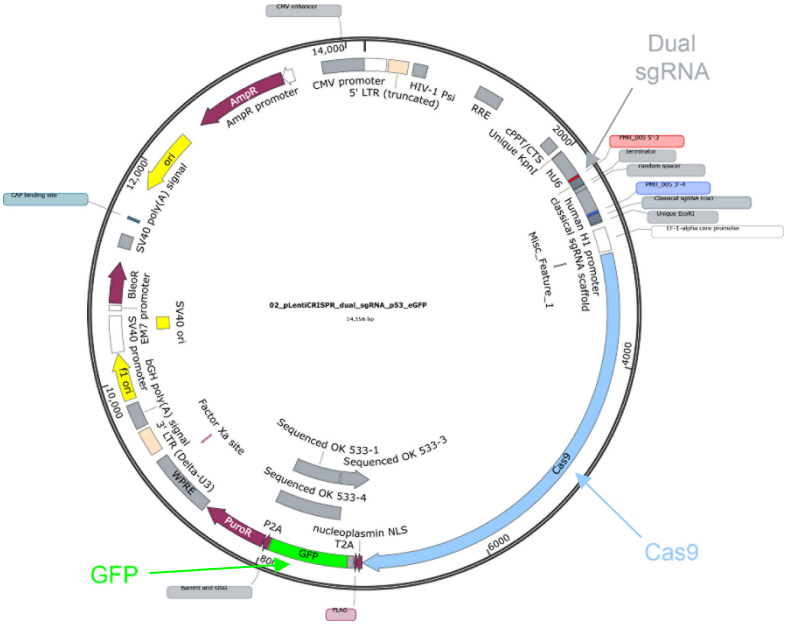
11.0 Potential Impacts of Genetic Manipulation on Organoids

Aforementioned studies on the impact of alcohol on haematopoietic precursors and about the genetic and environmental predispositions to hepatic steatosis indicate that we can effectively characterise genes by combining gene knockout with relevant physiological stress (Garaycoechea *et al.*, 2018; Hendriks *et al.*, 2023). To model HGSOC, first a library of passage-matched hFT organoids is required from each patient, comprising of driver mutations (*TP53*, *BRCA1* & *PTEN*) individually and in combination (Lõhmussaar *et al.*, 2020). Exposure to endogenous mutagens may then reveal secrets about the carcinogenetic process.

11.1 Genetic Manipulation Using a Lentiviral Approach

To develop the library of genetically manipulated organoids, a method of delivery of CRISPR/Cas9 machinery to enable gene deletion had to be determined. Owing to poor transfection efficacy with larger constructs, lipofection, a process in which genetic material is complexed with cationic lipids, was not considered (Schwank *et al.*, 2013). Electroporation, in which transient high-voltage shocks can be used to transfect complexes of ribonucleoprotein (RNP) and sgRNA/Cas9, avoids integration and minimises off-target hits, so is appealing (Matano *et al.*, 2015; Menche and Farin, 2021). However, the low efficacy and high toxicity in certain cell types, and absence of selection cassette integration point towards lentiviral transduction as a faster and more economical option (Cao *et al.*, 2010). With lentivirus, the risk of insertional mutagenesis means that regardless, clonal selection and genomic survey is necessary after purification of the mutant population (Shalem *et al.*, 2014). However, careful sgRNA design can mitigate the risk of off-target deletions, and such disturbances can now be reliably predicted and quantified (Gilbert *et al.*, 2013; Replogle, Bonnar *et al.*, 2022). With the slight advantage that, post-synthesis, lentiviruses can be easily aliquoted and frozen, ready-for-use in library generation, this approach was chosen.

A



B

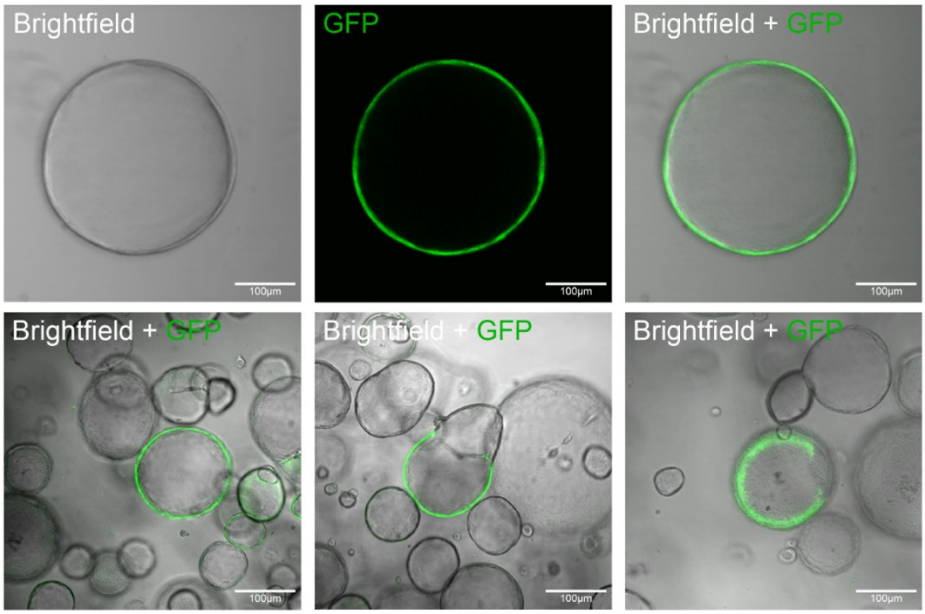


Figure 34. *Successful integration of a lentiviral vector.* ***(A)*** *Construct design of the Transfer Plasmid carrying a dual sgRNA CRISPR/Cas9 system targeting TP53 at two sites ~300 bases apart, reporting GFP.* ***(B)*** *Top Row – Brightfield, GFP and Overlay confocal images of an EV-GFP transduced hFT organoid (fluorophore-only control); Bottom Row – three Overlay confocal images of PTEN-KO_GFP transduced hFT organoids. [Credit for synthesis, transduction and imaging in (B): May Sallam]*

To robustly cause loss-of-function, lentiviruses were designed to deliver dual sgRNA (with Cas9), introducing double-nicks in a single gene (Figure 34A), with corresponding selection fluorophores. The gene would therefore lose a large fragment, with possible further frameshift upon NHEJ repair (Menche and Farin, 2021). After transduction, FACS was used to select Cas9 transactivated cells, and then clonal selection to isolate true mutants. Studies suggest that with this dual sgRNA pipeline, most populations should be hetero- or homozygotes, assuming viability (Replogle, Saunders *et al.*, 2022). Given this likelihood, the decision was made to use third over second generation lentiviral vectors, owing to the safety advantages despite lower typical synthetic yields (Table 3) (Addgene, 2021).

Safety-Related Feature	***2nd Generation***	***3rd Generation***	***Impact***
5' LTR in Transfer Plasmid	Wild Type	Chimeric – Tat independent	Eliminates need for Tat, reducing risk of accidental virus activation
3' LTR in Transfer Plasmid	Truncated, replication incompetent	Truncated, replication incompetent	Both result in a self-inactivating lentivirus post-integration
Packaging Plasmid	Single plasmid: Includes Gag, Pol, Tat, Rev	Split into two plasmids: Gag & Pol; Rev	Use of multiple plasmids decreases the odds of generating a competent lentivirus
Envelope Plasmid	Codes for VSV-G	Codes for VSV-G	Both carry the risk that VSV-G can infect many cell types
Synthesis and Transduction Efficiency	Easier to produce, but higher titres needed	Harder to produce, but lower titres sufficient	Reduced titre requirements lessens the risk of a lentivirus gaining comptence

Table 3. *3rd generation lentiviral vectors have many advantages over the 2nd generation. For the initial syntheses, based on availability, 2nd generation packaging plasmids were used, meaning certain safety benefits (light green) were not accessed. Overall, both systems are widely regarded as being safe. Note: LTR = Long Terminal Repeat; Tat = Transactivation; Gag = Glycosaminoglycan; Pol = Polymerase; Rev = Reverse Transcriptase; VSV-G = Vesicular Stomatitis Virus Glycoprotein [Information from (Addgene, 2021)]*

Transduction was then successfully performed for *TP53*, *BRCA1* and *PTEN*-targeting lentiviral vectors, along with blank controls, as illustrated in Figure 34B (Credit: May Sallam). Although promising, three points of concern were reported within the group: 1) The transduction efficacy, as estimated by the proportion of fluorophore expressing cells, was extremely low – typically ~5%; 2) Transduced organoids rarely survived beyond 2 passages, and when so, could often only be split at 1:1 – 1:1.5 ratio, leaving little scope for expansion; 3) The stress of organoid trypsinisation and FACS frequently led to all transduced cells dying. These findings had been recurrent across several attempts. To target these problems, we revisit some of the former results in this thesis. In Figure 20D, it was shown that 10pM Surrogate Wnt with 1µg/ml insulin in organoid medium increased confluence, and enabled organoids to establish sooner. In Figure 32B it was found that 500pM Surrogate Wnt alone supported the growth of organoids under sublethal oxidative stress. This knowledge was leveraged for rescue and expansion. Firstly, post-transduction, with precious numbers of struggling cells, 500pM Surrogate Wnt best promoted survival beyond passage 2. When thriving, organoids were expanded with the assistance of the far more cost-effective 10pM Surrogate Wnt and 1µg/ml insulin. Once upwards of 50,000 transduced cells were present (~1-2 million total cells), FACS was performed, with cells cultured in 500pM Surrogate Wnt one week either side, ensuring fresh medium the day prior. Putative *TP53*, *BRCA1* and *PTEN* mutants were successfully nurtured in this way.

11.2 Purifying *TP53*-Mutant hFT Organoids

With cells robustly proliferating post-transduction, the *TP53*-KO_GFP population were stringently selected by FACS, to minimise contamination with non-transduced, auto-fluorescent cells (Figure 35A). As previous works show, Nutlin-3a, an MDM2 inhibitor, potently activates p53, enabling p21-mediated cell-cycle arrest and PUMA/NOXA-mediated apoptosis in wild-type but not mutant cells, selecting for the latter (Kucab *et al.*, 2017; Aubrey *et al.*, 2018). However, it is worth recalling the lability of MDM2; during stress, it can switch to negatively regulate the cell cycle through Rb,

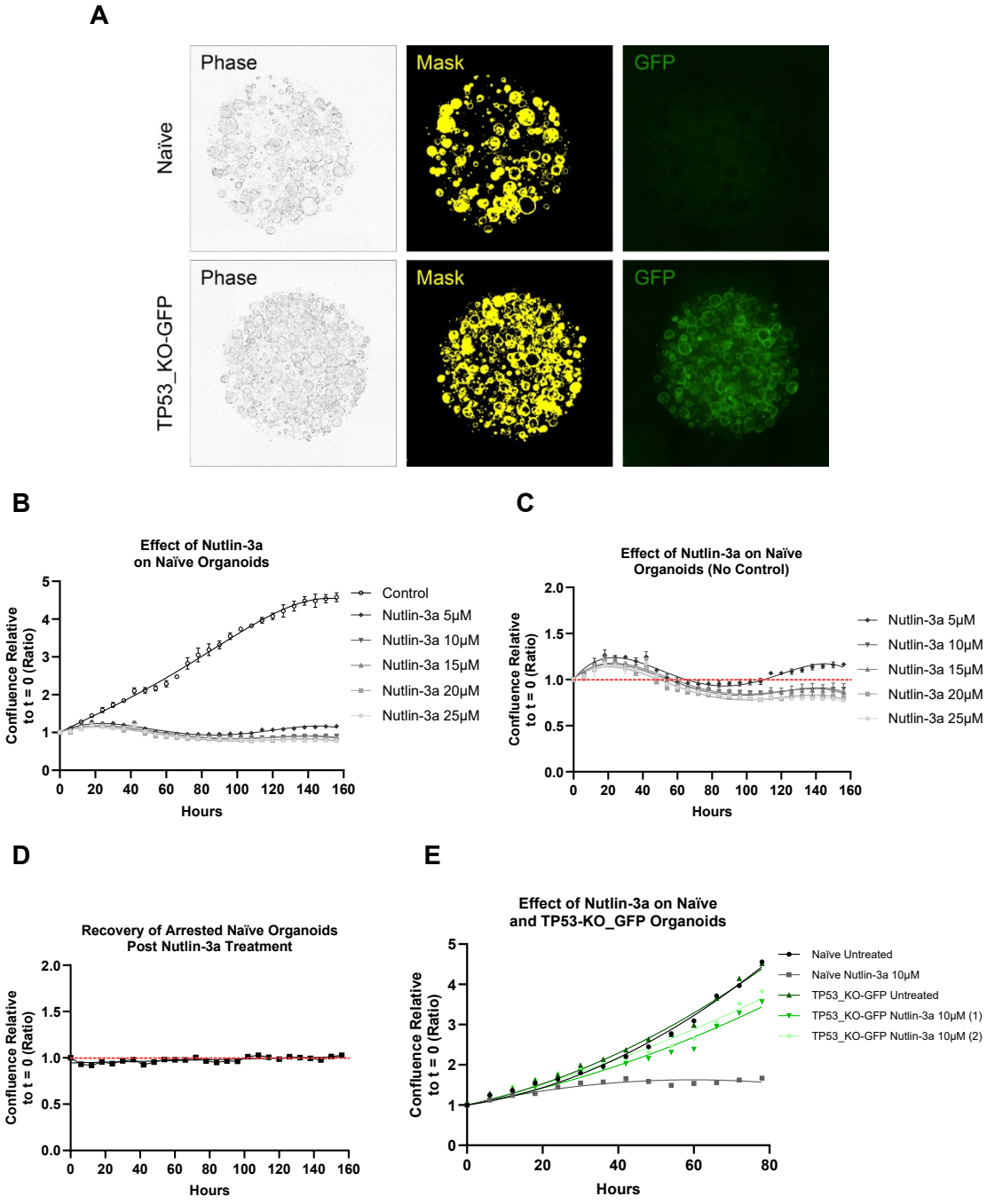


Figure 35. *Purification of TP53 mutant hFT organoids.* ***(A)*** *Post-transduction and expansion, the top 25% of suspected green cells were selected by FACS (3% overall). Phase, Selection Mask (for clarity) and GFP images show that no naïve hFT organoids fluoresced green, whereas the signal was bright for the TP53-KO_GFP transduced population.* ***(B)*** *Nutlin-3a 5-25μM throttled the growth of naïve hFT organoids relative to the control.* ***(C)*** *The lowest Nutlin-3a dose arresting organoid growth with no increases from baseline was 10μM.* ***(D)*** *Since Nutlin-3a halted growth but did not induce apoptosis, it was confirmed that after 5 days of treatment in Nutlin-3a 10μM, organoids could not re-grow in fresh medium.* ***(E)*** *Untreated naïve/TP53-KO_GFP organoids grew at a similar fast rate, treated TP53-KO_GFP organoids grew at ~70% of this rate (in 2 batches), and treated naïve organoids fully arrested.*

and ATM can convert it into a p53 transcription factor (Hernandez-Monge *et al.*, 2021). There is evidence to the contrary, that in p53 wild-type cancers under hypoxic stress, MDM2 inhibition has therapeutic value (Lerma Clavero *et al.*, 2023).

First, the minimum Nutlin-3a dose to cause complete arrest of hFT organoid growth was determined as 10μM (Figure 35B), with the test range based on typical organoid culture (5-10μM) and cancer cell line ranges (15-25μM) (Sachs *et al.*, 2019; Dekkers *et al.*, 2020; Warrington *et al.*, 2022). Although more rapid effect was seen at higher doses (15-25μM), the potential for off-target DNA damage effects is well recognised, so was avoided (Figure 35C) (Lerma Clavero *et al.*, 2023). To minimise the risk of stress altering the selection preference, media was changed 3 times weekly, with a treatment course of 2 weeks. Since organoids did not involute, it was next confirmed that halted organoids could not recover (Figure 35D). Finally, with Nutlin-3a treatment of FACS-sorted (GFP+) putative mutants, ~70% of the population survived (Figure 35E). As a provisional finding, qPCR on this same population corroborates ~70% *TP53* deletion (May Sallam, *personal communication*).

11.3 Optimising a hFT Organoid Clonal Selection Protocol

Within local laboratories, development of a cost-effective routine for clonal expansion of hFT or colorectal epithelial organoids has been unsuccessful (Alsaadi, 2021). In literature, seeding of FACS-sorted single hFT cells into 10μL Matrigel® ECMdomes achieved organoid forming efficiency (OFE) of 0.5-1%, at a likely cost of ~£400/clone, without even considering the difficulty of organoid picking and expansion (Kessler *et al.*, 2015). To make this feasible to conduct in bulk, several changes were made to this protocol: 1) Organoids were expanded for at least 3 passages post-FACS, and then trypsinised to single cells, as this greatly reduced cell stress; 2) Organoids were cultured with 500pM of Surrogate Wnt to promote OFE; 3) Single cells were pelleted in a 1.5ml Eppendorf Safe-Lock Tube, enabling complete aspiration of medium; 4) Cells were resuspended in Cultrex® ECM rather than Matrigel® ECM – its

wetter nature allowed 1µL domes to be polymerised without instant dehydration; 5) Considering the improved OFE of 4% for unstressed cells in optimised medium, putative mutant cells were seeded at 200/100/50/25 cells/dome, to increase the frequency of single organoid domes, as well preserve cell density effects to promote healthy organoid growth.

With this method, 10 clones were established from 96-wells of putative *BRCA1*-KO_GFP mutants (Figures 36A and 36B), at a cost of ~£4/clone. Although 25-50 cells/dome appeared to be the optimal seeding density, even in Cultrex® ECM, several domes dehydrated if medium was not added within 5 minutes. With this corrected, as high as 30 clones/96 wells may be expected. The biggest challenge, however, was harvesting single organoids, for which two methods were trialled: 1) Maceration with Dispase (1Unit/ml) alone led to 0/6 single organoids being grabbed; 2) Maceration with a pre-mix of Trypsin (0.05%) and Dispase (1Unit/ml) fully dissolved ECM, allowing organoids to instantly pellet as single cells, leading to 2/4 clones being successfully split. In brief – failure to fully digest ECM at first passage led to cells being lost. After utilising immunofluorescence microscopy to confirm GFP+ status (Figure 36C), one clone was successfully expanded in enhanced growth medium (with 10pM Surrogate Wnt and 1µg/ml insulin) to 2 million cells, ready for sequencing and study. Given that *Brca1* homozygous KO is embryonically lethal in mice, and not viable even on the background of *Trp53* KO in easier-to-culture murine oviduct organoids, the success of this clonal selection protocol in hFT organoids is particularly exciting (Liu *et al.*, 2007; Lõhmussaar *et al.*, 2020).

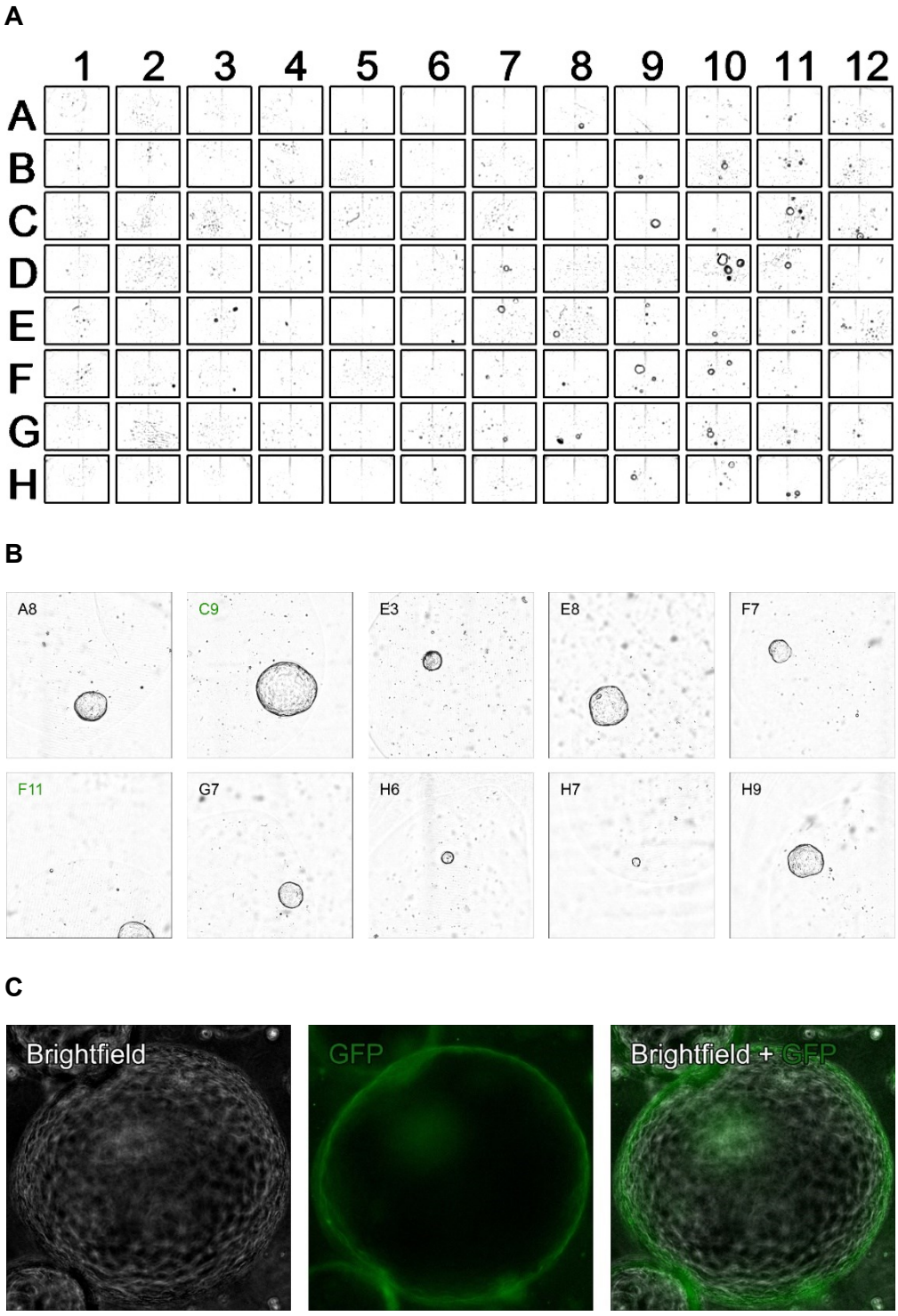


Figure 36. *Successful clonal selection in BRCA1-KO_GFP transduced hFT organoids.* ***(A)*** *Serial dilutions of 200/100/50/25 cells per 1µl dome were respectively seeded in columns 1-3/4-6/7-9/10-12. Columns 1-6 dried out rapidly, nevertheless 25-50 cells/dome appeared optimal to generate single organoids.* ***(B)*** *10 wells were identified to host exactly 1 organoid, from which C9 and F11 were successfully harvested and expanded.* ***(C)*** *Fluorescence microscopy showing GFP expression in the expanded clone C9.*

CHAPTER 12

Summary and Discussion

12.0 Creating an Early Detection Tool for HGSOC

The rising incidence of ovarian cancer demands enhanced early detection methods (Bray *et al.*, 2018). This study focused on hFT multipotent stem cells, the putative HGSOC origin, cultivated in organoids and exposed to both inherent mutagens and genetic alteration. Creating an accurate physiological mutagenesis model permits multi-omic analyses at each phase, potentially revealing unique DNA fragments and proteins – potential biomarkers – that enter the bloodstream. Used alongside patient data, these biomarker levels can help develop epithelial cancer screening panels with customised sensitivity and specificity (Liberto *et al.*, 2022). For affordability and safety, a positive predictive value (PPV) of 10% is essential for routine screening (Bast *et al.*, 2019). Despite the implication that 9 out of 10 positive tests might be false, it is a necessary trade-off to detect the 1 in 10 true cases. Given the low prevalence of ovarian cancer (0.07%) a cost-effective test with exceptional specificity and reasonable sensitivity is crucial (Bast *et al.*, 2019; SEER, 2020). For example, CancerSEEK, which examines select ctDNA and glycoproteins, offers 96.0% sensitivity and 99.0% specificity for ovarian cancer, but only achieves a PPV of 6.3%. It is also ~50% less effective for early-stage cancers (Cohen *et al.*, 2018). By contrast, PapSEEK, a combined plasma and Pap test for ctDNA, has a lower sensitivity at 63.0% but an impressive 99.9% specificity, resulting in a PPV of 30.6% (Wang *et al.*, 2018). However, with this sensitivity, ~4700 screenings are needed to prevent one case, and with current ctDNA test costs of ~£400, this equates to ~£1.8 million per case, a prohibitive amount (Kowalchuk *et al.*, 2022).

