Early serous ovarian carcinogenesis:

Understanding the genetic and lifestyle factors

Thesis Submitted

In Fulfilment of the Requirements for the Degree of

Doctor of Philosophy

By

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B.Eng (Biotechnology); M.Sc. (Molecular Genetics)

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AUSTRALIA

To

Faculty of Health,

School of Biomedical Sciences and Pharmacy

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DECLARATIONS PART A

TESTIMONY OF ORIGINALITY

*I hereby certify that this thesis is my own work and contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due references and acknowledgement has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository**, subject to the provisions of the Copyright Act 1968.*

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I hereby certify that the work embodied in this thesis contains a published paper/s/scholarly work of which I am a joint author. I have included as part of the thesis, written statements for each published work, endorsed by my supervisor, attesting to my contribution to the joint publication/s/scholarly work.

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I hereby certify that this thesis is in the form of series of published papers of which I am a joint author. I have included as part of the thesis written statement from each co-author endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

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I hereby certify that that the work embodied in this thesis has been done in collaboration with other researchers. I have included as part of the thesis a statement clearly outlining the extent of collaboration and under what auspices.

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Prathima B Nagendra dd/mm/yyyy

DECLARATIONS PART B

Acknowledgement of authorship

I hereby certify that the work embodied in this thesis contains submitted/published papers of which I am a joint author. I have included a written declaration below endorsed in writing by my supervisor, attesting to my contribution to the joint publication/s/scholarly work.
Publications included as a part of this thesis are listed below:

I the undersigned corresponding author of following publications:

1. Nagendra PB and Tanwar PS. Role of Fallopian tube in pelvic serous ovarian carcinogenesis. Gynaecology Oncology (Submitted)
2. Nagendra PB, Goad J, Nielsen S, Rassam L, Lombard JM, Nahar P, et al. Ovarian hormones through Wnt signalling regulate the growth of human and mouse ovarian cancer initiating lesions. Oncotarget. 2016;7(40):64836-53.
3. Nagendra PB and Tanwar PS. Evidence of early Fallopian tube precursor escape in omental serous ovarian carcinoma. Cancer research (Submitted)
4. Nagendra PB and Tanwar PS. Molecular characterisation of papillary tubal hyperplasia: the putative precursors of low grade serous carcinoma. Gynaecology Oncology (submitted)

Authorize the inclusion of these works and declare that Research Higher Degree candidate, Prathima B Nagendra contributed to the paper/publication. Outlined below are the items that the candidate has contributed towards the fulfilment of these papers:

- Conducted and designed most of the experiments
- Critical analysis and interpretation of results
- Prepared and organized the figures
- Drafting and conceptualizing the manuscripts
- Contributed in formatting initial and revised versions of manuscripts

Signature:
Pradeep S. Tanwar

Date: 05/09/2019
dd/mm/yyyy

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LIST OF PUBLICATIONS INCLUDED AS A PART OF THIS THESIS

Contained in:

CHAPTER 1

Nagendra PB and Tanwar PS. Role of Fallopian tube in pelvic serous ovarian carcinogenesis. Gynaecology Oncology (Submitted)

CHAPTER 2

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List of abbreviations

A	
ANZGOG	Australia New Zealand Gynaecology Oncology Group
ALDH1	Aldehyde dehydrogenase 1 family, member A1
APE	Atypical proliferative endosalpingiosis
B	
BRCA1	Breast cancer type 1 susceptibility protein 1
BRCA2	Breast cancer type 1 susceptibility protein 2
BSA	Bovine serum albumin
BRaf	serine/threonine-protein kinase B-Raf kinase
C	
CK8	Cytokeratin 8
CNV	Copy number variations
D	
DAB	Diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DEG	Differentially expressed genes
2D	Two dimensional
3D	Three dimensional
E	
EGFR	Epidermal growth factor receptor
EDTA	Ethylenediamine tetraacetic acid
EOC	Epithelial Ovarian Cancer
4E-BP1	Eukaryotic initiation factor 4E-binding protein 1
EZH2	Enhancer of zeste homolog 2
ERBB2	Erythroblastic oncogene B, a gene isolated from avian genome
ER	Estrogen Receptor
F	
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
FTE	Fallopian tube epithelium
FIGO	International Federation of Gynecology and Obstetrics
FTSEC	Fallopian Tube Secretory Epithelial Cells
FTCEC	Fallopian tube Ciliated Epithelial Cells
G	
GRH	Gonadotropin releasing hormone
H	
HGSOC	High grade serous ovarian carcinoma
HGSC	High grade serous carcinoma
I	
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IRS1	Insulin receptor substrate 1

IMP3	U3 small nucleolar ribonucleoprotein

K	
KDa	Kilodalton
KRas	Kirsten RAt Sarcoma virus protein
L	
LH	Luteinizing hormone
LKB1	Liver Kinase B 1
LGSC	Low Grade Serous Carcinoma
LGSOC	Low Grade Serous Ovarian Carcinoma
LEF1	Lymphoid enhancer-binding factor 1
LOH	Loss of heterozygosity
M	
Mg	Milligram
Ml	Millilitre
mM	Millimolar
µg	Microgram
µl	Microlitre
µM	Micromolar
nM	Nanomolar
N	
NIH	National Institute of Health
O	
OvCa	Ovarian cancer
OSE	Ovarian surface epithelium
P	
Pax2	Paired box gene 2
Pax8	Paired box gene 8
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol-4,5-biphosphate-3-kinase
PIP2	Phosphatidylinositol 4, 5-biphosphate
PSC	Pelvic Serous Carcinoma
PTEN	Phosphatase and tensin homolog
PCGA	Pre-Cancerous Genome Atlas
PTH	Papillary tubal hyperplasia
p16	p16INK4a, cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1
PR	Progesterone Receptor
R	
RPMI media	Rosewell Park Memorial Institute media
RTK	Receptor tyrosine kinase
RT	Room Temperature
RCN1	Reticulocalbin 1
RRSO	Risk Reducing Salpingo Oophorectomy
RNASeq	RNA Sequencing

S	
SDS- PAGE	Sodium dodecyl sulfate Polyacrylamide gel electrophoresis
SE	Standard error
SCE	Secretory Cell Expansions
SCOUTS	Secretory Cell Outgrowths
STIC	Serous Tubal Intraepithelial Carcinoma
Stmn1	Stathmin-1/ Metablastin /Oncoprotein 18
SBT	Serous Borderline Tumours

T	
TBS	Tris buffered saline
TCGA	The Cancer Genome Atlas
TP53	Tumour Protein 53
TAH-BSO	Total Abdominal Hysterectomy-Bilateral Salpingo Oophorectomy
W	
WES	Whole exome sequencing

Abstract

For the last century, carcinoma has been a consistent Emperor of all maladies. Carcinoma is not a single disease, but a family of diseases which have one common characteristic. Cancer is the loss of cellular regulation, norm and function in a particular cell, leading to uncontrolled growth and disruption of the normal physiology and biology of the organ where it occurs. In the last century, we have succeeded in recognising each of the carcinomas that humans encounter, and have characterised their anatomical, physiological, histological and in most cases molecular features. This has greatly enhanced our ability to treat carcinomas.

40% of all cancer incidences are today treatable with more than 60% of the patients surviving 5 years post incidence. We have not had this kind of success in cases of heterogenous tumours. These are carcinomas which depend on multiple signalling cascades for their growth advantage, thus circumventing traditional chemotherapy and targeted therapies. The other failure is lack of accurate and scalable detection mechanisms, especially in deep seated organs. The combination of these two factors leads to high mortality rates in such tumours.

One such example is the serous ovarian carcinoma. It has two histological subtypes, High grade serous ovarian carcinoma (HGSOC) and Low grade serous ovarian carcinoma (LGSOC). HGSOC is a high heterogeneity carcinoma due to which relapse rates are 60% within 5 years. LGSOC although mostly detected in early stages is inherently chemoresistant leading to poor prognosis. The best way to circumvent such carcinomas is early detection. This needs evolutionary understanding of the disease. Our work focuses on understanding the earliest stages of SOC, drivers of these changes and deciphering the mechanisms of prevention. To achieve this, we probe dysplasia and preneoplasia in the Fallopian tubes, which are one of the sites of origin of SOC. The Fallopian tube secretory epithelial cells (FTSEC) are the closest molecular phenotype to SOC.

Secretory cell outgrowths (SCOUTS), p53 signatures and papillary tubal hyperplasia are the dysplastic lesions acting as precursors of SOC in the Fallopian tube epithelium (FTE). FTE is primarily constituted of secretory and ciliated cells and the ratio between these cells is key to maintaining homeostasis. The disruption of this balance is the first step to SOC. The secretory cell type dominates and outgrows ciliated cells leading to dysplasia. All three aforementioned preneoplasia are made up of secretory cells.

We undertook molecular characterisation of these three lesions. The morphological and immunohistochemical aspects of the lesions are well charted. Through histopathological analysis, use

of microdissection, next generation animal models and next generation sequencing we sought to characterise molecular nature of these lesions.

We found that Wnt signalling pathway is the driver of SCOUTS. We have established an accurate mouse model by constitutive activation of β-Catenin specifically in the FTSECs. We have further used this model to probe the role of ovarian hormonal milieu in progression of HGSOC. We found progesterone mitigates progression of SCOUT lesions and oestrogen enhances this progression. This is the first mouse model to accurately mimic early serous ovarian carcinogenesis.

By molecular probing of morphologically normal Fallopian tubes in a case of p53 null-omental high grade serous carcinoma three years post risk reducing salpingo oophorectomy (RRSO), we found tumour associated aberrations, specifically identical *TP53* mutations in FTE before RRSO and the omental tumour. This establishes the clonal identity and proves the precursor escape model in pelvic serous ovarian carcinomas.

Papillary tubal hyperplasia are known to be putative precursors for LGSOC, atypical endosalpingiosis (AES) in the peritoneum and also intermediate Serous Borderline tumours (SBT). They are the only known precursors in the FT. LGSC is also the closest to the FTSECs. As no molecular attributes were known, through Whole exome sequencing (WES) and RNA sequencing (RNASeq) we have characterised molecular aberrations and also have established them as precursors for the accompanying AES. Study of these three lesions suggests, salpingectomy can be a good preventive measure in at least patients with high risk of ovarian cancer incidence. Progesterone can also be used as an effective preventive measure.

Thesis overview

Life is a series of processes appropriately timed and patterned with repeatability and reliability of very high order. Each step of this process is conserved both in terms of its timing and its players. Cancer is the highest form of distortion of this process. This is because cancer involves deviations from the norm at the very fundamental cellular and molecular steps of life. Cancer has been long looked at as an outcome rather than a process, mostly because of its late detection.

The evolution of a cancer cell from a single cell or a clone of a few hundred cells to a mass of few cms takes a few decades in most cases. This long process involves cellular decisions in fate and cellular signalling which are diversions from the norm. The last phase of at least three of these diversions would lead to a neoplastic state. Over the last two decades, most of the focus has been on the last two of these phases due to their enormous implications in morbidity and mortality, but the first of these steps has often been discounted as it lacks cellular, morphological and pathological footprints. But molecular footprints must surely be available and merit investigations.

In between the normal epithelia and the carcinomas, there are a host of dysplastic lesions, which are basically intermediate steps or neoplasia in transition. To study the first molecular footprints, the best object of study would be the dysplasia. When normal cells display a change in the cell fate or change of gene expression, cellular proliferation, cell size, nuclear size, or other changes in nuclear attributes, but are not so distant from the nomal that they are regarded as neoplastic and/or lose their function, they are termed dysplasia or preneoplasia or precursors. These are footprints of active or stopped carcinomas. The changes in the dysplasia are generally hallmarks of the first of the changes the cells will take towards neoplasia. This thesis is an attempt in that direction.

The cells of origin, sites of origin and the sites of metastasis are generally evolutionarily conserved for specific types of cancers. Understanding cells and sites of origin has significant impact on the detection, treatment and prognosis of patients. Thus, to understand the cells of origin and their specific steps of transformations, spatio-temporal and longitudinal studies are warranted. This thesis is an attempt in understanding the first steps in the evolution of a cancer cell from a normal cell.

The model of this study is serous ovarian cancer. It acts as a good candidate due to its very late detection (generally post metastasis) and the inherent heterogeneity of the disease.

Aims:

The goal of this PhD work is to understand the first step of neoplastic transformation in the serous ovarian carcinoma of the Fallopian tube. We achieve this by investigating three major precursor lesions of serous ovarian carcinoma in the Fallopian tube. They are SCOUTS or secretory cell outgrowths, p53 signatures and papillary tubal hyperplasia. This work has direct and indirect

implications in diagnostic, prognostic and therapeutic benefits to patients particularly in early detection and prevention of the progression of this disease.

Specifying the aims further,

1. To understand the mechanism of transition of normal Fallopian tube epithelium to secretory cell expansions and secretory cell outgrowths.
2. To determine the role of ovarian hormones, oestrogen and progesterone in the progression of secretory cell expansions/outgrowths into serous ovarian carcinoma.
3. To decipher the role of the Fallopian tube as the site of origin and to verify the precursor escape model.
4. To investigate the role of papillary tubal hyperplasia as a putative precursor to low grade serous ovarian carcinoma.

Organisation of the thesis:

This thesis starts with a thesis overview, which introduces and summarizes the chapters presented further. There are four chapters. Each chapter is preluded by a preface which briefly outlines the rationale and results contained in the studies. Each chapter is an individual study published or submitted as original research article.

Chapter1 titled "Role of Fallopian tube in pelvic serous ovarian carcinogenesis" summarizes the existing literature. The nature of pelvic serous ovarian carcinoma, its histological subtypes: high grade- and low grade-serous ovarian carcinoma, their sites of origin, cells of origin, putative precursors, the clinical and molecular evidences for the same, the embryological basis of the disease and pathobiology of early serous ovarian carcinogenesis is outlined.

Chapter 2 is titled "Ovarian hormones through Wnt signalling regulate the growth of human and mouse ovarian cancer initiating lesions". This chapter addresses aims, 1 and 2. This chapter investigates the role of Wnt signalling in maintenance of Fallopian tube epithelial cell fate. It establishes role of the canonical Wnt pathway in the transition from the normal intermittent secretory and ciliated cell fate to the secretory fate which marks the first step in serous ovarian carcinogenesis. It also deciphers the role of ovarian hormones, oestrogen and progesterone in the progression of these precursor lesions to carcinoma.

Chapter 3 is titled "Evidence of early Fallopian tube precursor escape in omental serous ovarian carcinoma". So far evidences have established that approximately 50-70% of all high grade serous ovarian carcinomas originate from the Fallopian tube. This chapter addresses aim 3. In this chapter we provide evidence for very early precursor escape from the Fallopian tube, making a case for earlier

salpingectomy for genetically susceptible women (with *BRCA*1/2 germline mutations) aged less than 40, wanting to conserve their fertility. Evidences presented in this chapter also question the notion that presence of precursor lesions is the deterministic evidence in establishing the site of origin in carcinomas.

Chapter 4 is titled "Molecular characterisation of papillary tubal hyperplasia: the putative precursors of low grade serous carcinoma". This chapter aims in identifying molecular markers of low grade serous carcinoma in putative precursors, the papillary tubal hyperplasia. It also establishes the Fallopian tube origin in a case of peritoneal low grade serous carcinoma.

Chapter 1: Role of Fallopian tube in pelvic serous ovarian carcinogenesis

Chapter 1

Preface

Pelvic serous ovarian carcinoma includes two histological subtypes of epithelial ovarian carcinoma, the high grade serous ovarian carcinoma and the low grade serous ovarian carcinoma. Together they constitute 90% of all ovarian cancer incidences and ~80% of ovarian cancer induced mortality. The mortality to incidence ratio is 2:3, the highest for any cancer in women. This is due to two main reasons: a) diagnosed at later FIGO Stages-III/IV when the disease has metastasized to the ovaries, Fallopian tubes and the peritoneum and/or b) development of chemoresistance upon reoccurrence of tumours in case of high grade serous carcinoma; and inherent chemoresistance in case of low grade serous carcinoma.

Due to late diagnosis, the sites, cells and mechanisms of origin are not well deciphered. This chapter serves with all the background information on the nature of serous ovarian carcinoma, the two histological subtypes, their pathobiology, sites of origin, molecular and clinical evidences for the same, the developmental aspects of different putative cells of origin, and specifically the different documented dysplasia and atypia in the Fallopian tube and their role in serous ovarian carcinogenesis as precancerous lesions.

UON Graduate research

Co-authorship declaration

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

Statement of Author's contributions

This statement summarizes the intellectual inputs by all the authors stated in the review article titled "Role of Fallopian tube in pelvic serous ovarian carcinogenesis". This review article is submitted in the journal, Gynaecology Oncology.

Authors	Statement of contribution
Prathima B Nagendra (First author)	Wrote the initial draft and made figures for the manuscript Edited and revised the manuscript
Pradeep S Tanwar (Corresponding author)	Edited the manuscript and provided intellectual inputs

Prathima B Nagendra

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Role of Fallopian tube in pelvic serous ovarian carcinogenesis

Abstract

This review deals with pelvic serous ovarian carcinomas. These constitute 90% of all ovarian cancer incidences and 80% of deaths due to ovarian cancer. There are two histological subtypes, high grade- and low grade-serous ovarian carcinomas. High grade serous ovarian carcinoma has high genetic instability and multiple genomic and proteomic changes. It is a highly heterogenous disease, with very high intra- and inter-patient heterogeneity. Thus, there are no targeted therapies available yet. Low grade serous carcinomas are highly chemoresistant. Thus, both these types need better diagnostic and treatment strategies. This review summarises the clinical nature of pelvic serous ovarian carcinoma, the aetiology, the sites of origin, cells of origin, putative precursors, the epidemiological, clinical and molecular evidences, the embryological basis of the disease and pathobiology of early serous ovarian carcinogenesis.

1. Introduction

Ovarian cancer is the most lethal of all gynaecological diseases and has the highest death to incidence ratio with two thirds of women succumbing to the disease within 5 years of incidence. Since the first case of ovarian cancer surgery in 1809 [1-3], the rate of incidence and deaths due to the disease has only increased with the growing population control and industrialization [1].

Ovarian cancer has about 4000 perturbations-gene copy number variations, single nucleotide polymorphisms, protein upregulation, downregulation, transcriptional repression or overactivation, epigenetic modifications in proteins or methylation silencing of the chromatin regions, all of which contribute to the phenomenal patient to patient variation and lack of unifying disease specific markers irrespective of patient clinical history [4-6]. There is an urgent need to find early stage specific markers which have a diagnostic, prognostic and therapeutic value.

Ovarian cancer is not a single disease, but a multitude of diseases. They are distinguishable not only based on morphological and histopathological differences but are also distinct in their molecular mechanisms of pathogenesis [7-9]. Research on molecular insights into the disease over the years has established cellular and molecular diversity in the presentation of the disease [8, 10, 11]. It is an asymptomatic disease in earlier stages and is often diagnosed at very late phases, when the disease has metastasized to other organs. Due to this very reason, the earlier phases of the disease are uncharacterised making the probability of early diagnosis meagre.

The current clinical practice of treatment is debulking surgery, platinum based chemotherapy and radiotherapy. The five year survival rate has been stagnant since the advent of platinum based chemotherapies [12, 13].

Current methods of Ovarian cancer screening such as transvaginal ultrasound examination and serum CA-125 concentration lack in sensitivity and specificity and have not had positive effects in reducing ovarian cancer mortality. The prognostic indicators in use are the tumour load at the time of surgery and post-operative residual disease indicated by CA-125 radioimmunographs [14, 15]. Prophylactic bilateral salpingo-oophorectomy is the current practice of prevention. Salpingectomy is the removal of Fallopian tubes and oophorectomy is the removal of ovaries [16, 17]. There are many documented side effects of surgical menopause such as vasomotor symptoms, vaginal dryness, osteoporosis and higher risk for heart disease. Thus, tubal ligation and salpingectomy can act as a good alternative known to have considerable benefits for delay in the onset of the disease [18, 19].

Ovarian cancers are further classified into epithelial, germ cell and sex cord stromal cancers. Tumours of epithelial origin constitute 85-90% of all incidences of the disease [4]. The epithelial cancers are

classified into four molecular subtypes: serous, endometrioid, clear cell and mucinous. The different molecular subtypes of ovarian cancer are histologically similar to the epithelia of different Mullerian organs. Serous histotype is similar to the Fallopian tube, clear cell to the vaginal rests, mucinous to the intestinal mucosa and the endometrioid to the endometrium. All of these epithelia are derived from a single coelomic mesothelium [20, 21].

The staging and grading methods for ovarian cancer are the recommendations from International Federation of Gynecology and Obstetrics (FIGO), the World Health Organization (WHO), and the Gynecologic Oncology Group (GOG). The FIGO system uses three grades on the basis of: the proportion of glandular or papillary structures relative to areas of solid tumor growth. Grades 1: <5%, 2: 5–50% and 3: >50%. The WHO system uses architectural and cytological criteria for grading, whereas the GOG system varies for each histological type [22, 23]. Most patients undergoing chemotherapy have short remission periods and the disease relapses with higher malignant potency. Many resistance mechanisms have been reported [24, 25]. Since chemotherapy is inadequate in handling the disease there is a need for targeted therapies and early detection.

2. The aetiology of ovarian cancer

Two major factors impact the likelihood of ovarian cancer: lifestyle and genetics.

2.1 Lifestyle: Many epidemiological and molecular studies have uncovered the role of life style factors in predisposition to developing ovarian cancer. Incessant ovulation without pauses created by lack of pregnancies is postulated to increase the risk of ovarian cancer. Studies in hens which replicate the model aptly strengthen this hypothesis [26]. Factors related to ageing: cyclic or chronic inflammation, stromal changes and follicle depletion are also shown to increase risk of ovarian cancer [27, 28]. Retrograde menstrual flow and endometriosis are specifically attributed to endometrioid subtype [29, 30]. As the epithelia of the female reproductive tract undergo drastic changes in response to ovarian hormones, their influence has been extensively studied. Androgens, estrogen, progesterone, gonadotropins are all known to play a role in ovarian cancer pathogenesis [31-34].

Studies in large cohorts of women showed that breast-feeding, pregnancy/parity and combined oral contraceptive use significantly decreases, whereas, infertility and nulliparity increases their risk of developing ovarian cancer. First full term pregnancy confers a 40% reduction in ovarian cancer risk, and every subsequent pregnancy after the first birth provides further risk reduction of 14% [32]. Combined oral contraceptive use is the most effective preventive measure with 50% reduction in ovarian cancer risk after 3-5 years of use. The protective effects of oral contraceptive use and pregnancy against ovarian cancer are postulated to occur due to high levels of progesterone hormone

as combined oral contraceptives with high progestin are known to reduce ovarian cancer risk, whereas, low progestin and high oestrogen formulations have an opposite effect [34, 35]. Most ovarian cancer incidences are known to skew at a median age of 58, which is post menopause and this can be attributed to ageing, follicle depletion, excessive exposure to estrogen and androgens during menopause. Other known causes of ovarian cancer are intake of carcinogens such as talcum powder through retrograde menstruation or other means [36].

2.2 Genetics: The idea of ovarian cancer inheritance was postulated and established by Paul Broca, an oncologist and anatomist who first documented HBOC (Heriditary breast and ovarian cancer) [37]. 99% of all incidences are in the serous ovarian cancer histotype. HBOC results from mutations of *BRCA1* and *BRCA2*. It is the most common cause of hereditary breast and ovarian cancers and occurs in all ethnic and racial populations. The overall prevalence of *BRCA1/2* pathogenic variants in the general population is estimated at 1 in 4000, excluding the Ashkenazi Jews [38]. Women are screened for *BRCA1/2* mutations if one or more 1st, 2nd, or 3rd degree relative in either lineage is a mutation carrier. Women with *BRCA1* germline mutations have a higher risk of ovarian cancer compared to women with germline *BRCA2* mutations (34% and 19% respectively) [39, 40]. Also, women with mutations in the ovarian cancer cluster region (OCCR) of exon 11 of *BRCA2* have a higher ratio of ovarian to breast cancer. Thus, the site of variants plays a major role as well. Other genes that are known to exhibit germline mutations in case of ovarian cancer incidences (although at a low frequency of 6%) are *BARD1, BRIP1, CHEK2, MRE11A, MSH6, NBN, PALB2, RAD50, RAD51C*, or *TP53*, all belonging to the homologous recombination pathway, leading to very high genomic instability, a hallmark of serous ovarian cancer [41, 42].

As this thesis addresses the serous histo-subtypes, this review elaborates on only these two subtypes.

3. Histological subtypes of epithelial serous ovarian cancer.

Two major histological subtypes of ovarian cancer that fall under "serous" histotype are high grade serous ovarian cancer and low grade serous ovarian cancer. The name serous is derived from serum secreting. These are tumours predominantly of the secretory cells and in many cases causes cystic tumours filled with serum, known as ascites.

3.1 High grade serous ovarian cancer

Clinical aspects: 90% of the cases are diagnosed in stages III/IV. As this disease presents in three organs at the same time when diagnosed in Stages III/ IV, depending on which of these organs present detectable precursor lesions or higher tumour mass, they are classified into primary organs in that category empirically rather than evidentially (not necessarily with certainty of the cancer progression

routes). High grade serous ovarian carcinoma (HGSOC), serous tubal intraepithelial cancer (STIC) and serous primary peritoneal carcinoma (PPC) occur primarily in ovary, Fallopian tube and peritoneum respectively [43]. They are collectively termed peritoneal or pelvic serous ovarian carcinoma (PSOC). This review will use the term PSOC henceforth to address the disease, unless a specific site needs to be invoked. These constitute approximately 80% of all ovarian cancer incidences. The diagnostic criteria of each of these carcinomas vary. They all share considerable molecular and morphological characteristics and respond to debulking and chemotherapy in the same way.

Histological aspects: Silverberg grading system has taken prominence in the last decade. In this method, three criteria are scored and the overall score determines the grade of the tumour. The three categories are architecture (glandular, papillary, or solid), degree of nuclear atypia, and mitotic index.

The PSC cells are genetically, immunotypically and morphologically the most similar to FTSEC (Fallopian tube secretory epithelial cells). Efforts in the last decade have generated mouse models which reflect formation of STIC and HGSOC from the FTE. Piek et al was the first study to identify the Fallopian tube as a source for HGSOC [44]. They reported dysplastic epithelial lesions in FTs similar to the serous histotype.

Genetic aspects: There are very few genetic mutations identified as drivers in HGSOC and the disease is characterized by high copy number variations, through acquired DNA damage, chromosomal gains and losses and higher heterochromatin. Most of these changes can be attributed to perturbations in the Homologous Recombination (HR) pathway, showing very high rates of DNA damage [8, 42, 45]. The high levels of DNA damage are known to lead to chromatin instability.

Numerous large-scale tumour sequencing studies have consistently reasserted the driver mutations. *TP53, BRCA1, BRCA2, RB1, NF1, FAT3, CSMD3, GABRA6* and *CDK12* are the most frequently mutated genes [42, 45]. *TP-53, BRCA1* and *BRCA2* are the three genes with highest mutational spectra and frequencies. TP53 mutations are a hallmark of PSC, with greater than 95% patients displaying mutational status in tumours and are mostly used to bifurcate them from low grade or borderline tumours. Interestingly they are constituents of the HR pathway. Members of the HR pathway are one of the most frequently mutated genes in HGSOC. A study screened for mutations in 21 tumour suppressor genes, including point mutations and large genomic deletions and insertions. Of 360 subjects, 24% carried germ-line loss-of-function mutations: 18% in *BRCA1* or *BRCA2* and 6% in *BARD1, BRIP1, CHEK2, MRE11A, MSH6, NBN, PALB2, RAD50, RAD51C*, or *TP53* [46].

3.2 Low grade serous ovarian carcinoma

Clinical aspects: This is a rare disease as it constitutes around 5-7% of all ovarian cancer incidences. It is detected mostly in early stages, I and II, which constitute 80-90% of all disease incidences. Although the disease is detected early-on in its progression and is therefore restricted to a single organ, the disease is extremely chemoresistant. This makes the treatment of the disease hard. Current standard of care is removal of ovaries and/or Fallopian tubes. However, in around 40% of the cases the disease is present in the peritoneum, which makes the surgeries complicated. There is an immediate need for alternatives and Ras pathway inhibitors are in clinical trials.