12.1 The Importance of Identifying a hFT Stem Cell Marker

The major current limitation to targeting the putative cell of origin, the hFT stem cell, is that a stemness marker is yet to be found. Studies of breast and pancreatic cancer validate that de-differentiation or metaplasia could well define the origin (Molyneux *et al.*, 2010; Flowers *et al.*, 2021). Transforming the correct cell increases the chances of identifying relevant ctDNA or protein biomarkers. Since activation of the

WβC axis is necessary for hFT organoid formation, the stem cell marker is likely to be a component of this axis (Alsaadi, 2021). One approach to finding this marker is as follows: 1) In Wnt-reporter organoids, delete Ser45 in β-catenin to increase Wnt-activity in a 'just-right' manner, whilst retaining dependence on a Wnt ligand (Parker, Rudeen and Neufeld, 2020); 2) Stimulate these manipulated organoids with 500pM Surrogate Wnt to boost stemness (Figure 19A); 3) Conduct scRNA-seq on FACS-isolated Wnt-active and Wnt-inactive PAX8+ (secretory) cells, and identify differentially expressed Wnt genes; 4) Conduct stem cell lineage tracing with putative markers (Barker *et al.*, 2009). To leverage this information to find the cell of origin, we must individually target candidate cells of origin. The possibilities are stem cells (PAX8+StemMarker+), non-stem secretory cells (PAX8+StemMarker-), Peg cells (CD44+ITGA+) and ciliated cells (FOXJ1+TUBB4+) (Paik *et al.*, 2012). In organoids, conducting inducible homozygous knockout of TP53 conditional to each of these cell types in the presence of continuous sublethal oxidative stress should most readily transform the true cell of origin.

12.2 Contextualising hFT Stemness to HGSOC

Promotion of the WNT7A-FZD5 axis is essential to the maintenance of hFT stem cells in organoids (Alsaadi *et al.*, 2022). Factors that activate this axis, including Surrogate Wnt and CHIR99021, boost Wnt-activity (Figure 19A), implying an induction of stemness, and likely through this mechanism, promote the growth of unstressed organoids and the rescue of organoids exposed to physiological oxidative stress (Figures 19B, 32A and 32B). Thus, during ovulation, WβC activators in FF likely enrich hFT stem cells available, and reduce death signalling following DNA damage – a recipe for carcinogenesis. It has already been discussed that RSPO2 concentrates in FF, and FF exosomes carry portfolios of miRNAs regulating Wnt genes, along with *miR-31-5p* that potently suppresses *SFRP4* (da Silveira *et al.*, 2012; Kadim, Al-Wasiti and Qader, 2017; Yuan *et al.*, 2021). However, we must equally consider the loss of WβC inhibitors. During follicular expansion, maturing oocytes secrete Growth Differentiation Factor 9 (GDF9) and BMP15, TGFβ superfamily members that activate SMAD2/3 and

SMAD1/5/8 differentiation pathways respectively (Reader *et al.*, 2011). Attrition of these factors within follicles is observed as feature of ovarian ageing, replicating the *in vitro* effects of A83.01 and Noggin in maintaining stem cells (Park *et al.*, 2020). Ageing may therefore lead to increasingly dangerous FF. The active metabolite of another FF component, Vitamin D, downregulates canonical WβC activity through its receptor by recruiting β-catenin to compete with TCF-binding, upregulating Vitamin D target genes including *DKK1* (Larriba *et al.*, 2013). Indeed, knockdown of Vitamin D receptor increases WβC signalling and aggression in colon cancer, and Vitamin D deficiency has been associated with ovarian cancer risk (Larriba *et al.*, 2011; Dovnik and Dovnik, 2020). Additionally, hyaluronic acid in FF may positively regulate components of WβC signalling, as in amniotic mesenchymal stem cells (Liu *et al.*, 2016). This is particularly interesting, as hyaluronic acid, along with its binding partners CD44 and versican, are heavily implicated in invasion (Ween, Oehler and Ricciardelli, 2011); thus it may in particular mediate successful STIC seeding to the ovary.

12.3 Follicular Fluid Growth Factors and their Impact on the hFT

Multiple growth factors exert their effects through PI3K-AKT and RAS-MAPK signalling, including IGF2 and insulin in FF (Hsu *et al.*, 2019). These factors again potently confer survival benefits on unstressed and stressed organoids (Figures 32C and 32D), and similar to WβC activators the latter is the dangerous phenomenon. Poor synergy between IGF1R and insulin suggests saturation of IGF1R (Figure 21F), however FF contains several further growth factors that bind alternative receptors. For instance, amphiregulin is the most enriched EGF receptor activator in FF, along with epiregulin and betacellulin at high levels, and trace quantities of TGFα and EGF (Hsieh, Zamah and Conti, 2009; Inoue *et al.*, 2009). Alongside wider proliferative advantages, other growth factor-receptor pathways that converge on these survival pathways, effected by FF, include IGF1-IGF1R, BDNF-TrkB, VEGF-VEGR, FGF-FGFR, LIF-LIFR and Leptin-LEPR (Hsieh, Zamah and Conti, 2009). Thus, it would be of great value to quantify the individual and collective efficacy of these growth factors in

activating AKT *in vitro*, using an AKT Kinase Activity Assay Kit (Abcam #ab139436). Moreover, fluorogenic dyes are available that can measure intracellular ROS, such as H_2DCF-DA (ThermoFisher #D399). Subsequent studies may include treating hFT organoids with FF from IVF patients, and through γH2AX, ROS, apoptosis, AKT and Wnt-activity survey, understand how extensively growth/survival factors, Wnt-activators and hormones enable tolerance of physiological stress.

12.4 The Influence of Follicular Fluid Hormones on the hFT

Great confusion surrounds the potential roles of E2 and P4 in the hFT, owing to inconsistent responses of tubal cells to hormones in different *in vitro* conditions (Table 4) (Chang, Ding and Chu, 2019; Alsaadi, 2021; Zhu *et al.*, 2023). Despite this, they point in unanimity to a picture for E2; in hFT monolayer, porcine oviduct (pOv) monolayer / air-liquid interface, and pOv organoid culture, a co-activator may be maintained that enables E2-ERα targeting of TCF/LEF to hijack transcription of Wnt-target genes. In hFT organoids, sustenance of this factor may be subordinate to continuous mesenchymal-to-epithelial signalling, perhaps via WNT2/WNT9A/RSPO3 (Alsaadi *et al.*, 2022). On the other hand, E2-ERα may drive ciliation without need for this factor. As a result, without mesenchyme, hFT organoids lose stem cells, differentiate and ciliate, leading to crippling (Figure 22C). By contrast, in unimpaired signalling, an equilibrium of stemness and differentiation promotion may be reached, resulting in proliferation and ciliogenesis simultaneously. In endometrial organoids, E2-ERα has been evidenced to create a permissive setting for multi-ciliogenesis through downregulating *NOTCH1*, and then upregulating the master co-ordinator, *GMNC* (Haider *et al.*, 2019). Since WβC signalling in hFT organoids is understood to promote Notch1 expression by contrast, and since there is no evidence *GMNC* or *NOTCH1* are direct Wnt target genes, it is likely that E2-ERα-determined ciliogenesis is unrelated to β-catenin, supporting the hypothesis (Kessler *et al.*, 2015; Nusse, 2023).

	hFT (2D)	***hFT (3D)***	***pOv (2D)***	***pOv (3D)***
	(Alsaadi, 2021) (Chang, Ding and Chu, 2019)	(Alsaadi, 2021) This Thesis	Zhu *et al.*, 2023	Zhu *et al.*, 2023
ERα presence	✓ ✓	✓ ?	?	?
WNT7A suppression	E2 ✓ E2 ?	E2 ✓ E2 ?	E2 ?	E2 ?
Wnt-related markers or TOPFlash assay	E2 ↑ P4 ↓ E2 ↑ P4 ↑↑	E2 ↓ P4 ↓ E2 ─ P4 ─	E2 ? P4 ?	E2 ? P4 ?
Ciliation	E2 ? E2 ✓	E2 ? E2 ?	E2 ✓	E2 ?
Secretory or stemness markers (PAX8)	E2 ? P4 ? E2 ─ P4 ─	E2 ? P4 ? E2 ↓ P4 ↓	E2 ─ P4 ↑	E2 ? P4 ?
Ciliated / differentiation markers (TUBB4 / Ac-T / FOXJ1 / CAPS)	E2 ? P4 ? E2 ─ P4 ─	E2 ↑↑ P4 ↑ E2 ? P4 ?	E2 ↑↑ P4 ↑	E2 ? P4 ?
Proliferative effect	E2 ? P4 ? E2 ? P4 ?	E2 ↓ P4 ? E2 ↓ P4 ↓↓	E2 ↑↑ P4 ↑	E2 ↑↑ P4 ↑

Table 4. *Varied effects of E2 and P4 on stemness, ciliation and growth in tubal tissue have been observed.* ***(Column 1)*** *In 2D monolayer hFT studies, E2 and P4 both promote stemness and differentiation simultaneously.* ***(Column 2)*** *In 3D hFT organoids both E2 and P4 oppose stemness, causing differentiation and reduced growth.* ***(Column 3)*** *In 2D pig oviduct air-liquid interface and monolayer cultures, both E2 and P4 increased differentiation yet increased proliferation.* ***(Column 4)*** *This proliferative gain was recapitulated in 3D pig oviduct organoids.*

There is another apparent contradiction. P4 increases Wnt-related markers in 2D hFT and has a proliferative effect in pOV organoids (Chang, Ding and Chu, 2019; Zhu *et al.*, 2023). Meanwhile, it inhibits TOPFlash activity in 2D hFT and suppresses Wnt-related markers in hFT organoids (Alsaadi, 2021). Firstly, in 2D, LGR5/6 may simply be priming the cell for later WβC signalling, whilst AXIN2, DKK1 and FOXO1 robustly shut off the pathway (Wang *et al.*, 2010; Bernkopf, Hadjihannas and Behrens, 2015). Secondly, hFT organoids required P4 co-treatment with E2 to induce progesterone receptors (PR). Since P4-PR induction of DKK1 is especially rapid, in the first hours with low PR expression, suppression of Wnt-related markers may have been the initial effect (Tulac *et al.*, 2006). Thirdly, with Wnt-independent growth, mOv organoids have different stemness physiology to hFT organoids (Alsaadi, 2021). If this is true for pOv organoids, an altered P4 response may be expected.

12.5 The Therapeutic Potential of P4

A similar dilemma exists for the aforementioned p53-defective FE25 cells, which are selectively killed by high doses of P4 (30μg/ml) through PR/TNFα/RIPK1/RIPK3/MLKL-mediated necroptosis, with no killing of p53 wild-type primary cells (Wu *et al.*, 2017). FE25 cells did not express PR-A, likely due to epigenetic repression of ERα and PR-A that occurs in *in vitro* cultures. It is likely that the retained toxicity to high-dose P4 occurred through residual weakly expressed PR-B. Indeed, transfection of PR-A doubled toxicity, and *in vivo* diestrus, when PR expression is prolific, cytolytic doses of P4 to *Trp53*-/- mOv epithelium were ~1000-fold lower than *in vitro* (Wu *et al.*, 2017). In this thesis, it was found that primary hFT cells, which are fully resistant to 30μg/ml P4 in 2D, are unable to form organoids in as comparatively little as 2.5μg/ml P4 (Figure 23C). The previously confirmed absence of PR-A means the effects are likely effected through minimal PR-B (Alsaadi, 2021). A logical reason could be that P4-mediated DKK1 and FOXO1 inhibition of WβC signalling is more relevant to organoid than 2D proliferation.

The therapeutic potential of this is vast; *TP53*-mutant hFT stem cells, the cells that likely transform to HGSOC, *in vivo* will have combined vulnerability to PR-mediated stemness suppression and necroptosis. Although ovulation provides a burst of P4 at likely supra-clinical concentration (5-15μg/ml) when PR-A/PR-B is abundantly available, surviving mutants have 28-days to transform before being re-exposed to such a toxic effect (Mangal *et al.*, 1997). By combining the findings that even with no PR-A/low PR-B, FF concentrations of P4 can suppress stemness (Figure 22B and 23B) and in similar context induce necroptosis in *TP53* mutants, a window of opportunity opens (Wu *et al.*, 2017). During menses, even when PR-A/PR-B are low, if a topical burst of FF-dose P4 could be applied to the fimbriae this would serve as a second layer to erase any expanding *TP53* mutants. This could be delivered as laparoscopically implanted chips grafted onto distal hFTs that, during menstruation, release a burst of a progestin designed to have ultra-short half-life to action only imminently local effects

(Figure 37A). This could be especially preventative for high-risk BRCA mutant carriers who get HGSOC early, whilst preserving ongoing fertility, and critically avoiding systemic progestin (MPA) that increases breast cancer risk despite potently protecting against ovarian cancer (C. I. Li *et al.*, 2012; Wilailak *et al.*, 2012).

With wild-type and *TP53*-knockout populations of passage-matched organoids purified in this thesis (Figures 35A – 35E), the next experimental step is to recapitulate the differential killing effect observed in 2D, at lower P4 doses. Following this, for *in vivo* study, as the only animal other than humans to naturally develop HGSOC, the laying hen could be an excellent model. Importantly, hens are matched to humans with

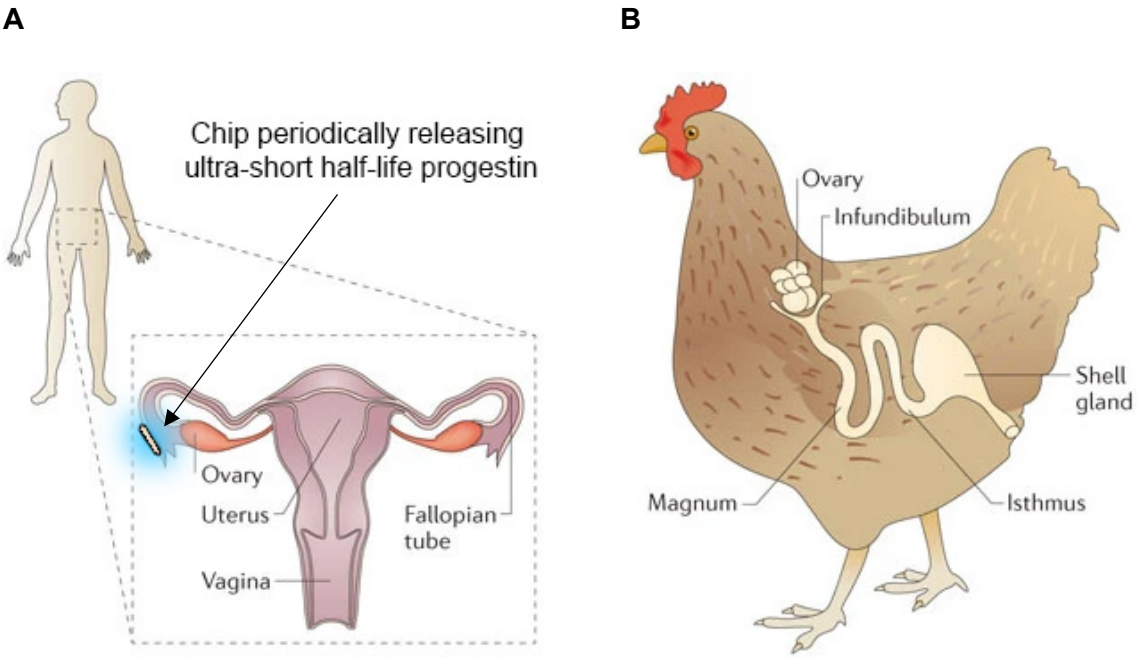


Figure 37. *A future hen model could validate a local progestin-releasing chip to prevent HGSOC.* ***(A)*** *By tuning burst release of an ultra-short half-life progestin to the onset of menstruation TP53 mutants generated during ovulations can perhaps be selectively killed.* ***(B)*** *In the laying hen, there is one ovary and oviduct, with follicles hierarchically arranged for daily ovulations. Albumen is only added later in the magnum, and the shell develops in the shell gland, meaning that infundibular folds are directly exposed to yolk-imbued mutagenic follicular fluid. [(A) and (B) taken from a figure in (Johnson and Giles, 2013), edited to insert the chip].*

oviductal ageing, histology, and peritoneal nature of metastatic spread (Johnson and Giles, 2013). Moreover, while mice have an ovarian bursa to protect the oviduct from follicular fluid, hens must capture a large yolk, and do so with complex infundibular folds that are exposed to mutagens (Figure 37B) (Apperson *et al.*, 2017; Hosotani *et al.*, 2021). Given that hens ovulate every 24-36 hours, it is no surprise that 5-35% of them spontaneously develop ovarian cancer (Hawkridge, 2014). Thus, the prophylactic efficacy of a local progestin-releasing chip could be demonstrated in wild-type and *BRCA* mutant hens, with/without inducible *PAX8*-driven *TP53* knockout.

12.6 The Mutagenic Orchestra in HGSOC Tumour Initiation

In this thesis, along with literature, an interesting possible model of HGSOC initiation is coming together. During ovulation, ROS/H_2O_2 is transduced by NOX as ROS intracellularly wherein it can cause oxidative DNA adducts (Figure 26A) (Huang *et al.*, 2016). Some ROS is neutralised by intracellular antioxidants such as superoxide dismutase (SOD), wherein it is stabilised as H_2O_2. Holo-transferrin (hTf), through TfR1, STEAP and DMT1, deliver Fe^{2+} intracellularly, to undergo a Fenton reaction with H_2O_2, regenerating ROS, increasing DNA damage (Figure 30A) (Rockfield *et al.*, 2017). If high quantities of Fe^{3+} and sufficient Tf are concomitantly delivered, then Fe^{2+} can be shuttled copiously into cells (Figure 30C). At such excess, additional to the Fenton reaction, it can drive lipid peroxidation creating aldehyde-DNA adducts independent of H_2O_2 (Gentile *et al.*, 2017). E2 can downregulate Hepcidin/Ferroportin export of Fe^{2+}, amplifying these effects (Hou *et al.*, 2012). During mutagenesis, as cells develop a HR deficit, E2 metabolites may form quinone-based DNA adducts, causing further damage (Cavalieri and Rogan, 2021).

While dsDNA breaks should lead to p53-mediated death, the FF contains IGF2, insulin and a portfolio of growth factors including amphiregulin, which facilitate survival through PI3K/AKT signalling (Figures 32C and 32D) (Hsieh, Zamah and Conti, 2009). For hFT stem cells, FF exosomes and microvesicles contain Wnt activators (miRNAs

and proteins), and E2 is capable *in vivo* of Wnt-hijack, increasing stemness and conferring proliferative benefits (Figures 32A and 32B) (da Silveira *et al.*, 2012; Alsaadi, 2021; Yuan *et al.*, 2021). Additionally, *in vivo*, mesenchymal-to-epithelial signalling may support growth and survival, perhaps particularly through WNT2, WNT9A and RSPO3 (Alsaadi *et al.*, 2022). Although some patients have particularly high FF ROS levels, the combination of these growth advantages may enable cells to survive and maximally accrue DNA damage. Hb and insulin may reduce ROS – either they are protective, or they may reduce ROS levels to a 'Goldilocks Zone' where the dot product of viability and DNA damage is maximal (Huang *et al.*, 2016).

The repair of DNA adducts by BER, MMR, NER or HR in close proximity lead to dsDNA breaks forming (Fuchs *et al.*, 2021). Stochastically, this leads to *TP53* knockout in a single cell. This can better resist cellular stress, and so is clonally selected for during ovulations, developing a p53 signature with genomic scars indicating recurrent oxidative DNA damage. Eventually, a subclone acquires the loss of HR apparatus, such as through *BRCA1* mutation, after which cells cannot undertake error-free repair. With NHEJ as the dsDNA break repair mechanism, genomic instability and chromosomal rearrangements result (Rodgers and McVey, 2016). Further mutations in pathways such as PI3K-RAS may provide independence from growth factor signalling, issuing a proliferative advantage. This constitutes a STIC in the hFT epithelium, which may depend on further FF chemicals such as hyaluronic acid to metastasise to the ovary, and beyond (Ween, Oehler and Ricciardelli, 2011).

12.7 Approaches to Comprehensively Study DNA Adducts

Despite the elegance of the mutagenic orchestra model, its validation will depend on more thorough study of the mechanisms leading to DNA damage. While treating with the endogenous mutagens we must quantify the nature of adducts they induce. For oxidative stress, the DNA Damage Competitive ELISA Kit (ThermoFisher #EIADNAD) comprises of a HRP-conjugated detection antibody capable of labelling

both 8-OHdG and 8-OH-G directly. However, other adducts are more complex to quantify. LPO of PUFAs generates reactive aldehydes, of which MDA is a frequent product. This commonly forms the M_1dG adduct (pyrimidopurinone), which one group has developed a monoclonal antibody (Mab) for (Sevilla *et al.*, 1997). Another team manufactured a panel of four Mabs for the stereoisomers of the OHPdG adducts (cyclic 1,N_2-propanodeoxyguanosine) formed by crotonaldehyde and acrolein, further LPO products (Foiles, Chung and Hecht, 1987; Chung *et al.*, 2012). Finally, a Mab has been generated to quantify E2-3,4-Q, the most common estrogenic adduct to any intracellular macromolecule, as a surrogate for DNA adducts (Casale *et al.*, 2004). Moreover, in culture, the intracellular ROS and labile iron pools can be quantified using H_2DCF-DA (ThermoFisher #D399) and Calcein-AM (ThermoFisher #C1430) respectively. However, for a more complete characterisation of mutagenic processes, treatment of organoids with actual FF will be required, alongside siRNA knockdown of signalling pathways, and depletion of FF components with antibody-bound beads.

To understand the exact portfolio of mutations that follicular fluid components induce, and to understand the exact genomic sites that they target, we must rely on advancing technologies in DNA adductomics (Boysen and Nookaew, 2022). Since DNA adducts are usually rare, at $<1/10^7$ bases, high-fidelity quantification with mass spectrometry is difficult. It usually relies on base isolation (thermal/acid hydrolysis or enzymatic digestion), followed by purification with high-performance liquid chromatography (HPLC), which leads to loss of genome-site information (Figure 38A) (Yun *et al.*, 2020). However, genome-wide DNA damage mapping is possible. The general approach is to localise molecules that recognise specific adducts, such as DNA repair enzymes, and then cleave, amplify and sequence DNA (Figure 38B). For instance, in Click-Code-Seq, BER machinery is used to excise 8-oxodG adducts, which are then replaced by a synthetic nucleotide (O-3′-propargyl-dGTP). A synthetic code sequence is coupled to each site, and library preparation and Sanger sequencing undertaken (Wu, McKeague and Sturla, 2018). Alternatively, for MDA, mitochondrial DNA damage has been mapped by shearing DNA by sonication, immunolabelling with

anti-M_1dG Mab, and then eluting and sequencing fragments (Wauchope *et al.*, 2018). Separately, a promising advent is nanopore-type technology; the transmission of DNA sequences through a protein channel, generating a current signal, with a characteristic signature for individual adducts (Figure 38C). The capability for this to recognise 8-oxodG adducts has been demonstrated (Zeng *et al.*, 2018). Most efficiently, however, with Oxford Nanopore Technologies, DNA molecules larger than 2Mb can be directly sequenced, and new tools are in development that identify specific 'errors' in the base-calling algorithm as being particular DNA adducts (Nookaew *et al.*, 2020).