Histological aspects: The low grade serous carcinoma is characterised by either focal or extensive invasive components characterized by micropapillae constituted of small round nests of cells that may or may not infiltrate the stroma in a haphazard pattern. Unlike the HGSOC, the micropapillae lack fibrovascular cores and are frequently surrounded by a clear space or cleft. The tumours can be elongated and generally display branched appearance [47]. The most common feature is the presence of psammonna bodies and is found in most patients. Coming to the morphological appearance of the cells, they display low mitotic entities (less than 12%- criteria in distinction between PSC and LGSC), have higher nuclear to cytoplasmic ratio compared to normal epithelia, but the nuclei are uniform and round and the chromatin is even, unlike the hyper chromatin in high grade serous carcinoma (<2:1 for low grade and >3:1 for high grade and also the number of total cells with hyperchromasia are low). The nucleoli are small and do not vary greatly [48].

Genetic aspects: LGSCs typically constitute mutations of the *KRAS, BRAF,* or *ERBB2* genes, with 80% of the tumors having a mutation in either one of these genes. Mutations in each of these genes are mutually exclusive. *KRAS*, *BRAF*, and *ERBB2* are upstream regulators of mitogen-activated protein kinase (MAPK). Mutations found in LGSC tumours lead to constitutive activation of the MAPK pathway resulting in uncontrolled proliferation [49, 50]. *TP53* mutations are very rare in LGSC (between 3 to 8% in the reports) and generally such tumours in cases unintervened will transform to the more malignant PSOCs [48].

4. The sites of incidence and sites of origin

Identifying the right site of origin is very important clinically for both prophylactic large scale efforts in decreasing the disease incidence and prognostic decisions both in previvor women who are genetically susceptible to HGSOCs and general population who have incurred the disease. Serous ovarian carcinoma is known to occur in the ovaries, Fallopian tubes, omentum, peritoneum and is different from the serous carcinomas which originate in the uterus. 80% of ovarian cancer incidences

are in Stage III and greater, which means that the tumours are either metastatic or found in multiple organs during detection. This either indicates a) independent routes of neoplastic transformation in multiple organs as each of these routes can be mutually exclusive [51, 52] or b) the tumour originates in one site and metastasizes to other sites [52, 53] .

In case of independent neoplastic transformation in multiple sites, there are certain clinical and molecular evidences in support. A study has found the tumours found in the ovary and the Fallopian tubes belong to different molecular classes, thus must have had modes of independent neoplastic transformation [54]. This was based on extensive bioinformatics analysis of gene and protein expression analysis. In this study tumours were classified into two types, type I and type II. Type I were tumours most likely originating from Fallopian tubes as they lacked epithelial to mesenchymal transition markers, were associated with p53 signatures in continuity (in some cases), showed DNA damage and genetic instability and showed histological markers specific to the Fallopian tube. The type II tumours in contrast showed EMT markers, immune invasion markers which are hallmarks of the post ovulatory wound healing. Some patients showed tumours with both the features and in multiple sites. This proves the hypothesis of independent routes of neoplastic transformation.

The second scenario is the origin of tumour in one site and metastasis to other sites. The three main sites of origin are the Fallopian tubes, the ovaries and the peritoneum/omentum. There are no cases of non-metastatic in-situ serous ovarian carcinomas reported outside the peritoneal cavity.

4.1 Evidences in support of the ovarian surface epithelium as the site of origin

The first line of evidence is the presence of dysplasia or halted cancers in contralateral ovaries of patients with unilateral incidence of SOC. The prophylactically removed ovarian tissues also present themselves with dysplastic lesions in the ovary. Specifically, Auersperg *et al* have demonstrated a transition from the mesenchymal type ovarian surface epithelium to the Mullerian epithelium (similar to Fallopian tube epithelium) in [51]. These lesions display an array of cytological and histological atypia common with the carcinomas [55-57]. The monthly ovulation process leads to rupture of the ovarian surface epithelium and release of the oocyte. The repair terminates in the epithelial cells dismantled to the stroma during the rupture process, undergoing epithelial to mesenchymal transition. Sometimes, this repair is not very efficient leading to epithelial invaginations and cortical cysts, which are plausible precursors of SOC. Ageing, follicle depletion and changes in stroma due to highly reactive oxygen species enriched follicular fluid which promotes inflammatory environment could also lead to changes in the ovarian surface epithelium, leading to hyperplasia, deep cleft invaginations, cortical inclusion cysts and papillary hyperplastic growths on the ovarian surface epithelium [28, 31]. This is specifically likely in case of *BRCA1/2* mutation carriers who have

significantly compromised DNA damage repair response. This theory is known as the incessant ovulation hypothesis [26, 30, 58]. There have been multiple models in hen and through cell culture models, which demonstrate this phenomenon, invivo [34, 51, 59-61]. The second line of evidences are epidemiological in nature. The million women study on the use of combined contraceptives (with estrogen) demonstrates a reduced risk of occurrence of ovarian cancer through halting ovulations in women who took contraceptives for long term basis [32]. Ovarectomies in high risk patients are also known to decrease the incidence of PSOC.

The third evidence is the regulation of the ovarian surface epithelial stem cells [59, 62, 63]. Their ability of metaplastic transformation to the Mullerian epithelium has been suggested by several groups [64-68]. This has an embryonic origin as its basis. The phenomenon of metaplasia is not unlikely as it has been proven through models in multiple organs including the cervix, colon and kidneys within the peritoneal cavity itself.

4.2 Evidences in support of Fallopian tube epithelium as the site of origin

The first line of evidence is the presence of intraepithelial carcinomas in the Fallopian tubes (STIC). STICs are the most commonly occurring lesions together with serous ovarian carcinomas in the ovaries and the peritoneum (in a range of 30-70% of the cases in multiple reports). The incidence of STIC is the highest in the genetically susceptible women reported between 60-85% of all incidences. Incidental STIC cases are often low volume tumours and have no ovarian counterparts. Unilateral incidences of HGSOC also present with STIC cases in ipsilateral and contralateral tubes [8, 69-72]. The transformation of the normal Fallopian tube epithelium to high grade serous ovarian carcinomas is well established through multiple mouse models which are generated by mutations in Fallopian tube secretory epithelial cells(FTSEC) in *TP53, RB1, PI3KCA, BRCA1, CCNE1, NF1,* etc, thus establishing the transformation potential of Fallopian tube epithelium [6, 44, 73, 74]. Next range of evidence are an array of dysplasia found in genetically susceptible women with *BRCA1/2* mutations undergoing risk reducing salpingo oophorectomy (removal of ovaries and Fallopian tubes) or post-menopausal aged women. These include secretory cell expansions, secretory cell outgrowths, p53 signatures and STICs [75, 76]. These dysplasia share substantial number of immunohistochemical changes in common with high grade serous ovarian carcinomas. Recent studies have demonstrated a continuum of these dysplasia which transform into the neoplasia [6, 53, 73]. Example of this is illustrated in **Figure1**. Such dysplasia have also been reported in contralateral Fallopian tubes in case of unilateral HGSOC. The Fallopian tube secretory epithelial cells themselves are the closest epithelia to the serous ovarian carcinomas (both high grade and low grade). They are similar genetically, in gene expression, in

protein expression, morphologically and immunohistochemically. Some studies have also established clonal link between p53 signatures and ovarian tumours as they shared the same *TP53* mutations [8, 10, 71, 73]. However, in many cases such continuum is not demonstrated. There are no dysplasia found in the Fallopian tubes in such cases. To this the counter justifications have been that the dysplasia have been engulfed by the tumours of the Fallopian tubes. There have also been cases where women have undergone RRSO, despite which they have encountered incidences of High grade serous ovarian carcinomas in the peritoneum [77]. Such cases need further immunohistochemical and genetic screening to establish biological possibilities.

In terms of epidemiology, many studies have identified the benefits of tubal ligation and salpingectomy on ovarian cancer incidence. These studies have also recommended earlier salpingectomy for women who want to preserve their ovaries and fertility [19, 78].

The current recommendations of FIGO suggests that if there is presence of invasive tumours or intra epithelial carcinomas in the Fallopian tubes, the Fallopian tubes are to be designated as the site of origin.

4.3 Evidences for peritoneum or omentum as site of origin

As mentioned earlier, there have been reports of patients undergoing bilateral salpingo oophorectomy who have had incidents of high grade serous ovarian cancers 2-7 years post surgery [77]. In many of these cases the surgery was either conducted much before dysplasia development or the precursors had already escaped the site of origin leaving no traces to implant in peritoneum. Thus, it is hard to find a link between either Fallopian tubes or ovaries and the peritoneal tumours in such cases. The site of origin is labelled peritoneum in such cases, known as primary peritoneal serous carcinomas.

There are four possibilities here. A) Early precursor escape either from the Fallopian tube epithelium or the ovarian surface epithelium. B) Endosalpingiosis- either as cellular droppings from fimbriae due to tear during ovulation and/or dissemination of Fallopian tube epithelia due to exposure to high estrogen or highly reactive oxygen species rich ovulation fluid. Many studies have indirectly established the clonal link. C) Endosalpingiosis due to metaplasia of the peritoneum into the Mullerian epithelium as both have common embryonic origin [61]. This is at this stage a biological plausibility and lacks in vivo evidence. D) Ovary-Fallopian tube and peritoneum-Fallopian tube transition zones have higher propensity to neoplastic transformation as they are poorly differentiated. Particularly their stem cell niche are highly susceptible to transformation. Some transition zones have been

reported in [5, 7, 51] and also in other cancers. Again, this scenario is likely, but has not been proven as proving metaplastic transformation is hard with invivo or cell culture models.

5. Embryonic origin

To better understand why this disease has multiple sites and cells of origin, we need to understand the genesis of these organs. The intraembryonic coelum develops as a single void from a lateral plate mesoderm at week 3. This cavity further develops into pericardium, pleural cavity and peritoneum at week 5 of gestation. The entire coelum forming the gonadal ridge thickens and forms the coelomic epithelium. At around 10 weeks into development the ovarian surface epithelium is formed out of the gonadal ridge in the coelomic epithelium [51, 79, 80]. This thickened epithelium forms papillary structures, some of the cells migrate to the centre of the ovary and form the follicular lining. The point of significance here is that early reports suggest this intermediary migratory papillary state is initiated by intragonadal steroid hormones, which is very similar to the serous ovarian carcinomas. Atleast 35%-50% of the cases are papillary in nature. The coelomic epithelium right beside the gonadal ridge at week 5 also gives rise to the peritoneum, Fallopian tube epithelium, uterine epithelium and cervical epithelium. The differences between each of these epithelia at week 5 is a singular cell fate decision which is executed by specific molecular cues (are not discussed in this review). Which of these cues are activated later in the adult stage leading to neoplastic transformation needs detailed investigations. One elegant study which clearly proves this theory of embryonic origins of carcinomas is the Cheng W *et al* study on Hox family of genes ([81]). This study explains the conundrum of ovarian tumours mimicking the morphological features of the Mullerian duct derived epithelial features. The serous subtype is similar to the Fallopian tube epithelium, the endometrioid subtype to the endometrium and mucinous type resembles the endocervix. This study proves that ectopic expression of *Hoxa9* in mouse ovarian surface epithelial cells gave rise to papillary tumors resembling serous type, *Hoxa10* induced the endometrioid type and *Hoxa11* induces the mucinous subtype. These are the very genes which induce differentiation in the embryonic state as well. Also, these specific genes have been found to be over expressed in the aforementioned histological subtypes of carcinomas. Thus, genes which control molecular patterning of embryonic cell fate can also dictate specific neoplastic transformation routes. This explains the propensity of specific sites showing high incidences of specific tumours. In this way the embryonic origin hypothesis has merit. **Figure2** summarises these implications in the context of serous ovarian carcinoma.

6. The dysplasia

Tumour cells have multiple differences compared to normal counterparts. They accumulate these changes over a course of decades. In most carcinomas the timing, sequence and sites of these cellular

changes are conserved. Not all of these series of changes culminate in carcinomas. Some women despite genetic susceptibility never develop ovarian carcinomas. Such prophylactically collected samples show an array of cellular, histological and morphological changes known as dysplasia. All the dysplasia with potential of transformation or the ones that contribute to serous ovarian carcinomas have been listed below. The site of interest of this thesis is Fallopian tubes, thus, the dysplasia in the FTs are detailed elaborately.

6.1 Ovarian dysplasia

Studies so far have identified ovarian dyplasia in either prophylactically removed ovaries in genetically susceptible women such as *BRCA1/2* mutation carriers, or normal women who underwent ovarectomies due to other medical conditions, or unilateral unaffected ovaries in women who had single ovary affected by ovarian cystadenomas or higher forms of malignancies [56, 57]. The ovarian dysplasia so far documented can be summarised as follows: epithelial multilayering (ovarian surface epithelium is a monolayer of squamous or cuboidal cells), surface papillomatosis-outgrowth of the OSE resembling papillae, deep epithelial cortical invaginations, tufting, inclusion cysts, psammoma and stromal hyperplasia [17, 51, 57, 82]. Of the listed dysplasia; cortical inclusion cysts, epithelial invaginations, epithelial hyperplasia and epithelial papillary growths are the most reported and are ordered based on descending frequency of occurrence. Examples of these dysplasia are summarised in **Figure3**. These dysplasia are typically characterised by the following cellular atypia: epithelial pseudostratification, epithelial proliferation, irregular nuclear chromatin pattern, irregular nuclear contour, cellular pleiomorphism, increase in nuclear size, inclusion cysts, deep epithelial invaginations, psammoma, stromal hyperplasia. Cortical inclusion cysts express differential features. They can be broadly classified into 3 types: 1. Calretinin positive lining marking the ovarian surface epithelium; 2. Monolayer of Pax8 positive cells similar to Fallopian tube epithelium with intermittent ciliated cells and 3. Pseudostratified Fallopian tube epithelial lining with only Pax8 positive cells. The mesothelium like ovarian surface epithelium transitioning into Mullerian type Fallopian tube epithelium in a single cyst lining has also been reported [51, 64]. All these cysts may or may not contain cystic fluid. Calretinin marks OSE and Pax8 marks FTE, thus immunophenotypically ovarian dysplasia are either Calretinin positive or Pax8 positive. Ovarian dysplasia are also shown to be positive for KI-67, Hox family of proteins, STMN1 or STATHMIN1 and TP53, LEF1 and phosphoS6 [81, 83-85].

6.2 Fallopian tube epithelial dysplasia

Tubal dysplasia reported so far are: p53 signatures, SCOUTS (Secretory cell outgrowths), IMP3 signatures, TILs or TINs (Tubal Intraepithelial Lesions or Tubal Intraepithelial Neoplasms), STICs (Serous

Tubal Intraepithelial Carcinomas), papillary tubal hyperplasia and the extra tubal depositions in the peritoneum are termed endosalpingiosis [70, 77, 84-86].

The histopathological characteristics of the tubal dysplasia are epithelial pseudostratification, nuclear atypia, nucleomegaly, tufting, loss of nuclear polarity, increase in nuclear density and loss of ciliation [65, 74, 87]. Examples of these lesions are summarised in **Figure 4.**

6.2.1 Fallopian tube epithelium

Before getting into the characteristics of the preneoplastic lesions in the FT, it is important to understand the nature of the normal Fallopian tube. The Fallopian tube is made up of the fimbriae, ampulla, isthmus and the uterotubal junction [80]. The fimbriae are the finger like projections responsive to the ovarian cues through hormones and approaches the ovary at the site of ovulation only during ovulation and collects the ovum. The ampulla is the most likely site of fertilization, has higher epithelial folding and higher epithelia to muscle tissue ratio. Isthmus is the region closest to the uterus, is smaller in diameter compared to the rest of the tube and has much lesser epithelial foldings. The Fallopian tube epithelium comprises of secretory cells and ciliated cells intermittently placed. The ratio of secretory to ciliated cells is the highest in the uterotubal junction and is the least in the fimbriae[80, 86]. Other than the morphological characteristics, the epithelial cells can be distinguished based on immunohistochemical markers. The Fallopian tube epithelium comprises of secretory cells and ciliated cells which are intermittently placed. The ratio of secretory to ciliated cells is the highest in the uterotubal junction and is the least in the fimbriae. The secretory cells exclusively express BCL2, PAX2, PAX8, HMFG2 and lack expression of P73, FOXJ1, and acetylated α-Tubulin [87, 88]. Whereas the ciliated cells express P73, FOXJ1, acetylated α-Tubulin and LHS28 and do not express BCL2, PAX2, PAX8, HMFG2. PAX2, a transcription factor expression is observed during the differentiation of Mullerian duct from the coelomic epithelium and remains so in all adult stages in secretory cells. PAX8 is another member of the Pax family which is ubiquitously used marker for the secretory cells across many organs. P73, a tumour suppressor induces cell cycle arrest. HMFG2 or MUC-1 is a cell cycle regulation protein involved in the epithelial-mesenchymal transition. BCL2 is a anti apoptotic protein which maintains the mitochondrial membrane integrity. Forkhead Box J1 (FOXJ1) is a forkhead family transcription factor. It regulates the transcription of genes that control the production of motile cilia.

6.2.2 Secretory cell expansions

The number of tubal secretory cells increases with age, specifically in the fimbriae. Two studies have specifically shown an increase of secretory cells in genetically susceptible candidates and also in contralateral and ipsilateral tubes of patients with PSCs and have shown that the SCEs are sensitive

markers for early serous carcinogenesis in patients with coexisting PSC [75, 89]. SCE is prevalent throughout the FTE and not specifically in the serous carcinoma prone fimbriae. This would mean that the earliest preneoplastic changes occur throughout the FTE of all regions but the required microenvironment and the niche is probably present in the fimbriae [90]. This study suggested that SCE is a far better predictor of occurrence of PSOC than other proven preneoplastic lesions such as p53 signatures[90]. Secretory cell expansions can be identified by PAX2 and PAX8 expression and are also positive for LEF1.

6.2.3 Secretory Cell Outgrowths (SCOUTS)

Secretory cell outgrowths are commonly known with the acronym, SCOUTS. They are defined as a series of 30 consecutive secretory cells. Similar to the SCEs, these lesions are also associated with genetic predisposition and increasing age [76]. There is no evidence to suggest that these are the lesions which progress to become STIC due to two observations. One, SCOUTS have been shown to be present throughout the FTE across all parts and with no part exhibiting differential frequency compared to others [75, 91]. Unlike the p53 signatures that share common mutations with STIC or HGSOC, SCOUTs are not proven to share common mutations, as yet. Secondly in patients with STIC or HGSOC with substantial amount of normal FTE present for examination, there is no decline in number of SCOUTS due to presence of STIC or HGSOC. Due to lack of evidence of direct progression from SCOUTS to either STIC or HGSOC, SCOUTS are considered surrogate precursors of PSC [83, 92, 93]. But they do share substantial histological markers in common with PSC [83, 84, 91, 92], which implies need to study SCOUTS as they may provide important clues in early serous ovarian carcinogenesis. The SCOUTs are positive for PAX8, STATHMIN1, BCL2 [94], EZH2, LEF1 and β-CATENIN [95] and are negative for PAX2 [92], ALDH1 [96], TP53, P73, KI-67 and γ-H2AX [56, 97]. Some of these SCOUTs are also positive for proliferation markers such as Ki67 (at a frequency of 1-4%). β-CATENIN is a dual function protein, regulating the coordination of cell-cell adhesion and gene transcription and is a regulator of canonical Wnt pathway. LEF1 is a transcription factor similar to high mobility group protein-1 and is involved in the Wnt signaling pathway. RCN1- Reticulocalbin 1 is a calcium-binding protein present in the Endoplasmic reticulum. In cancer cells, the protein is known to show membrane expression. EZH2 is a member of the Polycomb-group (PcG) family involved in the embryonic ectoderm development and acts as a transcriptional repressor. Although many histological markers are identified, the driver genetic event was unknown. It was deciphered in our study elaborated in ***chapter2***.

6.2.4 p53 signatures

p53 signatures are defined as a row of >12 consecutive morphologically normal Fallopian tube epithelial cells which overexpress TP53 [97, 98]. This implies that there is substantial amounts of DNA

damage accumulated which needs to be tackled by the overexpression of TP53. p53 signatures have been shown to be the first of lesions in the progression of serous ovarian carcinogenesis. They are known to share identical mutations in the *TP53* gene as in the HGSC tumours. This acts as conclusive proof that they are precursors of PSCs [53, 73, 98, 99]. As the lesion is mostly absent in presence of STIC or HGSC of the FT, the hypothesis is that the tumours grow from the p53 signatures and these precursors get engulfed into the tumours. In case of unilateral tumours, these lesions have been found in the contralateral FTs [53, 99]. These lesions are also commonly seen in women with genetic predisposition to OvCa and also in women who have got their FTs removed due to other pathologies [97, 98]. The p53 signatures are positive for TP53, BCL2, PAX8, STMN1 [76, 84, 97, 100] as is the case with SCOUTS, have low expression of KI-67 (<10% cells are positive). There are two types of *TP53* mutations, one type which leads to overexpression of TP53 which can be detected in p53 signatures and the second type which truncate the protein leading to loss of protein expression. Such truncation mutations leading to loss of TP53 expression cannot be detected in morphologically normal samples. This issue has been addressed in ***chapter3***.

6.2.5 IMP3 signatures

IMP3 signatures are defined as >10 secretory cells in a row positive for the protein IMP3. IMP3, also known as IGF2BP3 is a member of insulin-like growth factor II mRNA binding proteins. It is a fetal protein specifically known to be overexpressed in carcinomas of the FRT, including endometrium, cervix and vagina. According to [103], IMP3 expression was specifically present in certain SCEs and SCOUTS but not p53 signatures. Just as is the case of the SCEs and SCOUTs, IMP3 signatures increase with age and genetic susceptibility. All IMP3 signatures either fall under SCEs or SCOUTs. But not all SCEs and SCOUTs express IMP3 [103].

6.2.6 Papillary tubal hyperplasia

As the name suggests, papillary tubal hyperplasia are characterized by papillary growths in the Fallopian tube epithelia with psammonna bodies as a salient feature. They generally feature epithelial pseudostratification and epithelial tufting and have 4-10% mitotic index. They constitute micropapillary architecture and can be present as widespread or focal lesions [70, 103]. They often form papillae rings which separate from the tubal epithelium and float in the tubal fluid. For this reason, they are considered plausible precursors for borderline serous tumours and low grade serous ovarian carcinomas. There are no well charecterised immunohistochemical markers. The molecular analysis of these lesions has been accomplished in ***chapter4***.

6.2.7 Serous Tubal Intraepithelial Carcinomas or STICs

STICs are known to be confirmed precursors of HGSOC. As the name suggests they are intraepithelial carcinomas and are neoplastic in nature [76]. They are characterised by higher mitotic indices (KI67, phosphoH3 or PCNA), *TP53* mutations (marked by overexpression or complete loss of TP53) and also share multitude of immunohistochemical markers with HGSOC. STICs also display loss of cellular polarity, sometimes with multiple stratifications, higher epithelial hyperchromasia and telomere shortening [10, 56, 63, 69]. Upon following the SEM-FEE protocol, STICs have been found to co-occur with HGSC between 50-70% of the times. One report found two patients in which mutational evolution analysis found that STICs were indeed metastasis of the HGSCs in the ovary or peritoneum [71]. Between 7-22% women who have undergone prophylactic removal of FTs have displayed incidental STIC which were in the range of 1-20mm in size and were either uni- or multi-focal and either uni-or bi-lateral [10, 69, 73]. *TP53* mutations either lead to overexpression or truncation of the protein. The latter type of mutations with truncated protein cannot be detected using Immunohistochemical techniques and are therefore known as null phenotype (indicated by complete loss of immunohistochemical expression of TP53) [101, 102].

High grade serous carcinoma express most of the immunohistochemical markers specifically expressed in the aforementioned preneoplastic lesions which is substantial evidence of their precursor attributes. These include the presence of TP53, BCL2, PAX8, γ-H2AX, STMN1, EZH2, HMGA2, Ki67, CCNE1 and exhibit absence of PAX2 and ALDH1A1 [72, 76, 83, 92, 103, 104]. Of all the dysplasia mentioned here, STICs and p53 signatures are the only lesions which are confirmed to be the precursors of HGSOC as in some cases reported they share the same *TP53* mutations as the HGSC confirming their precursor nature in the cancer continuum. Although these lesions are proven precursors of HGSOC, they are frank carcinomas and not precancerous. SCE, SCOUTS and IMP3 signatures are putative precursor lesions as they are present in the HGSC patients [10, 55, 56, 76] and may very well contribute to the serous carcinogenesis in the FT as a field effect and also share several molecular markers, but do not have conclusive proof as to how they progress to become HGSC. The diversity of these dysplasia could be one of the reasons behind clonal diversity and heterogeneity of PSOC.

6.2.8 Endosalpingiosis

Endosalpingiosis is the growth of Fallopian tube epithelial tissue outside the Fallopian tube. They are mostly found in the peritoneum or the omentum [105, 106]. These are very hard to screen as even in patients who undergo RRSO, screening the entire peritoneum and omentum is surgically implausible. Similar to its more known counterpart, endometriosis, endosalpingiosis occurs due to retrograde

menstruation, or due to accidental discharge of fimbrial cells during ovulation. These can also occur due to metaplastic differentiation of the peritoneal mesothelium, as both share same embryonic origins (the coelomic epithelium). Both these theories are difficult to prove as it is hard to generate animal models for such pre-metastatic spread of tissue [61, 105].

7. Conclusions

HGSOC is detected very late leading to poor prognosis in patients. The cells and sites of origin for the disease have been well characterised. The morphology, immunohistotype and the genetic makeup of the advanced disease is also well characterised. Although the dysplasia are well established, the molecular mechanisms behind their manifestation are still unknown. The disease displays heterogeneity at three levels: heterogeneity at different organs it affects, intra tumour heterogeneity (cell to cell differences) and tumour progression (molecular differences in grades and stages), making it a difficult candidate for targeted therapies. Thus, better prognosis can be achieved only by early diagnosis of the disease. To achieve this, deciphering the chronological aspects of carcinogenic events, especially early drivers of serous ovarian cancer is necessary to identify plausible molecular targets for treating the disease. Study of precancerous lesions would decipher the first stages of the disease and lead to specific biomarkers which will lead to better detection and treatment strategies.

8. Figure legends

Figure1: The normal to neoplastic transformation in high grade serous ovarian carcinoma in the Fallopian tube. A represents cross section of the Fallopian tube ampulla, showing a transition from the normal epithelium with lack of TP53 expression, as shown in **B**, to STIC (blue arrow shows the transition point, and black arrow represents the adjacent normal epithelia). **C** represents the enlarged images of the stromal invasions shown by red arrows, transitioning from STIC to high grade serous carcinoma.

Figure2: Embryonic origins of high grade serous ovarian carcinoma. A represents the Waddington plot modified to represent the embryonic origins of the Mullerian epithelia. At week 5 of embryonic development, the coelum differentiates to the the peritoneum, ovarian surface epithelium covering the genital ridge and undergoes mesothelium to epithelial transition and forms the Mullerian epithelia including the Fallopian tube epithelium. The tufting in the Waddington plot represents clear molecular cues leading to accurately timed transitioning. The point of differentiation here is the coelomic epithelium. **B** represents the scenario of carcinogenesis. The lack of tufting as in the case of **A**

represents loss of accurate molecular cues. The peritoneum, ovarian surface epithelium and the Fallopian tube epithelium acquire molecular changes leading to either trans differentiation to Fallopian tube epithelium, or dedifferentiation to a version closer to embryonic states leading to carcinogenesis.

Figure3: The ovarian dysplasia. **A** represents the schematic of prominently occurring ovarian dysplasia of prognostic implications. Each dysplasia is also marked by specific markers as indicated. **B** shows the normal ovarian surface epithelium marked by Calretinin, examples of dysplasia with specific markers are as follows: hyperplasia positive for Pax8 (**C**), Calretinin positive invagination (**D**), Calretinin positive papillary growth (**E**), Pax8 positive inclusion cysts (**F, G**) and Calretinin positive inclusion cysts (**H, I**).

Figure4: The Fallopian tube dysplasia. A represents a schematic of all the Fallopian tube dysplasia. Each are distinguishable by specific molecular markers stated. **B** is a cross section of morphologically normal Fallopian tube epithelium marked by Pax8. The staining is intermittent as Pax8 marks secretory cells and is absent in ciliated cells. **C** shows secretory cell outgrowths (SCOUTS) marked by nuclear β-Catenin staining in contrast to membrane staining in adjacent normal epithelia (marked by black arrows). **D** shows a p53 signature positive for p53 staining. **E** shows a serous tubal intraepithelial carcinoma marked by Pax8 and **F** is a HandE staining of papillary tubal hyperplasia as molecular markers are unknown. Some psammonna bodies which are hallmarks are marked by red arrows.

9. References

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Figure 1

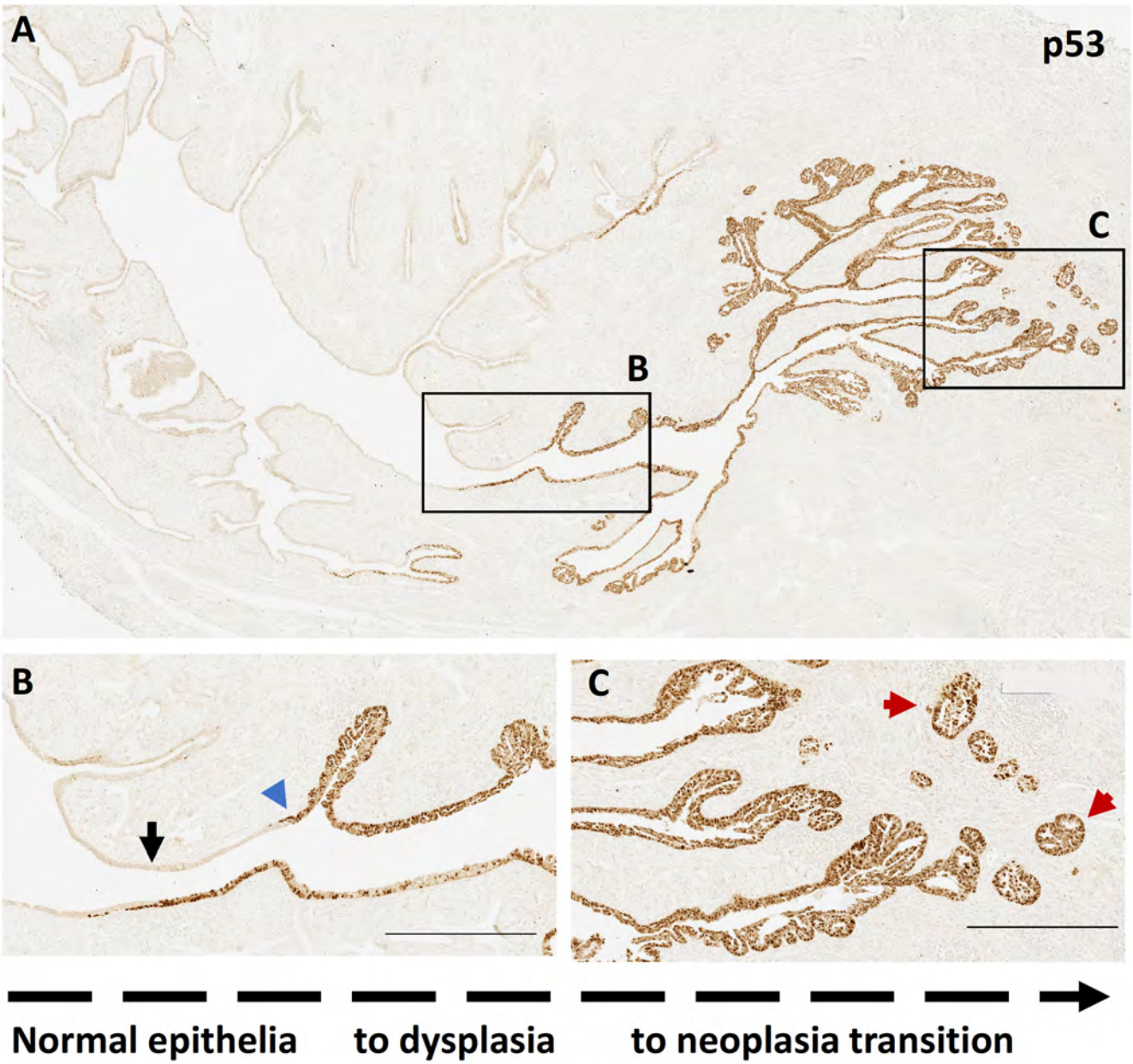

A
p53
C
B
B
C
Normal epithelia
to dysplasia
to neoplasia transition

Figure 2

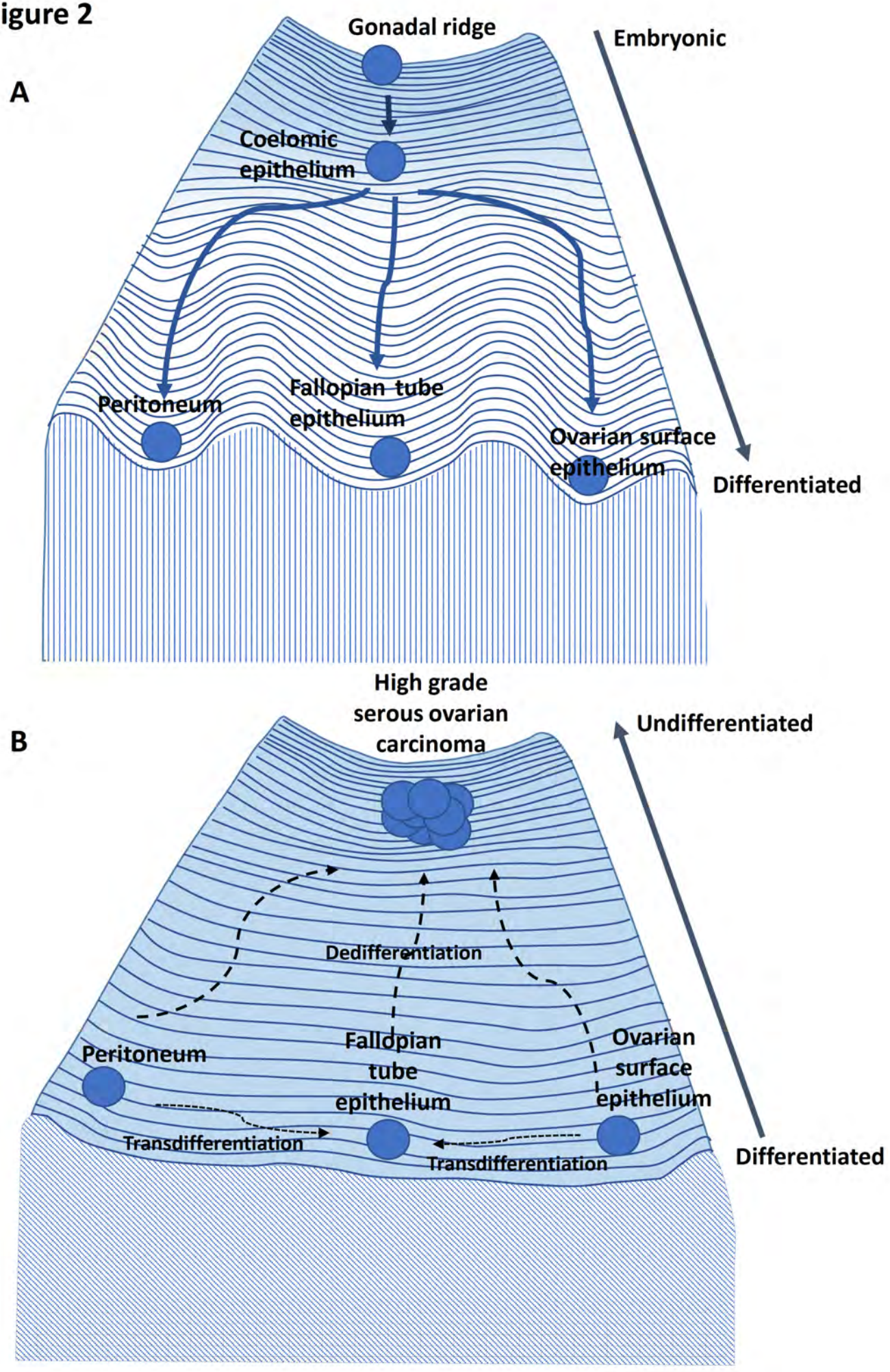
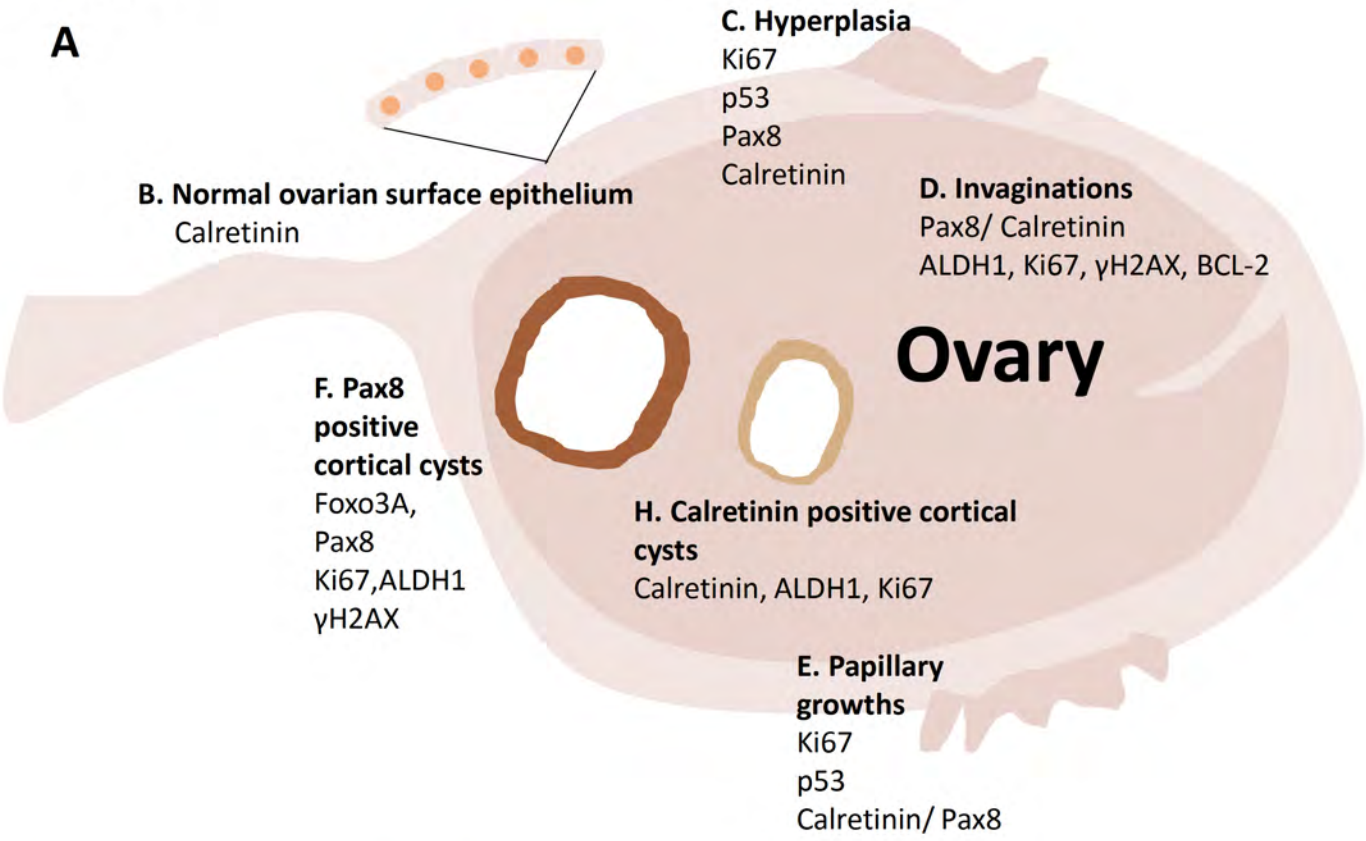

A
Gonadal ridge
Embryonic
Coelomic epithelium
Peritoneum
Fallopian tube epithelium
Ovarian surface epithelium
Differentiated
B
High grade serous ovarian carcinoma
Undifferentiated
Dedifferentiation
Peritoneum
Fallopian tube epithelium
Ovarian surface epithelium
Transdifferentiation
Transdifferentiation
Differentiated

Figure 3


A
C. Hyperplasia
Ki67
p53
Pax8
Calretinin
B. Normal ovarian surface epithelium
Calretinin
D. Invaginations
Pax8/ Calretinin
ALDH1, Ki67, γH2AX, BCL-2
Ovary
F. Pax8 positive cortical cysts
Foxo3A,
Pax8
Ki67,ALDH1
γH2AX
H. Calretinin positive cortical cysts
Calretinin, ALDH1, Ki67
E. Papillary growths
Ki67
p53
Calretinin/ Pax8

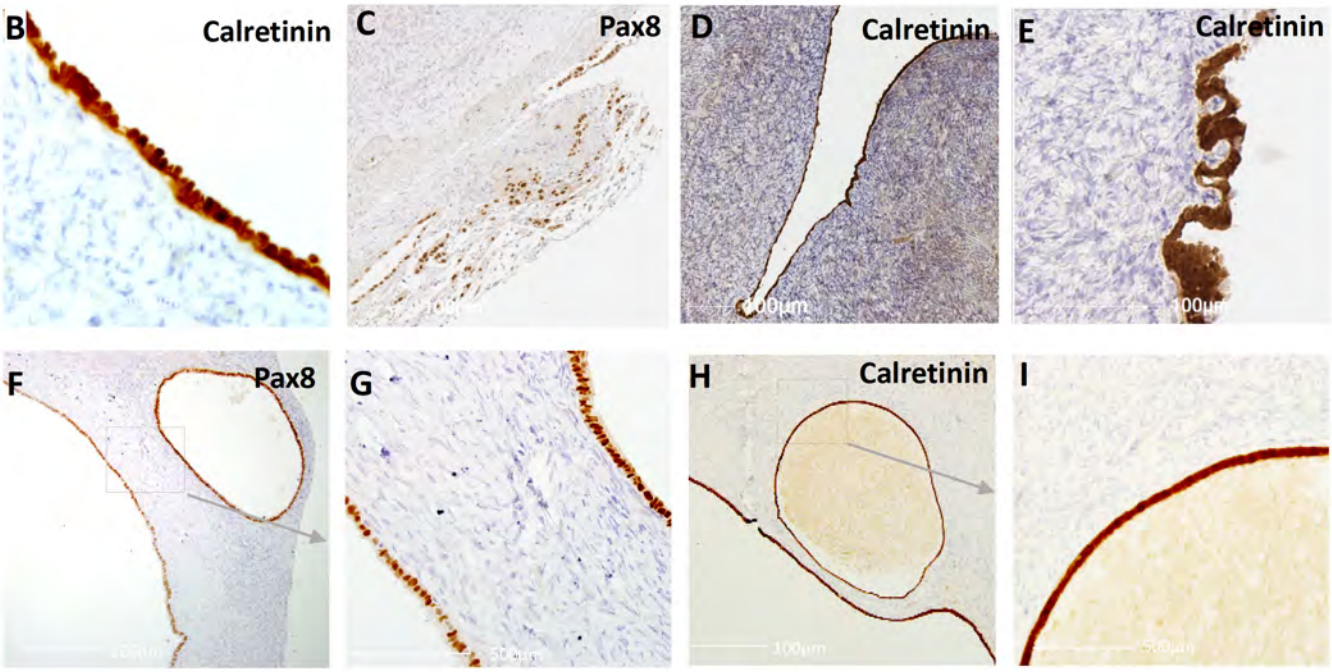
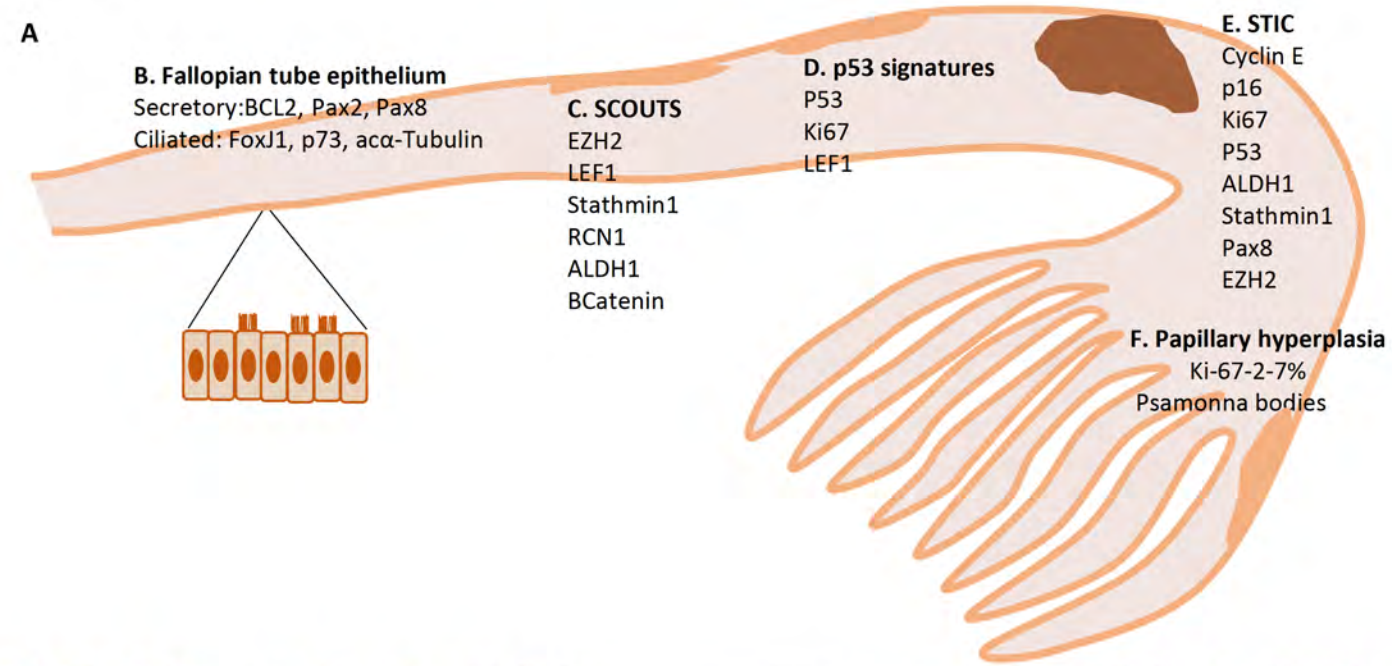
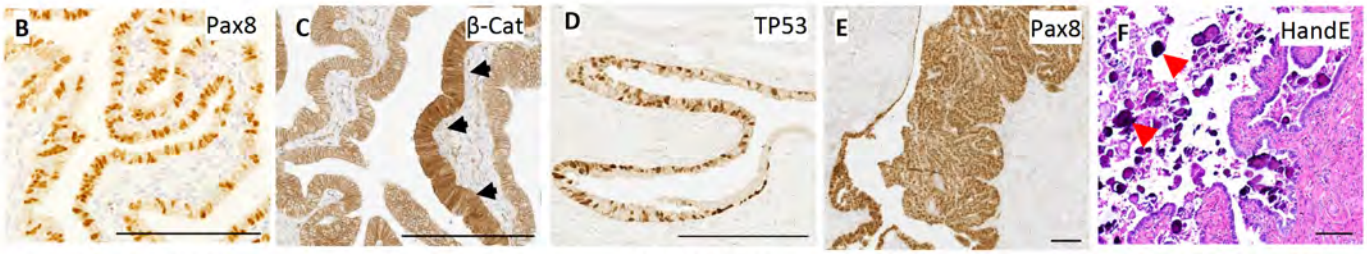

B
Calretinin
C
Pax8
D
Calretinin
E
Calretinin
F
Pax8
G
H
Calretinin
I

Figure 4





Normal FT epithelium | SCOUTS | p53 signatures | STIC | Papillary hyperplasia

Chapter 2: Ovarian hormones through Wnt signalling regulate the growth of human and mouse ovarian cancer initiating lesions

Chapter 2

Preface

As an epithelial cancer, HGSOC is the closest to the Fallopian tube secretory cell epithelium genetically, transcriptionally, immunohistochemically and phenotypically. SOC is essentially a shift from the normal fallopian tube epithelium which is structured as a balance between secretory and ciliated cell fate to the expansion or outgrowth of the secretory cell fate. One of the first such changes leads to secretory cell expansions and secretory cell outgrowths. What are the signalling aberrations that lead to this change? These forms of dysplasia are found throughout the Fallopian tubes of women who are predisposed to developing OvCa. Not all of these women progress to develop OvCa. What are the factors that determine the further progression of these lesions? What is the role of ovarian hormones in mitigating the microenvironment in favour of tumour progression?

LGR5, a member of the Wnt family is known to be one of the important signalling cues, specifically expressed in the stem cells of the Fallopian tube epithelium, thus is an important determinant in Fallopian tube epithelial cell fate determination. As it is a member of the canonical Wnt signalling, we wanted to determine the role of Wnt/βcatenin signalling in early stages of human SOC. We developed a mouse model with constitutive activation of βcatenin specifically in Fallopian tube epithelial secretory cells. Using human SOC cells and our mouse model, we have also defined the role of ovarian hormones, oestrogen and progesterone in the development of secretory cell expansions/ secretory cell outgrowths.

Statement of Author's contributions

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

This statement summarizes the intellectual inputs by all the authors stated in the article titled "Ovarian hormones through Wnt signalling regulate the growth of human and mouse ovarian cancer initiating lesions". This article is published in the journal 'Oncotarget'.

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Prathima B Nagendra (First author)	Performed experiments, Wrote the initial draft and made figures Edited and revised the manuscript
Pradeep S Tanwar (Corresponding author)	Edited the manuscript and provided intellectual inputs
Jyoti Goad	Performed some in-vivo experiments,
Sarah Nielsen	Provided with ovarian cancer tissue sample sections
Loui Rassam	Analysed mouse and ovarian cancer tissue samples and provided inputs to the manuscript
Janine M.Lombard	Provided with inputs on the manuscript
Pravin Nahar	Provided ovarian cancer tissue samples and inputs to the manuscript

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Ovarian hormones through Wnt signalling regulate the growth of human and mouse ovarian cancer initiating lesions

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ABSTRACT

Ovarian cancer (OC) is the most deadly gynaecological disease largely because the majority of patients are asymptomatic and diagnosed at later stages when cancer has spread to other vital organs. Therefore, the initial stages of this disease are poorly characterised. Women with *BRCA1/2* mutations have a genetic predisposition for developing OC, but not all of these women develop the disease. Epidemiological findings show that lifestyle factors such as contraceptive use and pregnancy, a progesterone dominant state, decrease the risk of getting OC. How ovarian hormones modify the risk of OC is currently unclear. Our study identifies activated Wnt signalling to be a marker for precursor lesions of OC and successfully develops a mouse model that mimics the earliest events in pathogenesis of OC by constitutively activating βcatenin. Using this model and human OC cells, we show that oestrogen promotes and progesterone suppresses the growth of OC cells.

INTRODUCTION

Ovarian cancer is the most deadly gynaecological cancer and the fourth leading cause of cancer death in women [1]. Every year, approximately 238,700 new cases are diagnosed and 151,900 deaths occur worldwide due to ovarian cancer [2]. Chemotherapy combined with debulking surgery is a standard treatment for ovarian cancer [3]. Ovarian cancer is one of the most chemosensitive solid malignancies and the initial response rate to standard treatment exceeds 80% [3]. However, most of these women will develop recurrent disease and eventually die because their cancer becomes resistant to chemotherapy, or has inherent chemo-resistance [3]. The 5-year survival rate of ovarian cancer patients has not significantly improved and is around 40% over the last 20 years [4], highlighting the need to understand the signalling pathways involved in the pathogenesis of this disease. This will allow us to identify novel therapeutic targets and thus develop novel means of treating this disease.

There are four major subtypes of ovarian cancer, namely, Clear cell, Endometrioid, Mucinous, and Serous [4]. The serous subtype is the most prevalent form of ovarian cancer and is responsible for 70-80% of ovarian cancer deaths [5]. Both ovary and fallopian tube are considered as the site of origin of ovarian cancer [6-9]. However, the majority of serous ovarian carcinomas are suspected to originate from the distal fallopian tube and then spread to the rest of peritoneal organs including the ovary [5]. Fallopian tube epithelium mainly consists of two cells types, secretory and ciliated cells. Secretory cells are believed to be the progenitors of serous ovarian cancer (SOC) [5, 9]. Extensive histopatholgical examination of fallopian tubes collected from patients that are predisposed to developing ovarian cancer revealed

secretory cell expansions/outgrowths (SCE/SCOUTs) and/or serous tubal intraepithelial neoplasia/carcinoma (STINs/STICs) [10]. SCE/SCOUTs/STINs/STICs share many histological and molecular features with SOC. Engraftment of transformed human secretory cells into the peritoneum of immunocompromised mice leads to the development of tumours that are grossly, histologically, immunophenotypically, and genetically similar to SOC [11]. Additionally, secretory cell-specific genetic alterations in the *Brca1/2*, *Tp53*, and *Pten* genes using a Pax8-driven promoter causes development of tumors that are similar to human SOC [9]. Collectively, these findings suggest that deregulated signaling in the fallopian tube secretory cells induces SOC.

Various epidemiological and molecular studies have associated genetic and life style factors with the predisposition to developing ovarian cancer [5]. Germline mutations in breast and ovarian cancer susceptibility genes, *BRCA1* and *BRCA2*, significantly increase lifetime risk of developing ovarian cancer compared to the general population [5]. Patients with hereditary mutations in *BRCA1/2* genes have very high risk of developing SOC and are recommended to undergo risk-reducing salpingo-oophorectomy (RRSO) by age 40 [12]. Studies in large cohorts of women showed that breast-feeding, pregnancy/parity and combined oral contraceptive use significantly decreases, whereas, infertility and nulliparity increases their risk of developing ovarian cancer [13]. Combined oral contraceptive use is the most effective preventive measure against ovarian cancer and approximately 50% reduction in ovarian cancer risk occurs after 3-5 years of use [13, 14]. First full term pregnancy confers a 40% reduction in ovarian cancer risk, and every subsequent pregnancy after the first birth provides further risk reduction of 14% [15]. The protective effects of oral contraceptive use and pregnancy against ovarian cancer are postulated to occur due to high levels of progesterone hormone as combined oral contraceptive formulations with high progestin, synthetic progesterone agonists, are known to reduce ovarian cancer risk, whereas, low progestin and high oestrogen formulations have opposite effect [13]. How progesterone provides protection against developing ovarian cancer is currently unclear. In this study, we investigated the role of Wnt/βcatenin signalling in pathogenesis of SOC and showed the presence of active Wnt/βcatenin signalling in SCOUTs/STICs of human fallopian tubes. We have developed a mouse model by altering Wnt/βcatenin signalling that mimics the early stages of human SOC. Using human SOC cells and our mouse model, we have defined the role of oestrogen and progesterone in ovarian cancer development. Collectively, our data provides evidence that progesterone suppresses the growth of ovarian cancer initiating lesions and thereby provides protection against developing ovarian cancer.