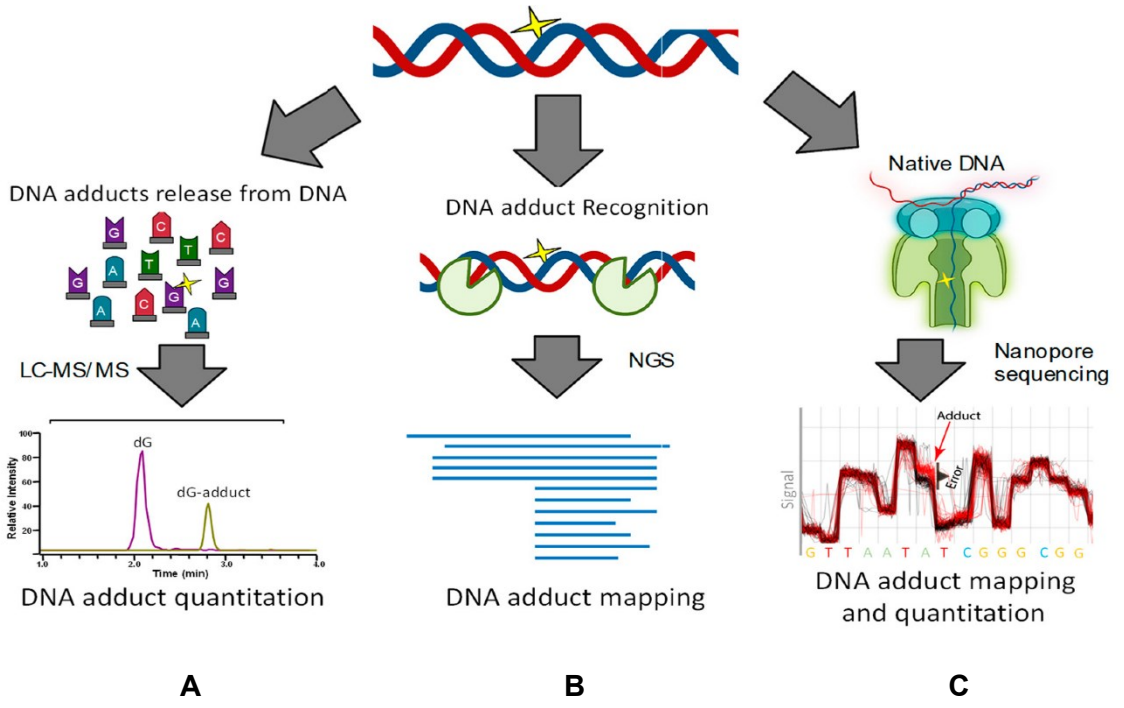


Figure 38. *Methods to quantify different DNA adducts and map their genomic locations.* ***(A)*** *To study the levels of adducts by mass spectrometry, bases must be excised and chromatographically purified, resulting in loss of genome position information.* ***(B)*** *Adduct-specify DNA excisions, library preparation and next-generation sequencing are the current strategy to localise DNA adducts.* ***(C)*** *Nanopore sequencing with optimised base-adduct calling algorithms is a rapid, cheaper method to both quantify and localise adducts, but this sub-field is in its infancy. [Figure taken from (Boysen and Nookaew, 2022)]*

12.8 Strategies to Select for Genetically Modified Organoids

Culture conditions can be altered to gain insights into how hFT cells gain selective advantages through acquired genetic changes throughout carcinogenesis. For instance, there is evidence from 2D models that *TP53* mutant cells may be selected for with oxidative stress from FF, owing to lack of p53-mediated cell cycle arrest and apoptosis (Huang *et al.*, 2015). Clonal expansion could then lead to the p53 signature, and characteristic dsDNA breaks based on the tendencies of oxidative adducts (Yun *et al.*, 2020). This process is more physiologically relevant than the current *in vitro* approach utilising Nutlin-3a to kill p53-functional cells (Figure 35), to generate the 'p53 signature' without exposure to extensive stress (Kucab *et al.*, 2017).

Another discussed gene that is frequently dysregulated in HGSOC is *PTEN*. PTEN dephosphorylates PIP3 to PIP2, switching off the PI3K-RAS pathway, de-activating AKT-mediated effects which include apoptosis inhibition (BAD) and proliferation (mTORC1) (Carnero and Paramio, 2014). Although *PTEN* knockout cells would be expected to harbour an innate proliferative advantage, ROS treatment would likely select for them strongly. However, since PTEN also initiates RAD51-mediated HR, there may be reduced resolution of bulky estrogenic DNA adducts, leading to a signature of genomic scarring (Savage *et al.*, 2014; Sharma and Almasan, 2021). In organoids, since *PTEN* mutants may have constitutive PI3K activity, which is normally driven by EGF-EGFR signalling, withdrawing EGF from medium may effectively kill non-mutants without causing stress to mutants.

Third, there is evidence that *BRCA1* mutation, a frequent occurrence in HGSOC, promotes stemness and induces the cancer stem cell marker ALDH1 (Liu *et al.*, 2008). *BRCA1* mutation may also reduce ERα, protecting niche-conferring hFT cells against E2-ERα-mediated suppression of WNT7A (Gorski *et al.*, 2009). Moreover, in Wnt-active cells, which are likely WNT7A-, in the context of no endogenous Wnt production, *BRCA1* mutation may lead to constitutive expression of the classical ERα

targets Wnt4, Wnt5a and Wnt7a, with repression of Sfrp2 (Das *et al.*, 2000). Thus, *in vivo*, *BRCA1* mutants may be at advantage through Wnt-independence, whilst *in vitro*, reducing RSPO1 could encourage their selection. Moreover, with complete loss of HR, estrogenic DNA scarring may be emphasised further than with partial loss via the PTEN/RAD51 axis (Savage *et al.*, 2014).

12.9 Next Steps to Validate Genetic Manipulation Efficacy

Before interrogating putative *TP53*, *BRCA1* or *PTEN* knockouts, it is important to validate the genetic manipulation process. Lentiviral transduction of dual sgRNA-Cas9 can entail various genetic edits. The induction of two proximal dsDNA breaks can result in deletion of the intermediate fragment, as intended, or inversions are possible. Moreover, at each crRNA-directed excision site, indels are frequent, and if dual excision fails, this may be the only event (Salonia *et al.*, 2022). Post-transduction and post-FACS, Next Generation Sequencing (NGS) should be conducted on the heterogenous population. PCR amplification of a genetic region flanking both target sites, followed by amplicon library sequencing on an Illumina MiSeq platform is an effective strategy (Quail *et al.*, 2012). The resultant FASTQ files can be rapidly assembled with the CRISPResso2 computational pipeline to visualise the nature and frequency of CRISPR-Cas9 events (Pinello *et al.*, 2016).

However, quality checking lentivirus genetic manipulation efficacy with NGS is expensive; cross-comparison of data with High-Resolution Melt Assay Analysis (HRMA) should enable its validation as a cost-effective surrogate tool (Denbow, Ehivet and Okumoto, 2018). Substantial shifts or gradient changes in the melt curve would indicate homozygous or heterozygous large fragment deletion respectively, whilst subtle changes would correspondingly represent site-specific indels. However, since the ultimate goal is to isolate homozygous fragment knockouts, a cheaper and faster approach could be to skip NGS/HRMA, and jump directly to clonal selection. Using primers inbound of the ~300bp target sequence, PCR and gel electrophoresis should

yield no band with homozygous fragment deletion. Given that deletions in the 300-500bp range with dual sgRNA-Cas9 are often successful, NGS price is ~£1000/sample, and clonal selection cost is reduced to £4 in this thesis, this strategy is sensible (Salonia *et al.*, 2022).

12.10 Building a Screening Tool with Genetically Edited and Stressed Organoids

The complete survey of transformative features acquired in purified, validated knockouts, individually and in combination, may give clues for developing a screening tool. To assess morphological impacts, organoids can be assessed for multilayering, sphericity/solidity, necrotic/non-hollow-cores and invagination frequency, using the ImageJ Canny Edge Detector plugin. Moreover, IHC for ovarian tumour specific markers (PAX8, CK7, CK20 and c-Myc), alongside proliferation (Ki67) and differentiation (TUBB4) markers may indicate how cells are altering. Whole Genome Sequencing (WGS) will be required to study copy number aberrations (CNAs), to assess chromosomal instability. With a thorough accompanying interrogation of survival, DNA damage and apoptosis in response to individual mutagens, we can then explore how vulnerabilities develop (Garaycoechea *et al.*, 2018; Hendriks *et al.*, 2023). Furthermore, withdrawal of organoid media components should give insights into acquired pathway independences (Lannagan *et al.*, 2019).

It is likely that deletion of *TP53*, *BRCA1* and *PTEN* will be required in combination to yield STIC organoids (Lõhmussaar *et al.*, 2020). Bulk RNA-seq and differential expression analysis between these and healthy patient-matched organoids can be used to identify differentially expressed genes encoding for secreted proteins. Separately, as knockouts are sequentially conducted, the relevant mutagenic stress should be applied. This should generate DNA adducts across the genome, which can be mapped to particular genetic sites of preponderance (Boysen and Nookaew, 2022). Scrutiny of these sites may identify likely ctDNAs, which along with secreted proteins may formulate a cervical smear and/or blood screening panel, similar to PapSEEK

(Wang *et al.*, 2018). Since typically STIC takes ~7 years to migrate to the ovary, there is a wide window of opportunity for such a screening method (Labidi-Galy *et al.*, 2017).

12.11 Wider Prospective Pursuits with these Approaches

With toolkits in place for genetic manipulation alongside the study of mutagens, there are several other avenues which warrant investigation. For instance, the finding of *NOTCH1/2/3* genes carrying more frequent mutations in benign hFT than HGSOC remains unexplained (Wietek *et al.*, Pre-Publication). It is noted that Notch promotes stemness in hFT organoids, and that Notch can support or undermine p53 depending on context (Kessler *et al.*, 2015; Aster, Pear and Blacklow, 2017). Perhaps specific *NOTCH1/2/3* mutations simultaneously issue gain-of-function for both stemness and p53 effects, generating clones with increased proliferative capacity but prompt arrest with oxidative stress, that can safely outcompete *TP53* mutants. By generating a library of *NOTCH1/2/3* edits and contextualising their response to oxidative stress, this hypothesis can be explored. On a separate note, it has been curiously found that SOX2 is frequently overexpressed in ciliated cells of the distal fallopian tube in the imminently pre-malignant state. Frequent inactivating mutations in a distal repressor region at the *BB5* element, which recruits EZH2, a subunit of Polycomb Repressive Complex 2, are responsible (Hellner *et al.*, 2016). Whilst applying oxidative stress and conducting transforming sequential knockouts, it will be intriguing to monitor if a particular step leads to SOX2 overexpression, and to interrogate the impact of its silencing.

Bibliography

Abud, H.E., Chan, W.H. and Jardé, T. (2021) 'Source and Impact of the EGF Family of Ligands on Intestinal Stem Cells', *Frontiers in Cell and Developmental Biology*, 9, p. 685665. Available at: https://doi.org/10.3389/fcell.2021.685665.

Addgene (2021) *Addgene: Lentiviral Guide*. Available at: https://www.addgene.org/guides/lentivirus/ (Accessed: 22 September 2023).

Adler, E. *et al.* (2015) 'PAX8 expression in ovarian surface epithelial cells', *Human Pathology*, 46(7), pp. 948–956. Available at: https://doi.org/10.1016/j.humpath.2015.03.017.

Ahmed, A.A. *et al.* (2010) 'Driver mutations in *TP53* are ubiquitous in high grade serous carcinoma of the ovary: *TP53* mutation in high-grade pelvic serous carcinoma', *The Journal of Pathology*, 221(1), pp. 49–56. Available at: https://doi.org/10.1002/path.2696.

Akahane, T. *et al.* (2022) '*TP53* variants in p53 signatures and the clonality of STICs in RRSO samples', *Journal of Gynecologic Oncology*, 33(4), p. e50. Available at: https://doi.org/10.3802/jgo.2022.33.e50.

Akatsuka, S. *et al.* (2006) 'Contrasting genome-wide distribution of 8-hydroxyguanine and acrolein-modified adenine during oxidative stress-induced renal carcinogenesis', *The American Journal of Pathology*, 169(4), pp. 1328–1342. Available at: https://doi.org/10.2353/ajpath.2006.051280.

Alberts, B. *et al.* (2002) *Molecular Biology of the Cell*. 4th edn. Garland Science.

Albuquerque, C. *et al.* (2002) 'The "just-right" signaling model: APC somatic mutations are selected based on a specific level of activation of the β-catenin signaling cascade', *Human Molecular Genetics*, 11(13), pp. 1549–1560. Available at: https://doi.org/10.1093/hmg/11.13.1549.

Alsaadi, A. (2021) *Identification of Fallopian Tube Stem Cells using Patient-derived Organoids*. University of Oxford. Available at: https://ethos.bl.uk/OrderDetails.do?uin=uk.bl.ethos.826444.

Alsaadi, A. *et al.* (2022) 'Single Cell Transcriptomics identifies a WNT7A-FZD5 Signaling Axis that maintains Fallopian Tube Stem Cells in Patient-derived Organoids'. bioRxiv, p. 2022.08.02.502319. Available at: https://doi.org/10.1101/2022.08.02.502319.

Amit, M. *et al.* (2004) 'Feeder Layer- and Serum-Free Culture of Human Embryonic Stem Cells1', *Biology of Reproduction*, 70(3), pp. 837–845. Available at: https://doi.org/10.1095/biolreprod.103.021147.

Amouroux, R. *et al.* (2010) 'Oxidative stress triggers the preferential assembly of base excision repair complexes on open chromatin regions', *Nucleic Acids Research*, 38(9), pp. 2878–2890. Available at: https://doi.org/10.1093/nar/gkp1247.

Antfolk, M. and Jensen, K.B. (2020) ‘A bioengineering perspective on modelling the intestinal epithelial physiology in vitro’, *Nature Communications*, 11(1), p. 6244. Available at: https://doi.org/10.1038/s41467-020-20052-z.

Apperson, K.D. *et al.* (2017) ‘Histology of the Ovary of the Laying Hen (Gallus domesticus)’, *Veterinary Sciences*, 4(4), p. 66. Available at: https://doi.org/10.3390/vetsci4040066.

Arce, L., Pate, K.T. and Waterman, M.L. (2009) ‘Groucho binds two conserved regions of LEF-1 for HDAC-dependent repression’, *BMC cancer*, 9, p. 159. Available at: https://doi.org/10.1186/1471-2407-9-159.

Artegiani, B. *et al.* (2019) ‘Probing the Tumor Suppressor Function of BAP1 in CRISPR-Engineered Human Liver Organoids’, *Cell Stem Cell*, 24(6), pp. 927-943.e6. Available at: https://doi.org/10.1016/j.stem.2019.04.017.

Asghar, W. *et al.* (2015) ‘Engineering cancer microenvironments for in vitro 3-D tumor models’, *Materials Today*, 18(10), pp. 539–553. Available at: https://doi.org/10.1016/j.mattod.2015.05.002.

Aster, J.C., Pear, W.S. and Blacklow, S.C. (2017) ‘The Varied Roles of Notch in Cancer’, *Annual Review of Pathology: Mechanisms of Disease*, 12(1), pp. 245–275. Available at: https://doi.org/10.1146/annurev-pathol-052016-100127.

Atcha, F.A. *et al.* (2007) ‘A unique DNA binding domain converts T-cell factors into strong Wnt effectors’, *Molecular and Cellular Biology*, 27(23), pp. 8352–8363. Available at: https://doi.org/10.1128/MCB.02132-06.

Attar, E. *et al.* (2009) ‘Prostaglandin E2 Via Steroidogenic Factor-1 Coordinately Regulates Transcription of Steroidogenic Genes Necessary for Estrogen Synthesis in Endometriosis’, *The Journal of Clinical Endocrinology & Metabolism*, 94(2), pp. 623–631. Available at: https://doi.org/10.1210/jc.2008-1180.

Aubrey, B.J. *et al.* (2018) ‘How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression?’, *Cell Death & Differentiation*, 25(1), pp. 104–113. Available at: https://doi.org/10.1038/cdd.2017.169.

Auersperg, N. *et al.* (2001) ‘Ovarian Surface Epithelium: Biology, Endocrinology, and Pathology’, 22(2).

Axelrod, J.D. *et al.* (1996) ‘Interaction Between Wingless and Notch Signaling Pathways Mediated by Dishevelled’, *Science*, 271(5257), pp. 1826–1832. Available at: https://doi.org/10.1126/science.271.5257.1826.

Babaier, A. and Ghatage, P. (2020) ‘Mucinous Cancer of the Ovary: Overview and Current Status’, *Diagnostics*, 10(1), p. 52. Available at: https://doi.org/10.3390/diagnostics10010052.

Backx, E. *et al.* (2022) ‘On the Origin of Pancreatic Cancer: Molecular Tumor Subtypes in Perspective of Exocrine Cell Plasticity’, *Cellular and Molecular Gastroenterology and Hepatology*, 13(4), pp. 1243–1253. Available at: https://doi.org/10.1016/j.jcmgh.2021.11.010.

Bakkenist, C.J. and Kastan, M.B. (2003) 'DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation', *Nature*, 421(6922), pp. 499–506. Available at: https://doi.org/10.1038/nature01368.

Ban, J. *et al.* (2008) 'EWS-FLI1 Suppresses NOTCH-Activated p53 in Ewing's Sarcoma', *Cancer Research*, 68(17), pp. 7100–7109. Available at: https://doi.org/10.1158/0008-5472.CAN-07-6145.

Bänziger, C. *et al.* (2006) 'Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells', *Cell*, 125(3), pp. 509–522. Available at: https://doi.org/10.1016/j.cell.2006.02.049.

Barger, C. *et al.* (2019) 'Pan-Cancer Analyses Reveal Genomic Features of FOXM1 Overexpression in Cancer', *Cancers*, 11(2), p. 251. Available at: https://doi.org/10.3390/cancers11020251.

Barker, N. *et al.* (2007) 'Identification of stem cells in small intestine and colon by marker gene Lgr5', *Nature*, 449(7165), pp. 1003–1007. Available at: https://doi.org/10.1038/nature06196.

Barker, N. *et al.* (2009) 'Crypt stem cells as the cells-of-origin of intestinal cancer', *Nature*, 457(7229), pp. 608–611. Available at: https://doi.org/10.1038/nature07602.

Bartscherer, K. *et al.* (2006) 'Secretion of Wnt ligands requires Evi, a conserved transmembrane protein', *Cell*, 125(3), pp. 523–533. Available at: https://doi.org/10.1016/j.cell.2006.04.009.

Bast, R.C. *et al.* (2019) 'Critical questions in ovarian cancer research and treatment: Report of an American Association for Cancer Research Special Conference', *Cancer*, 125(12), pp. 1963–1972. Available at: https://doi.org/10.1002/cncr.32004.

Behrens, J. *et al.* (1996) 'Functional interaction of β-catenin with the transcription factor LEF-1', *Nature*, 382(6592), pp. 638–642. Available at: https://doi.org/10.1038/382638a0.

Bernkopf, D.B., Hadjihannas, M.V. and Behrens, J. (2015) 'Negative-feedback regulation of the Wnt pathway by conductin/axin2 involves insensitivity to upstream signalling', *Journal of Cell Science*, 128(1), pp. 33–39. Available at: https://doi.org/10.1242/jcs.159145.

Beurel, E., Grieco, S.F. and Jope, R.S. (2015) 'Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases', *Pharmacology & therapeutics*, 0, pp. 114–131. Available at: https://doi.org/10.1016/j.pharmthera.2014.11.016.

Beyaz, S. *et al.* (2016) 'High-fat diet enhances stemness and tumorigenicity of intestinal progenitors', *Nature*, 531(7592), pp. 53–58. Available at: https://doi.org/10.1038/nature17173.

Bhat, R.A. *et al.* (2007) 'Structure–Function analysis of secreted frizzled-related protein-1 for its Wnt antagonist function', *Journal of Cellular Biochemistry*, 102(6), pp. 1519–1528. Available at: https://doi.org/10.1002/jcb.21372.

BioTechne (2023) *Recombinant Human R-Spondin 2 Protein*, *www.rndsystems.com*. Available at: https://www.rndsystems.com/products/recombinant-human-r-spondin-2-protein_3266-rs (Accessed: 8 September 2023).

Blaine, S.A. *et al.* (2010) 'Adult pancreatic acinar cells give rise to ducts but not endocrine cells in response to growth factor signaling', *Development (Cambridge, England)*, 137(14), pp. 2289–2296. Available at: https://doi.org/10.1242/dev.048421.

Boj, S.F. *et al.* (2017) 'Forskolin-induced Swelling in Intestinal Organoids: An In Vitro Assay for Assessing Drug Response in Cystic Fibrosis Patients', *Journal of Visualized Experiments : JoVE*, (120), p. 55159. Available at: https://doi.org/10.3791/55159.

Bonner-Weir, S. *et al.* (2004) 'The pancreatic ductal epithelium serves as a potential pool of progenitor cells', *Pediatric Diabetes*, 5(s2), pp. 16–22. Available at: https://doi.org/10.1111/j.1399-543X.2004.00075.x.

Bonner-Weir, S. and Sharma, A. (2002) 'Pancreatic stem cells', *The Journal of Pathology*, 197(4), pp. 519–526. Available at: https://doi.org/10.1002/path.1158.

Borowiecka, M. *et al.* (2012) 'Oxidative stress markers in follicular fluid of women undergoing in vitro fertilization and embryo transfer', *Systems Biology in Reproductive Medicine*, 58(6), pp. 301–305. Available at: https://doi.org/10.3109/19396368.2012.701367.

Bourhis, E. *et al.* (2010) 'Reconstitution of a frizzled8.Wnt3a.LRP6 signaling complex reveals multiple Wnt and Dkk1 binding sites on LRP6', *The Journal of Biological Chemistry*, 285(12), pp. 9172–9179. Available at: https://doi.org/10.1074/jbc.M109.092130.

Bowen, N.J. *et al.* (2007) 'Emerging roles for PAX8 in ovarian cancer and endosalpingeal development', *Gynecologic Oncology*, 104(2), pp. 331–337. Available at: https://doi.org/10.1016/j.ygyno.2006.08.052.

Boysen, G. and Nookaew, I. (2022) 'Current and Future Methodology for Quantitation and Site-Specific Mapping the Location of DNA Adducts', *Toxics*, 10(2), p. 45. Available at: https://doi.org/10.3390/toxics10020045.

Braconi, C. and Patel, T. (2010) 'Cholangiocarcinoma: New insights into disease pathogenesis and biology', *Infectious disease clinics of North America*, 24(4), pp. 871–884. Available at: https://doi.org/10.1016/j.idc.2010.07.006.

Bray, F. *et al.* (2018) 'Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries', *CA: A Cancer Journal for Clinicians*, 68(6), pp. 394–424. Available at: https://doi.org/10.3322/caac.21492.

Brennan, K. *et al.* (1999) 'Wingless Modulates the Effects of Dominant Negative Notch Molecules in the Developing Wing of Drosophila', *Developmental Biology*, 216(1), pp. 210–229. Available at: https://doi.org/10.1006/dbio.1999.9502.

Brett M., R. *et al.* (2017) 'Epidemiology of ovarian cancer: a review', *Cancer Biology & Medicine*, 14(1), pp. 9–32. Available at: https://doi.org/10.20892/j.issn.2095-3941.2016.0084.

Buchanan, F.G. and DuBois, R.N. (2006) 'Connecting COX-2 and Wnt in cancer', *Cancer Cell*, 9(1), pp. 6–8. Available at: https://doi.org/10.1016/j.ccr.2005.12.029.

Bulun, S.E., Wan, Y. and Matei, D. (2019) 'Epithelial Mutations in Endometriosis: Link to Ovarian Cancer', *Endocrinology*, 160(3), pp. 626–638. Available at: https://doi.org/10.1210/en.2018-00794.

Bunting, S.F. *et al.* (2010) '53BP1 Inhibits Homologous Recombination in Brca1-Deficient Cells by Blocking Resection of DNA Breaks', *Cell*, 141(2), pp. 243–254. Available at: https://doi.org/10.1016/j.cell.2010.03.012.

Byrne, S.L. *et al.* (2013) 'Ferristatin II Promotes Degradation of Transferrin Receptor-1 In Vitro and In Vivo', *PLOS ONE*, 8(7), p. e70199. Available at: https://doi.org/10.1371/journal.pone.0070199.

Cadet, J. and Wagner, J.R. (2013) 'DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation', *Cold Spring Harbor Perspectives in Biology*, 5(2), p. a012559. Available at: https://doi.org/10.1101/cshperspect.a012559.

Campana, L. *et al.* (2021) 'Liver regeneration and inflammation: from fundamental science to clinical applications', *Nature Reviews Molecular Cell Biology*, 22(9), pp. 608–624. Available at: https://doi.org/10.1038/s41580-021-00373-7.

Candelaria, P.V. *et al.* (2021) 'Antibodies Targeting the Transferrin Receptor 1 (TfR1) as Direct Anti-cancer Agents', *Frontiers in Immunology*, 12, p. 607692. Available at: https://doi.org/10.3389/fimmu.2021.607692.

Cao, F. *et al.* (2010) 'Comparison of Gene-Transfer Efficiency in Human Embryonic Stem Cells', *Molecular Imaging and Biology*, 12(1), pp. 15–24. Available at: https://doi.org/10.1007/s11307-009-0236-x.

Carbone, C. *et al.* (2018) 'Adipocytes sustain pancreatic cancer progression through a non-canonical WNT paracrine network inducing ROR2 nuclear shuttling', *International Journal of Obesity*, 42(3), pp. 334–343. Available at: https://doi.org/10.1038/ijo.2017.285.

Carmon, K.S. and Loose, D.S. (2010) 'Development of a bioassay for detection of Wnt-binding affinities for individual frizzled receptors', *Analytical Biochemistry*, 401(2), pp. 288–294. Available at: https://doi.org/10.1016/j.ab.2010.03.009.

Carmona, R. *et al.* (2013) 'Cells Derived from the Coelomic Epithelium Contribute to Multiple Gastrointestinal Tissues in Mouse Embryos', *PLoS ONE*. Edited by R. Dettman, 8(2), p. e55890. Available at: https://doi.org/10.1371/journal.pone.0055890.

Carnero, A. and Paramio, J.M. (2014) 'The PTEN/PI3K/AKT Pathway in vivo, Cancer Mouse Models', *Frontiers in Oncology*, 4. Available at: https://www.frontiersin.org/articles/10.3389/fonc.2014.00252 (Accessed: 22 September 2023).

Carta, L. and Sassoon, D. (2004) 'Wnt7a Is a Suppressor of Cell Death in the Female Reproductive Tract and Is Required for Postnatal and Estrogen-Mediated Growth1', *Biology of Reproduction*, 71(2), pp. 444–454. Available at: https://doi.org/10.1095/biolreprod.103.026534.

Casale, G.P. *et al.* (2004) 'A monoclonal antibody for macromolecular adducts of estradiol-3,4-quinone, a suspected initiator of breast and prostate cancers', *Cancer Research*, 64(7_Supplement), p. 937.

Castellano, E. and Downward, J. (2011) 'RAS Interaction with PI3K: More Than Just Another Effector Pathway', *Genes & Cancer*, 2(3), pp. 261–274. Available at: https://doi.org/10.1177/1947601911408079.

Cavalieri, E. and Rogan, E. (2021) 'The 3,4-Quinones of Estrone and Estradiol Are the Initiators of Cancer whereas Resveratrol and N-acetylcysteine Are the Preventers', *International Journal of Molecular Sciences*, 22(15), p. 8238. Available at: https://doi.org/10.3390/ijms22158238.

Cavalieri, E.L. and Rogan, E.G. (2017) 'Etiology and prevention of prevalent types of cancer', *Journal of rare diseases research & treatment*, 2(3), pp. 22–29. Available at: https://doi.org/10.29245/2572-9411/2017/3.1093.

Cavallo, R.A. *et al.* (1998) 'Drosophila Tcf and Groucho interact to repress Wingless signalling activity', *Nature*, 395(6702), pp. 604–608. Available at: https://doi.org/10.1038/26982.

Cavodeassi, F. *et al.* (2005) 'Early Stages of Zebrafish Eye Formation Require the Coordinated Activity of Wnt11, Fz5, and the Wnt/β-Catenin Pathway', *Neuron*, 47(1), pp. 43–56. Available at: https://doi.org/10.1016/j.neuron.2005.05.026.

Chae, W.-J. and Bothwell, A.L.M. (2018) 'Canonical and Non-Canonical Wnt Signaling in Immune Cells', *Trends in immunology*, 39(10), pp. 830–847. Available at: https://doi.org/10.1016/j.it.2018.08.006.

Chang, T.-H. *et al.* (2015) 'Structure and functional properties of Norrin mimic Wnt for signalling with Frizzled4, Lrp5/6, and proteoglycan', *eLife*. Edited by J. Kuriyan, 4, p. e06554. Available at: https://doi.org/10.7554/eLife.06554.

Chang, Y.-H., Ding, D.-C. and Chu, T.-Y. (2019) 'Estradiol and Progesterone Induced Differentiation and Increased Stemness Gene Expression of Human Fallopian Tube Epithelial Cells', *Journal of Cancer*, 10(13), pp. 3028–3036. Available at: https://doi.org/10.7150/jca.30588.

Chantalat, E. *et al.* (2020) 'Estrogen Receptors and Endometriosis', *International Journal of Molecular Sciences*, 21(8), p. 2815. Available at: https://doi.org/10.3390/ijms21082815.

Charbonneau, B. *et al.* (2013) 'The Immune System in the Pathogenesis of Ovarian Cancer', *Critical reviews in immunology*, 33(2), pp. 137–164.

Cheasley, D. *et al.* (2022) 'Molecular characterization of low-grade serous ovarian carcinoma identifies genomic aberrations according to hormone receptor expression', *npj Precision Oncology*, 6(1), p. 47. Available at: https://doi.org/10.1038/s41698-022-00288-2.

Chen, B. *et al.* (2005) 'Progesterone Inhibits the Estrogen-Induced Phosphoinositide 3-Kinase→AKT→GSK-3β→Cyclin D1→pRB Pathway to Block Uterine Epithelial Cell Proliferation', *Molecular Endocrinology*, 19(8), pp. 1978–1990. Available at: https://doi.org/10.1210/me.2004-0274.

Chen, C. and Struhl, G. (1999) 'Wingless transduction by the Frizzled and Frizzled2 proteins of Drosophila', *Development*, 126(23), pp. 5441–5452. Available at: https://doi.org/10.1242/dev.126.23.5441.

Chen, E.Y. *et al.* (2010) ‘Secretory cell outgrowth, PAX2 and serous carcinogenesis in the fallopian tube’, *The Journal of pathology*, 222(1), pp. 110–116. Available at: https://doi.org/10.1002/path.2739.

Chen, H. *et al.* (2020) ‘Development of Potent, Selective Surrogate WNT Molecules and Their Application in Defining Frizzled Requirements’, *Cell Chemical Biology*, 27(5), pp. 598-609.e4. Available at: https://doi.org/10.1016/j.chembiol.2020.02.009.

Chen, P.-H. *et al.* (2013) ‘The structural basis of R-spondin recognition by LGR5 and RNF43’, *Genes & Development*, 27(12), pp. 1345–1350. Available at: https://doi.org/10.1101/gad.219915.113.

Chen, P.-M. *et al.* (2013) ‘MnSOD Promotes Tumor Invasion via Upregulation of FoxM1–MMP2 Axis and Related with Poor Survival and Relapse in Lung Adenocarcinomas’, *Molecular Cancer Research*, 11(3), pp. 261–271. Available at: https://doi.org/10.1158/1541-7786.MCR-12-0527.

Chen, Q. *et al.* (2016) ‘Different populations of Wnt-containing vesicles are individually released from polarized epithelial cells’, *Scientific Reports*, 6, p. 35562. Available at: https://doi.org/10.1038/srep35562.

Chen, S. *et al.* (2001) ‘WNT-1 Signaling Inhibits Apoptosis by Activating β-Catenin/T Cell Factor–Mediated Transcription’, *The Journal of Cell Biology*, 152(1), pp. 87–96.

Chen, Y. *et al.* (2021) ‘The involvement of noncanonical Wnt signaling in cancers’, *Biomedicine & Pharmacotherapy*, 133, p. 110946. Available at: https://doi.org/10.1016/j.biopha.2020.110946.

Cheng, Q. *et al.* (2023) ‘Establishing and characterizing human stem cells from the apical papilla immortalized by hTERT gene transfer’, *Frontiers in Cell and Developmental Biology*, 11, p. 1158936. Available at: https://doi.org/10.3389/fcell.2023.1158936.

Cheung, E.C. *et al.* (2013) ‘TIGAR is required for efficient intestinal regeneration and tumorigenesis’, *Developmental Cell*, 25(5), pp. 463–477. Available at: https://doi.org/10.1016/j.devcel.2013.05.001.

Cheung, E.C. *et al.* (2020) ‘Dynamic ROS Control by TIGAR Regulates the Initiation and Progression of Pancreatic Cancer’, *Cancer Cell*, 37(2), pp. 168-182.e4. Available at: https://doi.org/10.1016/j.ccell.2019.12.012.

Choi, Y.S. *et al.* (2006) ‘Comparison of follicular fluid IGF-I, IGF-II, IGFBP-3, IGFBP-4 and PAPP-A concentrations and their ratios between GnRH agonist and GnRH antagonist protocols for controlled ovarian stimulation in IVF-embryo transfer patients’, *Human Reproduction*, 21(8), pp. 2015–2021. Available at: https://doi.org/10.1093/humrep/del091.

Chomarat, P. *et al.* (2000) ‘IL-6 switches the differentiation of monocytes from dendritic cells to macrophages’, *Nature Immunology*, 1(6), pp. 510–514. Available at: https://doi.org/10.1038/82763.

Chung, F.-L. *et al.* (2012) ‘Regioselective Formation of Acrolein-Derived Cyclic 1,N2-Propanodeoxyguanosine Adducts Mediated by Amino Acids, Proteins, and Cell Lysates’, *Chemical Research in Toxicology*, 25(9), pp. 1921–1928. Available at: https://doi.org/10.1021/tx3002252.

Cochrane, D.R. *et al.* (2017) 'Clear cell and endometrioid carcinomas: are their differences attributable to distinct cells of origin?: Distinct cellular origins for endometrioid and clear cell cancers', *The Journal of Pathology*, 243(1), pp. 26–36. Available at: https://doi.org/10.1002/path.4934.

Cohen, J.D. *et al.* (2018) 'Detection and localization of surgically resectable cancers with a multi-analyte blood test', *Science*, 359(6378), pp. 926–930. Available at: https://doi.org/10.1126/science.aar3247.

Cohen, S. *et al.* (2016) 'Reactive Oxygen Species and Serous Epithelial Ovarian Adenocarcinoma', *Cancer Research Journal*, 4(6), p. 106. Available at: https://doi.org/10.11648/j.crj.20160406.13.

Cooke, P.S. *et al.* (1997) 'Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium', *Proceedings of the National Academy of Sciences of the United States of America*, 94(12), pp. 6535–6540. Available at: https://doi.org/10.1073/pnas.94.12.6535.

Coombs, G.S. *et al.* (2010) 'WLS-dependent secretion of WNT3A requires Ser209 acylation and vacuolar acidification', *Journal of Cell Science*, 123(Pt 19), pp. 3357–3367. Available at: https://doi.org/10.1242/jcs.072132.

Coscia, F. *et al.* (2016) 'Integrative proteomic profiling of ovarian cancer cell lines reveals precursor cell associated proteins and functional status', *Nature Communications*, 7(1), p. 12645. Available at: https://doi.org/10.1038/ncomms12645.

Criscimanna, A. *et al.* (2011) 'Duct Cells Contribute to Regeneration of Endocrine and Acinar Cells Following Pancreatic Damage in Adult Mice', *Gastroenterology*, 141(4), pp. 1451-1462.e6. Available at: https://doi.org/10.1053/j.gastro.2011.07.003.

CRUK (2013) *Cancer survival for common cancers*, *Cancer Research UK*. Available at: https://www.cancerresearchuk.org/health-professional/cancer-statistics/survival/common-cancers-compared (Accessed: 19 August 2023).

CRUK (2018) 'CRUK Ovarian Cancer Survival Statistics'. https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/ovarian-cancer/survival#heading-Three.

Crum, C.P. and Xian, W. (2010) 'Bringing the p53 signature into focus', *Cancer*, 116(22), pp. 5119–5121. Available at: https://doi.org/10.1002/cncr.25450.

Dajani, R. *et al.* (2003) 'Structural basis for recruitment of glycogen synthase kinase 3beta to the axin-APC scaffold complex', *The EMBO journal*, 22(3), pp. 494–501. Available at: https://doi.org/10.1093/emboj/cdg068.

Daniels, D.L. and Weis, W.I. (2005) 'Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation', *Nature Structural & Molecular Biology*, 12(4), pp. 364–371. Available at: https://doi.org/10.1038/nsmb912.

Dann, C.E. *et al.* (2001) 'Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains', *Nature*, 412(6842), pp. 86–90. Available at: https://doi.org/10.1038/35083601.

Das, S.K. *et al.* (2000) 'Estrogen targets genes involved in protein processing, calcium homeostasis, and Wnt signaling in the mouse uterus independent of estrogen receptor-

alpha and -beta', *The Journal of Biological Chemistry*, 275(37), pp. 28834–28842. Available at: https://doi.org/10.1074/jbc.M003827200.

Davis, H. *et al.* (2015) 'Aberrant epithelial GREM1 expression initiates colonic tumorigenesis from cells outside the stem cell niche', *Nature Medicine*, 21(1), pp. 62–70. Available at: https://doi.org/10.1038/nm.3750.

Day, T.F. and Yang, Y. (2008) 'Wnt and hedgehog signaling pathways in bone development', *The Journal of Bone and Joint Surgery. American Volume*, 90 Suppl 1, pp. 19–24. Available at: https://doi.org/10.2106/JBJS.G.01174.

De, A. (2011) 'Wnt/Ca2+ signaling pathway: a brief overview', *Acta Biochimica Et Biophysica Sinica*, 43(10), pp. 745–756. Available at: https://doi.org/10.1093/abbs/gmr079.

De Calisto, J. *et al.* (2005) 'Essential role of non-canonical Wnt signalling in neural crest migration', *Development (Cambridge, England)*, 132(11), pp. 2587–2597. Available at: https://doi.org/10.1242/dev.01857.

Dekkers, J.F. *et al.* (2020) 'Modeling Breast Cancer Using CRISPR-Cas9–Mediated Engineering of Human Breast Organoids', *JNCI: Journal of the National Cancer Institute*, 112(5), pp. 540–544. Available at: https://doi.org/10.1093/jnci/djz196.

Denbow, C., Ehivet, S.C. and Okumoto, S. (2018) 'High Resolution Melting Temperature Analysis to IdentifyCRISPR/Cas9 Mutants from Arabidopsis', *Bio-protocol*, 8(14), p. e2944. Available at: https://doi.org/10.21769/BioProtoc.2944.

DeNicola, G.M. *et al.* (2011) 'Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis', *Nature*, 475(7354), pp. 106–109. Available at: https://doi.org/10.1038/nature10189.

Denu, J.M. (2005) 'Vitamin B3 and sirtuin function', *Trends in Biochemical Sciences*, 30(9), pp. 479–483. Available at: https://doi.org/10.1016/j.tibs.2005.07.004.

Diamond, M.P. *et al.* (1985) 'Human follicular fluid insulin concentrations', *The Journal of Clinical Endocrinology and Metabolism*, 61(5), pp. 990–992. Available at: https://doi.org/10.1210/jcem-61-5-990.

Dong, C. *et al.* (2021) 'Activation of PI3K/AKT/mTOR Pathway Causes Drug Resistance in Breast Cancer', *Frontiers in Pharmacology*, 12, p. 628690. Available at: https://doi.org/10.3389/fphar.2021.628690.

Dotto, G.P. (2009) 'Crosstalk of Notch with p53 and p63 in cancer growth control', *Nature Reviews Cancer*, 9(8), pp. 587–595. Available at: https://doi.org/10.1038/nrc2675.

Dovnik, A. and Dovnik, N.F. (2020) 'Vitamin D and Ovarian Cancer: Systematic Review of the Literature with a Focus on Molecular Mechanisms', *Cells*, 9(2), p. 335. Available at: https://doi.org/10.3390/cells9020335.

Drapkin, R. and Hecht, J. (2002) 'The origins of ovarian cancer: hurdles and progress'.

Duan, L. and Maki, C.G. (2016) 'The IGF-1R/AKT pathway determines cell fate in response to p53', *Translational cancer research*, 5(6), pp. 664–675. Available at: https://doi.org/10.21037/tcr.2016.09.16.

Duh, G. *et al.* (2000) ‘EGF Regulates Early Embryonic Mouse Gut Development in Chemically Defined Organ Culture’, *Pediatric Research*, 48(6), pp. 794–802. Available at: https://doi.org/10.1203/00006450-200012000-00016.

Duronio, R.J. and Xiong, Y. (2013) ‘Signaling Pathways that Control Cell Proliferation’, *Cold Spring Harbor Perspectives in Biology*, 5(3), p. a008904. Available at: https://doi.org/10.1101/cshperspect.a008904.

Elias, K.M. *et al.* (2018) ‘Primordial germ cells as a potential shared cell of origin for mucinous cystic neoplasms of the pancreas and mucinous ovarian tumors’, *The Journal of Pathology*, 246(4), pp. 459–469. Available at: https://doi.org/10.1002/path.5161.

van Es, J.H. *et al.* (2019) ‘Enteroendocrine and tuft cells support Lgr5 stem cells on Paneth cell depletion’, *Proceedings of the National Academy of Sciences*, 116(52), pp. 26599–26605. Available at: https://doi.org/10.1073/pnas.1801888117.

Espinosa, L. *et al.* (2003) ‘Phosphorylation by Glycogen Synthase Kinase-3β Down-regulates Notch Activity, a Link for Notch and Wnt Pathways’, *Journal of Biological Chemistry*, 278(34), pp. 32227–32235. Available at: https://doi.org/10.1074/jbc.M304001200.

Essers, M.A.G. *et al.* (2005) ‘Functional interaction between beta-catenin and FOXO in oxidative stress signaling’, *Science (New York, N.Y.)*, 308(5725), pp. 1181–1184. Available at: https://doi.org/10.1126/science.1109083.

Ewa, B. and Danuta, M.-Š. (2017) ‘Polycyclic aromatic hydrocarbons and PAH-related DNA adducts’, *Journal of Applied Genetics*, 58(3), pp. 321–330. Available at: https://doi.org/10.1007/s13353-016-0380-3.

Fabbro, M. *et al.* (2004) ‘BRCA1-BARD1 Complexes Are Required for p53Ser-15 Phosphorylation and a G1/S Arrest following Ionizing Radiation-induced DNA Damage’, *Journal of Biological Chemistry*, 279(30), pp. 31251–31258. Available at: https://doi.org/10.1074/jbc.M405372200.

Fagotto, F. *et al.* (1999) ‘Domains of axin involved in protein-protein interactions, Wnt pathway inhibition, and intracellular localization’, *The Journal of Cell Biology*, 145(4), pp. 741–756. Available at: https://doi.org/10.1083/jcb.145.4.741.

Fagotto, F., Glück, U. and Gumbiner, B.M. (1998) ‘Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of beta-catenin’, *Current biology: CB*, 8(4), pp. 181–190. Available at: https://doi.org/10.1016/s0960-9822(98)70082-x.

Fan, S. *et al.* (2001) ‘Role of direct interaction in BRCA1 inhibition of estrogen receptor activity’, *Oncogene*, 20(1), pp. 77–87. Available at: https://doi.org/10.1038/sj.onc.1204073.

Fang, D. *et al.* (2007) ‘Phosphorylation of β-Catenin by AKT Promotes β-Catenin Transcriptional Activity’, *Journal of Biological Chemistry*, 282(15), pp. 11221–11229. Available at: https://doi.org/10.1074/jbc.M611871200.

Farooq, M. *et al.* (2021) ‘The Role of Fibroblast Growth Factor (FGF) Signaling in Tissue Repair and Regeneration’, *Cells*, 10(11), p. 3242. Available at: https://doi.org/10.3390/cells10113242.

Fata, C.R. *et al.* (2015) ‘Are clear cell carcinomas of the ovary and endometrium phenotypically identical? A proteomic analysis’, *Human Pathology*, 46(10), pp. 1427–1436. Available at: https://doi.org/10.1016/j.humpath.2015.06.009.

Fathalla, M.F. (1971) ‘Incessant Ovulation - A Factor in Ovarian Neoplasia?’, *The Lancet*, 298(7716), p. 163.

Fayard, E. *et al.* (2005) ‘Protein kinase B/Akt at a glance’, *Journal of Cell Science*, 118(24), pp. 5675–5678. Available at: https://doi.org/10.1242/jcs.02724.

Feeley, K.M. and Wells, M. (2001) ‘Precursor lesions of ovarian epithelial malignancy: *Ovarian cancer precursors*’, *Histopathology*, 38(2), pp. 87–95. Available at: https://doi.org/10.1046/j.1365-2559.2001.01042.x.

Feigelson, H.S. and Henderson, B.E. (1996) ‘Estrogens and breast cancer’, *Carcinogenesis*, 17(11), pp. 2279–2284. Available at: https://doi.org/10.1093/carcin/17.11.2279.

Ferreira, R.M.M. *et al.* (2017) ‘Duct- and Acinar-Derived Pancreatic Ductal Adenocarcinomas Show Distinct Tumor Progression and Marker Expression’, *Cell Reports*, 21(4), pp. 966–978. Available at: https://doi.org/10.1016/j.celrep.2017.09.093.

Flentke, G.R. *et al.* (2014) ‘CaMKII Represses Transcriptionally-Active β-Catenin to Mediate Acute Ethanol Neurodegeneration and Can Phosphorylate β-Catenin’, *Journal of neurochemistry*, 128(4), pp. 523–535. Available at: https://doi.org/10.1111/jnc.12464.

Fleszar, A.J. *et al.* (2018) ‘The extracellular matrix of ovarian cortical inclusion cysts modulates invasion of fallopian tube epithelial cells’, *APL Bioengineering*, 2(3), p. 031902. Available at: https://doi.org/10.1063/1.5022595.

Flier, L.G.V. der *et al.* (2007) ‘The Intestinal Wnt/TCF Signature’, *Gastroenterology*, 132(2), pp. 628–632. Available at: https://doi.org/10.1053/j.gastro.2006.08.039.

Flowers, B.M. *et al.* (2021) ‘Cell-of-origin influences pancreatic cancer subtype’, *Cancer discovery*, 11(3), pp. 660–677. Available at: https://doi.org/10.1158/2159-8290.CD-20-0633.