RESULTS

Sustained activation of Wnt/βcatenin signalling in the precursor lesions of human SOC

Putative SOC precursor lesions (SCE/SCOUTs/STINs/STICs) are present in the fallopian tubes of patients at a high risk of developing ovarian cancer [10]. We collected whole fallopian tubes from 11 patients (*BRCA1/2* mutation positive or with a family history of breast or ovarian cancer) who underwent RRSO and performed extensive sectioning (~3000 slides) to detect SOC precursor lesions under the supervision of a pathologist (L.R.) (Figure 1A-1E and Table 1). Wnt signalling plays a significant role in fallopian tube development and differentiation [16, 17]. Deregulated Wnt signalling is involved in carcinogenesis of various other organ systems [18]. To investigate if overactive Wnt signalling contributes to the pathogenesis of SOC, we performed immunohistochemical localization of βcatenin and LEF1, well-known targets of Wnt signalling [19], on serial sections of human fallopian tubes. We found patches of epithelial cells showing strong nuclear accumulation of βcatenin, which is indicative of active Wnt signalling (N: 11/11 patients; Figure 1F-1H). Staining of serial tissue sections revealed presence of LEF1 expression in same cells (N: 11/11 patients; Figure 1I-1K). These nuclear βcatenin and LEF1-positive epithelial patches were devoid of cilia, which were present on the normal looking adjacent epithelial cells (Figure 1H and 1K), suggesting that these epithelial patches are primarily consisting of secretory cells. As normal healthy women rarely undergo salpingo-oophorectomy, we were unable to procure the whole fallopian tubes from these females. We examined representative sections from the distal and the proximal end of fallopian tubes of young ($N = 3$; Age: 21-22 yrs; BRCA1/2 mutation status unknown) and adult ($N = 3$; Age: 33-65 yrs; *BRCA1/2* mutation negative) patients (Figure 1L-1S). We were unable to find epithelial lesions co-expressing nuclear βcatenin and LEF1 in these patients (Figure 1L-1S).

To prove that the patches of epithelial cells presented with nuclear βcatenin and LEF1 expression represent SOC precursor lesions, we performed immunostaining for two well-established markers of SCE/SCOUTs/STINs/STICs and SOC, Stathmin 1 and Pax8 [9, 20, 21]. Fallopian tube epithelial cells with nuclear βcatenin and LEF1 expression were also positive for both stathmin 1 and Pax8 staining confirming their identity as SOC precursor lesions (Figure 2A-2F). These lesions also co-express oestrogen receptor α (ERα; Figure 2G-2I) and progesterone receptor (PR; Figure 2J-2L), suggesting that ovarian hormones might regulate their growth. Assessment of representative

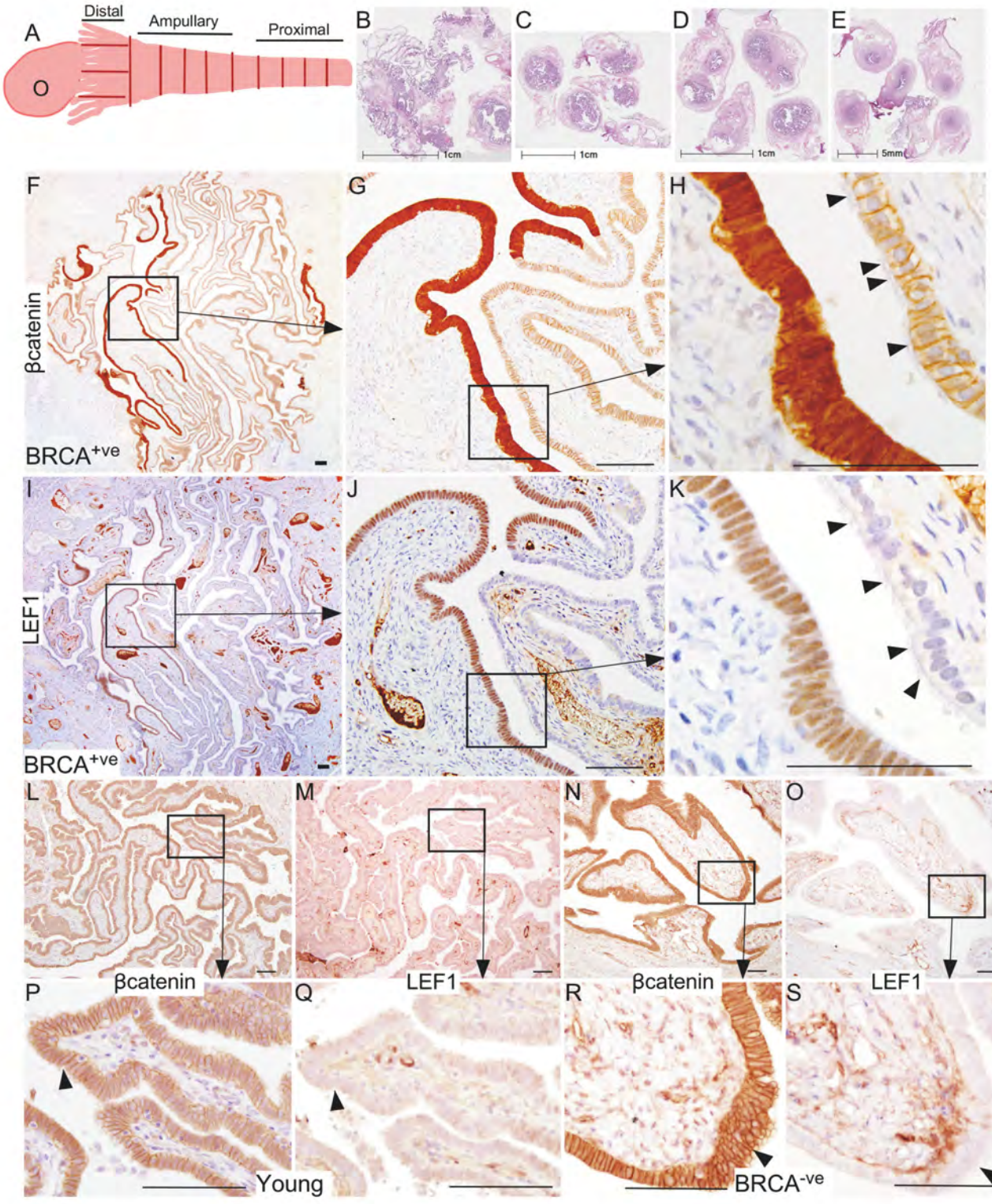

Figure 1: Hyperactive Wnt/βcatenin signalling is present in the Fallopian tube epithelium of *BRCA1/2* mutation positive women. Whole fallopian tubes were collected from the *BRCA1/2* positive women who underwent risk-reducing salpingo-oophorectomy and were histologically examined for the presence of ovarian cancer precursor lesions using an established protocol described by Moorman *et al* [46] **A.** Representative tissue sections of the distal end (fimbriae, B), the middle region (ampulla, **C.** and **D.**) and the proximal end (isthmus, **E.**) are shown in panel **B.-E.** A typical ovarian cancer precursor lesion presented with nuclear/cytoplasmic (active form) βcatenin **F.-H.** and LEF1 **I.-K.** staining. Arrowheads in panel H and K are marking cilia. Representative sections from the fallopian tubes of young or *BRCA1/2* mutation negative women showed membranous/cytoplasmic βcatenin **L.**, **P.**, **N.**, **R.**; arrowhead) and absence of LEF1 staining **M.**, **Q.**, **O.**, **S.**; arrowhead). Bars: 100um.

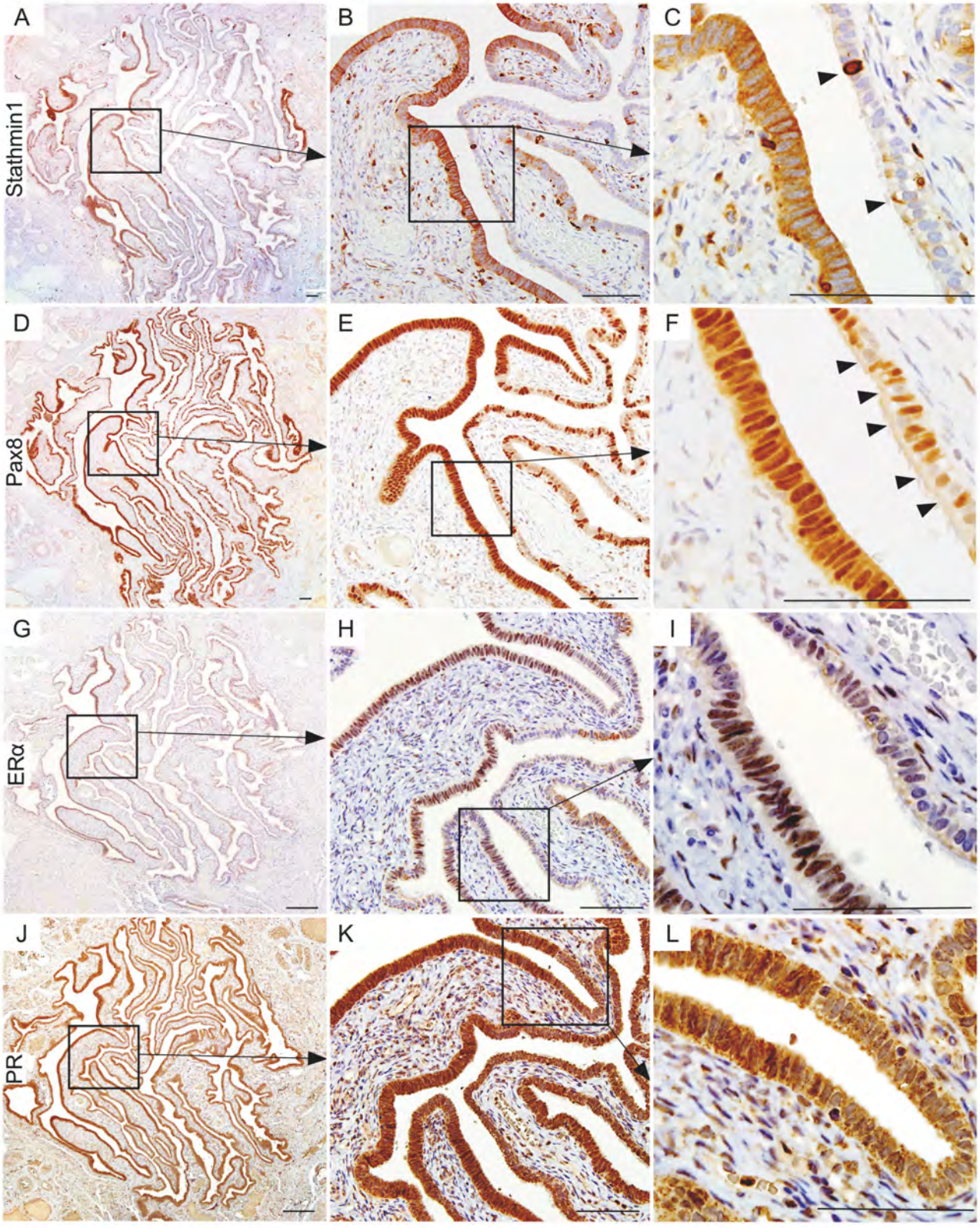

Figure 2: Wnt activation marks the human ovarian cancer precursor lesions. Serial tissue sections of the *BRCA1/2* mutation positive fallopian tube epithelia with hyperactive Wnt signalling (nuclear βcatenin and LEF expression) also showed Stathmin1 **A.**, **B.** and **C.** and Pax8 **D.**, **E.** and **F.** expression, which are known markers of ovarian cancer precursor lesions. Arrowheads in panel C show intermittent positive cells for Stathmin1. Arrowheads in panel F are marking Pax8-negative ciliated cells, a typical feature of normal fallopian tube epithelia. These putative ovarian cancer precursor lesions also expressed ovarian hormone receptors, oestrogen receptor α (ERα; **G.**, **H.** and **I.**), and progesterone receptor (PR; **J.**, **K.** and **L.**). Bars: 100um.

Table 1: Patient information

Patient number	Age (years)	*BRCA 1/2* mutation status
1	42	*BRCA1*
2	52	*BRCA2*
3	48	*BRCA1*
4	36	*BRCA1*
5	57	*BRCA2*
6	56	*BRCA2*
7	45	N.A. (history of breast cancer)
8	37	*BRCA1*
9	47	*BRCA2*
10	54	*BRCA2*
11	47	*BRCA2*
12	22	N.A.
13	21	N.A.
14	22	N.A.
15	63	Negative
16	55	Negative
17	33	Negative

N.A.: information not available or *BRCA1/2* mutation status was not determined.

sections from the entire fallopian ducts of 11 patients with high risk of developing ovarian cancer revealed presence of cytoplasmic/nuclear βcatenin, LEF1, Pax8 and Stathmin 1-positive precursor lesions in all of the patients (SFigure 1). We observe variability in the number of lesions between different patients (SFigure 1). However, no correlation was observed between the number of lesions, patients' age, and the stage of menstrual cycle at the time of surgery. We also examined the Cancer Genome Atlas serous ovarian cancer database [22] and found genetic alterations in the Wnt pathway members in 62% (113/182) of the patients (Figure 3). In summary, these data showed that activation of Wnt/βcatenin signalling occurs in human SOC precursor lesions.

Constitutive activation of Wnt/βcatenin signalling in mouse fallopian tube epithelium

Studies using human and animal models have provided evidence that uncontrolled growth of fallopian

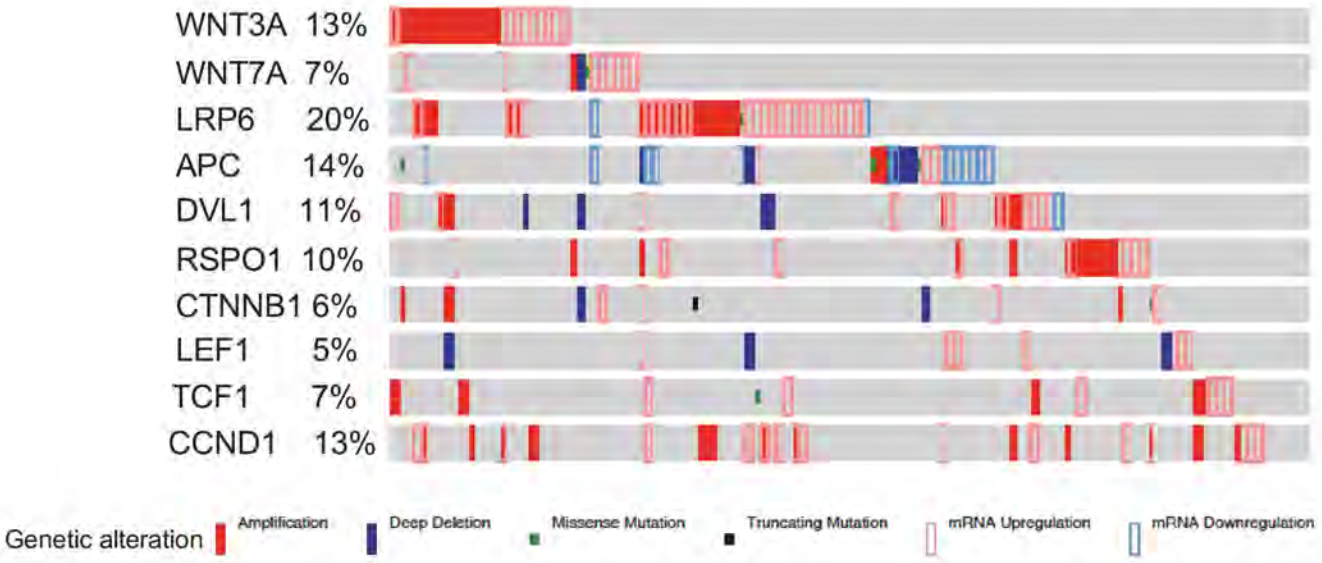

Figure 3: Alterations in the members of Wnt signalling pathway in human ovarian cancer patients. Interrogation of the cancer genome atlas serous ovarian cancer dataset showed significant alterations (113 out of total 182 patients) in the Wnt pathway members.

tube secretory cells is responsible for the pathogenesis of SOC [9]. Pax8 is a bona fide marker of both human and mouse secretory cells [9]. To test if hyperactive Wnt signalling in secretory cells is responsible for the development of SCE/SCOUTs/STINs/STICs, we developed a mouse model (βcatenin^{ex3}cko) in which constitutively active form of βcatenin is expressed under the control of a Pax8-driven reverse tetracycline-controlled transactivator combined with a tetracycline-responsive Cre recombinase (*Pax8^{rtta}Tetocre or LC1cre*;

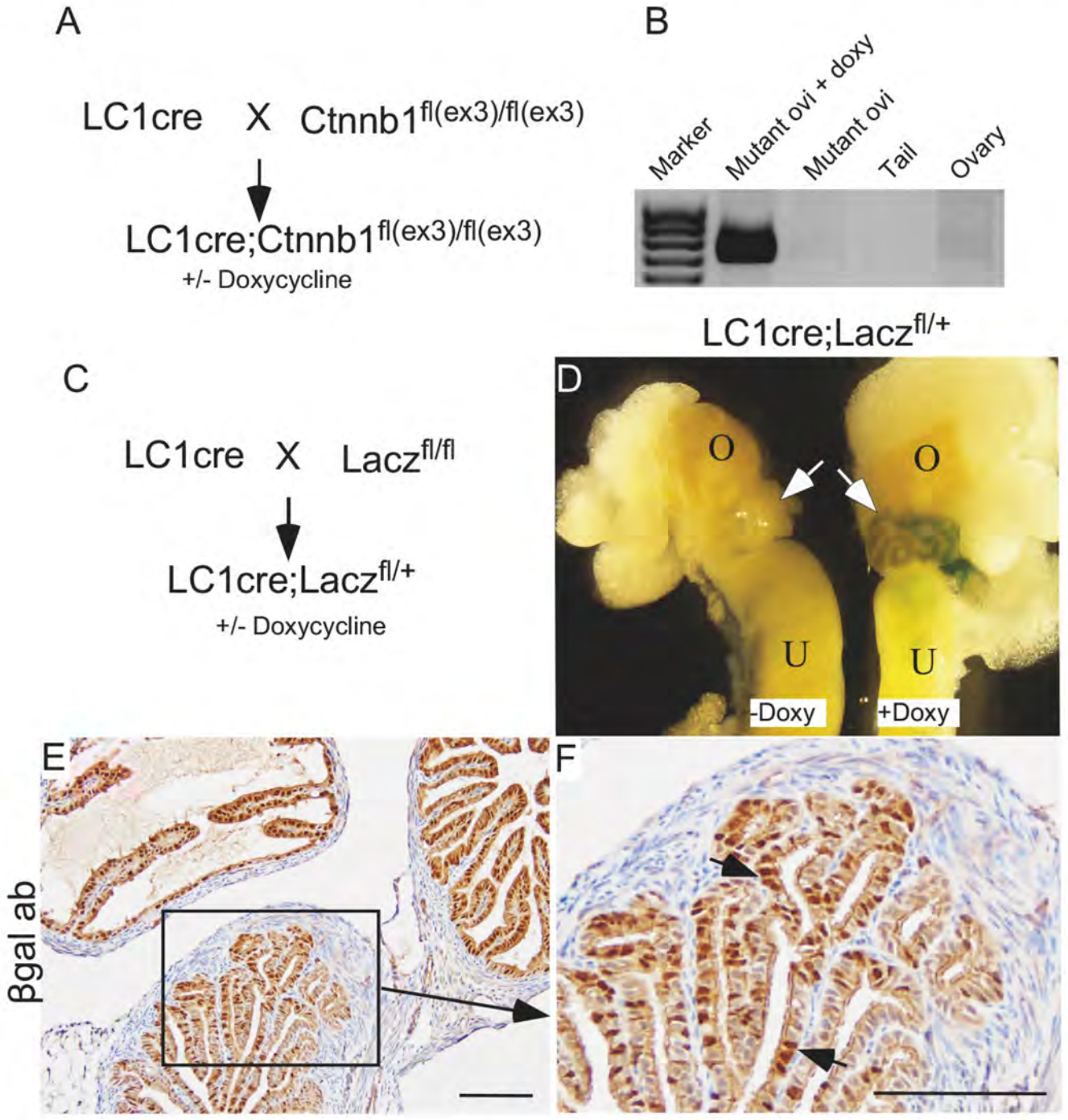

Figure 4: Constitutive activation of Wnt/βcatenin signalling in mouse fallopian tube secretory cells. LC1cre mice were crossed with Ctnnb1$^{fl(ex3)/fl(ex3)}$ to generate LC1 cre;Ctnnb1$^{fl(ex3)/fl(ex3)}$ (βcatenin^{ex3}cko; **A.**). Recombination PCR confirmed presence of floxed allele (band size: 0.7kb) in mutant oviduct but not in ovaries **B.** Mouse tails were used as negative controls **B.** Breeding strategy used for developing LC1cre driven lacZ reporter mouse model **C.** The gross image of the female reproductive tract isolated from mice treated with and without doxycycline showed recombination specifically in the fallopian tubes (arrows) of doxycycline treated LC1cre; Lacz$^{fl/+}$ mice, but not in the ovary **D.** Fallopian tube section from LC1cre; Lacz$^{fl/+}$ mouse stained for βgalactosidase showed secretory cell specific expression (arrows; **E.** and **F.**). Bars: 100um.

Figure 4A). βcatenin^{ex3}cko mice were given doxycycline in their drinking water (0.2mg/ml, adlibitum) to induce recombination of the flox alleles. Using primers directed to detect the recombined knock-in alleles of the *βcatenin*, we showed that recombination of the *βcatenin^{ex3}* allele occurs in fallopian tube (Figure 4B). Mouse Tail DNA was used as a negative control as Pax8, and thus Cre expression is absent in this tissue [9] (Figure 4B). To confirm that *Pax8* promoter driven *Cre recombinase* is specifically targeting the flox alleles in the mouse fallopian

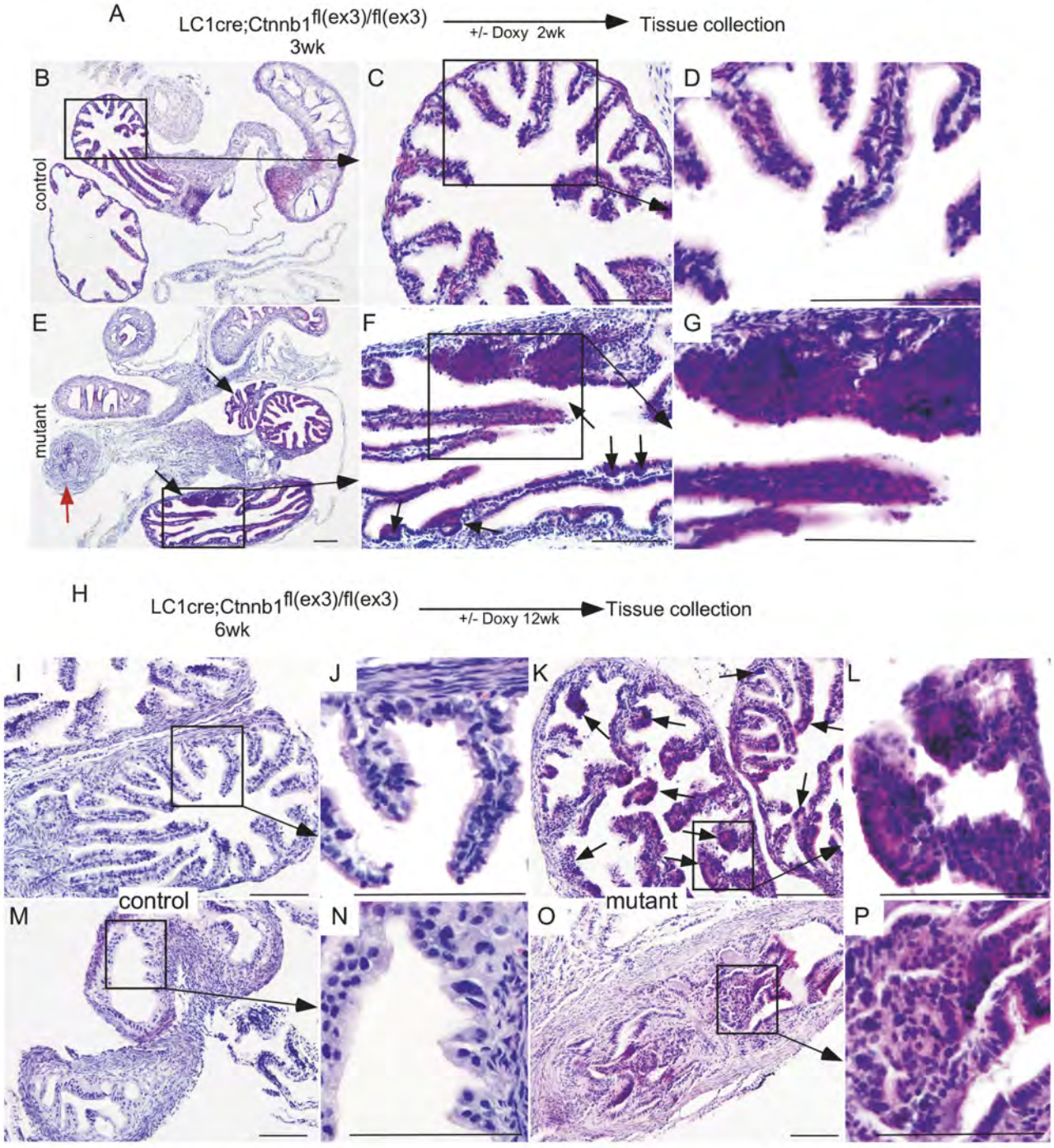

Figure 5: Sustained Wnt/βcatenin signalling in the fallopian tube secretary cells leads to abnormal epithelial growths similar to human SCOUT/STIC. Short-term (2wk) doxycycline treatment of βcatenin^{ex3}cko mice **A.** H&E stained Fallopian tube sections from control and short-term doxycycline treated mutant mice **B.-G.** The mutant fallopian tubes showed epithelial hyperplasia (black arrows) and occluded lumen (red arrow; **E.-G.** Long-term treatment with doxycycline of mutant mice resulted in wide spread epithelial dysplasia, focal growths (arrows, **K.** and **L.**), and blockage of the fallopian tube lumen (**O.** and **P.**). Morphologically normal fallopian tubes from control mice (**I.**, **J.**, **M.** and **N.**). Bars: 100um.

tube secretory cells, we developed another mouse model (LC1cre;Lacz$^{fl/+}$) by crossing LC1cre mice with Lacz reporter mice (Lacz$^{fl/fl}$; Figure 4C). Whole mount βgalactosidase (βgal) staining of the female reproductive tracts collected from LC1cre;Lacz$^{fl/+}$ mice treated with or without doxycycline showed Lacz expression in fallopian tubes but not in the ovaries of mutant mice that were exposed to doxycycline (Figure 4D). No Lacz expression was observed in untreated LC1cre;Lacz$^{fl/+}$ mice, which was used as negative control (Figure 4D). Using an antibody against βgal, we confirmed that LC1cre driven recombination was limited to the fallopian tube secretory cells of LC1cre;Lacz$^{fl/+}$ mice (Figure 4E and 4F).

Sustained activation of βcatenin leads to abnormal outgrowths of secretory cells

To determine the effects of overactive Wnt/βcatenin signalling, we collected female reproductive tracts from βcatenin^{ex3}cko mice treated with doxycycline for 2wks (short-term; Figure 5A). Compared to controls (Figure 5B-5D), histological examination of mutant mice showed nodular and focal expansion of epithelial cells in the distal fallopian tubes (Figure 5E-5G; N = 5/5). Abnormal growth of epithelial cells was also observed in the proximal fallopian tubes (Figure 5E, marked with a red arrow). To test if long-term administration of doxycycline would increase the severity of mutant mice phenotype, we examined fallopian tubes collected from βcatenin^{ex3}cko mice exposed to doxycycline for 12wks and found intraepithelial tumorous growth similar to human STIC in both the distal and the proximal fallopian tubes (Figure 5K, 5L, 5O and 5P; N = 7/7). No such growths were present in control mice (Figure 5I, 5J, 5M and 5N; N = 5/5).

Studies in fallopian tubes collected from asymptomatic women with germ line mutations in *BRCA1/2* genes have suggested stepwise progression to SOC [10]. The earliest lesions identified in these patients' fallopian tubes are SCEs followed by SCOUTs and then STINs/STICs [10]. To confirm that epithelial growths in mutant mice originated from the secretory cells and phenocopy human SOC precursor lesions, we performed colocalization of βcatenin and Pax8 (Figure 6A-6L). In controls, a discrete pattern of Pax8 expression was observed in the distal fallopian tubes where positive secretory cells were interspersed between negative ciliated cells (Figure 6A-6F). In mutants, abnormal outgrowths present in the fallopian tube epithelium were Pax8-positive confirming their identity as secretory cells (Figure 6G and 6J). Examination of βcatenin expression revealed nuclear accumulation of βcatenin in these Pax8-positive lesions (Figure 6H and 6K). In contrast, mainly membranous βcatenin localization was seen in fallopian tubes of control mice (Figure 6B and 6E).

LEF1, TCF1 and Cyclin d1 are well known downstream targets of Wnt/βcatenin signalling [18, 19]. Examination of their expressions showed increased LEF1, TCF1 and Cyclin d1-positive cells in mutant fallopian tube epithelial cells compared to controls (Figure 6M-6T), which further provide evidence for hyperactivation of this pathway in mutant mice compared to controls. Collectively, these results showed that constitutive activation of Wnt/βcatenin signalling leads to abnormal secretory cell outgrowths that are similar to those observed in human patients who are predisposed to developing ovarian cancer.

Oestrogen promotes and progesterone suppresses SOC precursor lesions growth

Studies in human patients suggest that hormones play a key role in the development of ovarian cancer [15]. Differences in the rate of fallopian tube epithelial cell proliferation rate are observed between the follicular and the luteal phase of the ovarian cycle suggesting that oestrogen and progesterone signalling is an important regulator of the fallopian tube functions [23]. Analysis of oestrogen and progesterone receptor (ER and PR) showed normal expression in both the control and the mutant fallopian tube epithelial cells (Figure 6U-6X). Interestingly, both ER and PR were also present in abnormal epithelial outgrowths of the mutant fallopian tubes suggesting that their development might be regulated by the changes in the levels of ovarian hormones (Figure 6V and 6X).

To test if oestrogen and progesterone supplementation influences the initiation of SOC, we surgically removed both ovaries from βcatenin^{ex3}cko mice (N = 10, age: 6wks) and allowed the mice to rest for 14 days to remove any trace of circulating ovarian hormones (Figure 7). After the resting period, oestrogen (0.72 mg/90 day release) or oestrogen and progesterone (0.72 mg + 100mg/90 day release) pellets were subcutaneously placed in these mice (N = 5/group). For controls, sham surgeries were performed on βcatenin^{ex3}cko mice of the same age and tissues were collected at the same time with two other groups (N = 5; Figure 7A). Histological examination and Pax8 immunolocalization showed abnormal enlargement and intraepithelial cancerous growth in both the distal and the proximal end of fallopian tubes of the oestrogen treated group (N = 5/5; Figure 7H-7M). These epithelial tumours invaded and occluded the lumen of the mutant fallopian tubes (N = 5/5; Figure 7H and 7K). In contrast, oestrogen and progesterone treated mice fallopian tubes were comparable to the control group (N = 5/5; Figure 7N-7S and 7B-7G). These tumours in oestrogen treated group were highly invasive as epithelial cells were present in the muscular and serosal layers of fallopian tubes (N= 5/5; SFigure 2). However, no evidence of metastasis was

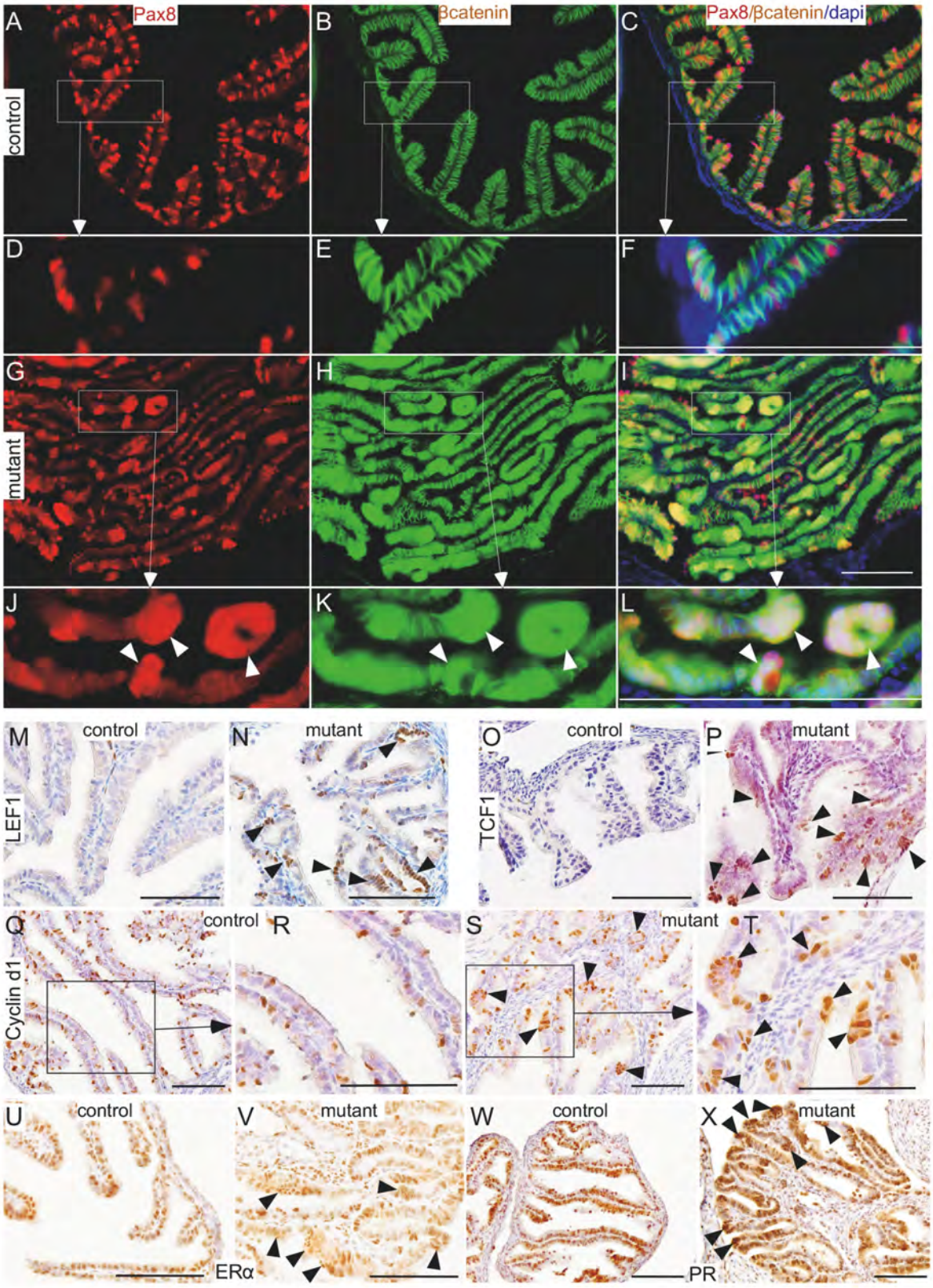

Figure 6: Histopathological analysis of abnormal changes in the mutant fallopian tube epithelium. Pax8 and βcatenin expression in fallopian tubes collected from control mice **A.-F.** Nuclear/cytoplasmic accumulation of βcatenin in the Pax8-positive epithelial lesions (arrowheads) present in βcatenin^{ex3}cko fallopian tubes **G.-L.** Expression of LEF1 (**M.** and **N.**), TCF1 (**O.** and **P.**), cyclin d1 (**Q.-T.**), ERα (**U.** and **V.**), and PR (**W.** and **X.**) in control and mutant fallopian tubes. Arrowheads mark the epithelial lesions that are positive for the probed proteins. Bars: 100um.

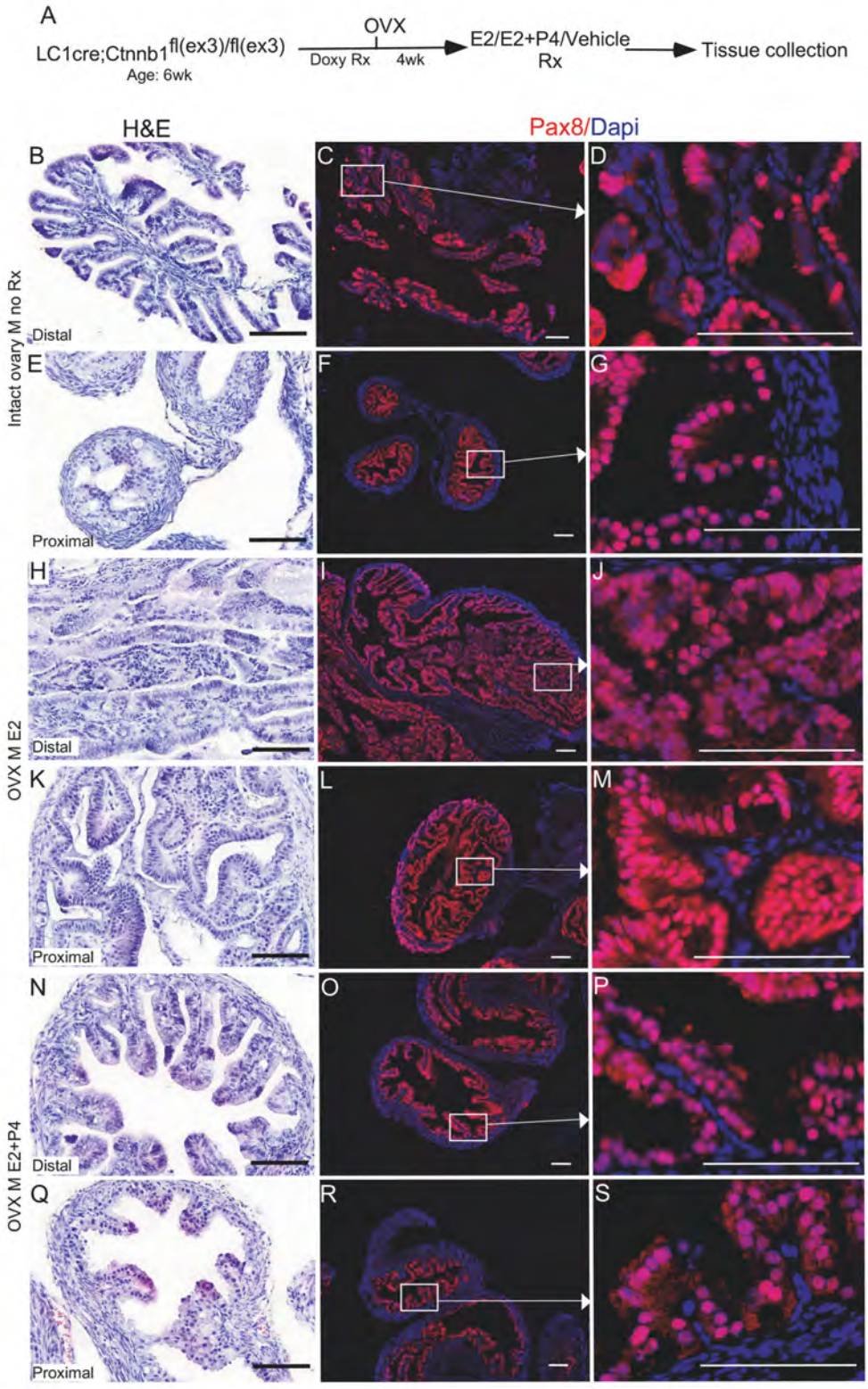

Figure 7: Oestrogen promotes and progesterone suppresses the growth of precancerous lesions in the mutant fallopian tube epithelium. βcatenin^{ex3}cko mice were ovariectomised to remove endogenous hormones and were subjected to doxycycline and hormonal treatments sequentially, as shown in **A.** Hyperplasia in the fallopian tube epithelium of mutant mice belonging to the control group **B.-D.** The Pax8 expression showed focal expansions in the fallopian tube of βcatenin^{ex3}cko mice **C.** and **D.** The cohort subjected to oestrogen treatment developed intraepithelial carcinoma in both the distal **H.** and proximal **K.** fallopian tube. Panel **I.**, **J.**, **L.** and **M.** shows the secretory cell positive epithelial outgrowths. Mice treated with both oestrogen and progesterone displayed reduced abnormal epithelial growths **N.-S.** and were comparable to controls **B.-G.** Bars: 100um.

observed in any of these mutant mice, suggesting that additional genetic or molecular events are required for the metastatic spread of SOC cells. These murine tumours expressed markers of early and late human SOC, Pax8 and Stathmin 1 (Figure 7 and 8A-8C). Immunostaining for Ki67 depicted increase in proliferating cells in oestrogen treated group compared to oestrogen and progesterone and control (mutant mice with intact ovaries) mice (Figure 8D-8F). In summary, these experiments have shown that ovarian hormones are key regulators of SOC growth and provide an explanation for the effectiveness of combined oral contraception and pregnancy, high progesterone conditions, in suppressing the development of SOC.

Ovarian hormones affect Wnt/βcatenin signalling for regulating the growth of SOC

To examine if changes in ovarian hormone levels affect Wnt signalling, we analysed the expression of βcatenin and its downstream targets (LEF1, TCF1 and Cyclin d1) in the fallopian tube samples collected from the βcatenin^{ex3}cko mice treated with oestrogen, oestrogen and progesterone, and control group. The assessment of βcatenin expression revealed increase in nuclear/cytoplasmic localization of this protein, indicative of active Wnt signalling, in oestrogen treated group compared to controls (Figure 9A-9C). Co-treatment with progesterone mitigated the effects of oestrogen on βcatenin localization and reduced its expression compared to the oestrogen treated and control group (Figure 9A-9C). We have previously established that cells with active βcatenin signalling show increased nuclear expression of LEF1, TCF1 and Cyclin d1 [19]. Examination of LEF1, TCF1 and Cyclin d1 expression showed significant increase in their expression in oestrogen treated group compared to controls (Figure 9D-9L). Interestingly, co-treatment with progesterone reduced the expression of all these three markers in the mutant fallopian tube epithelial cells compared to the oestrogen treated group (Figure 9D-9L). These findings suggest that the inhibitory effect of progesterone treatment on the initiation of SOC might involve suppression of Wnt signalling.

To assess whether ovarian hormones regulate the growth of SOC, we treated PEO1 cells, a well-characterized SOC cell line that is known to express oestrogen and progesterone receptors [24], with varying doses of oestradiol (100nM, 250nM and 500nM) and/or medroxyprogesterone acetate (MPA; 100nM, 250nM and 500nM). Drug doses were determined on the basis of previous studies [25, 26]. Consistent with our observations in the mouse model, oestradiol treatment significantly increased PE-01 cell viability in a dose dependent manner (Figure 10A). The SOC cell viability was decreased with incremental doses of MPA alone (Figure 10A). Co-treatment with MPA mitigated the effect of oestradiol (Figure 10B). Furthering this, the current standard of

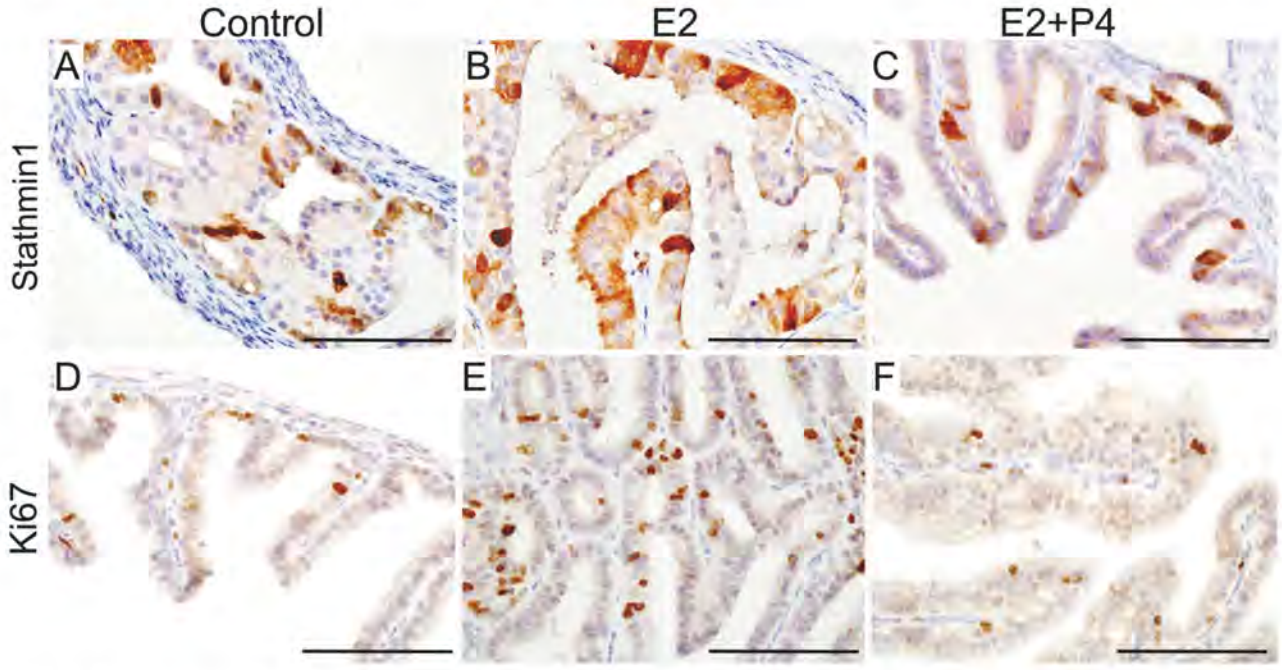

Figure 8: Expression of human ovarian cancer markers in the tumorous lesions observed in the fallopian tube of mutant mice. Expression of Stathmin 1, a well established marker of serous ovarian cancer, in the fallopian tube epithelial growths of control (mutant mice with intact ovaries; **A.**), oestrogen treated group (E2; **B.**) and oestrogen plus progesterone treated group (E2+P4; **C.**). Ki67, a marker of proliferating cells, showed an increase in the rate of proliferation of the fallopian tube epithelial cells in E2 treated group **E.** compared to control **D.** and E2+P4 group **F.** Bars: 100um.

care chemotherapeutic drug, carboplatin was combined with incremental doses of MPA and our data showed that MPA treatment enhances the efficacy of carboplatin, the higher doses of MPA being as efficient as the lower doses of carboplatin (Figure 10C). As expected, no response to oestradiol treatment was observed in another ovarian cancer cell line, COV362, which lacks the expression of oestrogen and progesterone receptors (data not shown).

To understand whether ovarian hormones affect Wnt signalling in ovarian cancer cells, PE01 cells were plated in six well plates and were treated with either oestradiol (500nM) or MPA (500nM) or oestradiol (500nM) + MPA (500nM) or DMSO. Compared to controls, treatment with oestradiol increased the level of βcatenin protein (Figure 10D), whereas, co-treatment with MPA had an opposite effect (Figure 10D). These results indicate that ovarian hormones regulate the growth of human SOC by modulating Wnt/βcatenin signalling.

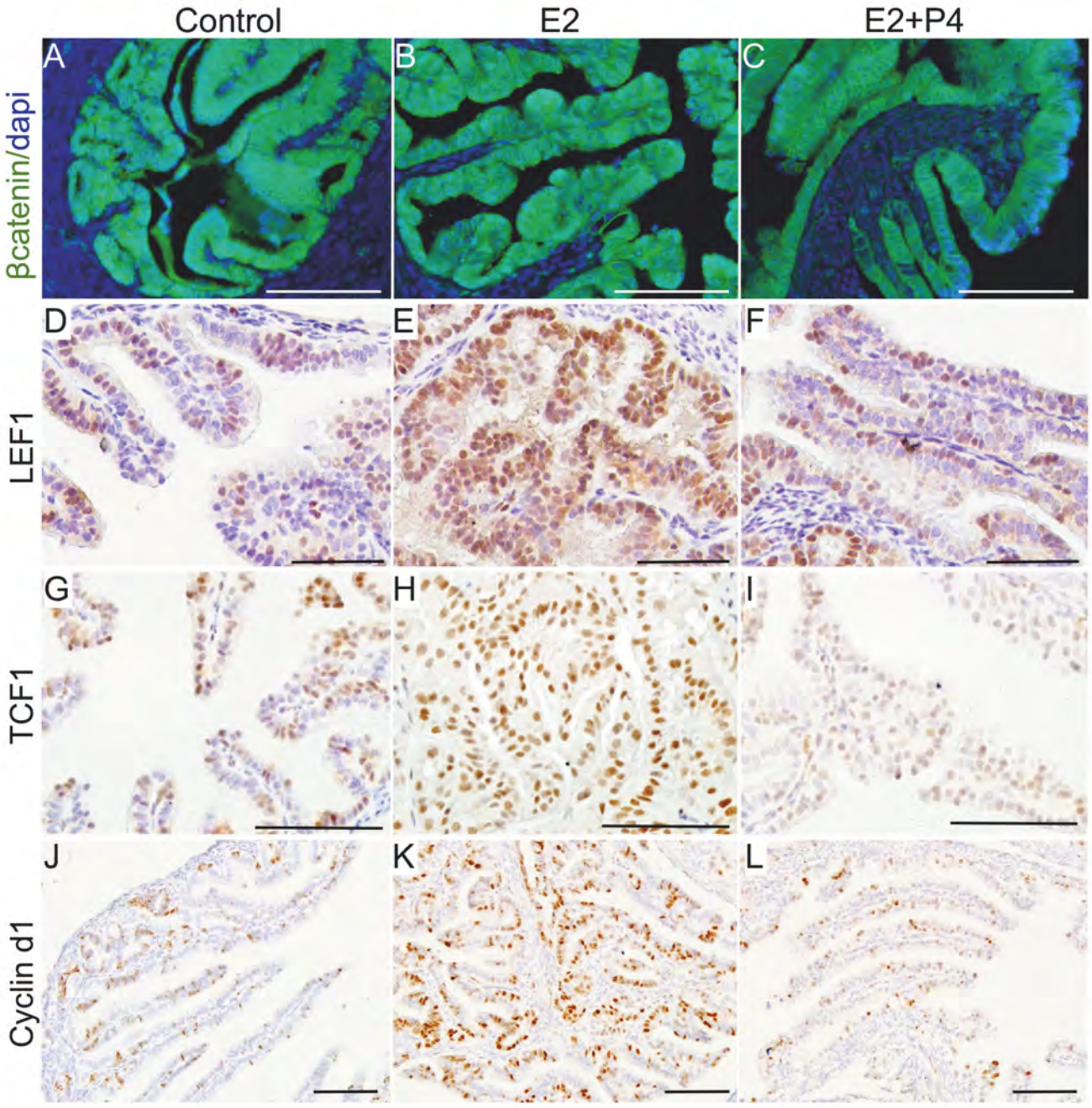

Figure 9: Ovarian hormones modulate Wnt/βcatenin signalling to affect the growth of precancerous lesions. Increased expression of βcatenin and its downstream targets, LEF1, TCF1 and Cyclin d1, in the fallopian tubes of oestrogen (E2; **B.**, **E.**, **H.** and **K.**) treated group compared control (mutant mice with intact ovaries; **A.**, **D.**, **G.** and **J.**) and oestrogen plus progesterone (E2+P4; **C.**, **F.**, **I.** and **L.**) group. Bars: 100um.

DISCUSSION

The fallopian tubes are the tubal organs that physically connect the ovaries to the uterus. These organs are the sites for egg sperm fusion and provide an appropriate environment for early embryonic development and transport. Studies from human patients with increased risk (mainly with germ line mutations in the *BRCA1/2* genes) to ovarian cancer have provided evidence for the fallopian tube epithelium being one of the sites of origin for SOC [5, 27]. This is further supported by the findings that the surgical removal of fallopian tubes (bilateral salpingectomy) significantly lowers the risk of developing ovarian cancer compared to women with no surgery [28]. Similar to the breasts and the ovaries, fallopian tubes are affected by changes in hormone levels during the oestrus cycle. Examination of fallopian tubes from women with or without *BRCA1/2* mutations revealed increased proliferation of epithelial cells during the follicular phase, an oestrogen dominant phase, compared to the luteal phase of the oestrous cycle, a progesterone dominant phase [23]. Epidemiological studies in human

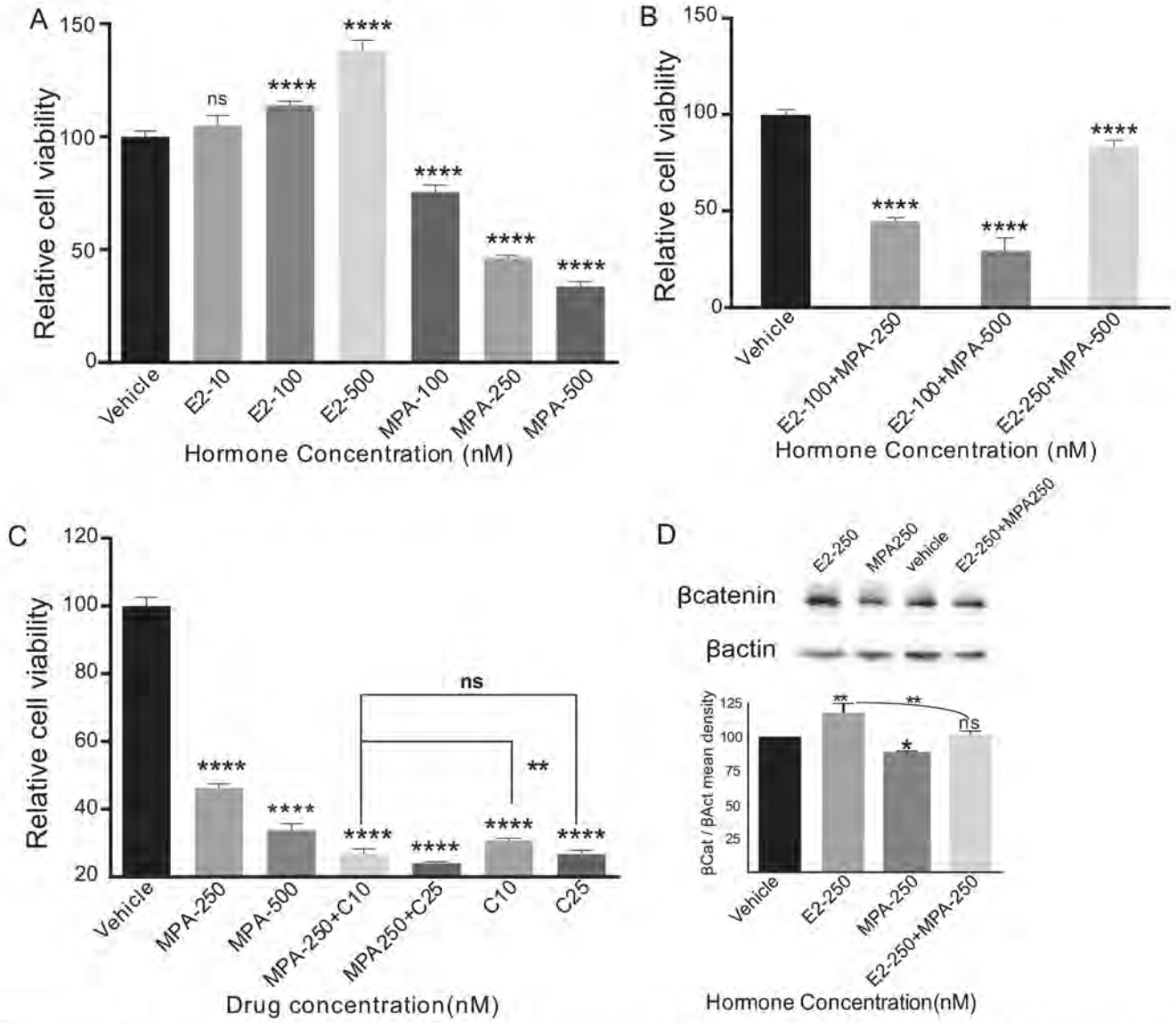

Figure 10: Oestrogen promotes and progesterone suppresses the growth of human serous ovarian cancer cells. PE01 cells treated with incremental doses of 17-β-oestradiol (E2) showing increase in cellular viability in a dose dependent fashion **A.** Medroxyprogesterone acetate (MPA) treatment had an opposite effect **A.** Co-treatment of PEO1 cells with E2 and MPA resulted in suppression of the growth promoting effect of oestrogen by MPA **B.** However, surplus concentrations of 17-β-oestradiol with lower concentrations of MPA further increased the cellular viability of PEO1 cells **B.** Cellular viability of PE-01 cells treated with incremental doses of MPA and MPA+Carboplatin **C.** E2 and/or MPA treatment affects the βcatenin levels **D.** βactin was used as a loading control **D.** The data shown are representative of three individual Western blot analyses. *p* value *panel A-C* ***P* = *0.0018*, *****P* = *0.0001*, *ns P* = *0.1053 (panel A)*, *P* = *0.9998 (panel C)*, *P* = *0.4771*, *panel D*, **P* = *0.0115*, ***P* = *0.0020*, *ns P* = *0.9534*, *One-Way Anova test.*

patients with hereditary predisposition to ovarian cancer have indicated that increased number of ovulatory cycles confers high risk of developing ovarian cancer [29]. It has been hypothesised that with every ovulatory cycle, the fallopian tube epithelial cells are bathed with oestrogen rich follicular fluid and that the unopposed mitogenic effects of oestrogen on these epithelial cells lead to neoplastic growth [30]. This hypothesis is supported by *in vitro* findings showing that exposure of the fallopian tube epithelial cells to follicular fluid induces DNA damage and increases proliferation mimicking the early events leading to SOC [31].