Foiles, P.G., Chung, F.L. and Hecht, S.S. (1987) ‘Development of a monoclonal antibody-based immunoassay for cyclic DNA adducts resulting from exposure to crotonaldehyde’, *Cancer Research*, 47(2), pp. 360–363.

Fousek, K., Horn, L.A. and Palena, C. (2021) ‘Interleukin-8: a Chemokine at the Intersection of Cancer Plasticity, Angiogenesis, and Immune Suppression’, *Pharmacology & therapeutics*, 219, p. 107692. Available at: https://doi.org/10.1016/j.pharmthera.2020.107692.

Fredriksson, R. *et al.* (2003) ‘The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints’, *Molecular Pharmacology*, 63(6), pp. 1256–1272. Available at: https://doi.org/10.1124/mol.63.6.1256.

Frey, M.R. *et al.* (2006) ‘p38 kinase regulates epidermal growth factor receptor downregulation and cellular migration’, *The EMBO journal*, 25(24), pp. 5683–5692. Available at: https://doi.org/10.1038/sj.emboj.7601457.

Fu, N.Y. *et al.* (2017) 'Identification of quiescent and spatially restricted mammary stem cells that are hormone responsive', *Nature Cell Biology*, 19(3), pp. 164–176. Available at: https://doi.org/10.1038/ncb3471.

Fu, N.Y. *et al.* (2020) 'Stem Cells and the Differentiation Hierarchy in Mammary Gland Development', *Physiological Reviews*, 100(2), pp. 489–523. Available at: https://doi.org/10.1152/physrev.00040.2018.

Fuchs, R.P. *et al.* (2021) *Crosstalk between repair pathways elicits double-strand breaks in alkylated DNA and implications for the action of temozolomide*, *eLife*. eLife Sciences Publications Limited. Available at: https://doi.org/10.7554/eLife.69544.

Fujii, J., Iuchi, Y. and Okada, F. (2005) 'Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system', *Reproductive biology and endocrinology : RB&E*, 3, p. 43. Available at: https://doi.org/10.1186/1477-7827-3-43.

Fujii, M. *et al.* (2015) 'Efficient genetic engineering of human intestinal organoids using electroporation', *Nature Protocols*, 10(10), pp. 1474–1485. Available at: https://doi.org/10.1038/nprot.2015.088.

Fujii, S. *et al.* (2016) 'PGE2 is a direct and robust mediator of anion/fluid secretion by human intestinal epithelial cells', *Scientific Reports*, 6, p. 36795. Available at: https://doi.org/10.1038/srep36795.

Funston, G. *et al.* (2020) 'The diagnostic performance of CA125 for the detection of ovarian and non-ovarian cancer in primary care: A population-based cohort study', *PLOS Medicine*. Edited by S.D. Shapiro, 17(10), p. e1003295. Available at: https://doi.org/10.1371/journal.pmed.1003295.

Gamallo, C. *et al.* (1999) 'β-Catenin Expression Pattern in Stage I and II Ovarian Carcinomas', *The American Journal of Pathology*, 155(2), pp. 527–536. Available at: https://doi.org/10.1016/S0002-9440(10)65148-6.

Gannon, P.O. *et al.* (2011) 'Impact of hemochromatosis gene (HFE) mutations on epithelial ovarian cancer risk and prognosis', *International journal of cancer*, 128(10), pp. 2326–2334. Available at: https://doi.org/10.1002/ijc.25577.

Garaycoechea, J.I. *et al.* (2018) 'Alcohol and endogenous aldehydes damage chromosomes and mutate stem cells', *Nature*, 553(7687), pp. 171–177. Available at: https://doi.org/10.1038/nature25154.

Gazon, H. *et al.* (2018) 'Hijacking of the AP-1 Signaling Pathway during Development of ATL', *Frontiers in Microbiology*, 8. Available at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02686 (Accessed: 22 August 2023).

Gentile, F. *et al.* (2017) 'DNA damage by lipid peroxidation products: implications in cancer, inflammation and autoimmunity', *AIMS Genetics*, 4(2), pp. 103–137. Available at: https://doi.org/10.3934/genet.2017.2.103.

Giebel, N. *et al.* (2021) 'USP42 protects ZNRF3/RNF43 from R-spondin-dependent clearance and inhibits Wnt signalling', *EMBO Reports*, 22(5), p. e51415. Available at: https://doi.org/10.15252/embr.202051415.

Gilbert, L.A. *et al.* (2013) 'CRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes', *Cell*, 154(2), pp. 442–451. Available at: https://doi.org/10.1016/j.cell.2013.06.044.

Gong, Y. *et al.* (2010) 'Wnt Isoform-Specific Interactions with Coreceptor Specify Inhibition or Potentiation of Signaling by LRP6 Antibodies', *PLOS ONE*, 5(9), p. e12682. Available at: https://doi.org/10.1371/journal.pone.0012682.

Goodman, R.M. *et al.* (2006) 'Sprinter: a novel transmembrane protein required for Wg secretion and signaling', *Development (Cambridge, England)*, 133(24), pp. 4901–4911. Available at: https://doi.org/10.1242/dev.02674.

Gorski, J.J. *et al.* (2009) 'The Complex Relationship between BRCA1 and ERα in Hereditary Breast Cancer', *Clinical Cancer Research*, 15(5), pp. 1514–1518. Available at: https://doi.org/10.1158/1078-0432.CCR-08-0640.

Grabinger, T. *et al.* (2014) 'Ex vivo culture of intestinal crypt organoids as a model system for assessing cell death induction in intestinal epithelial cells and enteropathy', *Cell Death & Disease*, 5(5), p. e1228. Available at: https://doi.org/10.1038/cddis.2014.183.

Griffin, J.N. *et al.* (2018) 'RAPGEF5 Regulates Nuclear Translocation of β-Catenin', *Developmental Cell*, 44(2), pp. 248-260.e4. Available at: https://doi.org/10.1016/j.devcel.2017.12.001.

Gross, J.C. *et al.* (2012) 'Active Wnt proteins are secreted on exosomes', *Nature Cell Biology*, 14(10), pp. 1036–1045. Available at: https://doi.org/10.1038/ncb2574.

Grossmann, J. *et al.* (2003) 'Progress on isolation and short-term ex-vivo culture of highly purified non-apoptotic human intestinal epithelial cells (IEC)', *European Journal of Cell Biology*, 82(5), pp. 262–270. Available at: https://doi.org/10.1078/0171-9335-00312.

Gwak, J. *et al.* (2006) 'Protein-kinase-C-mediated β-catenin phosphorylation negatively regulates the Wnt/β-catenin pathway', *Journal of Cell Science*, 119(22), pp. 4702–4709. Available at: https://doi.org/10.1242/jcs.03256.

Ha, N.-C. *et al.* (2004) 'Mechanism of Phosphorylation-Dependent Binding of APC to β-Catenin and Its Role in β-Catenin Degradation', *Molecular Cell*, 15(4), pp. 511–521. Available at: https://doi.org/10.1016/j.molcel.2004.08.010.

Habas, R., Kato, Y. and He, X. (2001) 'Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel Formin homology protein Daam1', *Cell*, 107(7), pp. 843–854. Available at: https://doi.org/10.1016/s0092-8674(01)00614-6.

Haider, S. *et al.* (2019) 'Estrogen Signaling Drives Ciliogenesis in Human Endometrial Organoids', *Endocrinology*, 160(10), pp. 2282–2297. Available at: https://doi.org/10.1210/en.2019-00314.

Hamilton, E. and Infante, J.R. (2016) 'Targeting CDK4/6 in patients with cancer', *Cancer Treatment Reviews*, 45, pp. 129–138. Available at: https://doi.org/10.1016/j.ctrv.2016.03.002.

Han, E.-S. *et al.* (2008) 'The in vivo gene expression signature of oxidative stress', *Physiological Genomics*, 34(1), pp. 112–126. Available at: https://doi.org/10.1152/physiolgenomics.00239.2007.

Han, Y. *et al.* (2019) 'Insulin mitigates apoptosis of porcine follicular granulosa cells by downregulating BimEL', *Reproductive Biology*, 19(3), pp. 293–298. Available at: https://doi.org/10.1016/j.repbio.2019.08.003.

Hanahan, D. and Weinberg, R.A. (2011) 'Hallmarks of cancer: the next generation', *Cell*, 144(5), pp. 646–674. Available at: https://doi.org/10.1016/j.cell.2011.02.013.

Hao, D. *et al.* (2017) 'Integrated Analysis Reveals Tubal- and Ovarian-Originated Serous Ovarian Cancer and Predicts Differential Therapeutic Responses', *Clinical Cancer Research*, 23(23), pp. 7400–7411. Available at: https://doi.org/10.1158/1078-0432.CCR-17-0638.

Hao, H.-X. *et al.* (2012) 'ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner', *Nature*, 485(7397), pp. 195–200. Available at: https://doi.org/10.1038/nature11019.

Hao, H.-X., Jiang, X. and Cong, F. (2016) 'Control of Wnt Receptor Turnover by R-spondin-ZNRF3/RNF43 Signaling Module and Its Dysregulation in Cancer', *Cancers*, 8(6), p. 54. Available at: https://doi.org/10.3390/cancers8060054.

Havrilesky, L.J. *et al.* (2008) 'Reducing ovarian cancer mortality through screening: Is it possible, and can we afford it?', *Gynecologic Oncology*, 111(2), pp. 179–187. Available at: https://doi.org/10.1016/j.ygyno.2008.07.006.

Hawkridge, A.M. (2014) 'The Chicken Model of Spontaneous Ovarian Cancer', *Proteomics. Clinical applications*, 8(9–10), pp. 689–699. Available at: https://doi.org/10.1002/prca.201300135.

Hecht, A. *et al.* (2000) 'The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates', *The EMBO journal*, 19(8), pp. 1839–1850. Available at: https://doi.org/10.1093/emboj/19.8.1839.

Hellner, K. *et al.* (2016) 'Premalignant SOX2 overexpression in the fallopian tubes of ovarian cancer patients: Discovery and validation studies', *eBioMedicine*, 10, pp. 137–149. Available at: https://doi.org/10.1016/j.ebiom.2016.06.048.

Henderson, B.R. (2000) 'Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover', *Nature Cell Biology*, 2(9), pp. 653–660. Available at: https://doi.org/10.1038/35023605.

Hendriks, D. *et al.* (2023) 'Engineered human hepatocyte organoids enable CRISPR-based target discovery and drug screening for steatosis', *Nature Biotechnology*, pp. 1–15. Available at: https://doi.org/10.1038/s41587-023-01680-4.

Hermida, M.A., Dinesh Kumar, J. and Leslie, N.R. (2017) 'GSK3 and its interactions with the PI3K/AKT/mTOR signalling network', *Advances in Biological Regulation*, 65, pp. 5–15. Available at: https://doi.org/10.1016/j.jbior.2017.06.003.

Hernandez-Monge, J. *et al.* (2021) 'MDM2 regulates RB levels during genotoxic stress', *EMBO reports*, 22(1), p. e50615. Available at: https://doi.org/10.15252/embr.202050615.

Hofmann, C. *et al.* (2007) ‘Cell-Cell Contacts Prevent Anoikis in Primary Human Colonic Epithelial Cells’, *Gastroenterology*, 132(2), pp. 587–600. Available at: https://doi.org/10.1053/j.gastro.2006.11.017.

Holmberg, F.E. *et al.* (2017) ‘Culturing human intestinal stem cells for regenerative applications in the treatment of inflammatory bowel disease’, *EMBO Molecular Medicine*, 9(5), pp. 558–570. Available at: https://doi.org/10.15252/emmm.201607260.

Hoppler, S. and Kavanagh, C.L. (2007) ‘Wnt signalling: variety at the core’, *Journal of Cell Science*, 120(Pt 3), pp. 385–393. Available at: https://doi.org/10.1242/jcs.03363.

van der Horst, P.H. *et al.* (2012) ‘Interaction between sex hormones and WNT/β-catenin signal transduction in endometrial physiology and disease’, *Molecular and Cellular Endocrinology*, 358(2), pp. 176–184. Available at: https://doi.org/10.1016/j.mce.2011.06.010.

Hosotani, M. *et al.* (2021) ‘Anatomy and histology of the foramen of ovarian bursa opening to the peritoneal cavity and its changes in autoimmune disease-prone mice’, *Journal of Anatomy*, 238(1), pp. 73–85. Available at: https://doi.org/10.1111/joa.13299.

Hou, Y. *et al.* (2012) ‘Estrogen regulates iron homeostasis through governing hepatic hepcidin expression via an estrogen response element’, *Gene*, 511(2), pp. 398–403. Available at: https://doi.org/10.1016/j.gene.2012.09.060.

Hsieh, M., Zamah, A.M. and Conti, M. (2009) ‘Epidermal Growth Factor-Like Growth Factors in the Follicular Fluid: Role in Oocyte Development and Maturation’, *Seminars in reproductive medicine*, 27(1), pp. 52–61. Available at: https://doi.org/10.1055/s-0028-1108010.

Hsu, C.-F. *et al.* (2019) ‘IGF-axis confers transformation and regeneration of fallopian tube fimbria epithelium upon ovulation’, *EBioMedicine*, 41, pp. 597–609. Available at: https://doi.org/10.1016/j.ebiom.2019.01.061.

Hsu, C.-F. *et al.* (2022) ‘The Double Engines and Single Checkpoint Theory of Endometriosis’, *Biomedicines*, 10(6), p. 1403. Available at: https://doi.org/10.3390/biomedicines10061403.

Hu, Z. *et al.* (2020) ‘The Repertoire of Serous Ovarian Cancer Non-genetic Heterogeneity Revealed by Single-Cell Sequencing of Normal Fallopian Tube Epithelial Cells’, *Cancer Cell*, 37(2), pp. 226-242.e7. Available at: https://doi.org/10.1016/j.ccell.2020.01.003.

Huang, H.-C. and Klein, P.S. (2004) ‘The Frizzled family: receptors for multiple signal transduction pathways’, *Genome Biology*, 5(7), p. 234. Available at: https://doi.org/10.1186/gb-2004-5-7-234.

Huang, H.-S. *et al.* (2015) ‘Mutagenic, surviving and tumorigenic effects of follicular fluid in the context of p53 loss: initiation of fimbria carcinogenesis’, *Carcinogenesis*, 36(11), pp. 1419–1428. Available at: https://doi.org/10.1093/carcin/bgv132.

Huang, H.-S. *et al.* (2016) ‘Haemoglobin in pelvic fluid rescues Fallopian tube epithelial cells from reactive oxygen species stress and apoptosis’, *The Journal of Pathology*, 240(4), pp. 484–494. Available at: https://doi.org/10.1002/path.4807.

Huang, H.-S., Chu, S.-C. and Chu, T.-Y. (2015) 'Efficient analyses of DNA double-strand breaks and the cell cycle in the secretory epithelial cells of fallopian tube fimbriae', *Tzu Chi Medical Journal*, 27(3), pp. 102–106. Available at: https://doi.org/10.1016/j.tcmj.2015.05.004.

Huang, Y. *et al.* (2019) 'Inflamm-Aging: A New Mechanism Affecting Premature Ovarian Insufficiency', *Journal of Immunology Research*, 2019, pp. 1–7. Available at: https://doi.org/10.1155/2019/8069898.

Huch, M. *et al.* (2013) 'Unlimited in vitro expansion of adult bi-potent pancreas progenitors through the Lgr5/R-spondin axis', *The EMBO Journal*, 32(20), pp. 2708–2721. Available at: https://doi.org/10.1038/emboj.2013.204.

Huels, D.J. and Sansom, O.J. (2015) 'Stem vs non-stem cell origin of colorectal cancer', *British Journal of Cancer*, 113(1), pp. 1–5. Available at: https://doi.org/10.1038/bjc.2015.214.

Ikeda, S. *et al.* (2000) 'GSK-3beta-dependent phosphorylation of adenomatous polyposis coli gene product can be modulated by beta-catenin and protein phosphatase 2A complexed with Axin', *Oncogene*, 19(4), pp. 537–545. Available at: https://doi.org/10.1038/sj.onc.1203359.

Inoue, Y. *et al.* (2009) 'Amphiregulin is much more abundantly expressed than transforming growth factor-alpha and epidermal growth factor in human follicular fluid obtained from patients undergoing in vitro fertilization-embryo transfer', *Fertility and Sterility*, 91(4), pp. 1035–1041. Available at: https://doi.org/10.1016/j.fertnstert.2008.01.014.

Jaber, S. *et al.* (2016) 'p53 downregulates the Fanconi anaemia DNA repair pathway', *Nature Communications*, 7, p. 11091. Available at: https://doi.org/10.1038/ncomms11091.

Janda, C.Y. *et al.* (2012) 'Structural basis of Wnt recognition by Frizzled', *Science (New York, N.Y.)*, 337(6090), pp. 59–64. Available at: https://doi.org/10.1126/science.1222879.

Jennings, R.E. *et al.* (2015) 'Human pancreas development', *Development*, 142(18), pp. 3126–3137. Available at: https://doi.org/10.1242/dev.120063.

Jensen, C. and Teng, Y. (2020) 'Is It Time to Start Transitioning From 2D to 3D Cell Culture?', *Frontiers in Molecular Biosciences*, 7. Available at: https://www.frontiersin.org/articles/10.3389/fmolb.2020.00033 (Accessed: 25 August 2023).

Ji, J.X. *et al.* (2018) 'Clear cell carcinomas of the ovary and kidney: clarity through genomics: Ovarian and renal clear cell carcinomas', *The Journal of Pathology*, 244(5), pp. 550–564. Available at: https://doi.org/10.1002/path.5037.

Jin, Y.H. *et al.* (2009) 'Beta-catenin modulates the level and transcriptional activity of Notch1/NICD through its direct interaction', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1793(2), pp. 290–299. Available at: https://doi.org/10.1016/j.bbamcr.2008.10.002.

Jin, Y.-R. and Yoon, J.K. (2012) ‘The R-spondin family of proteins: emerging regulators of WNT signaling’, *The international journal of biochemistry & cell biology*, 44(12), pp. 2278–2287. Available at: https://doi.org/10.1016/j.biocel.2012.09.006.

Johnen, S. *et al.* (2011) ‘Presence of Xenogenic Mouse RNA in RPE and IPE Cells Cultured on Mitotically Inhibited 3T3 Fibroblasts’, *Investigative Ophthalmology & Visual Science*, 52(5), pp. 2817–2824. Available at: https://doi.org/10.1167/iovs.10-6429.

Johnson, P.A. and Giles, J.R. (2013) ‘The hen as a model of ovarian cancer’, *Nature Reviews Cancer*, 13(6), pp. 432–436. Available at: https://doi.org/10.1038/nrc3535.

Jridi, I. *et al.* (2021) ‘Inflammation and Wnt Signaling: Target for Immunomodulatory Therapy?’, *Frontiers in Cell and Developmental Biology*, 8. Available at: https://www.frontiersin.org/articles/10.3389/fcell.2020.615131 (Accessed: 23 August 2023).

Ju, B. *et al.* (2018) ‘Morphologic and Immunohistochemical Study of Clear Cell Carcinoma of the Uterine Endometrium and Cervix in Comparison to Ovarian Clear Cell Carcinoma’:, *International Journal of Gynecological Pathology*, 37(4), pp. 388–396. Available at: https://doi.org/10.1097/PGP.0000000000000430.

Kabiri, Z. *et al.* (2014) ‘Stroma provides an intestinal stem cell niche in the absence of epithelial Wnts’, *Development*, 141(11), pp. 2206–2215. Available at: https://doi.org/10.1242/dev.104976.

Kadim, E., Al-Wasiti, E. and Qader, H. (2017) ‘Concentration of R-spondin 2 in the Follicular Fluid is Correlated with Oocyte Number and Metaphase II Oocytes in Iraqi Women Undergo ICSI’, *International Journal of Science and Research (IJSR)*, 6, pp. 9–2017. Available at: https://doi.org/10.21275/ART20176210.

Kadowaki, T. *et al.* (1996) ‘The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing’, *Genes & Development*, 10(24), pp. 3116–3128. Available at: https://doi.org/10.1101/gad.10.24.3116.

Kaidi, A., Williams, A.C. and Paraskeva, C. (2007) ‘Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia’, *Nature Cell Biology*, 9(2), pp. 210–217. Available at: https://doi.org/10.1038/ncb1534.

Kang, S. *et al.* (2003) ‘Insulin can block apoptosis by decreasing oxidative stress via phosphatidylinositol 3-kinase- and extracellular signal-regulated protein kinase-dependent signaling pathways in HepG2 cells’, *European Journal of Endocrinology*, 148(1), pp. 147–155. Available at: https://doi.org/10.1530/eje.0.1480147.

Kapałczyńska, M. *et al.* (2018) ‘2D and 3D cell cultures – a comparison of different types of cancer cell cultures’, *Archives of Medical Science : AMS*, 14(4), pp. 910–919. Available at: https://doi.org/10.5114/aoms.2016.63743.

Karst, A.M. and Drapkin, R. (2010) ‘Ovarian Cancer Pathogenesis: A Model in Evolution’, *Journal of Oncology*, 2010, p. 932371. Available at: https://doi.org/10.1155/2010/932371.

Kessler, M. *et al.* (2015) ‘The Notch and Wnt pathways regulate stemness and differentiation in human fallopian tube organoids’, *Nature Communications*, 6(1), p. 8989. Available at: https://doi.org/10.1038/ncomms9989.

Kim, H. and D'Andrea, A.D. (2012) 'Regulation of DNA cross-link repair by the Fanconi anemia/BRCA pathway', *Genes & Development*, 26(13), pp. 1393–1408. Available at: https://doi.org/10.1101/gad.195248.112.

Kim, J., Koo, B.-K. and Knoblich, J.A. (2020) 'Human organoids: model systems for human biology and medicine', *Nature Reviews Molecular Cell Biology*, 21(10), pp. 571–584. Available at: https://doi.org/10.1038/s41580-020-0259-3.

Kim, K. *et al.* (2000) 'Overexpression of beta-catenin induces apoptosis independent of its transactivation function with LEF-1 or the involvement of major G1 cell cycle regulators', *Molecular Biology of the Cell*, 11(10), pp. 3509–3523. Available at: https://doi.org/10.1091/mbc.11.10.3509.

Kim, K.-A. *et al.* (2005) 'Mitogenic Influence of Human R-Spondin1 on the Intestinal Epithelium', *Science*, 309(5738), pp. 1256–1259. Available at: https://doi.org/10.1126/science.1112521.

Kinzler, K.W. and Vogelstein, B. (1996) 'Lessons from Hereditary Colorectal Cancer', *Cell*, 87(2), pp. 159–170. Available at: https://doi.org/10.1016/S0092-8674(00)81333-1.

Kioussi, C. *et al.* (2002) 'Identification of a Wnt/Dvl/beta-Catenin --> Pitx2 pathway mediating cell-type-specific proliferation during development', *Cell*, 111(5), pp. 673–685. Available at: https://doi.org/10.1016/s0092-8674(02)01084-x.

Kitagawa, M. *et al.* (1999) 'An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of β-catenin', *The EMBO Journal*, 18(9), pp. 2401–2410. Available at: https://doi.org/10.1093/emboj/18.9.2401.

Kobayashi, H. *et al.* (2009) 'The role of iron in the pathogenesis of endometriosis', *Gynecological Endocrinology*, 25(1), pp. 39–52. Available at: https://doi.org/10.1080/09513590802366204.

Köbel, M. and Kang, E.Y. (2022) 'The Evolution of Ovarian Carcinoma Subclassification', *Cancers*, 14(2), p. 416. Available at: https://doi.org/10.3390/cancers14020416.

Komiya, Y. and Habas, R. (2008) 'Wnt signal transduction pathways', *Organogenesis*, 4(2), pp. 68–75. Available at: https://doi.org/10.4161/org.4.2.5851.

Kordon, E.C. and Smith, G.H. (1998) 'An entire functional mammary gland may comprise the progeny from a single cell', *Development (Cambridge, England)*, 125(10), pp. 1921–1930. Available at: https://doi.org/10.1242/dev.125.10.1921.

Kormish, J.D., Sinner, D. and Zorn, A.M. (2010) 'Interactions between SOX factors and Wnt/beta-catenin signaling in development and disease', *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, 239(1), pp. 56–68. Available at: https://doi.org/10.1002/dvdy.22046.

Kowalchuk, R.O. *et al.* (2022) 'Estimated Cost of Circulating Tumor DNA for Posttreatment Surveillance of Human Papillomavirus–Associated Oropharyngeal Cancer', *JAMA Network Open*, 5(1), p. e2144783. Available at: https://doi.org/10.1001/jamanetworkopen.2021.44783.

Koyanagi, M. *et al.* (2008) 'Inhibition of the Rho/ROCK pathway reduces apoptosis during transplantation of embryonic stem cell-derived neural precursors', *Journal of*

Neuroscience Research, 86(2), pp. 270–280. Available at: https://doi.org/10.1002/jnr.21502.

Kucab, J.E. *et al.* (2017) 'Nutlin-3a selects for cells harbouring TP53 mutations', *International Journal of Cancer*, 140(4), pp. 877–887. Available at: https://doi.org/10.1002/ijc.30504.

Kurman, R.J. and Shih, I.-M. (2008) 'Pathogenesis of Ovarian Cancer: Lessons From Morphology and Molecular Biology and Their Clinical Implications', *International Journal of Gynecological Pathology*, PAP. Available at: https://doi.org/10.1097/PGP.0b013e318161e4f5.

Kurman, R.J. and Shih, I.-M. (2010) 'The Origin and Pathogenesis of Epithelial Ovarian Cancer: A Proposed Unifying Theory', *American Journal of Surgical Pathology*, 34(3), pp. 433–443. Available at: https://doi.org/10.1097/PAS.0b013e3181cf3d79.

Kurman, R.J. and Shih, I.-M. (2016) 'The Dualistic Model of Ovarian Carcinogenesis', *The American Journal of Pathology*, 186(4), pp. 733–747. Available at: https://doi.org/10.1016/j.ajpath.2015.11.011.

Labidi-Galy, S.I. *et al.* (2017) 'High grade serous ovarian carcinomas originate in the fallopian tube', *Nature Communications*, 8(1), p. 1093. Available at: https://doi.org/10.1038/s41467-017-00962-1.

Lamlum, H. *et al.* (1999) 'The type of somatic mutation at APC in familial adenomatous polyposis is determined by the site of the germline mutation: a new facet to Knudson's "two-hit" hypothesis', *Nature Medicine*, 5(9), pp. 1071–1075. Available at: https://doi.org/10.1038/12511.

Lannagan, T.R. *et al.* (2019) 'Genetic editing of colonic organoids provides a molecularly distinct and orthotopic preclinical model of serrated carcinogenesis', *Gut*, 68(4), pp. 684–692. Available at: https://doi.org/10.1136/gutjnl-2017-315920.

Larriba, M.J. *et al.* (2011) 'Vitamin D Receptor Deficiency Enhances Wnt/β-Catenin Signaling and Tumor Burden in Colon Cancer', *PLOS ONE*, 6(8), p. e23524. Available at: https://doi.org/10.1371/journal.pone.0023524.

Larriba, M.J. *et al.* (2013) 'Vitamin D Is a Multilevel Repressor of Wnt/β-Catenin Signaling in Cancer Cells', *Cancers*, 5(4), pp. 1242–1260. Available at: https://doi.org/10.3390/cancers5041242.

de Lau, W. *et al.* (2014) 'The R-spondin/Lgr5/Rnf43 module: regulator of Wnt signal strength', *Genes & Development*, 28(4), pp. 305–316. Available at: https://doi.org/10.1101/gad.235473.113.

Lawrenson, K. *et al.* (2013) 'In vitro three-dimensional modeling of fallopian tube secretory epithelial cells', *BMC Cell Biology*, 14, p. 43. Available at: https://doi.org/10.1186/1471-2121-14-43.

Lecarpentier, Y. *et al.* (2019) 'Multiple Targets of the Canonical WNT/β-Catenin Signaling in Cancers', *Frontiers in Oncology*, 9, p. 1248. Available at: https://doi.org/10.3389/fonc.2019.01248.

Ledermann, J.A. *et al.* (2014) 'Gynecologic Cancer InterGroup (GCIG) Consensus Review for Mucinous Ovarian Carcinoma', *International Journal of Gynecologic*

Cancer, 24(Supp 3), pp. S14–S19. Available at: https://doi.org/10.1097/IGC.0000000000000296.

Lee, A.Y.L. *et al.* (2019) 'Cell of origin affects tumour development and phenotype in pancreatic ductal adenocarcinoma', *Gut*, 68(3), pp. 487–498. Available at: https://doi.org/10.1136/gutjnl-2017-314426.

Lee, Y. *et al.* (2007) 'A candidate precursor to serous carcinoma that originates in the distal fallopian tube', *The Journal of Pathology*, 211(1), pp. 26–35. Available at: https://doi.org/10.1002/path.2091.

Lee-Six, H. *et al.* (2019) 'The landscape of somatic mutation in normal colorectal epithelial cells', *Nature*, 574(7779), pp. 532–537. Available at: https://doi.org/10.1038/s41586-019-1672-7.

Lerma Clavero, A. *et al.* (2023) 'MDM2 inhibitors, nutlin-3a and navtemadelin, retain efficacy in human and mouse cancer cells cultured in hypoxia', *Scientific Reports*, 13(1), p. 4583. Available at: https://doi.org/10.1038/s41598-023-31484-0.

Leushacke, M. and Barker, N. (2012) 'Lgr5 and Lgr6 as markers to study adult stem cell roles in self-renewal and cancer', *Oncogene*, 31(25), pp. 3009–3022. Available at: https://doi.org/10.1038/onc.2011.479.

Levine, D.A. *et al.* (2005) 'Frequent Mutation of the *PIK3CA* Gene in Ovarian and Breast Cancers', *Clinical Cancer Research*, 11(8), pp. 2875–2878. Available at: https://doi.org/10.1158/1078-0432.CCR-04-2142.

Li, A. *et al.* (2020) 'Transferrin Insufficiency and Iron Overload in Follicular Fluid Contribute to Oocyte Dysmaturity in Infertile Women With Advanced Endometriosis', *Frontiers in Endocrinology*, 11, p. 391. Available at: https://doi.org/10.3389/fendo.2020.00391.

Li, C.I. *et al.* (2012) 'Effect of depo-medroxyprogesterone acetate on breast cancer risk among women 20–44 years of age', *Cancer Research*, 72(8), pp. 2028–2035. Available at: https://doi.org/10.1158/0008-5472.CAN-11-4064.

Li, H., Lee, T.-H. and Avraham, H. (2002) 'A Novel Tricomplex of BRCA1, Nmi, and c-Myc Inhibits c-Myc-induced Human Telomerase Reverse Transcriptase Gene (hTERT) Promoter Activity in Breast Cancer', *Journal of Biological Chemistry*, 277(23), pp. 20965–20973. Available at: https://doi.org/10.1074/jbc.M112231200.

Li, J. *et al.* (2012) 'Ovarian serous carcinoma: recent concepts on its origin and carcinogenesis', *Journal of Hematology & Oncology*, 5(1), p. 8. Available at: https://doi.org/10.1186/1756-8722-5-8.

Liberto, J.M. *et al.* (2022) 'Current and Emerging Methods for Ovarian Cancer Screening and Diagnostics: A Comprehensive Review', *Cancers*, 14(12), p. 2885. Available at: https://doi.org/10.3390/cancers14122885.

Lim, X. *et al.* (2016) 'Axin2 marks quiescent hair follicle bulge stem cells that are maintained by autocrine Wnt/β-catenin signaling', *Proceedings of the National Academy of Sciences*, 113(11), pp. E1498–E1505. Available at: https://doi.org/10.1073/pnas.1601599113.

Lindemans, C.A. *et al.* (2015) 'Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration', *Nature*, 528(7583), pp. 560–564. Available at: https://doi.org/10.1038/nature16460.

Littler, D.R. *et al.* (2010) 'Structure of the FoxM1 DNA-recognition domain bound to a promoter sequence', *Nucleic Acids Research*, 38(13), pp. 4527–4538. Available at: https://doi.org/10.1093/nar/gkq194.

Liu, C. *et al.* (2002) 'Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism', *Cell*, 108(6), pp. 837–847. Available at: https://doi.org/10.1016/s0092-8674(02)00685-2.

Liu, C., Barger, C.J. and Karpf, A.R. (2021) 'FOXM1: A Multifunctional Oncoprotein and Emerging Therapeutic Target in Ovarian Cancer', *Cancers*, 13(12), p. 3065. Available at: https://doi.org/10.3390/cancers13123065.

Liu, H. *et al.* (2015) 'FOXO3a modulates WNT/β-catenin signaling and suppresses epithelial-to-mesenchymal transition in prostate cancer cells', *Cellular Signalling*, 27(3), pp. 510–518. Available at: https://doi.org/10.1016/j.cellsig.2015.01.001.

Liu, J. *et al.* (2004) 'A Genetically Defined Model for Human Ovarian Cancer', *Cancer Research*, 64(5), pp. 1655–1663. Available at: https://doi.org/10.1158/0008-5472.CAN-03-3380.

Liu, R.-M. *et al.* (2016) 'Hyaluronic acid enhances proliferation of human amniotic mesenchymal stem cells through activation of Wnt/β-catenin signaling pathway', *Experimental Cell Research*, 345(2), pp. 218–229. Available at: https://doi.org/10.1016/j.yexcr.2016.05.019.

Liu, S. *et al.* (2008) 'BRCA1 regulates human mammary stem/progenitor cell fate', *Proceedings of the National Academy of Sciences*, 105(5), pp. 1680–1685. Available at: https://doi.org/10.1073/pnas.0711613105.

Liu, S. and Wang, Y. (2015) 'Mass spectrometry for the assessment of the occurrence and biological consequences of DNA adducts', *Chemical Society Reviews*, 44(21), pp. 7829–7854. Available at: https://doi.org/10.1039/c5cs00316d.

Liu, X. *et al.* (2007) 'Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated basal-like breast cancer', *Proceedings of the National Academy of Sciences*, 104(29), pp. 12111–12116. Available at: https://doi.org/10.1073/pnas.0702969104.

Liu, X. *et al.* (2012) 'ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells', *The American Journal of Pathology*, 180(2), pp. 599–607. Available at: https://doi.org/10.1016/j.ajpath.2011.10.036.

Liu, Y. *et al.* (2018) 'The Role of PPAR-δ in Metabolism, Inflammation, and Cancer: Many Characters of a Critical Transcription Factor', *International Journal of Molecular Sciences*, 19(11), p. 3339. Available at: https://doi.org/10.3390/ijms19113339.

Llames, S. *et al.* (2015) 'Feeder Layer Cell Actions and Applications', *Tissue Engineering. Part B, Reviews*, 21(4), pp. 345–353. Available at: https://doi.org/10.1089/ten.teb.2014.0547.

Lõhmussaar, K. *et al.* (2020) ‘Assessing the origin of high-grade serous ovarian cancer using CRISPR-modification of mouse organoids’, *Nature Communications*, 11(1), p. 2660. Available at: https://doi.org/10.1038/s41467-020-16432-0.

Long, Y. *et al.* (2021) ‘GLUT4 in Mouse Endometrial Epithelium: Roles in Embryonic Development and Implantation’, *Frontiers in Physiology*, 12. Available at: https://doi.org/10.3389/fphys.2021.674924.

Longacre, T.A. *et al.* (2005) ‘Ovarian Serous Tumors of Low Malignant Potential (Borderline Tumors): Outcome-Based Study of 276 Patients With Long-Term (≥5-Year) Follow-Up’, *American Journal of Surgical Pathology*, 29(6), pp. 707–723. Available at: https://doi.org/10.1097/01.pas.0000164030.82810.db.

López-Reig, R. and López-Guerrero, J.A. (2020) ‘The hallmarks of ovarian cancer: proliferation and cell growth’, *European Journal of Cancer Supplements*, 15, pp. 27–37. Available at: https://doi.org/10.1016/j.ejcsup.2019.12.001.

Lozano-Velasco, E. *et al.* (2022) ‘Post-Transcriptional Regulation of Molecular Determinants during Cardiogenesis’, *International Journal of Molecular Sciences*, 23(5), p. 2839. Available at: https://doi.org/10.3390/ijms23052839.

Lu, W. *et al.* (2004) ‘Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth’, *Cell*, 119(1), pp. 97–108. Available at: https://doi.org/10.1016/j.cell.2004.09.019.

Luo, W. *et al.* (2007) ‘Protein phosphatase 1 regulates assembly and function of the beta-catenin degradation complex’, *The EMBO journal*, 26(6), pp. 1511–1521. Available at: https://doi.org/10.1038/sj.emboj.7601607.

MacDonald, B.T., Tamai, K. and He, X. (2009) ‘Wnt/β-catenin signaling: components, mechanisms, and diseases’, *Developmental cell*, 17(1), pp. 9–26. Available at: https://doi.org/10.1016/j.devcel.2009.06.016.

Mancini, M. and Toker, A. (2009) ‘NFAT Proteins: Emerging Roles in Cancer Progression’, *Nature reviews. Cancer*, 9(11), pp. 810–820. Available at: https://doi.org/10.1038/nrc2735.

Mangal, R.K. *et al.* (1997) ‘Differential expression of uterine progesterone receptor forms A and B during the menstrual cycle’, *The Journal of Steroid Biochemistry and Molecular Biology*, 63(4), pp. 195–202. Available at: https://doi.org/10.1016/S0960-0760(97)00119-2.

Mansour, W.Y. *et al.* (2018) ‘Loss of PTEN-assisted G2/M checkpoint impedes homologous recombination repair and enhances radio-curability and PARP inhibitor treatment response in prostate cancer’, *Scientific Reports*, 8(1), p. 3947. Available at: https://doi.org/10.1038/s41598-018-22289-7.

Matano, M. *et al.* (2015) ‘Modeling colorectal cancer using CRISPR-Cas9–mediated engineering of human intestinal organoids’, *Nature Medicine*, 21(3), pp. 256–262. Available at: https://doi.org/10.1038/nm.3802.

Matsui, T. *et al.* (2005) ‘Noncanonical Wnt signaling regulates midline convergence of organ primordia during zebrafish development’, *Genes & Development*, 19(1), pp. 164–175. Available at: https://doi.org/10.1101/gad.1253605.

McCrea, P.D., Turck, C.W. and Gumbiner, B. (1991) ‘A Homolog of the *armadillo* Protein in *Drosophila* (Plakoglobin) Associated with E-Cadherin’, *Science*, 254(5036), pp. 1359–1361. Available at: https://doi.org/10.1126/science.1962194.

McKie, A.T. *et al.* (2001) ‘An iron-regulated ferric reductase associated with the absorption of dietary iron’, *Science (New York, N.Y.)*, 291(5509), pp. 1755–1759. Available at: https://doi.org/10.1126/science.1057206.

McMahon, A.P. and Moon, R.T. (1989) ‘Ectopic expression of the proto-oncogene int-1 in Xenopus embryos leads to duplication of the embryonic axis’, *Cell*, 58(6), pp. 1075–1084. Available at: https://doi.org/10.1016/0092-8674(89)90506-0.

Means, A.L. *et al.* (2005) ‘Pancreatic epithelial plasticity mediated by acinar cell transdifferentiation and generation of nestin-positive intermediates’, *Development*, 132(16), pp. 3767–3776. Available at: https://doi.org/10.1242/dev.01925.

Medina, A., Reintsch, W. and Steinbeisser, H. (2000) ‘Xenopus frizzled 7 can act in canonical and non-canonical Wnt signaling pathways: implications on early patterning and morphogenesis’, *Mechanisms of Development*, 92(2), pp. 227–237. Available at: https://doi.org/10.1016/S0925-4773(00)00240-9.

Melchor, L. and Benítez, J. (2008) ‘An integrative hypothesis about the origin and development of sporadic and familial breast cancer subtypes’, *Carcinogenesis*, 29(8), pp. 1475–1482. Available at: https://doi.org/10.1093/carcin/bgn157.

Mencalha, A.L. *et al.* (2012) ‘Forkhead Box M1 (FoxM1) Gene Is a New STAT3 Transcriptional Factor Target and Is Essential for Proliferation, Survival and DNA Repair of K562 Cell Line’, *PLoS ONE*. Edited by V.V. Kalinichenko, 7(10), p. e48160. Available at: https://doi.org/10.1371/journal.pone.0048160.

Menche, C. and Farin, H.F. (2021) ‘Strategies for genetic manipulation of adult stem cell-derived organoids’, *Experimental & Molecular Medicine*, 53(10), pp. 1483–1494. Available at: https://doi.org/10.1038/s12276-021-00609-8.

Meng, X. *et al.* (2020) ‘Fenton reaction-based nanomedicine in cancer chemodynamic and synergistic therapy’, *Applied Materials Today*, 21, p. 100864. Available at: https://doi.org/10.1016/j.apmt.2020.100864.

Menon, U. *et al.* (2021) ‘Ovarian cancer population screening and mortality after long-term follow-up in the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS): a randomised controlled trial’, *The Lancet*, 397(10290), pp. 2182–2193. Available at: https://doi.org/10.1016/S0140-6736(21)00731-5.

Mergo, P.J. *et al.* (1997) ‘Pancreatic neoplasms: MR imaging and pathologic correlation.’, *RadioGraphics*, 17(2), pp. 281–301. Available at: https://doi.org/10.1148/radiographics.17.2.9084072.

Mericskay, M., Kitajewski, J. and Sassoon, D. (2004) ‘Wnt5a is required for proper epithelial-mesenchymal interactions in the uterus’, *Development*, 131(9), pp. 2061–2072. Available at: https://doi.org/10.1242/dev.01090.

Miller, C., Degenhardt, K. and Sassoon, D.A. (1998) ‘Fetal exposure to DES results in de-regulation of Wnt7a during uterine morphogenesis’, *Nature genetics*, 20(3), pp. 228–230. Available at: https://doi.org/10.1038/3027.

Miller, C. and Sassoon, D.A. (1998) 'Wnt-7a maintains appropriate uterine patterning during the development of the mouse female reproductive tract', *Development*, 125(16), pp. 3201–3211. Available at: https://doi.org/10.1242/dev.125.16.3201.

Miller, L.D. *et al.* (2011) 'An iron regulatory gene signature predicts outcome in breast cancer', *Cancer Research*, 71(21), pp. 6728–6737. Available at: https://doi.org/10.1158/0008-5472.CAN-11-1870.

Molenaar, M. *et al.* (1996) 'XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos', *Cell*, 86(3), pp. 391–399. Available at: https://doi.org/10.1016/s0092-8674(00)80112-9.

Molyneux, G. *et al.* (2010) 'BRCA1 Basal-like Breast Cancers Originate from Luminal Epithelial Progenitors and Not from Basal Stem Cells', *Cell Stem Cell*, 7(3), pp. 403–417. Available at: https://doi.org/10.1016/j.stem.2010.07.010.

Momenimovahed, Z. *et al.* (2019) 'Ovarian cancer in the world: epidemiology and risk factors', *International Journal of Women's Health*, Volume 11, pp. 287–299. Available at: https://doi.org/10.2147/IJWH.S197604.

de la Monte, S.M. and Tong, M. (2009) 'Mechanisms of Nitrosamine–Mediated NNeurodegeneration: Potential Relevance to Sporadic Alzheimer's Disease', *Journal of Alzheimer's disease : JAD*, 17(4), pp. 817–825. Available at: https://doi.org/10.3233/JAD-2009-1098.

Moschetta, M. *et al.* (2016) 'BRCA somatic mutations and epigenetic BRCA modifications in serous ovarian cancer', *Annals of Oncology*, 27(8), pp. 1449–1455. Available at: https://doi.org/10.1093/annonc/mdw142.

Motohara, T. *et al.* (2019) 'An evolving story of the metastatic voyage of ovarian cancer cells: cellular and molecular orchestration of the adipose-rich metastatic microenvironment', *Oncogene*, 38(16), pp. 2885–2898. Available at: https://doi.org/10.1038/s41388-018-0637-x.

Muñoz-Cánoves, P. *et al.* (2013) 'Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword?', *The FEBS Journal*, 280(17), pp. 4131–4148. Available at: https://doi.org/10.1111/febs.12338.

Nagy, B. *et al.* (2019) 'Follicular fluid progesterone concentration is associated with fertilization outcome after IVF: a systematic review and meta-analysis', *Reproductive BioMedicine Online*, 38(6), pp. 871–882. Available at: https://doi.org/10.1016/j.rbmo.2018.12.045.

Nakamura, T. *et al.* (2004) 'Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders', *The British Journal of Ophthalmology*, 88(10), pp. 1280–1284. Available at: https://doi.org/10.1136/bjo.2003.038497.

Nakamuta, A., Yoshido, K. and Naoki, H. (2022) 'Stem cell homeostasis regulated by hierarchy and neutral competition', *Communications Biology*, 5(1), pp. 1–11. Available at: https://doi.org/10.1038/s42003-022-04218-7.

Nakanishi, Y. *et al.* (2013) 'Dclk1 distinguishes between tumor and normal stem cells in the intestine', *Nature Genetics*, 45(1), pp. 98–103. Available at: https://doi.org/10.1038/ng.2481.

Nam, J.-S. *et al.* (2006) 'Mouse Cristin/R-spondin Family Proteins Are Novel Ligands for the Frizzled 8 and LRP6 Receptors and Activate β-Catenin-dependent Gene Expression', *Journal of Biological Chemistry*, 281(19), pp. 13247–13257. Available at: https://doi.org/10.1074/jbc.M508324200.

Niehrs, C. and Shen, J. (2010) 'Regulation of Lrp6 phosphorylation', *Cellular and Molecular Life Sciences*, 67(15), pp. 2551–2562. Available at: https://doi.org/10.1007/s00018-010-0329-3.

Nishita, M. *et al.* (2006) 'Filopodia formation mediated by receptor tyrosine kinase Ror2 is required for Wnt5a-induced cell migration', *The Journal of Cell Biology*, 175(4), pp. 555–562. Available at: https://doi.org/10.1083/jcb.200607127.

Nookaew, I. *et al.* (2020) 'Detection and Discrimination of DNA Adducts Differing in Size, Regiochemistry, and Functional Group by Nanopore Sequencing', *Chemical research in toxicology*, 33(12), pp. 2944–2952. Available at: https://doi.org/10.1021/acs.chemrestox.0c00202.

Norquist, B.M. *et al.* (2010) 'The molecular pathogenesis of hereditary ovarian carcinoma: Alterations in the tubal epithelium of women with BRCA1 and BRCA2 mutations', *Cancer*, 116(22), pp. 5261–5271. Available at: https://doi.org/10.1002/cncr.25439.

Nusse, R. (2023) *Wnt Target genes | The Wnt Homepage*. Available at: http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes (Accessed: 22 August 2023).

Nusse, R. and Varmus, H.E. (1982) 'Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome', *Cell*, 31(1), pp. 99–109. Available at: https://doi.org/10.1016/0092-8674(82)90409-3.

Nüsslein-Volhard, C. and Wieschaus, E. (1980) 'Mutations affecting segment number and polarity in Drosophila', *Nature*, 287(5785), pp. 795–801. Available at: https://doi.org/10.1038/287795a0.

Okumura, N. *et al.* (2016) 'Rho kinase inhibitor enables cell-based therapy for corneal endothelial dysfunction', *Scientific Reports*, 6(1), p. 26113. Available at: https://doi.org/10.1038/srep26113.