Studies examining cancer risk in *BRCA1/2* mutation positive women have shown that approximately 50% of these women develop breast or ovarian cancer by the age of 70 years, but approximately 50% of these patients even in their late 80's don't develop these cancers [32], suggesting that in addition to genetic changes, other factors, such as lifestyle, influence the risk of developing reproductive cancers [13, 33]. A large study examining 31,658 catholic nuns showed that these women are more likely to die from reproductive cancers such as breast, ovarian and uterine cancers compared to the general population [33]. This study suggests that uninterrupted exposure to high levels of ovarian hormones for long durations might contribute to the pathogenesis of reproductive cancers. In support, a long-term follow up study of 17,032 women revealed that oral contraceptive use significantly lowers the risk of ovarian cancer without affecting the risk of breast cancer [34]. Breast-feeding, oral contraceptive use, and parity are associated with decreased risk of developing ovarian cancer in *BRCA1/2* mutation carriers [29]. High progestin oral contraceptive formulations provide greater protection compared to the low progestin formulations [13]. Similarly, twin pregnancies, a high progesterone physiological condition, provide better protection against ovarian cancer than singleton pregnancies [13]. These findings suggest progesterone and its mimetic provide protection against ovarian cancer. In contrast, oestrogen dominant conditions such as nulliparity and oestrogen-only hormone replacement therapy have opposite effects. How these ovarian hormones affect the development of ovarian cancer is currently unclear. In our study, we have shown that human SOC precursor lesions express oestrogen and progesterone receptors suggesting that these ovarian hormones affect their growth (Figure 1). Treatment of a SOC mouse model showed that oestrogen increases and progesterone decreases the growth of SOC precursor lesions (Figure 7). Collectively, these findings provide a molecular explanation to the epidemiological observations that high progesterone conditions provide protection against ovarian cancer.

Studies from our lab and others have shown that Wnt/βcatenin signalling plays a major role in female reproductive tract development and its deregulation leads to various reproductive tract diseases including ovarian cancer [35-37]. Mouse knockout studies have revealed that conditional loss of *βcatenin* results in shortening and defective coiling of the fallopian tubes, partially due to the lack of proliferation of epithelial cells [16, 36], indicating that normal Wnt/βcatenin signalling is essential for the fallopian tube development. Recently, two different studies in mice have identified that leucine-rich repeat-containing heterotrimeric guanine nucleotide-binding protein-coupled receptor 5 (LGR5), a member of the Wnt signalling receptor complex, marks stem/progenitor population of the ovarian surface epithelium and the distal fallopian tube [17, 38]. In this study, we have discovered that active Wnt/βcatenin signalling marks the precursor lesions of SOC in human fallopian tubes and the sustained activation of this signalling pathways leads to the development of similar lesions in mouse fallopian tubes (Figure 1-4). These findings raise an exciting possibility that ovarian cancer is a disease caused by the rogue stem/progenitor cells that have acquired changes culminating in overactive Wnt signalling. Similar to intestinal cancer development model [39], sustained activation of the Wnt pathway might cause defects in differentiation of the fallopian tube stem/progenitor cells leading to the abnormal expansion of these cells resulting in SOC. This is supported by observations in the fallopian tubes of human patients with the germline mutations in *BRCA1/2* genes that the earliest SOC precursor lesions are clonal expansions of few secretory cells [10]. In future studies, we plan to investigate the role of the fallopian tube stem/progenitor cells in the pathogenesis of ovarian cancer.

In summary, our examination of the fallopian tubes from women with hereditary predisposition to development of ovarian cancer revealed hyper activation of Wnt signalling in SOC precursor lesions. We developed a unique mouse model in which constitutive activation of βcatenin in the fallopian tube secretory cells causes development of similar precursor lesions confirming the involvement of deregulated Wnt/βcatenin involved in the initiation of SOC. Oestrogen treatment enhances and progesterone treatment suppresses tumorous growth in this mouse model by affecting Wnt/βcatenin signalling.

MATERIALS AND METHODS

Human fallopian tube tissue samples

This study is approved by the Institutional Human Research Ethics Committee at the University of Newcastle. Whole Fallopian tubes and ovaries were collected from the women who underwent risk reducing bilateral salpingo-oophorectomy at the John Hunter Hospital. Fallopian tube tissue samples were obtained from 17 patients. Patient information is presented in Table 1. Paraffin tissue blocks (10-16 blocks per high risk

Table 2: List of primer pairs used for PCR

Transgene	Forward Primer	Reverse Primer
tetOcre	5'GCGGTCTGGCAGTAAAAACTATC3'	5'GTGAAACAGCATTGCTGTCACTT3'
Pax8rtta	5'CCATGTCTAGACTGGACAAGA3'	5'CTCCAGGCCACATATGATTAG3'
Ctnnb1$^{Fl(ex3)}$	5'GACACCGCTGCGTGGACAATGA3'	5'GTGGCTGACAGCAGCTTTTCTA3'
(ROSA)26Sor^{tm1Joe}	5'AAA GTC GCT CTG AGT TGT TAT3' 5'TCC AGT TCA ACA TCA GCC GCT ACA3'	5'TAA GCC TGC CCA GAA GAC TC3'
Ctnnb1$^{Fl(ex3)}$ (Recombined allele)	5'-GGTAGGTGAAGCTCAGCGCAGAGC-3'	5'- ACGTGTGGCAAGTTCCGCGTCATCC-3'

patient and 2-3 blocks per normal healthy woman) were sectioned at 6um thickness and 20 serial tissue sections were collected from every tissue block. The tissue sections encompassing the proximal, the middle and the distal ends of fallopian tubes were used for immunohistochemical marker analysis.

Mouse genetics and husbandry

Mice used in the present study were maintained in standard animal housing conditions. The standard procedures and treatments performed on mouse models were approved by the Institutional Animal Care and Ethics Committee at the University of Newcastle. Pax8rtTA mice [40] were mated with tetOCre mice [41] to generate Pax8rtTa; tetOCre mice and referred to as LC1cre. LC1cre mice were crossed with Ctnnb1$^{fl(ex3)/fl(ex3)}$ mice [42] to develop LC1cre; Ctnnb1$^{fl(ex3)/fl(ex3)}$ (βcatenin^{ex3}cko). To generate lacZ reporter mice (LC1cre;lacZ$^{fl/+}$), LC1cre mice were bred with Gt(ROSA)26Sor^{tm1Joe} (lacZ$^{fl/fl}$) [43]. To induce recombination of flox alleles in βcatenin^{ex3}cko and LC1cre;lacZ$^{fl/+}$ mice, doxycycline (0.2-1mg/ml) was administrated in drinking water. Ear punch tissues were collected and genotyping PCRs were performed using REDExtract-N-Amp™ Tissue PCR Kit (Sigma, MO, USA). Recombination PCR to detect the floxed allele were done on DNA isolated from the fallopian tubes, ovaries and tails of βcatenin^{ex3}cko mice. Primer details are listed in Table 2.

βgalactosidase staining

βgalactosidase staining was performed as described by us in [44]. Briefly, female reproductive tracts were collected from adult LC1cre;lacZ$^{fl/+}$ mice and were fixed in 4% paraformaldehyde for 1hr at 4°C. Tissue were then washed in rinse buffer (0.1% sodium deoxycholate, 0.2% NP40, 2mM magnesium chloride in 0.1M phosphate buffer pH 7.3) for 30 min 3 times and stained with X-gal solution for 3-4 h at room temperature. Tissues were rinsed with PBS to remove excess of the solution and pictures were taken using Nikon SMZ25 stereoscope.

Hormonal treatments

βcatenin^{ex3}cko mice were treated with doxycycline for 4wks. These mutant mice were then ovariectomised and allowed to rest for 14 days to remove the traces of circulating hormones. 90-day slow releasing hormone pellets of 17-β-oestradiol (0.72 mg per pellet) or 17-β-oestradiol and Progesterone (0.72mg and 100mg per pellet; Innovative Research of America, Fl, USA) were subcutaneously inserted in mutant mice. Mutant mice with sham surgeries without any treatment were used as controls. After 46 days post-hormonal treatment, some of these mice presented with abnormal enlargement of peritoneum and all the mice were euthanized. The fallopian tubes were removed and fixed in 4% paraformaldehyde overnight at 4C.

Histology, immunohistochemistry (IHC) and immunofluorescence (IF)

Hematoxylin and Eosin (H&E) staining was carried out using a standard protocol. IHC and IF protocols are described in [44]. The primary antibodies used in this study are as follows: βcatenin (1:200; BD Biosciences, NJ, USA), Cyclin d1 (1:100), LEF1 (1:100), Stathmin 1 (1:1200), TCF1 (1:100; Cell Signalling Technologies, MA, USA), ERα (1:500), PR (1:200; Santa Cruz Biotechnology, CA, USA), βgal (1: 500, MP Biomedicals, CA, USA), Ki67 (ready to use; Biogenex, CA, USA), Pax8 (1:500, Proteintech, IL, USA). AlexaFluor (Jackson ImmunoResearch Labs, PA, USA) or biotinylated (Biogenex) secondary antibodies were used. Stained slides were imaged at high resolution with the Olympus DP72 microscope or the Aperio Scanscope slide scanner. The gain and exposure time were set constant across tissue samples. Analysis for intensity and number of cells was done using the Halo Image analysis software (Indica labs, NM, USA).

Western blotting

PE-01 cells were grown in 6-well plates and were treated with 17-β-oestradiol (500nM; Sigma, MO, USA)/

MPA (500nM; Sigma, MO, USA) or 17-β-estradiol (500nM) + MPA (500nM). Cells treated with DMSO were used as control. Protein was extracted using RIPA buffer. Equal amount of protein was loaded and β-actin was used as loading control. Primary antibodies: βcatenin (1:500; Cell Signalling Technology) and βactin (1:2000, Developmental Studies Hybridoma Bank, IA, USA). HRP conjugated secondary antibodies against mouse and rabbit were from Cell Signalling Technology or Jackson ImmunoResearch Laboratories. The mean density of the protein bands was determined using NIH Image J plugin.

Cell culture and treatments

COV362 and PE01, SOC cell lines [45], were cultured in DMEM and RPMI-1640 added with 10% Foetal Bovine Serum (FBS), respectively. 5000 Cells were seeded per well in 96 well plates and treated with 17-β-Oestradiol, (10nM, 100nM and 500nM) or medroxyprogesterone acetate (MPA; 100nM, 250nM and 500nM) or DMSO for 72 hours. For carboplatin and MPA experiments, PE01 cells were plated in 96 well plates and following day these cells were subjected to one of the following treatments: MPA (250nM, 500nM) or MPA (250nM) plus carboplatin (10nM/25nM; Hospira, Pfizer, Australia). All drugs were replenished every 24 hours and the cells were assessed for cellular viability using Vision Blue Cell Viability Fluorometric Assay kit (Biovision, CA, USA). These experiments were repeated thrice.

Statistical analysis

Graph Pad Prism 6.0 was used to conduct statistical analysis. All indicated values are in Mean ±SD and were subjected to one way ANOVA to assess differences between different groups with a P value less than 0.05 was considered statistically significant.

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

Author Contributions

P.B.N. and P.S.T. designed research. P.B.N., J.G. and S.N. performed the research. P.B.N., L.R., J.L., P.N. and P.S.T. analysed the data. P.B.N. and P.S.T. wrote the paper. P.S.T. provided financial support and final approval of the manuscript. All authors approved and commented on the manuscript.

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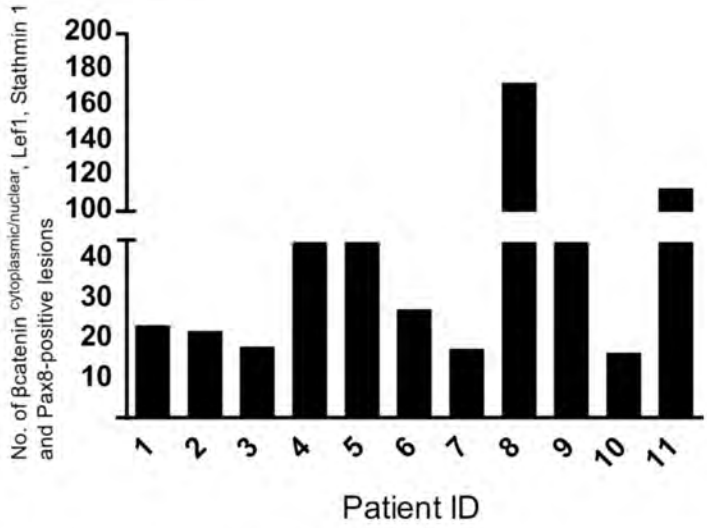
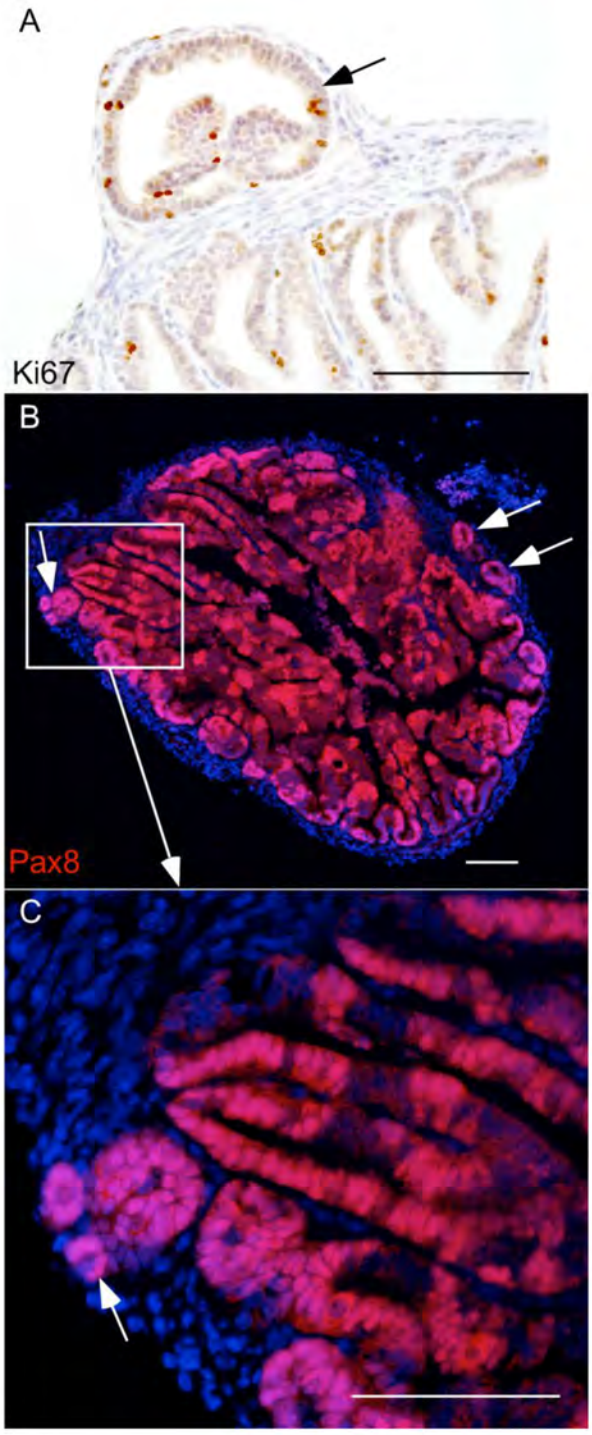
Ovarian hormones through Wnt signalling regulate the growth of human and mouse ovarian cancer initiating lesions

Supplementary Material



SFigure 1. Assessment of the βcatenin$^{nuclear/cytoplasmic}$, LEF1, Stathmin 1 and Pax8-positive ovarian cancer precursor lesions in the fallopian tubes collected from human patients with a hereditary predisposition to ovarian cancer development.



SFigure 2. Ki-67 staining showing invasive growth of the proliferating cells (arrow) in the serosal layer of oestrogen-treated mutant (bcatenin^{ex3} cko) oviducts (A). Pax8 staining of oestrogen-treated bcatenin^{ex3} cko oviducts (B). White arrows in panel B and C show Pax8-positive epithelial outgrowths in the serosal layer.

Chapter 3: Evidence of early precursor escape from the Fallopian tube in pelvic serous ovarian carcinoma

Chapter 3

Preface

Fallopian tube epithelium is the site of origin in approximately 50-70% of all high grade serous ovarian carcinomas (HGSOC). Especially in high risk women FTE shows multiple dysplasia and preneoplasia which show a continued progression and culminate in HGSOCs. However there have been cases of risk reducing salpingo oophorectomy post which there have been incidences of HGSOC in the peritoneum or omentum. This chapter probes one such patient samples to look for origin of such tumours. In this chapter we provide evidence for very early precursor escape from the Fallopian tube, making a case for earlier salpingectomy for genetically susceptible women (with *BRCA1/2* germline mutations) aged less than 40 wanting to conserve their fertility. This chapter also establishes site of origin despite the lack of preneoplasia or dysplasia in the FTs. It also provides evidence against precursor-carcinoma continuum as mandatory criteria to establish any site as site of origin.

UON Graduate research

Co-authorship declaration

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

Statement of Author's contributions

This statement summarizes the intellectual inputs by all the authors stated in the research article titled "Evidence of early Fallopian tube precursor escape in omental serous ovarian carcinoma". This article is submitted in the journal Cancer research.

Authors	Statement of contribution
Prathima B Nagendra (First author)	Conducted experiments, wrote the initial draft and made figures for the manuscript Edited and revised the manuscript
Pradeep S Tanwar (Corresponding author)	Edited the manuscript and provided intellectual inputs

Prathima B Nagendra

Pradeep S Tanwar

Dr Lesley MacDonald-Wicks-Faculty Assistant Dean Research training

Evidence of early precursor escape from the Fallopian tube in pelvic serous ovarian carcinoma

Abstract:

One of the cells of origin of high grade serous ovarian carcinoma are the secretory cells of the Fallopian tube epithelia. The Fallopian tube is an established site of origin in atleast half of the reported cases in the last decade. But in atleast 20-50% of the patients there is no evidence of precancerous or cancerous lesions present in the FT. One theory suggests that the precursors escape the site of origin, the Fallopian tubes, to go and adhere to the ovarian surface epithelium or the peritoneum, where there is a hormonal and reactive oxygen species milieu, conducive for serous ovarian carcinogenesis. To test this hypothesis, we investigated a case wherein retrospective in-vivo site tracing was possible. The patient underwent risk reducing salpingo oophorectomy and total abdominal hysterectomy as she had a family history of breast and ovarian carcinoma and was detected with a pathogenic variant of *BRCA1* gene. Three years later she developed high grade serous ovarian carcinoma in the omentum. The histological analysis of the ovaries and fallopian tubes indicated no morphological abnormalities. Further extensive immunohistochemical analysis indicated no precursor lesions in the Fallopian tubes. The goal of this project was to find any molecular evidences to the origin of omental serous ovarian carcinoma in the Fallopian tubes. To undertake this, we performed WES and RNA sequencing of microdissected epithelia of the fallopian tubes and the omental tumour. The sequencing showed clonal link between right Fallopian tube epithelium and the omental tumour as they shared common *TP53* mutations.

Introduction

High grade serous ovarian carcinoma (HGSOC) is the most lethal of all gynaecological cancers with a mortality to incidence ratio of 2:3 within 5 years of occurrence [1]. Due to mainly driver mutations in the homologous recombination and repair pathway and impending genomic instability, HGSOC displays thousands of molecular aberrations: copy number variations, single nucleotide polymorphisms, protein dysregulation, transcriptional changes, post translational modifications etc. This leads to large scale intra patient and inter patient variations and there is a lack of unifying disease specific markers irrespective of patient clinical history. Thus, HGSOC is a highly heterogenous disease [2-4]. This makes treatment of the disease very difficult. The current clinical treatment is combined chemotherapy (paclitaxel and platinum-based drugs) together with debulking surgery. Despite treatment around 60-70% women recur within 5 years of incidence. There is an urgent need to find early stage-disease specific markers which have a diagnostic, prognostic and therapeutic value.

For over a century the site of origin had been implicated to ovarian surface epithelium [5, 6]. In the last two decades many studies have presented multiple evidences implicating the Fallopian tube epithelium as an alternative site of origin [7, 8]. Around 30-70% of HGSOCs have originated in the Fallopian tubes in various studies [9-11]. Many studies also have investigated the various dysplasia in the FT and their role as precursors to HGSOC. These include the p53 signatures, STIN (serous tubal intraepithelial neoplasia) and STIC (serous tubal intraepithelial carcinoma) which act as direct precursors as they share multiple immunohistochemical markers with the HGSOC and also share common *TP53* (Tumour Protein 53) mutations indicating clonal identity [8, 11-14].

Many studies have also established a continuum of normal epithelia-preneoplasia-neoplasia transition in the FT [11-13]. These include morphologically normal benign entities such as p53 signatures, SCEs (secretory cell expansions) and SCOUTS (secretory cell outgrowths) and invasive but intraepithelial in-situ carcinomas, such as STIC and STIN. In rest of the patients, there is no evidence of precancerous or cancerous lesions present in the FT. This could of course be due to alternative sites of origin or the lack of extensive screening. Recent developments have mandated SEE-FIM protocol (Sectioning and extensively examining the fimbriated end of the fallopian tube) in screening the FT in cases of HGSOC and RRSO (risk reducing salpingo oophorectomy-prophylactic surgical removal of ovaries and Fallopian tubes in high risk women) [15, 16]. This protocol shows promise in better detection of STIC and has led to detection of sporadic cases of STIC in 4-24% of RRSO samples [9, 10, 17, 18].

However, few of the RRSO samples have no morphological aberrations during pathological examination but lead to incidences of HGSOC in the peritoneum or omentum 2-7 years post prophylactic surgeries [17-21]. This could be attributed to the fact that preneoplasia were already present during the time of surgery. This shows that depending on this continuum model and detection of precursor lesions cannot be the only criteria for determining the tumorigenicity of a tissue. Currently the most commonly attributed site of origin is the FT.

In such morphologically normal RRSO cases, there are no current recommendations as a prophylactic measure to further ensure non incidence of ovarian carcinomas. The timing of the surgery plays a very important role in such RRSO cases. The recommended age is below 40 years. The current genetic screening is available to *BRCA1* (Breast cancer type 1 susceptibility protein) and *BRCA2* (Breast cancer type 2 susceptibility protein) germline mutations. But multiple other genes have been implicated with risk in the homologous recombination-repair pathway [22, 23]. The RRSO is recommended within the age of 40 in high risk women.

One theory suggests that the precursors escape the site of origin, the Fallopian tubes at very early stages, to go and adhere to the ovarian surface epithelium or the peritoneum, where there is a hormonal and reactive oxygen species milieu, conducive for serous ovarian carcinogenesis [24, 25]. Beyond molecular evidences, there is accumulating epidemiological evidence of the benefits of salpingectomy in reducing the ovarian cancer risk, especially when performed earlier than oophorectomy, in a sequential manner [10, 26, 27]. There are also reported cases where the STIC lesions are the metastasis rather than the initial clones [6, 12]. The evolutionary time needed to transition from STIC to HGSOC is 1-5 years which is a very short period [11, 12]. The other well established precursors also support existence of preneoplastic tumour associated changes prior to STIC development due to field cancerization or otherwise.

To study the precursor escape hypothesis, we investigated a case where there was no evidence of precursor lesions present in the Fallopian tubes or ovaries. The patient underwent risk reducing salpingo oophorectomy and total abdominal hysterectomy as she had a family history of breast and ovarian carcinoma and was detected with a pathogenic variant of *BRCA1* gene. Three years later she developed high grade serous ovarian carcinoma in the omentum. The histological analysis of the ovaries and fallopian tubes indicated no morphological abnormalities. There were no dysplastic lesions either in ovaries or in the FT. Further immunohistochemical analysis indicated no precursor lesions in the Fallopian tubes. The goal of this project was to find any molecular evidences to the high grade serous ovarian carcinoma in the Fallopian tubes. To undertake this, we performed WES and RNA sequencing of microdissected epithelia of the Fallopian tubes and the omental tumour.

Materials and methods

Clinicopathological features

This study is approved by the Institutional Human Research Ethics Committee at the University of Newcastle. The patient samples were procured through the Hunter cancer biobank (HCB).

A woman with family history of breast and ovarian cancer underwent genetic testing for *BRCA* mutations at age 43. The patient carried a causative variant in *BRCA1*, c.3331_3334del which introduces a premature stop codon p.Gln1111Asnfs*5. This led to recommendation of TAHBSO (Total Abdominal Hysterectomy and Bilateral Salpingo oophorectomy). The patient underwent TAHBSO in 2014 (aged 43) but was diagnosed with omental serous ovarian carcinoma in 2017. Extensive histopatholgical examination of the Fallopian tubes, ovaries, uterus collected from TAHBSO showed no histological and morphological abnormalities. The omental tumour was positive for Pax8, WT-1, ER (focally), p16 (stromal positive) and negative for p53 (null phenotype), GCDFP 16, GATA-3, CDX2, CK-20, TTF1, confirming the High grade serous ovarian carcinoma.

Tissue microdissection: FFPE tissue blocks were collected. Approximately fifty 10-micron sections were taken for each sample and areas of interest were microdissected manually. A stereoscope was used for the purpose of microdissection, as it provides ample distance between the objective and the slides. A black sheet was used on the background which provided good contrast to distinguish stroma and epithelia. A sterile needle was used in case of FT epithelia, for low surface areas and the sharp edge of the scalpel for the tumour.

Histology and immunohistochemistry (IHC): Hematoxylin and Eosin (H&E) staining was carried out using a standard protocol. The TP53 IHC was conducted using the following protocol. The primary antibody used in this study was Anti p53 protein D07, ID:AM239-10M (ready to use; Biogenex, CA, USA). Biotinylated (Biogenex) secondary antibody was used. Embedded tissue samples were collected from Hunter cancer biobank, sectioned at 6 µm and mounted on slides. Antigen retrieval was performed in 1mM EDTA buffer (0.05% Tween-20, pH 8) followed by the endogenous peroxidase block using 3% (v/v) hydrogen peroxide in absolute methanol. Tissue sections were then blocked in blocking solution (10% Goat Serum in PBS, 0.1% Triton X-100) for 1 hr at room temperature. Following this, tissue sections were incubated overnight at 4°C with primary antibody followed by biotinylated secondary antibody for 1 hour with washes in between with PBS, 0.1% Triton X-100. Sections were incubated with horseradish peroxidase-conjugated streptavidin (Thermo Fischer Scientific). Sections were then exposed to Diaminobenzidine (DAB, Sigma) to develop colour. Sections were counterstained with hematoxylin. Stained slides were imaged at high resolution with the Olympus

DP72 microscope or the Aperio Scanscope slide scanner. The gain and exposure time were set constant across tissue samples.

The DNA and RNA isolation, WES and RNA sequencing were performed by BGI genomics, Hongkong.

DNA and RNA isolation: Deparaffinisation was carried out using the Qiagen deparaffinisation solution (ID: 19093). DNA and RNA were isolated using the QIAamp DNA FFPE Tissue Kit (ID: 56404) and RNeasy FFPE Kit (ID: 73504) respectively and were subjected to the sequencing pipeline.

Whole exome sequencing:

The quantified genomic DNA sample was randomly fragmented by Covaris technology with the size of library fragments averaging 150bp and 250bp (specifically as FFPE samples). DNA fragment ends were repaired, and an "A" base was added at the 3'-ends. Adapters were then ligated to both ends of the tailed DNA fragments for amplification and sequencing. DNA fragments were amplified by ligation-mediated PCR (LM- PCR), purified, and hybridized to the exome array for enrichment. Non-hybridized fragments were then washed out. Captured products were then circularized. The rolling circle amplification (RCA) was performed to produce DNA Nanoballs (DNBs). The captured library was loaded on BGISEQ-500 sequencing platforms, and high-throughput sequencing for each captured library was performed.

Bioinformatics pipeline: Briefly: BGISEQ-500 base calling Software was used for base-calling and data was generated in FASTQ format. Data was filtered to generate clean data. Burrows-Wheeler Aligner software was used to do the alignment. Local realignment around InDels and base quality score recalibration were performed using Genome Analysis Toolkit (GATK, https://www.broadinstitute.org/gatk/guide/bestpractices) and duplicate reads were removed by Picard tools. SNPs and InDels were detected by inhouse software, HaplotypeCaller of GATK (v3.6). The hard-filtering method was applied to get high-confident variant calls. Then the SnpEff tool (http://snpeff.sourceforge.net/SnpEff_manual.html) was applied to perform a series of annotations for variants. Data was filtered to remove adapters and low-quality base ratio. Clean reads were mapped by BWA-MEM method. The HaplotypeCaller of GATK(v3.6) was used to call SNPs and InDels simultaneously through a de-novo assembly of haplotypes, outputted into the VCF files. After high-confident SNPs and InDels were identified, the SnpEff tool (http://snpeff.sourceforge.net/SnpEff_manual.html) was used for gene based and platform based annotations.

RNA sequencing:

The poly-A containing mRNA molecules were separated using poly-T oligo-attached magnetic beads. Following purification, the mRNA were fragmented into smaller pieces using divalent cations under high temperature. The cleaved RNA fragments were copied into first strand cDNA using reverse transcriptase and random primers. Following this second strand cDNA was synthesized using DNA Polymerase I and RNase H. A single 'A' base was added and adapters were ligated. The resultant fragments were purified and enriched with PCR amplification. The final library was a single strand DNA circle. DNA nanoballs (DNBs) were generated with the ssDNA circle by rolling circle replication (RCR) to enhance fluorescent signals at the sequencing process. The DNBs were loaded into the patterned nanoarrays and pair-end reads of 100 bp were read through the BGISEQ-500 platform using Combinational Probe-Anchor Synthesis Sequencing Method.

Bioinformatics pipeline: Sequencing reads were filtered for low base ratio and adapters and clean data was generated. HISAT (Hierarchical Indexing for Spliced Alignment of Transcripts) was used to do genome mapping. StringTie was used to reconstruct transcripts, and Cuffcompare to compare transcripts to reference annotation. CPC tool was used to predict coding potential of novel transcripts and coding novel transcripts were merged with reference transcripts to get a complete reference. SNP and INDEL were called using GATK tool. Clean reads were mapped to reference using Bowtie2 and gene expression levels were calculated with RSEM tool. Pearson correlation between all samples were calculated using cor, perform hierarchical clustering between all samples using hclust, and plots were generated by ggplot2 on R.

Statistics and visualisation: For identifying differentially expressed genes, Poisson distributions were fitted on distributions of gene expression for each group and test statistics were calculated from mean and standard deviation of fitted Poisson distributions. Test statistics were then compared to a cut-off value (i.e. 1.45) to determine the significance of the genes among clinical groups. GO, KEGG, STRING tools were used for GO, KEGG and protein-protein interaction analysis, respectively. R version 3.4.0 was used for all analysis. Circos plot was generated using https://github.com/venyao/shinyCircos. p-values were set at either 0.01 or 0.05, varying upon number of genes detected. Statistical data was verified on Graphpad prism 8.

Results:

Experimental design: The goal of this study is to test the precursor escape model. Previous work has successfully shown the continuing lesions turning from preneoplasia to dysplasia [13, 14]. We have investigated a case where there was no evidence of precursor lesions present in the Fallopian tubes or ovaries, and the omental tumour is spatially and timewise independent of the FT tissue for three years. The patient underwent risk reducing salpingo oophorectomy and total abdominal hysterectomy

as she had a pathogenic variant of BRCA1 gene. Three years later she developed high grade serous ovarian carcinoma in the omentum. Further immunohistochemical analysis indicated no precursor lesions in the Fallopian tubes. The omental tumour was p53 null phenotype indicating loss of function *TP53* mutations. This makes it hard to detect p53 signatures using p53 IHC. Thus we used LEF1, PAX8, PAX2, Ki67, FOXJ1 and STMN1 to detect these lesions [28-31]. We found no p53 signatures in the Fallopian tube. The null phenotype, p53 and the strategy summary is indicated in **Figure1**. The goal of this project was to find any molecular evidences to the high grade serous ovarian carcinoma in the Fallopian tubes. To undertake this, we performed WES and RNA sequencing of microdissected epithelia of the Fallopian tubes and the omental tumour. This approach allowed us to identify nonsynonymous and synonymous sequence changes, including single base and small insertion or deletion mutations, as well as copy number alterations and heterozygosity in coding genes. We also found atleast 90.88% correlation between the RNA seq and WES data for each sample, further confirming our data. As the samples were FFPE and microdissected and 4 years old, a lot of care was taken to screen as many sections as possible

Tumour associated mutations found in morphologically normal Fallopian tubes

As expected, we identified sequence changes in the *TP53* gene, which were known pathogenic variants leading to loss of expression. Three key alterations were also present in the FT2 tissue. This indicates clear clonal identity and is evidence for site of origin. The site of origin of omental tumour was the right Fallopian tube. We also found pathogenic variants of *PI3KCA* gene, *MRE11, Rad51, CDK12, APC*, etc in the tumour. The affected genes have been summarised in **Figure2A** in the circus plot. The pathways with highest number of pathogenic variants are indicated in **Figure2B**.

Omental tumour displays high CNV and LOH load

There were 128 pathogenic CNVs in the omental tumour in relation to 6 in FT1 and 9 in FT2. Represented in **Figure3A, 3B and SFigure1A, B.** The large scale structural changes are indicated in the LOH plot for the omental tumour which is seen in **Figure3B**. HGSOC displays chromosomal gains, losses, instability, chromotrypsis and Loss of heterozygosity (LOH) specifically in chromosome 17p and 17p, which houses *TP53*. These changes were consistent with the omental tumour LOH data. This suggests the tumour evolution has mostly occurred in the omentum.

Oncogenic pathway activated in morphologically normal FT2

The Differentially expressed genes (DEGs) are shown in the Venn diagram. The fold change threshold was set to 1.5 and false discovery rate at 0.05 **(Figure4A).** The correlation between samples was determined by the Pearson's coefficient. Multiple genes and transcripts have been identified which

need further probing. As the samples are FFPE and have higher degradation, the samples were very small lesions and no repeats were available, very stringent criteria were set to verify the results. The hierarchical clustering indicated some DEGs common between FT2 and OT **(Figure 4D).**

The detailed pathway analysis was done through the KEGG method and gene ontology analysis and is illustrated in **Figure5B and C.** The most dysregulated pathways were pathways of cancer, RNA splicing processing, Wnt pathway, PI3K pathway, ER dysregulation, cell cycle dysregulation.

Mutational analysis indicates FT origin for HGSOC

Figure6 shows the tumour evolution pathway, which was generated by comparative analysis of pathogenic variants through SNPs and Indels. A comprehensive list was generated of which, the variants which have already been implicated to HGSOC through previous studies has been shown. The plot shows the branching from FT2 to the HGSOC. The common pathogenic variants between the two samples is indicated in red.

Discussion:

Fallopian tube secretory epithelial cells are the closest genetically, morphologically, immunohistochemically, embryologically, in terms of gene and protein expression [32]. The transition of the intermittent secretory and ciliated cell types to the secretory cell fate is the first step towards serous ovarian carcinogenesis [8, 31, 33, 34]. Thus, they display high propensity towards incidences of HGSOC than any other epithelia and also constitute highest number of incidences based on histotype.

Upon more studies coming to light which have conducted extensive examination of the FT for preneoplastic lesions, more number of sporadic cases of STIC had been detected and more patients were found to carry multitude of dysplasia and preneoplasia in cases of RRSO [9]. These lesions have been further investigated to probe any clonal links with co-occurring HGSOCs in the ovaries and peritoneum [12-14, 35]. Despite these conclusive evidences, some cases don't show any dysplasia in the FT during RRSO. This can be either due to origin of carcinogenesis in other sites such as ovary and peritoneum. Or another possibility is the precursor escape model implicating FT as the site of origin [24] even in cases which do not present the classical pathological model of normal epithelia-preneoplasia-neoplasia continuum. The continuum model especially has drawbacks as it is dependent on the classical sectioning and histology screening techniques. Most RRSO samples are not subjected to extensive immunohistochemical probing unless morphologically aberrant STIN/STIC are detected. Confirming the site of origin is of extreme prognostic and preventive significance in women with high risk to ovarian cancer.

Over 30% of all HGSOC incidences show p53 null phenotype. All of these cases may or may not have exhibited p53 signatures and/or STIC as they lacked TP53 expression as well. Normal FT does not express p53 protein as well. Thus, it becomes very hard to detect such p53 signatures. These cases form almost a third of all incidences of HGSOC and do not have any likelihood of being detected at the preneoplastic stage of p53 signatures, thus may create ambiguity in the site of origin of HGSOC in such cases.

There are also two other physiological reasons which support the precursor escape theory. One is the location and function of fimbriae. During ovulation the fimbrial tissue brushes on the ovarian surface epithelium creating an interaction between both the epithelia. This wear and tear may lead to dropping of the FT epithelia from the fimbriae either on the ovarian surface epithelium or the peritoneum. In this particular patient the FTs were adherent to the ovaries. Such cases are reported at around 30% of all BSO (Bilateral salpingo oophorectomies). The biological function of this adherence is unknown. The other reason is hormonal. The progesterone high, luteal phase leads to flushing of the Fallopian tube epithelia with progesterone leading to necroapoptosis of cells specifically high in DNA damage such as those that have acquired *TP53* mutations, the mechanisms of which are well established. Some cells can circumvent this effect and such cells may be exfoliated from the FT mucosa. This is known as the exfoliation effect [36]. The flux of the Fallopian tube fluid is from the utero tubal junction towards the ovary. If such cells are exfoliated, they do have higher physiological probability of landing on the ovarian surface epithelium or the peritoneum as the fimbriae opens into the peritoneum. If there are any inherent mechanisms leading to involutions of precursor lesions and their escape from the FT needs detailed investigations.

There are also genetic factors that create a more conducive environment for such escape. The inherited germline *BRCA1/2* mutations are of various nature. Specifically, pathological variants lead to severe change in the protein length, fold and function leading to compromised DNA damage repair mechanisms. Such cellular environment is conducive for accumulation of *TP53* mutations. The gene penetrance is also an important factor in *BRCA1/2* mutations [37, 38]. What determines acquisition of *TP53* loss of function mutations and the p53 null phenotype is unknown. What are the specific DNA damage mechanism failures which lead to loss of function *TP53* mutations over mutations leading to TP53 overexpression is unknown. Majority of BRCA1/2 related HGSOCs are implicated to be of FT origin [39]. Alternative detection systems and alternative markers such as LEF1 [28] need to be established for detection of such p53 signatures. Detailed studies need to address the correlation between the nature of BRCA1/2 mutations and TP53 mutations, if any.

Our work is the first effort in establishing in-vivo evidence for the biological plausibility of the precursor escape model. To achieve this, a spatiotemporal analysis of patient tissue has been done to achieve retrospective lineage tracing. The gap of three years between the RRSO samples and the omental tumour gave us a good opportunity for a time lapse analysis. With presence of identical *TP53* mutations in the right FT and the omental tumour, clonal identity is established, in samples separated between time and space. More such cases need to be investigated to help amplify proof for the benefits of sequential salpingectomy followed by oophorectomy [40]. Early *BRCA1/2* screening and execution of RRSO prior to age 40 is also very critical in avoiding such cases. Especially in cases where women prefer to postpone RRSO to save their fertility or to postpone surgical menopause, sequential salpingectomy followed by oophorectomy is a good alternative prophylactic mechanism.

Figure legends:

Figure1: Histopathology of patient samples. Enclosed within black dotted lines comprises the ovaries, FTs and uterus collected during the TAH-BSO. The histology of the two FT mucosa are shown in **B, E** and **D, G**. The omental tumour occurred three years later and its histology shown in **C, F.** The tumours were p53 null phenotype with complete absence of p53, shown in **I.** Inset positive control for p53. Histologically normal FTs show no expression of p53 as well, as seen in **H, J.**

Figure2: The mutational profile of high grade serous ovarian carcinoma in comparison to Fallopian tubes. A. The circos plots represent SNP and INDELs. Some pathological variants which lead to differentially expressed genes are named in the outer most rings. B. The mutational pathway analysis shows the top pathways with pathological variants belonged to the transcriptional dysregulation in cancer pathway, includes ribosomal complexes, splicing, histone demethylases. FT2 showed as many pathogenic variants as the omental tumour.

Figure3: The LOH and CNV profiles of the omental tumour. A. The circus plot shows the LOH variations for Left and right FT and the outermost circle depicts changes in genes due to LOH in the omental tumour. **B.** The copy number changes plot showing specific chromosomal positions which are either amplified or lost copies.

Figure4: The transcriptomic profile of histopathologically normal FT Vs omental tumour. A. The venn diagram summarizes the differences in gene expression between FT1, FT2 and omental tumour. Cut off in fold change set at 1.45. **B.** The heatmap shows the Pearson's coefficient plot showing FT1 and 2 are closer genomically in comparison to the omental tumour. The intensity of the colour indicates the Pearson value or correlation depth. **C.** The barplot summarises RNA sequencing results comprising total genes and transcripts identified which showed p value of <0.05. The dfferentially expressed genes are well correlated as FT samples skew together and more changes are found between OT Vs FT. **D.** The hierarchical clustering between Normal FT Vs OT. The colour scale represents log2 transformed fold change.

Figure5: The pathway analysis. A. The scatterplots indicate the fold change distribution of differentially expressed genes between samples, FT1 Vs OT, FT2 Vs OT, FT1 Vs FT2. **B.** The dotplots indicate the KEGG pathway analysis for the three comparisons. The gene count is indicated by the size of the dot and the colour scale indicates the p-values. **C.** The barplots indicate number of DEGs under different cellular compartments and functions. The colour scheme indicates the p-values.

Figure6: The genetic evolution plot. The root is the inherited BRCA1 mutation, and the FTs are less evolved in the timescale, followed by the omental tumour linked to FT2 as they share common

mutations. The OT has multitudes of pathogenic variants and CNVs, thus is placed at the forefront of the evolution plot.

SFigure1: The copy number changes plot. The FT samples, FT1 (**A.**) and FT2 (**B.**) showing specific chromosomal positions which are either amplified or have lost copies.

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Figure1

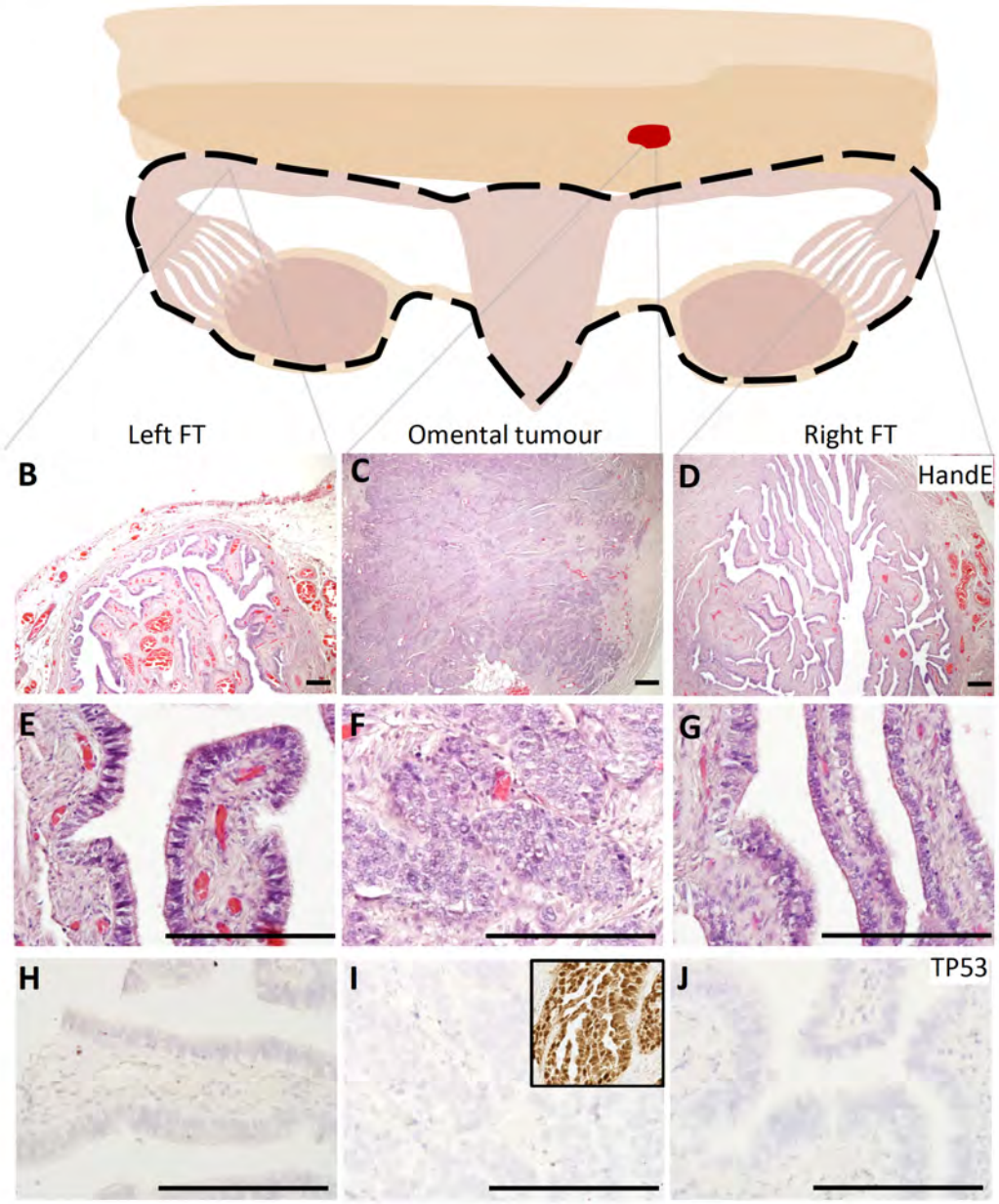
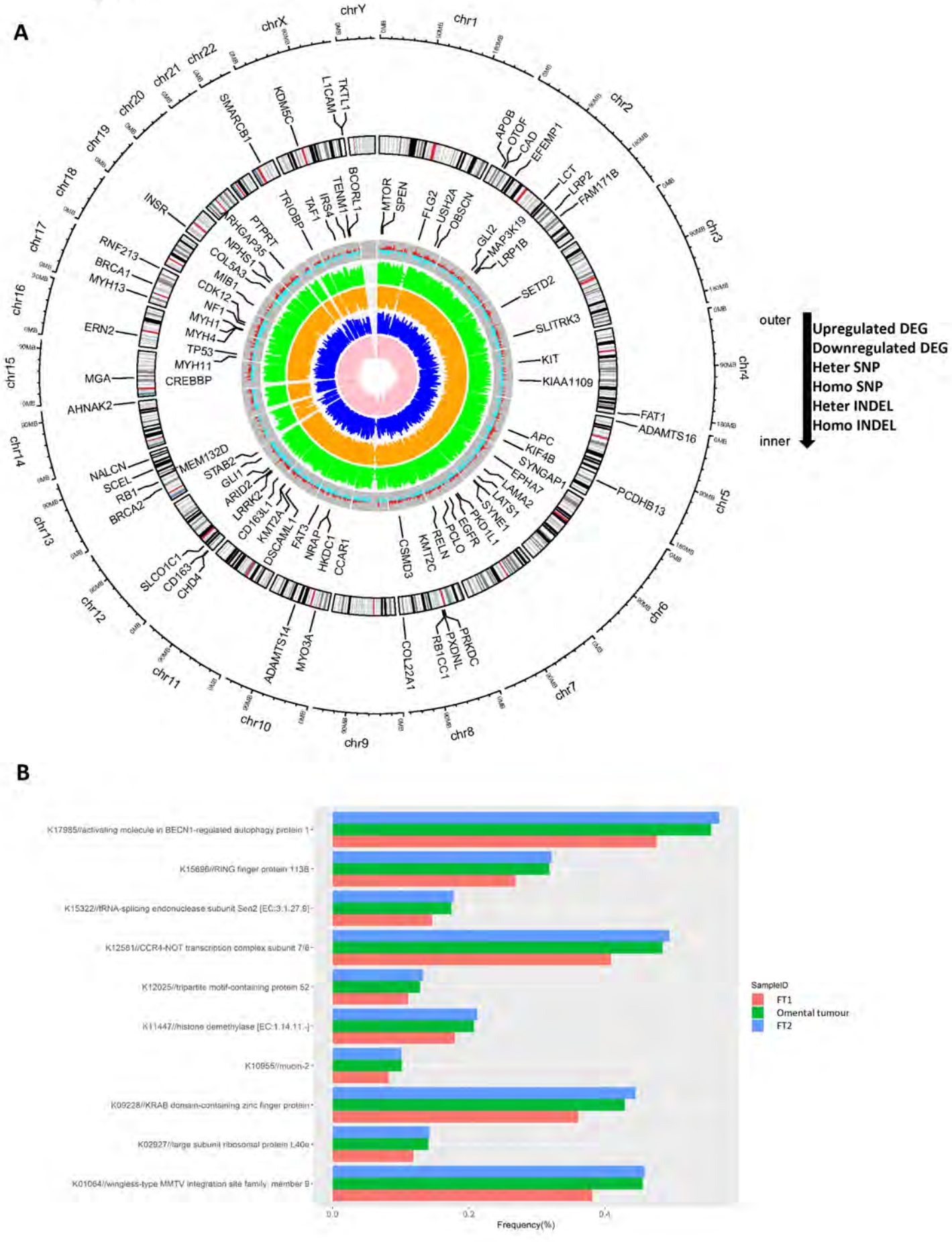


Figure2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Frequency(%)
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Omental tumour
FT2

Figure3

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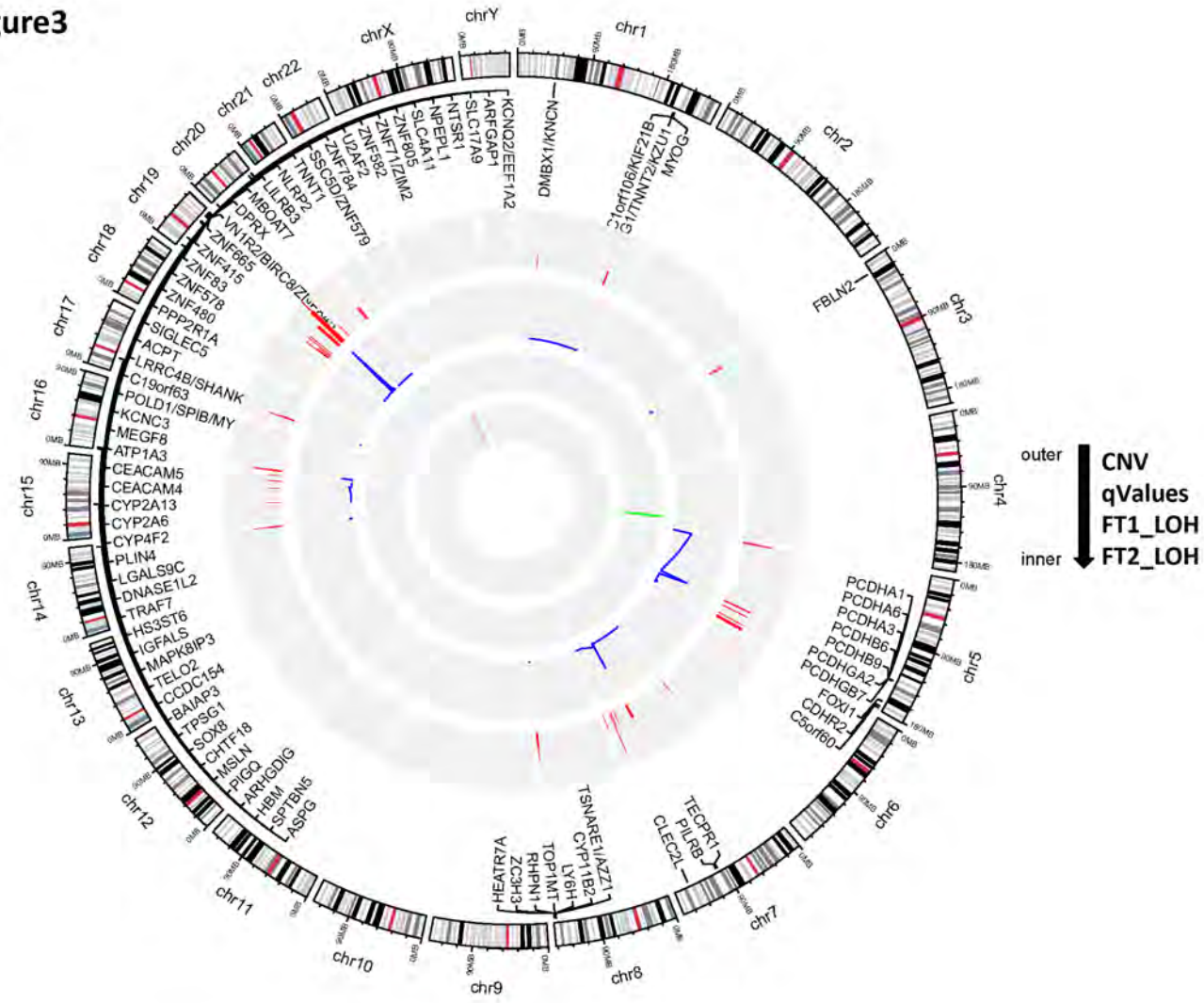
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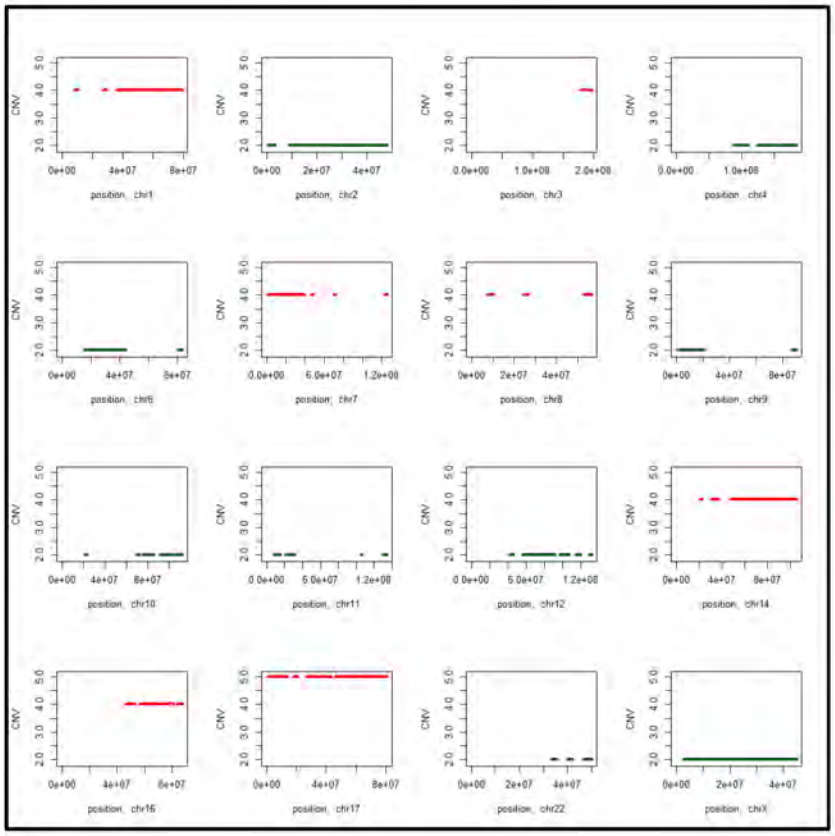
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position, chr3
position, chr4
position, chr5
position, chr6
position, chr7
position, chr8
position, chr9
position, chr10
position, chr11
position, chr12
position, chr14
position, chr16
position, chr17
position, chr22
position, chrX