Olson, L.E. *et al.* (2006) 'Homeodomain-mediated beta-catenin-dependent switching events dictate cell-lineage determination', *Cell*, 125(3), pp. 593–605. Available at: https://doi.org/10.1016/j.cell.2006.02.046.

Omoto, Y. and Iwase, H. (2015) 'Clinical significance of estrogen receptor β in breast and prostate cancer from biological aspects', *Cancer Science*, 106(4), pp. 337–343. Available at: https://doi.org/10.1111/cas.12613.

Ozawa, M., Baribault, H. and Kemler, R. (1989) 'The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species', *The EMBO journal*, 8(6), pp. 1711–1717. Available at: https://doi.org/10.1002/j.1460-2075.1989.tb03563.x.

Paik, D.Y. *et al.* (2012) 'Stem-like epithelial cells are concentrated in the distal end of the fallopian tube: a site for injury and serous cancer initiation', *Stem cells (Dayton, Ohio)*, 30(11), pp. 2487–2497. Available at: https://doi.org/10.1002/stem.1207.

Pan, H. *et al.* (2018) 'Transcription factor FoxM1 is the downstream target of c-Myc and contributes to the development of prostate cancer', *World Journal of Surgical Oncology*, 16(1), p. 59. Available at: https://doi.org/10.1186/s12957-018-1352-3.

Panciera, T. *et al.* (2016) 'Induction of Expandable Tissue-Specific Stem/Progenitor Cells through Transient Expression of YAP/TAZ', *Cell Stem Cell*, 19(6), pp. 725–737. Available at: https://doi.org/10.1016/j.stem.2016.08.009.

Park, H.J. *et al.* (2009) 'FoxM1, a critical regulator of oxidative stress during oncogenesis', *The EMBO Journal*, 28(19), pp. 2908–2918. Available at: https://doi.org/10.1038/emboj.2009.239.

Park, M.J. *et al.* (2020) 'Prediction of ovarian aging using ovarian expression of BMP15, GDF9, and C-KIT', *Experimental Biology and Medicine*, 245(8), pp. 711–719. Available at: https://doi.org/10.1177/1535370220915826.

Parker, T.W., Rudeen, A.J. and Neufeld, K.L. (2020) 'Oncogenic Serine 45-Deleted β-Catenin Remains Susceptible to Wnt Stimulation and APC Regulation in Human Colonocytes', *Cancers*, 12(8), p. 2114. Available at: https://doi.org/10.3390/cancers12082114.

Peng, W.C., de Lau, W., Forneris, F., *et al.* (2013) 'Structure of stem cell growth factor R-spondin 1 in complex with the ectodomain of its receptor LGR5', *Cell Reports*, 3(6), pp. 1885–1892. Available at: https://doi.org/10.1016/j.celrep.2013.06.009.

Peng, W.C., de Lau, W., Madoori, P.K., *et al.* (2013) 'Structures of Wnt-antagonist ZNRF3 and its complex with R-spondin 1 and implications for signaling', *PloS One*, 8(12), p. e83110. Available at: https://doi.org/10.1371/journal.pone.0083110.

Perets, R. *et al.* (2013) 'Transformation of the Fallopian Tube Secretory Epithelium Leads to High-Grade Serous Ovarian Cancer in Brca;Tp53;Pten Models', *Cancer Cell*, 24(6), pp. 751–765. Available at: https://doi.org/10.1016/j.ccr.2013.10.013.

Piek, J.M.J. *et al.* (2001) 'Dysplastic changes in prophylactically removed Fallopian tubes of women predisposed to developing ovarian cancer', *The Journal of Pathology*, 195(4), pp. 451–456. Available at: https://doi.org/10.1002/path.1000.

Pinello, L. *et al.* (2016) 'Analyzing CRISPR genome-editing experiments with CRISPResso', *Nature Biotechnology*, 34(7), pp. 695–697. Available at: https://doi.org/10.1038/nbt.3583.

Pinto, D. *et al.* (2003) 'Canonical Wnt signals are essential for homeostasis of the intestinal epithelium', *Genes & Development*, 17(14), pp. 1709–1713. Available at: https://doi.org/10.1101/gad.267103.

Pinzone, J.J. *et al.* (2009) 'The role of Dickkopf-1 in bone development, homeostasis, and disease', *Blood*, 113(3), pp. 517–525. Available at: https://doi.org/10.1182/blood-2008-03-145169.

Poetsch, A.R. (2020) 'The genomics of oxidative DNA damage, repair, and resulting mutagenesis', *Computational and Structural Biotechnology Journal*, 18, pp. 207–219. Available at: https://doi.org/10.1016/j.csbj.2019.12.013.

Powell, A.E. *et al.* (2014) 'Inducible loss of one Apc allele in Lrig1-expressing progenitor cells results in multiple distal colonic tumors with features of familial

adenomatous polyposis', *American Journal of Physiology. Gastrointestinal and Liver Physiology*, 307(1), pp. G16-23. Available at: https://doi.org/10.1152/ajpgi.00358.2013.

Prat, A. *et al.* (2014) 'Molecular features of the basal-like breast cancer subtype based on BRCA1 mutation status', *Breast Cancer Research and Treatment*, 147(1), pp. 185–191. Available at: https://doi.org/10.1007/s10549-014-3056-x.

Psilopatis, I. *et al.* (2022) 'Patient-Derived Organoids: The Beginning of a New Era in Ovarian Cancer Disease Modeling and Drug Sensitivity Testing', *Biomedicines*, 11(1), p. 1. Available at: https://doi.org/10.3390/biomedicines11010001.

Qi, Z. *et al.* (2017) 'BMP restricts stemness of intestinal Lgr5+ stem cells by directly suppressing their signature genes', *Nature Communications*, 8(1), p. 13824. Available at: https://doi.org/10.1038/ncomms13824.

Qian, J. *et al.* (2013) 'Twist1 Promotes Gastric Cancer Cell Proliferation through Up-Regulation of FoxM1', *PLoS ONE*. Edited by D. Sarkar, 8(10), p. e77625. Available at: https://doi.org/10.1371/journal.pone.0077625.

Qiu, C. *et al.* (2017) 'Gene expression profiles of ovarian low-grade serous carcinoma resemble those of fallopian tube epithelium', *Gynecologic Oncology*, 147(3), pp. 634–641. Available at: https://doi.org/10.1016/j.ygyno.2017.09.029.

Quail, M.A. *et al.* (2012) 'A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers', *BMC genomics*, 13, p. 341. Available at: https://doi.org/10.1186/1471-2164-13-341.

Rakha, E.A., Reis-Filho, J.S. and Ellis, I.O. (2008) 'Basal-like breast cancer: a critical review', *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, 26(15), pp. 2568–2581. Available at: https://doi.org/10.1200/JCO.2007.13.1748.

Rama, R. and Rodríguez, J. (2016) 'Excitotoxicity and Oxidative Stress in Acute Stroke', in. Available at: https://doi.org/10.5772/64991.

Reader, K.L. *et al.* (2011) 'Signalling pathways involved in the cooperative effects of ovine and murine GDF9+BMP15-stimulated thymidine uptake by rat granulosa cells', *Reproduction*, 142(1), pp. 123–131. Available at: https://doi.org/10.1530/REP-10-0490.

Replogle, J.M., Saunders, R.A., *et al.* (2022) 'Mapping information-rich genotype-phenotype landscapes with genome-scale Perturb-seq', *Cell*, 185(14), pp. 2559-2575.e28. Available at: https://doi.org/10.1016/j.cell.2022.05.013.

Replogle, J.M., Bonnar, J.L., *et al.* (2022) 'Maximizing CRISPRi efficacy and accessibility with dual-sgRNA libraries and optimal effectors', *eLife*. Edited by J.E. Carette, D.Y. Stainier, and M. Calabrese, 11, p. e81856. Available at: https://doi.org/10.7554/eLife.81856.

Rheinwald, J.G. and Green, H. (1975) 'Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells', *Cell*, 6(3), pp. 331–343. Available at: https://doi.org/10.1016/s0092-8674(75)80001-8.

Richter, M. *et al.* (2021) 'From Donor to the Lab: A Fascinating Journey of Primary Cell Lines', *Frontiers in Cell and Developmental Biology*, 9, p. 711381. Available at: https://doi.org/10.3389/fcell.2021.711381.

Rijsewijk, F. *et al.* (1987) ‘The Drosophila homology of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless’, *Cell*, 50(4), pp. 649–657. Available at: https://doi.org/10.1016/0092-8674(87)90038-9.

Rinne, N. *et al.* (2021) ‘Targeting the PI3K/AKT/mTOR pathway in epithelial ovarian cancer, therapeutic treatment options for platinum-resistant ovarian cancer’, *Cancer Drug Resistance* [Preprint]. Available at: https://doi.org/10.20517/cdr.2021.05.

Rizzo, A. *et al.* (2009) ‘Serum levels of reactive oxygen species (ROS) in the bitch’, *Immunopharmacology and Immunotoxicology*, 31(2), pp. 310–313. Available at: https://doi.org/10.1080/08923970802683954.

Rizzo, A. *et al.* (2012) ‘Roles of Reactive Oxygen Species in Female Reproduction: ROS and Reproduction’, *Reproduction in Domestic Animals*, 47(2), pp. 344–352. Available at: https://doi.org/10.1111/j.1439-0531.2011.01891.x.

Roberts, R.M. *et al.* (2022) ‘The role of BMP4 signaling in trophoblast emergence from pluripotency’, *Cellular and molecular life sciences: CMLS*, 79(8), p. 447. Available at: https://doi.org/10.1007/s00018-022-04478-w.

Rockfield, S. *et al.* (2017) ‘Iron Overload and Altered Iron Metabolism in Ovarian Cancer’, *Biological chemistry*, 398(9), pp. 995–1007. Available at: https://doi.org/10.1515/hsz-2016-0336.

Rockfield, S., Kee, Y. and Nanjundan, M. (2019) ‘Chronic iron exposure and c-Myc/H-ras-mediated transformation in fallopian tube cells alter the expression of EVI1, amplified at 3q26.2 in ovarian cancer’, *Oncogenesis*, 8(9), pp. 1–21. Available at: https://doi.org/10.1038/s41389-019-0154-y.

Rodgers, K. and McVey, M. (2016) ‘Error-Prone Repair of DNA Double-Strand Breaks’, *Journal of Cellular Physiology*, 231(1), pp. 15–24. Available at: https://doi.org/10.1002/jcp.25053.

Roig, A.I. *et al.* (2010) ‘Immortalized Epithelial Cells Derived From Human Colon Biopsies Express Stem Cell Markers and Differentiate In Vitro’, *Gastroenterology*, 138(3), pp. 1012-1021.e5. Available at: https://doi.org/10.1053/j.gastro.2009.11.052.

Ross, D.A. and Kadesch, T. (2001) ‘The Notch Intracellular Domain Can Function as a Coactivator for LEF-1’, *Molecular and Cellular Biology*, 21(22), pp. 7537–7544. Available at: https://doi.org/10.1128/MCB.21.22.7537-7544.2001.

Sachs, N. *et al.* (2019) ‘Long-term expanding human airway organoids for disease modeling’, *The EMBO Journal*, 38(4), p. e100300. Available at: https://doi.org/10.15252/embj.2018100300.

Salonia, F. *et al.* (2022) ‘A dual sgRNA-directed CRISPR/Cas9 construct for editing the fruit-specific β-cyclase 2 gene in pigmented citrus fruits’, *Frontiers in Plant Science*, 13, p. 975917. Available at: https://doi.org/10.3389/fpls.2022.975917.

Sampson, J.A. (1927) ‘Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation’, *The American Journal of Pathology*, 3(2), pp. 93-110.43.

Sampson, J.A. (1940) ‘The development of the implantation theory for the origin of peritoneal endometriosis’, *American Journal of Obstetrics and Gynecology*, 40(4), pp. 549–557. Available at: https://doi.org/10.1016/S0002-9378(40)91238-8.

Sanchez, A.M. *et al.* (2014) ‘The distinguishing cellular and molecular features of the endometriotic ovarian cyst: from pathophysiology to the potential endometrioma-mediated damage to the ovary’, *Human Reproduction Update*, 20(2), pp. 217–230. Available at: https://doi.org/10.1093/humupd/dmt053.

Sarabia-Sánchez, M.A. *et al.* (2023) ‘Non-canonical Wnt/Ca2+ signaling is essential to promote self-renewal and proliferation in colon cancer stem cells’, *Frontiers in Oncology*, 13, p. 1121787. Available at: https://doi.org/10.3389/fonc.2023.1121787.

Sartori, A.A. *et al.* (2007) ‘Human CtIP promotes DNA end resection’, *Nature*, 450(7169), pp. 509–514. Available at: https://doi.org/10.1038/nature06337.

Sasaki, T. *et al.* (2002) ‘Expression and Distribution of Laminin α1 and α2 Chains in Embryonic and Adult Mouse Tissues: An Immunochemical Approach’, *Experimental Cell Research*, 275(2), pp. 185–199. Available at: https://doi.org/10.1006/excr.2002.5499.

Sassoon, D. (1999) ‘Wnt genes and endocrine disruption of the female reproductive tract: a genetic approach’, *Molecular and Cellular Endocrinology*, 158(1–2), pp. 1–5. Available at: https://doi.org/10.1016/s0303-7207(99)00170-7.

Sato, T. *et al.* (2009) ‘Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche’, *Nature*, 459(7244), pp. 262–265. Available at: https://doi.org/10.1038/nature07935.

Sato, T., Stange, D.E., *et al.* (2011) ‘Long-term Expansion of Epithelial Organoids From Human Colon, Adenoma, Adenocarcinoma, and Barrett’s Epithelium’, *Gastroenterology*, 141(5), pp. 1762–1772. Available at: https://doi.org/10.1053/j.gastro.2011.07.050.

Sato, T., van Es, J.H., *et al.* (2011) ‘Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts’, *Nature*, 469(7330), pp. 415–418. Available at: https://doi.org/10.1038/nature09637.

Savage, K.I. *et al.* (2014) ‘BRCA1 Deficiency Exacerbates Estrogen-Induced DNA Damage and Genomic Instability’, *Cancer Research*, 74(10), pp. 2773–2784. Available at: https://doi.org/10.1158/0008-5472.CAN-13-2611.

Savage, K.I. and Harkin, D.P. (2015) ‘BRCA1, a “complex” protein involved in the maintenance of genomic stability’, *The FEBS Journal*, 282(4), pp. 630–646. Available at: https://doi.org/10.1111/febs.13150.

Schut, H.A.J. and Snyderwine, E.G. (1999) ‘DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis’, *Carcinogenesis*, 20(3), pp. 353–368. Available at: https://doi.org/10.1093/carcin/20.3.353.

Schwank, G. *et al.* (2013) ‘Generation of BAC transgenic epithelial organoids’, *PloS One*, 8(10), p. e76871. Available at: https://doi.org/10.1371/journal.pone.0076871.

Schwitalla, S. *et al.* (2013) ‘Intestinal Tumorigenesis Initiated by Dedifferentiation and Acquisition of Stem-Cell-like Properties’, *Cell*, 152(1), pp. 25–38. Available at: https://doi.org/10.1016/j.cell.2012.12.012.

SEER (2020) *Cancer of the Ovary - Cancer Stat Facts*. Available at: https://seer.cancer.gov/statfacts/html/ovary.html (Accessed: 12 September 2023).

Seidman, J.D. (2013) ‘The presence of mucosal iron in the fallopian tube supports the “incessant menstruation hypothesis” for ovarian carcinoma’, *International Journal of Gynecological Pathology*, 32(5), pp. 454–458. Available at: https://doi.org/10.1097/PGP.0b013e31826f5ce2.

Semenov, M.V. *et al.* (2007) ‘SnapShot: Noncanonical Wnt Signaling Pathways’, *Cell*, 131(7), p. 1378.e1-1378.e2. Available at: https://doi.org/10.1016/j.cell.2007.12.011.

Serov, S.F. and Scully, R.E. (1973) ‘Histological typing of ovarian tumours’, *International Histological Classification of Tumours*, 9.

Sevilla, C.L. *et al.* (1997) ‘Development of Monoclonal Antibodies to the Malondialdehyde−Deoxyguanosine Adduct, Pyrimidopurinone1’, *Chemical Research in Toxicology*, 10(2), pp. 172–180. Available at: https://doi.org/10.1021/tx960120d.

Shackleton, M. *et al.* (2006) ‘Generation of a functional mammary gland from a single stem cell’, *Nature*, 439(7072), pp. 84–88. Available at: https://doi.org/10.1038/nature04372.

Shalem, O. *et al.* (2014) ‘Genome-scale CRISPR-Cas9 knockout screening in human cells’, *Science (New York, N.Y.)*, 343(6166), pp. 84–87. Available at: https://doi.org/10.1126/science.1247005.

Sharma, A. and Almasan, A. (2021) ‘Chapter Six - Autophagy and PTEN in DNA damage-induced senescence’, in D.A. Gewirtz and P.B. Fisher (eds) *Advances in Cancer Research*. Academic Press (Autophagy and Senescence in Cancer Therapy), pp. 249–284. Available at: https://doi.org/10.1016/bs.acr.2021.01.006.

Sharma, R.K. and Agarwal, A. (2004) ‘Role of reactive oxygen species in gynecologic diseases: Oxidative stress and pregnancy’, *Reproductive Medicine and Biology*, 3(4), pp. 177–199. Available at: https://doi.org/10.1111/j.1447-0578.2004.00068.x.

Shaw, P.A. *et al.* (2009) ‘Candidate serous cancer precursors in fallopian tube epithelium of BRCA1/2 mutation carriers’, *Modern Pathology*, 22(9), pp. 1133–1138. Available at: https://doi.org/10.1038/modpathol.2009.89.

Shay, J.W., Pereira-Smith, O.M. and Wright, W.E. (1991) ‘A role for both RB and p53 in the regulation of human cellular senescence’, *Experimental Cell Research*, 196(1), pp. 33–39. Available at: https://doi.org/10.1016/0014-4827(91)90453-2.

Shi, B. *et al.* (2007) ‘Integration of estrogen and Wnt signaling circuits by the polycomb group protein EZH2 in breast cancer cells’, *Molecular and Cellular Biology*, 27(14), pp. 5105–5119. Available at: https://doi.org/10.1128/MCB.00162-07.

Shigeta, S. *et al.* (2016) ‘Transferrin facilitates the formation of DNA double-strand breaks via transferrin receptor 1: the possible involvement of transferrin in carcinogenesis of high-grade serous ovarian cancer’, *Oncogene*, 35(27), pp. 3577–3586. Available at: https://doi.org/10.1038/onc.2015.425.

Shkolnik, K. *et al.* (2011) ‘Reactive oxygen species are indispensable in ovulation’, *Proceedings of the National Academy of Sciences*, 108(4), pp. 1462–1467. Available at: https://doi.org/10.1073/pnas.1017213108.

da Silveira, J.C. *et al.* (2012) ‘Cell-Secreted Vesicles in Equine Ovarian Follicular Fluid Contain miRNAs and Proteins: A Possible New Form of Cell Communication Within the Ovarian Follicle1’, *Biology of Reproduction*, 86(3), pp. 71, 1–10. Available at: https://doi.org/10.1095/biolreprod.111.093252.

Simonetti, S., Veeck, L.L. and Jones, H.W. (1985) ‘Correlation of follicular fluid volume with oocyte morphology from follicles stimulated by human menopausal gonadotropin’, *Fertility and Sterility*, 44(2), pp. 177–180. Available at: https://doi.org/10.1016/s0015-0282(16)48731-5.

Slater, P.G. *et al.* (2013) ‘Frizzled-5 Receptor Is Involved in Neuronal Polarity and Morphogenesis of Hippocampal Neurons’, *PLOS ONE*, 8(10), p. e78892. Available at: https://doi.org/10.1371/journal.pone.0078892.

Slater, S.J. *et al.* (2001) ‘Interaction of protein kinase C isozymes with Rho GTPases’, *Biochemistry*, 40(14), pp. 4437–4445. Available at: https://doi.org/10.1021/bi001654n.

Slusarski, D.C. *et al.* (1997) ‘Modulation of Embryonic Intracellular Ca2+Signaling byWnt-5A’, *Developmental Biology*, 182(1), pp. 114–120. Available at: https://doi.org/10.1006/dbio.1996.8463.

Slusarski, D.C., Corces, V.G. and Moon, R.T. (1997) ‘Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling’, *Nature*, 390(6658), pp. 410–413. Available at: https://doi.org/10.1038/37138.

Smirnov, A., Melino, G. and Candi, E. (2023) ‘Gene expression in organoids: an expanding horizon’, *Biology Direct*, 18, p. 11. Available at: https://doi.org/10.1186/s13062-023-00360-2.

Smith Sehdev, A.E., Sehdev, P.S. and Kurman, R.J. (2003) ‘Noninvasive and Invasive Micropapillary (Low-Grade) Serous Carcinoma of the Ovary: A Clinicopathologic Analysis of 135 Cases’, *The American Journal of Surgical Pathology*, 27(6), pp. 725–736. Available at: https://doi.org/10.1097/00000478-200306000-00003.

Sneath, R.J. and Mangham, D.C. (1998) ‘The normal structure and function of CD44 and its role in neoplasia.’, *Molecular Pathology*, 51(4), pp. 191–200. Available at: https://doi.org/10.1136/mp.51.4.191.

Soares, C.P. *et al.* (2012) ‘2D and 3D-Organized Cardiac Cells Shows Differences in Cellular Morphology, Adhesion Junctions, Presence of Myofibrils and Protein Expression’, *PLOS ONE*, 7(5), p. e38147. Available at: https://doi.org/10.1371/journal.pone.0038147.

Sobrado, P. *et al.* (2005) ‘Basic region of residues 228-231 of protein kinase CK1alpha is involved in its interaction with axin: binding to axin does not affect the kinase activity’, *Journal of Cellular Biochemistry*, 94(2), pp. 217–224. Available at: https://doi.org/10.1002/jcb.20350.

Söderholm, S. and Cantù, C. (2021) ‘The WNT/β-catenin dependent transcription: A tissue-specific business’, *WIREs Mechanisms of Disease*, 13(3), p. e1511. Available at: https://doi.org/10.1002/wsbm.1511.

Stamos, J.L. and Weis, W.I. (2013) ‘The β-Catenin Destruction Complex’, *Cold Spring Harbor Perspectives in Biology*, 5(1), p. a007898. Available at: https://doi.org/10.1101/cshperspect.a007898.

van Steensel, B. and Belmont, A.S. (2017) ‘Lamina-Associated Domains: Links with Chromosome Architecture, Heterochromatin, and Gene Repression’, *Cell*, 169(5), pp. 780–791. Available at: https://doi.org/10.1016/j.cell.2017.04.022.

Stingl, J. *et al.* (2006) ‘Purification and unique properties of mammary epithelial stem cells’, *Nature*, 439(7079), pp. 993–997. Available at: https://doi.org/10.1038/nature04496.

Stone, I. (2021) ‘Ovacome Ovarian Cancer Support Charity - Written Evidence to Parliament’, 1 September. Available at: https://committees.parliament.uk/writtenevidence/38695/pdf/ (Accessed: 2 August 2023).

Sträter, J. *et al.* (1996) ‘Rapid onset of apoptosis in vitro follows disruption of beta 1-integrin/matrix interactions in human colonic crypt cells’, *Gastroenterology*, 110(6), pp. 1776–1784. Available at: https://doi.org/10.1053/gast.1996.v110.pm8964403.

Stronach, E.A. *et al.* (2011) ‘DNA-PK Mediates AKT Activation and Apoptosis Inhibition in Clinically Acquired Platinum Resistance’, *Neoplasia*, 13(11), pp. 1069-IN35. Available at: https://doi.org/10.1593/neo.111032.

Su, Y. *et al.* (2008) ‘APC Is Essential for Targeting Phosphorylated β-Catenin to the SCFβ-TrCP Ubiquitin Ligase’, *Molecular Cell*, 32(5), pp. 652–661. Available at: https://doi.org/10.1016/j.molcel.2008.10.023.

Sugino, N. (2006) ‘Roles of reactive oxygen species in the corpus luteum’, *Animal Science Journal*, 77(6), pp. 556–565. Available at: https://doi.org/10.1111/j.1740-0929.2006.00386.x.

Sugiyama, H. *et al.* (2008) ‘Human adipose tissue-derived mesenchymal stem cells as a novel feeder layer for epithelial cells’, *Journal of Tissue Engineering and Regenerative Medicine*, 2(7), pp. 445–449. Available at: https://doi.org/10.1002/term.111.

Sung, H. *et al.* (2021) ‘Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries’, *CA: A Cancer Journal for Clinicians*, 71(3), pp. 209–249. Available at: https://doi.org/10.3322/caac.21660.

Suprynowicz, F.A. *et al.* (2012) ‘Conditionally reprogrammed cells represent a stem-like state of adult epithelial cells’, *Proceedings of the National Academy of Sciences*, 109(49), pp. 20035–20040. Available at: https://doi.org/10.1073/pnas.1213241109.

Tadokoro, T. *et al.* (2014) ‘IL-6/STAT3 promotes regeneration of airway ciliated cells from basal stem cells’, *Proceedings of the National Academy of Sciences*, 111(35). Available at: https://doi.org/10.1073/pnas.1409781111.

Tafe, L.J. *et al.* (2016) ‘Molecular Genetic Analysis of Ovarian Brenner Tumors and Associated Mucinous Epithelial Neoplasms’, *The American Journal of Pathology*, 186(3), pp. 671–677. Available at: https://doi.org/10.1016/j.ajpath.2015.11.008.

Takada, R. *et al.* (2006) 'Monounsaturated Fatty Acid Modification of Wnt Protein: Its Role in Wnt Secretion', *Developmental Cell*, 11(6), pp. 791–801. Available at: https://doi.org/10.1016/j.devcel.2006.10.003.

Takada, S. *et al.* (2017) 'Differences in the secretion and transport of Wnt proteins', *The Journal of Biochemistry*, 161(1), pp. 1–7. Available at: https://doi.org/10.1093/jb/mvw071.

Takahashi, K. and Yamanaka, S. (2006) 'Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors', *Cell*, 126(4), pp. 663–676. Available at: https://doi.org/10.1016/j.cell.2006.07.024.

Takam Kamga, P. *et al.* (2020) 'The Role of Notch and Wnt Signaling in MSC Communication in Normal and Leukemic Bone Marrow Niche', *Frontiers in Cell and Developmental Biology*, 8, p. 599276. Available at: https://doi.org/10.3389/fcell.2020.599276.

Talora, C. *et al.* (2002) 'Specific down-modulation of Notch1 signaling in cervical cancer cells is required for sustained HPV-E6/E7 expression and late steps of malignant transformation', *Genes & Development*, 16(17), pp. 2252–2263. Available at: https://doi.org/10.1101/gad.988902.

Tanegashima, K., Zhao, H. and Dawid, I.B. (2008) 'WGEF activates Rho in the Wnt-PCP pathway and controls convergent extension in Xenopus gastrulation', *The EMBO journal*, 27(4), pp. 606–617. Available at: https://doi.org/10.1038/emboj.2008.9.

Teh, M.-T. *et al.* (2002) 'FOXM1 is a downstream target of Gli1 in basal cell carcinomas', *Cancer Research*, 62(16), pp. 4773–4780.

The Cancer Genome Atlas Research Network (2011) 'Integrated genomic analyses of ovarian carcinoma', *Nature*, 474(7353), pp. 609–615. Available at: https://doi.org/10.1038/nature10166.

ThermoFisher (2020) 'Wnt Surrogate-Fc Fusion Recombinant Protein'. Available at: https://www.thermofisher.com/proteins/product/Wnt-Surrogate-Fc-Fusion-Recombinant-Protein/PHG0401 (Accessed: 4 September 2023).

Tiwari, R.K. *et al.* (2016) 'Evaluation of Preoperative Serum Levels of CA 125 and Expression of p53 in Ovarian Neoplasms: A Prospective Clinicopathological Study in a Tertiary Care Hospital', *The Journal of Obstetrics and Gynecology of India*, 66(2), pp. 107–114. Available at: https://doi.org/10.1007/s13224-014-0611-7.

Topol, L. *et al.* (2009) 'Sox9 Inhibits Wnt Signaling by Promoting β-Catenin Phosphorylation in the Nucleus', *The Journal of Biological Chemistry*, 284(5), pp. 3323–3333. Available at: https://doi.org/10.1074/jbc.M808048200.

Touboul, C. *et al.* (2014) 'Role of mesenchymal cells in the natural history of ovarian cancer: a review', *Journal of Translational Medicine*, 12(1), p. 271. Available at: https://doi.org/10.1186/s12967-014-0271-5.

Tsai, C.-C. *et al.* (2010) 'Overexpression of hTERT increases stem-like properties and decreases spontaneous differentiation in human mesenchymal stem cell lines', *Journal of Biomedical Science*, 17(1), p. 64. Available at: https://doi.org/10.1186/1423-0127-17-64.

Tsutsumi, N. *et al.* (2023) ‘Structure of the Wnt–Frizzled–LRP6 initiation complex reveals the basis for coreceptor discrimination’, *Proceedings of the National Academy of Sciences*, 120(11), p. e2218238120. Available at: https://doi.org/10.1073/pnas.2218238120.

Tulac, S. *et al.* (2006) ‘Dickkopf-1, an Inhibitor of Wnt Signaling, Is Regulated by Progesterone in Human Endometrial Stromal Cells’, *The Journal of Clinical Endocrinology & Metabolism*, 91(4), pp. 1453–1461. Available at: https://doi.org/10.1210/jc.2005-0769.

Turco, M.Y. *et al.* (2017) ‘Long-term, hormone-responsive organoid cultures of human endometrium in a chemically-defined medium’, *Nature cell biology*, 19(5), pp. 568–577. Available at: https://doi.org/10.1038/ncb3516.

Tüysüz, N. *et al.* (2017) ‘Lipid-mediated Wnt protein stabilization enables serum-free culture of human organ stem cells’, *Nature Communications*, 8(1), p. 14578. Available at: https://doi.org/10.1038/ncomms14578.

Ugarte, F. *et al.* (2015) ‘Progressive Chromatin Condensation and H3K9 Methylation Regulate the Differentiation of Embryonic and Hematopoietic Stem Cells’, *Stem Cell Reports*, 5(5), pp. 728–740. Available at: https://doi.org/10.1016/j.stemcr.2015.09.009.

Ulrich, N.D. *et al.* (2022) ‘Cellular heterogeneity of human fallopian tubes in normal and hydrosalpinx disease states identified using scRNA-seq’, *Developmental Cell*, 57(7), pp. 914-929.e7. Available at: https://doi.org/10.1016/j.devcel.2022.02.017.

Umbhauer, M. *et al.* (2000) ‘The C-terminal cytoplasmic Lys-thr-X-X-X-Trp motif in frizzled receptors mediates Wnt/beta-catenin signalling’, *The EMBO journal*, 19(18), pp. 4944–4954. Available at: https://doi.org/10.1093/emboj/19.18.4944.

Van Gorp, T. *et al.* (2004) ‘Endometriosis and the development of malignant tumours of the pelvis. A review of literature’, *Best Practice & Research Clinical Obstetrics & Gynaecology*, 18(2), pp. 349–371. Available at: https://doi.org/10.1016/j.bpobgyn.2003.03.001.

Vang, R., Shih, I.-M. and Kurman, R.J. (2009) ‘Ovarian Low-grade and High-grade Serous Carcinoma: Pathogenesis, Clinicopathologic and Molecular Biologic Features, and Diagnostic Problems’, *Advances in Anatomic Pathology*, 16(5), pp. 267–282. Available at: https://doi.org/10.1097/PAP.0b013e3181b4fffa.

Varea, O. *et al.* (2009) ‘Estradiol Activates β-Catenin Dependent Transcription in Neurons’, *PLOS ONE*, 4(4), p. e5153. Available at: https://doi.org/10.1371/journal.pone.0005153.

Varga, J. *et al.* (2022) ‘Predictive factors of endometriosis progression into ovarian cancer’, *Journal of Ovarian Research*, 15(1), p. 5. Available at: https://doi.org/10.1186/s13048-021-00940-8.

Vitale, I. *et al.* (2017) ‘DNA Damage in Stem Cells’, *Molecular Cell*, 66(3), pp. 306–319. Available at: https://doi.org/10.1016/j.molcel.2017.04.006.

Wadman, M. (2023) ‘FDA no longer has to require animal testing for new drugs’, *Science (New York, N.Y.)*, 379(6628), pp. 127–128. Available at: https://doi.org/10.1126/science.adg6276.

Wagner, J. and Lehmann, L. (2006) ‘Estrogens modulate the gene expression of Wnt-7a in cultured endometrial adenocarcinoma cells’, *Molecular Nutrition & Food Research*, 50(4–5), pp. 368–372. Available at: https://doi.org/10.1002/mnfr.200500215.

Wang, D. *et al.* (2013) ‘Structural basis for R-spondin recognition by LGR4/5/6 receptors’, *Genes & Development*, 27(12), pp. 1339–1344. Available at: https://doi.org/10.1101/gad.219360.113.

Wang, X. *et al.* (2023) ‘Identification of iron metabolism-related predictive markers of endometriosis and endometriosis-relevant ovarian cancer’, *Medicine*, 102(15), p. e33478. Available at: https://doi.org/10.1097/MD.0000000000033478.

Wang, Y. *et al.* (2009) ‘Progesterone inhibition of Wnt/beta-catenin signaling in normal endometrium and endometrial cancer’, *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, 15(18), pp. 5784–5793. Available at: https://doi.org/10.1158/1078-0432.CCR-09-0814.

Wang, Y. *et al.* (2010) ‘Wnt/B-Catenin and Sex Hormone Signaling In Endometrial Homeostasis and Cancer’, *Oncotarget*, 1(7), pp. 674–684. Available at: https://doi.org/10.18632/oncotarget.201.

Wang, Y. *et al.* (2015) ‘Clonality analysis of combined Brenner and mucinous tumours of the ovary reveals their monoclonal origin: Monoclonal origin of combined Brenner and mucinous tumours of the ovary’, *The Journal of Pathology*, 237(2), pp. 146–151. Available at: https://doi.org/10.1002/path.4572.

Wang, Y. *et al.* (2018) ‘Evaluation of liquid from the Papanicolaou test and other liquid biopsies for the detection of endometrial and ovarian cancers’, *Science Translational Medicine*, 10(433), p. eaap8793. Available at: https://doi.org/10.1126/scitranslmed.aap8793.

Wang, Y. and Nathans, J. (2007) ‘Tissue/planar cell polarity in vertebrates: new insights and new questions’, *Development (Cambridge, England)*, 134(4), pp. 647–658. Available at: https://doi.org/10.1242/dev.02772.

Wang, Yiying *et al.* (2019) ‘Tubal Origin of “Ovarian” Low-Grade Serous Carcinoma: A Gene Expression Profile Study’, *Journal of Oncology*, 2019, pp. 1–9. Available at: https://doi.org/10.1155/2019/8659754.

Warrington, J.M. *et al.* (2022) ‘TP53 knockout in MCF7 breast cancer cells induces sensitivity to fluoropyrimidine drugs’. bioRxiv, p. 2022.02.10.479997. Available at: https://doi.org/10.1101/2022.02.10.479997.

Watanabe, S. *et al.* (2022) ‘Transplantation of intestinal organoids into a mouse model of colitis’, *Nature Protocols*, 17(3), pp. 649–671. Available at: https://doi.org/10.1038/s41596-021-00658-3.

Wauchope, O.R. *et al.* (2018) ‘Oxidative stress increases M1dG, a major peroxidation-derived DNA adduct, in mitochondrial DNA’, *Nucleic Acids Research*, 46(7), pp. 3458–3467. Available at: https://doi.org/10.1093/nar/gky089.

Ween, M.P., Oehler, M.K. and Ricciardelli, C. (2011) ‘Role of Versican, Hyaluronan and CD44 in Ovarian Cancer Metastasis’, *International Journal of Molecular Sciences*, 12(2), pp. 1009–1029. Available at: https://doi.org/10.3390/ijms12021009.

Wesley, C.S. (1999) ‘Notch and Wingless Regulate Expression of Cuticle Patterning Genes’, *Molecular and Cellular Biology*, 19(8), pp. 5743–5758. Available at: https://doi.org/10.1128/MCB.19.8.5743.

Westphalen, C.B. *et al.* (2014) ‘Long-lived intestinal tuft cells serve as colon cancer–initiating cells’, *The Journal of Clinical Investigation*, 124(3), pp. 1283–1295. Available at: https://doi.org/10.1172/JCI73434.

White, E. (2006) ‘Mechanisms of apoptosis regulation by viral oncogenes in infection and tumorigenesis’, *Cell Death & Differentiation*, 13(8), pp. 1371–1377. Available at: https://doi.org/10.1038/sj.cdd.4401941.

Widlund, H. (2012) ‘Notch Signalling’, *Cell Signal*, December. Available at: https://www.cellsignal.com/pathways/notch-signaling-pathway?_requestid=656370&_requestid=918090 (Accessed: 15 August 2023).

Widmer, C.C. *et al.* (2010) ‘Hemoglobin Can Attenuate Hydrogen Peroxide–Induced Oxidative Stress by Acting as an Antioxidative Peroxidase’, *Antioxidants & Redox Signaling*, 12(2), pp. 185–198. Available at: https://doi.org/10.1089/ars.2009.2826.

Wiegand, K.C. *et al.* (2010) ‘*ARID1A* Mutations in Endometriosis-Associated Ovarian Carcinomas’, *New England Journal of Medicine*, 363(16), pp. 1532–1543. Available at: https://doi.org/10.1056/NEJMoa1008433.

Wietek, N. *et al.* (Pre-Publication) ‘Ultra-deep sequencing reveals ubiquitous somatic mutations and the pervasive presence of pathogenic TP53 mutations in the non-cancerous fallopian tube epithelium’.

Wilailak, S. *et al.* (2012) ‘Depot medroxyprogesterone acetate and epithelial ovarian cancer: a multicentre case–control study’, *BJOG: An International Journal of Obstetrics & Gynaecology*, 119(6), pp. 672–677. Available at: https://doi.org/10.1111/j.1471-0528.2012.03298.x.

Willert, K. *et al.* (2003) ‘Wnt proteins are lipid-modified and can act as stem cell growth factors’, *Nature*, 423(6938), pp. 448–452. Available at: https://doi.org/10.1038/nature01611.

Wilson, R.B. *et al.* (2019) ‘Resistance to anoikis in transcoelomic shedding: the role of glycolytic enzymes’, *Pleura and Peritoneum*, 4(1), p. 20190003. Available at: https://doi.org/10.1515/pp-2019-0003.

Witte, C.J. de *et al.* (2020) ‘Patient-Derived Ovarian Cancer Organoids Mimic Clinical Response and Exhibit Heterogeneous Inter- and Intrapatient Drug Responses’, *Cell Reports*, 31(11). Available at: https://doi.org/10.1016/j.celrep.2020.107762.

Wu, C.H. *et al.* (2012) ‘Endocervical-type Mucinous Borderline Tumors are Related to Endometrioid Tumors Based on Mutation and Loss of Expression of ARID1A’:, *International Journal of Gynecological Pathology*, 31(4), pp. 297–303. Available at: https://doi.org/10.1097/PGP.0b013e31823f8482.

Wu, J., McKeague, M. and Sturla, S.J. (2018) ‘Nucleotide-Resolution Genome-Wide Mapping of Oxidative DNA Damage by Click-Code-Seq’, *Journal of the American Chemical Society*, 140(31), pp. 9783–9787. Available at: https://doi.org/10.1021/jacs.8b03715.

Wu, N.-Y. *et al.* (2017) 'Progesterone Prevents High-Grade Serous Ovarian Cancer by Inducing Necroptosis of p53-Defective Fallopian Tube Epithelial Cells', *Cell Reports*, 18(11), pp. 2557–2565. Available at: https://doi.org/10.1016/j.celrep.2017.02.049.

Wu, R. *et al.* (2019) 'Genomic landscape and evolutionary trajectories of ovarian cancer precursor lesions', *The Journal of Pathology*, 248(1), pp. 41–50. Available at: https://doi.org/10.1002/path.5219.

Wu, S. *et al.* (2020) 'Molecular Mechanisms of PALB2 Function and Its Role in Breast Cancer Management', *Frontiers in Oncology*, 10, p. 301. Available at: https://doi.org/10.3389/fonc.2020.00301.

Xiao, Q. *et al.* (2017) 'The many postures of noncanonical Wnt signaling in development and diseases', *Biomedicine & Pharmacotherapy*, 93, pp. 359–369. Available at: https://doi.org/10.1016/j.biopha.2017.06.061.

Xing, Y. *et al.* (2003) 'Crystal structure of a beta-catenin/axin complex suggests a mechanism for the beta-catenin destruction complex', *Genes & Development*, 17(22), pp. 2753–2764. Available at: https://doi.org/10.1101/gad.1142603.

Yamamoto, H. *et al.* (1999) 'Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3beta regulates its stability', *The Journal of Biological Chemistry*, 274(16), pp. 10681–10684. Available at: https://doi.org/10.1074/jbc.274.16.10681.

Yang, L. *et al.* (2021) 'The Role of Oxidative Stress and Natural Antioxidants in Ovarian Aging', *Frontiers in Pharmacology*, 11, p. 617843. Available at: https://doi.org/10.3389/fphar.2020.617843.

Yang, X. *et al.* (Pre-Publication) 'A 3D microtumour system that faithfully represents ovarian cancer minimal residual disease'.

Yanger, K. and Stanger, B.Z. (2011) 'Facultative stem cells in liver and pancreas: fact and fancy', *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, 240(3), pp. 521–529. Available at: https://doi.org/10.1002/dvdy.22561.

Yin, X. *et al.* (2014) 'Niche-independent high-purity cultures of Lgr5+ intestinal stem cells and their progeny', *Nature Methods*, 11(1), pp. 106–112. Available at: https://doi.org/10.1038/nmeth.2737.

Yoshihara, M. *et al.* (2014) 'Genome-wide profiling of 8-oxoguanine reveals its association with spatial positioning in nucleus', *DNA research: an international journal for rapid publication of reports on genes and genomes*, 21(6), pp. 603–612. Available at: https://doi.org/10.1093/dnares/dsu023.

Yu, Z. *et al.* (2021) 'A novel ferroptosis related gene signature is associated with prognosis in patients with ovarian serous cystadenocarcinoma', *Scientific Reports*, 11(1), p. 11486. Available at: https://doi.org/10.1038/s41598-021-90126-5.

Yuan, C. *et al.* (2021) 'Follicular fluid exosomes: Important modulator in proliferation and steroid synthesis of porcine granulosa cells', *The FASEB Journal*, 35(5), p. e21610. Available at: https://doi.org/10.1096/fj.202100030RR.

Yui, S. (2022) *World's first-in-human research using organoid transplantation yields good results*, *News-Medical.net*. Available at: https://www.news-medical.net/news/20220822/Worlds-first-in-human-research-using-organoid-transplantation-yields-good-results.aspx (Accessed: 25 August 2023).

Yun, B.H. *et al.* (2020) 'DNA Adducts: formation, biological effects, and new biospecimens for mass spectrometric measurements in humans', *Mass spectrometry reviews*, 39(1–2), pp. 55–82. Available at: https://doi.org/10.1002/mas.21570.

Yun, J. *et al.* (2015) 'p53 Modulates Notch Signaling in MCF-7 Breast Cancer Cells by Associating with the Notch Transcriptional Complex via MAML1', *Journal of cellular physiology*, 230(12), pp. 3115–3127. Available at: https://doi.org/10.1002/jcp.25052.

Zebisch, M. *et al.* (2013) 'Structural and molecular basis of ZNRF3/RNF43 transmembrane ubiquitin ligase inhibition by the Wnt agonist R-spondin', *Nature Communications*, 4, p. 2787. Available at: https://doi.org/10.1038/ncomms3787.

Zeng, T. *et al.* (2018) 'Nanopore Analysis of the 5-Guanidinohydantoin to Iminoallantoin Isomerization in Duplex DNA', *The Journal of Organic Chemistry*, 83(7), pp. 3973–3978. Available at: https://doi.org/10.1021/acs.joc.8b00317.

Zeng, X. *et al.* (2005) 'A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation', *Nature*, 438(7069), pp. 873–877. Available at: https://doi.org/10.1038/nature04185.

Zeng, X. *et al.* (2008) 'Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions', *Development (Cambridge, England)*, 135(2), pp. 367–375. Available at: https://doi.org/10.1242/dev.013540.

Zhai, B.-T. *et al.* (2022) 'Urokinase-type plasminogen activator receptor (uPAR) as a therapeutic target in cancer', *Journal of Translational Medicine*, 20(1), p. 135. Available at: https://doi.org/10.1186/s12967-022-03329-3.

Zhai, L., Chaturvedi, D. and Cumberledge, S. (2004) 'Drosophila wnt-1 undergoes a hydrophobic modification and is targeted to lipid rafts, a process that requires porcupine', *The Journal of Biological Chemistry*, 279(32), pp. 33220–33227. Available at: https://doi.org/10.1074/jbc.M403407200.

Zhang, H. *et al.* (2014) 'ROR1 expression correlated with poor clinical outcome in human ovarian cancer', *Scientific Reports*, 4(1), p. 5811. Available at: https://doi.org/10.1038/srep05811.

Zhang, Q.-G. *et al.* (2008) 'Role of Dickkopf-1, an antagonist of the Wnt/beta-catenin signaling pathway, in estrogen-induced neuroprotection and attenuation of tau phosphorylation', *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 28(34), pp. 8430–8441. Available at: https://doi.org/10.1523/JNEUROSCI.2752-08.2008.

Zhang, S. *et al.* (2019) 'Both fallopian tube and ovarian surface epithelium are cells-of-origin for high-grade serous ovarian carcinoma', *Nature Communications*, 10(1), p. 5367. Available at: https://doi.org/10.1038/s41467-019-13116-2.

Zhang, W. and Liu, H.T. (2002) ‘MAPK signal pathways in the regulation of cell proliferation in mammalian cells’, *Cell Research*, 12(1), pp. 9–18. Available at: https://doi.org/10.1038/sj.cr.7290105.

Zhou, Q. and Melton, D.A. (2018) ‘Pancreas regeneration’, *Nature*, 557(7705), pp. 351–358. Available at: https://doi.org/10.1038/s41586-018-0088-0.

Zhu, L. *et al.* (2009) ‘Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation’, *Nature*, 457(7229), pp. 603–607. Available at: https://doi.org/10.1038/nature07589.

Zhu, M. *et al.* (2023) ‘Effects of Follicular Fluid on Physiological Characteristics and Differentiation of Fallopian Tube Epithelial Cells Implicating for Ovarian Cancer Pathogenesis’, *International Journal of Molecular Sciences*, 24(12), p. 10154. Available at: https://doi.org/10.3390/ijms241210154.

Zipori, D. (2004) ‘The nature of stem cells: state rather than entity’, *Nature Reviews. Genetics*, 5(11), pp. 873–878. Available at: https://doi.org/10.1038/nrg1475.

Zona, S. *et al.* (2014) ‘FOXM1: An emerging master regulator of DNA damage response and genotoxic agent resistance’, *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, 1839(11), pp. 1316–1322. Available at: https://doi.org/10.1016/j.bbagrm.2014.09.016.