Figure4

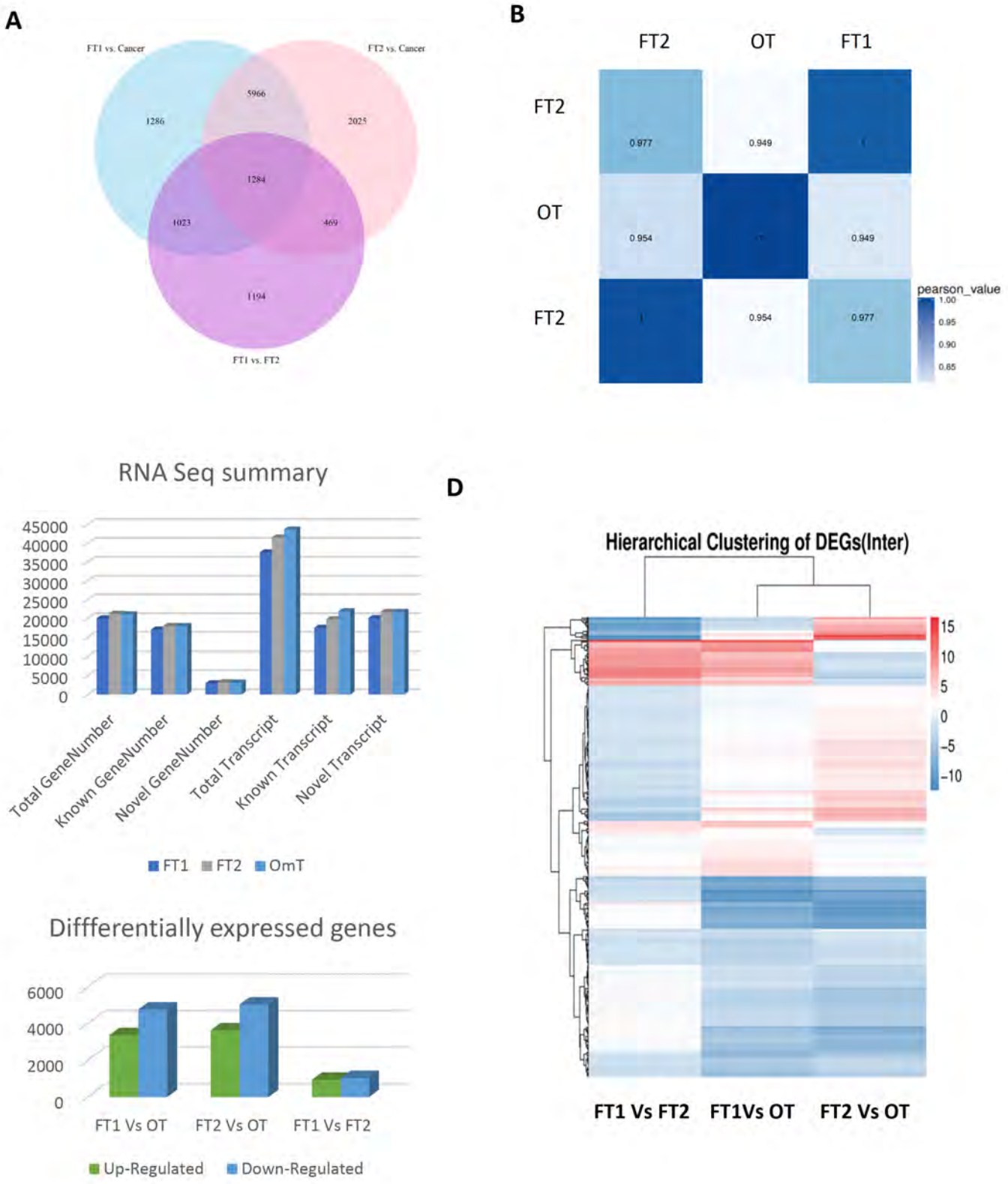

A
FT1 vs. Cancer
FT2 vs. Cancer
FT1 vs. FT2
5966
1286
2025
1284
1023
469
1194
B
FT2
OT
FT1
FT2
OT
FT2
0.977
0.949
0.954
0.949
0.954
0.977
pearson_value
1.00
0.95
0.90
0.85
C
RNA Seq summary
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Total GeneNumber
Known GeneNumber
Novel GeneNumber
Total Transcript
Known Transcript
Novel Transcript
FT1
FT2
OmT
Diffferentially expressed genes
6000
4000
2000
0
FT1 Vs OT
FT2 Vs OT
FT1 Vs FT2
Up-Regulated
Down-Regulated
D
Hierarchical Clustering of DEGs(Inter)
15
10
5
0
-5
-10
FT1 Vs FT2
FT1Vs OT
FT2 Vs OT

Figure5

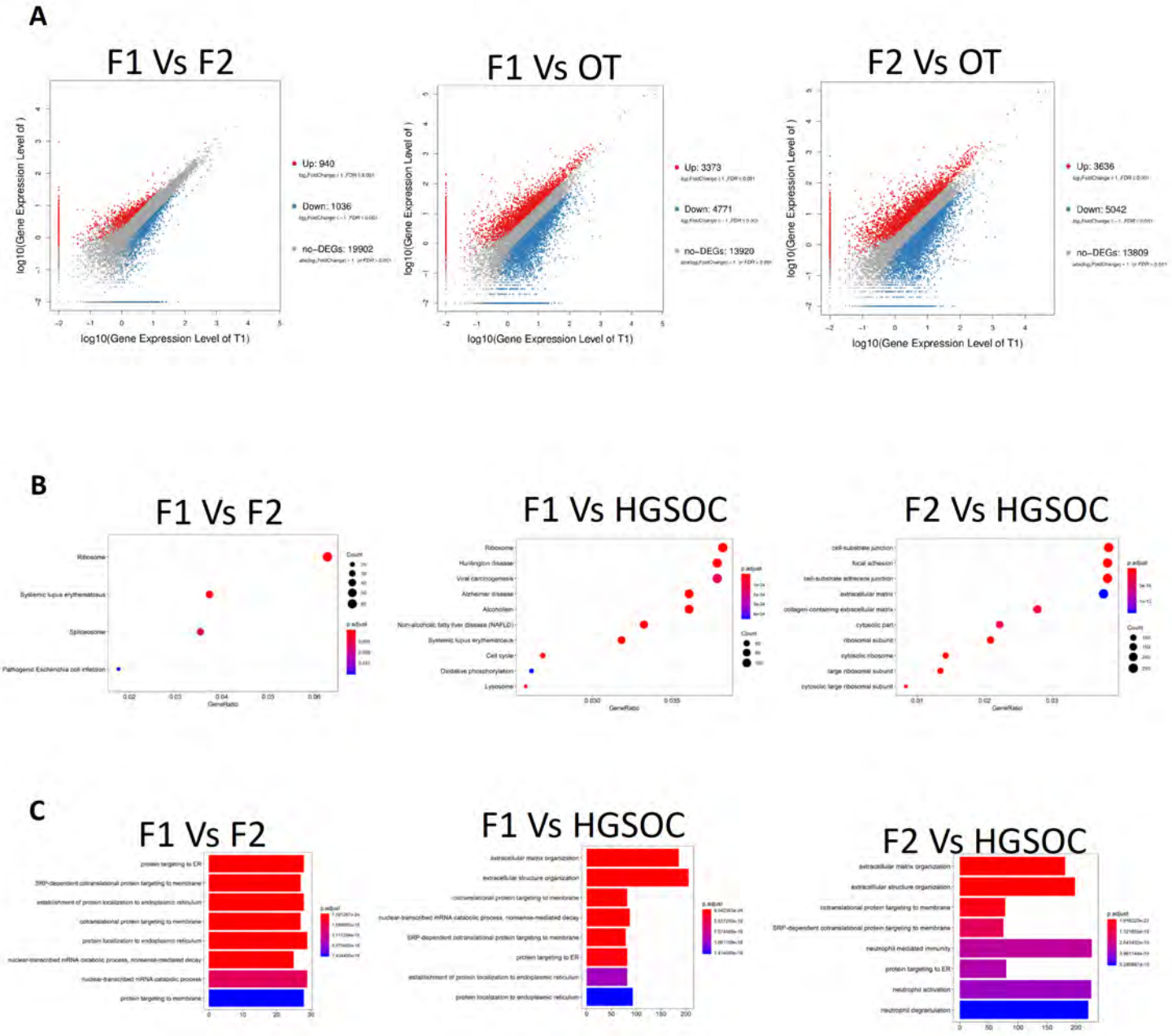
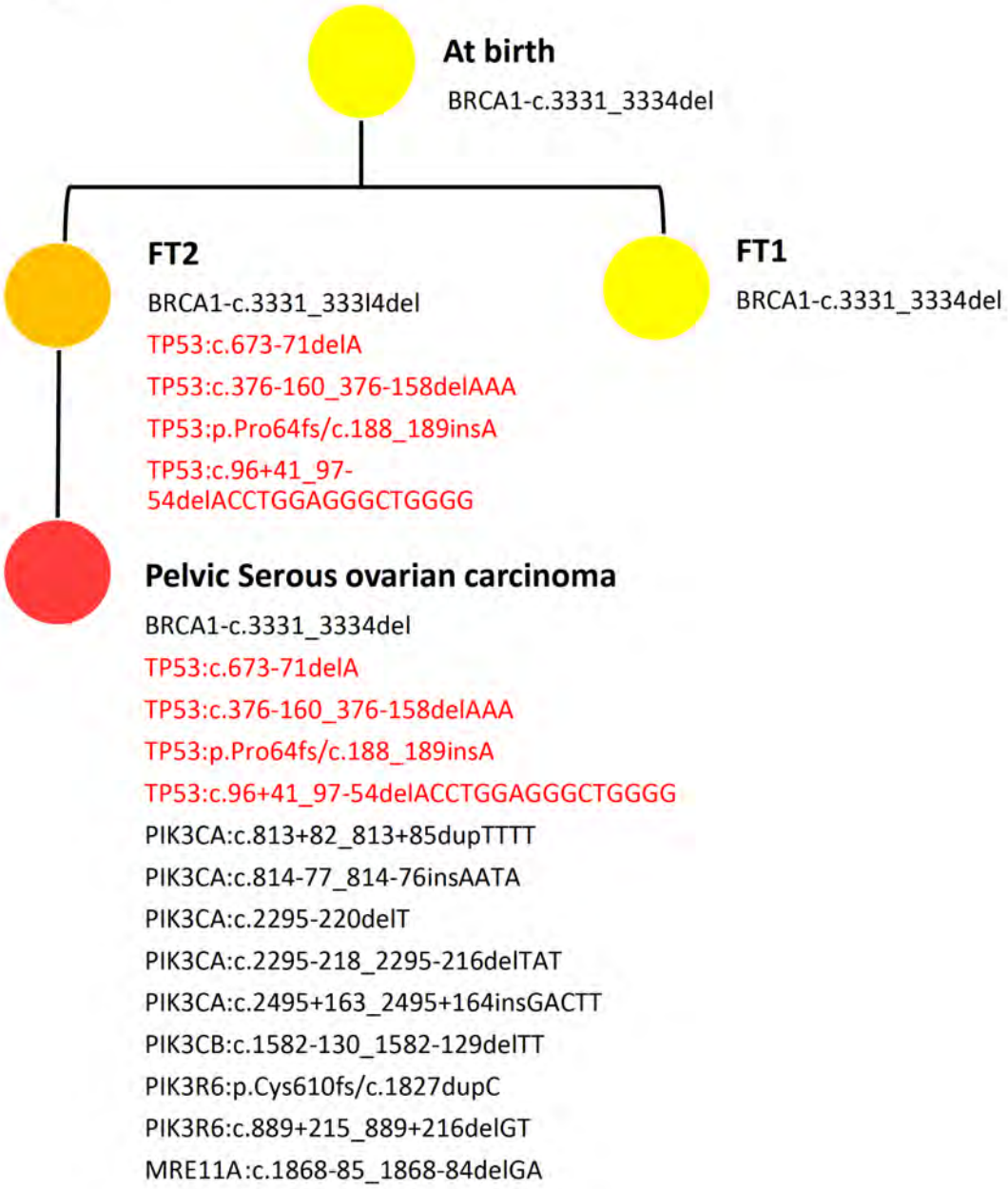
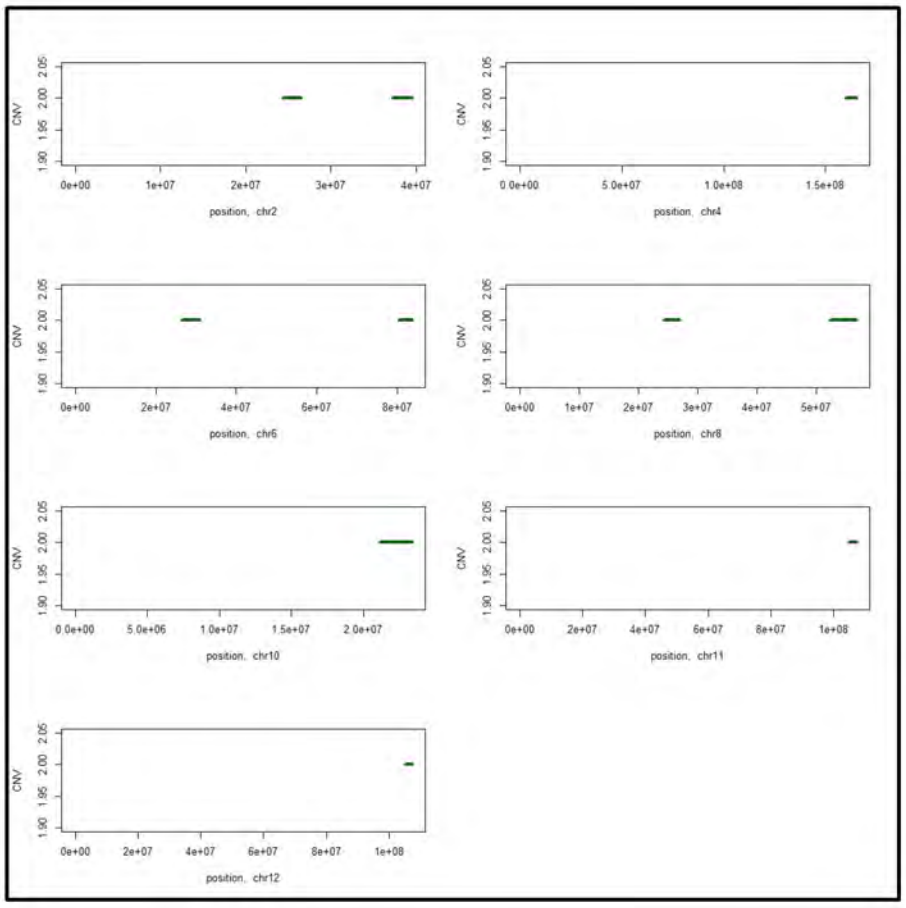

A
F1 Vs F2
log10(Gene Expression Level of)
log10(Gene Expression Level of T1)
Up: 940
Down: 1036
no-DEGs: 19902
F1 Vs OT
log10(Gene Expression Level of)
log10(Gene Expression Level of T1)
Up: 3373
Down: 4771
no-DEGs: 13920
F2 Vs OT
log10(Gene Expression Level of)
log10(Gene Expression Level of T1)
Up: 3636
Down: 5042
no-DEGs: 13809
B
F1 Vs F2
Ribosome
Systemic lupus erythematosus
Spliceosome
Pathogenic Escherichia coli infection
GeneRatio
F1 Vs HGSOC
Ribosome
Huntington disease
Viral carcinogenesis
Alzheimer disease
Alcoholism
Non-alcoholic fatty liver disease (NAFLD)
Systemic lupus erythematosus
Cell cycle
Oxidative phosphorylation
Lysosome
GeneRatio
F2 Vs HGSOC
cell-substrate junction
focal adhesion
cell-substrate adherens junction
extracellular matrix
collagen-containing extracellular matrix
cytosolic part
ribosomal subunit
cytosolic ribosome
large ribosomal subunit
cytosolic large ribosomal subunit
GeneRatio
C
F1 Vs F2
protein targeting to ER
SRP-dependent cotranslational protein targeting to membrane
establishment of protein localization to endoplasmic reticulum
cotranslational protein targeting to membrane
protein localization to endoplasmic reticulum
nuclear-transcribed mRNA catabolic process, nonsense-mediated decay
nuclear-transcribed mRNA catabolic process
protein targeting to membrane
F1 Vs HGSOC
extracellular matrix organization
extracellular structure organization
cotranslational protein targeting to membrane
nuclear-transcribed mRNA catabolic process, nonsense-mediated decay
SRP-dependent cotranslational protein targeting to membrane
protein targeting to ER
establishment of protein localization to endoplasmic reticulum
protein localization to endoplasmic reticulum
F2 Vs HGSOC
extracellular matrix organization
extracellular structure organization
cotranslational protein targeting to membrane
SRP-dependent cotranslational protein targeting to membrane
neutrophil mediated immunity
protein targeting to ER
neutrophil activation
neutrophil degranulation

Figure6

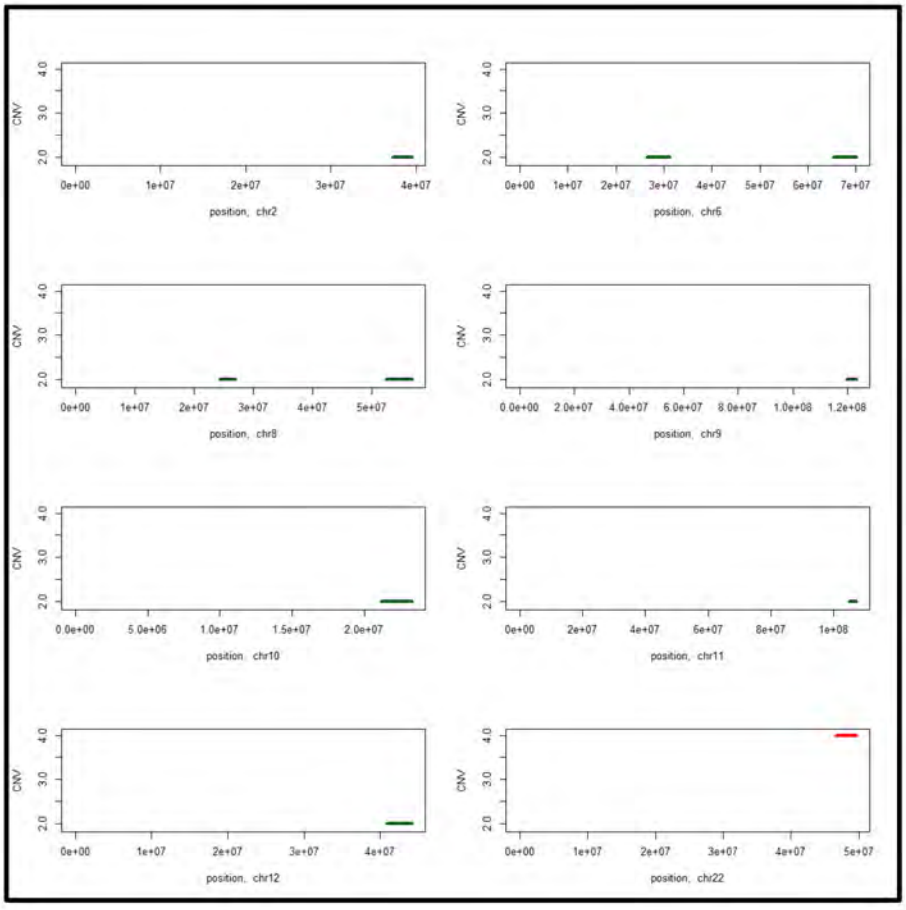

At birth
BRCA1-c.3331_3334del
FT2
BRCA1-c.3331_333I4del
TP53:c.673-71delA
TP53:c.376-160_376-158delAAA
TP53:p.Pro64fs/c.188_189insA
TP53:c.96+41_97-54delACCTGGAGGGCTGGGG
FT1
BRCA1-c.3331_3334del
Pelvic Serous ovarian carcinoma
BRCA1-c.3331_3334del
TP53:c.673-71delA
TP53:c.376-160_376-158delAAA
TP53:p.Pro64fs/c.188_189insA
TP53:c.96+41_97-54delACCTGGAGGGCTGGGG
PIK3CA:c.813+82_813+85dupTTTT
PIK3CA:c.814-77_814-76insAATA
PIK3CA:c.2295-220delT
PIK3CA:c.2295-218_2295-216delTAT
PIK3CA:c.2495+163_2495+164insGACTT
PIK3CB:c.1582-130_1582-129delTT
PIK3R6:p.Cys610fs/c.1827dupC
PIK3R6:c.889+215_889+216delGT
MRE11A:c.1868-85_1868-84delGA

SFigure1

A FT1



B FT2



Chapter 4: Molecular characterisation of papillary tubal hyperplasia: the putative precursors of low grade serous carcinoma

Chapter 4

Preface

Papillary tubal hyperplasia regularly co-occur with serous borderline tumours and low grade serous carcinomas. Unlike the High grade serous carcinomas, a continuing clonal link is not established between precursors and the carcinomas. The goal of this chapter is to see whether atypical endosalpingiosis found in a patient has any clonal link to bilateral papillary tubal hyperplasia in the FTs. To achieve this, we use whole exome sequencing (WES) and RNA sequencing. This chapter aims in identifying molecular markers of low grade serous carcinoma in putative precursors, the papillary tubal hyperplasia. It also establishes the Fallopian tube origin in a case of peritoneal low grade serous carcinoma.

UON Graduate research

Co-authorship declaration

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

Statement of Author's contributions

This statement summarizes the intellectual inputs by all the authors stated in the research article titled "Molecular characterisation of papillary tubal hyperplasia: the putative precursors of low grade serous carcinoma". This research article is submitted in the journal Gynaecology Oncology.

Authors	Statement of contribution
Prathima B Nagendra (First author)	Conducted experiments, wrote the initial draft and made figures for the manuscript Edited and revised the manuscript
Pradeep S Tanwar (Corresponding author)	Designed experiments, edited the manuscript and provided intellectual inputs

Prathima B Nagendra

Pradeep S Tanwar

Dr Lesley MacDonald-Wicks-Faculty Assistant Dean Research training

UON Graduate research

Co-authorship declaration

Molecular characterisation of papillary tubal hyperplasia: the putative precursors of low grade serous carcinoma

Abstract

Low grade serous carcinomas are very rare tumours, with lower growth rates but are extremely chemoresistant. Unlike high grade serous carcinomas, a well charted preneoplasia to carcinoma transition is not established. The only implicated preneoplasia of low-grade serous carcinomas are papillary tubal hyperplasia [8], which are further a rarity (0.1% incidence rate). These lesions also originate in the Fallopian tube secretory epithelial cells (FTSEC). No immunohistochemical markers are available and these lesions were not subjected to genetic and transcriptomic analysis so far. We found a clonal link between papillary tubal hyperplasia and atypical endosalpingiosis. We did not find any driver mutations in the Ras pathway in the papillary tubal hyperplasia or the endosalpingiosis, however we found differentially expressed genes in the Ras pathway, transcriptional regulation pathway and RNA splicing suggesting genomewide changes predate driver mutations and may be necessary to create an environment of mutagenesis.

This study aims to identify molecular markers of low-grade serous carcinoma in putative precursors, the papillary tubal hyperplasia. It also establishes the Fallopian tube origin in a case of peritoneal low grade serous carcinoma.

Introduction

Low grade serous carcinoma constitutes approximately 7-10% of all ovarian cancer incidences. They generally occur in low stage (80-90% of all cases in Stage I/II [1-3]. They have relatively low proliferation rates (>10 and <25% rate of proliferation), thus grow very slowly [4, 5]. Although the disease is slow in progression, the treatment is complicated due to the inherent chemoresistant nature of the disease. The current treatment includes surgical removal of affected organs, Fallopian tubes and ovaries combined with multimode chemotherapy [2, 6, 7]. The disease is multifocal and is either spread or present in the peritoneum in around 40-50% of all incidences at the time of presentation of the disease [2, 4, 8]. This leads to surgical complications and residual disease issues and higher relapse rates (around 40% within 5 years of incidence). This calls for a need for early detection and better therapeutic measures.

LGSCs are typical of mutations in the *KRAS* (Kirsten RAt Sarcoma virus gene), *BRAF* (Raf murine sarcoma viral oncogene homolog B), or *ERBB2* (V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2) genes [4, 6, 9-11]. At least 80% of the tumours probed for mutations have a mutation in one of these genes. Mutations in each of these genes are mutually exclusive. KRAS, BRAF, and ERBB2 are upstream regulators of mitogen-activated protein kinase (MAPK). Mutations found in LGSC tumours lead to constitutive activation of the MAPK pathway resulting in uncontrolled proliferation. ERBB2 or HER2 is a receptor tyrosine kinase family protein, which regulates outgrowth and stabilization of peripheral microtubules. It is known to have cross talk with the Estrogen signalling and also in transcriptional regulation of STAT genes and SRC. TP53 mutations are very rare in LGSC (between 3 to 8% in the reports) and generally marks the transition of LGSC to HGSOC [12].

LGSC is characterised by either focal or extensive invasive components characterized by micropapillae constituting small round nests of cells that have an ability to infiltrate the stroma in a haphazard pattern, after longer incubation times and during advanced stages. The disease is also found to be multifocal. In comparison to the HGSOC, the micropapillae donot have extensive fibrovascular cores and are surrounded by a clear space or cleft. The tumours are elongated and have branched appearance. The most common features are the psammonna bodies and are present in most patients [4, 13].

LGSC cells are low mitotic entities (less than 12%- criteria in distinction between PSC and LGSC), have higher nuclear to cytoplasmic ratio compared to normal epithelia, but the nuclei are uniform and round and the chromatin is even, unlike the hyper chromatin in high grade serous carcinoma (<2:1 for low grade and >3:1 for high grade and also the number of total cells with hyperchromasia are low). The nucleoli are small and do not vary greatly and display low levels of microsatellite instability and

chromosomal disruption. The intermediate stages are known to be cystadenomas in the ovaries and atypical proliferative serous tumors (APSTs) and serous borderline tumors (SBTs)]. A long dispute on the APSTs and SBTs ended when they were declared synonymous in 2003 by NCI, USA [11, 13, 14].

Unlike the high-grade SOCs which display an array of dysplastic and preneoplastic lesions which are well ordered in terms of their progression from dysplasia to preneoplasia to neoplasia, the dysplastic lesions of the ovaries and FTs are not implicated to LGSC. The secretory cell expansions (SCEs), secretory cell outgrowths (SCOUTs), p53 signatures and neoplastic Serous tubal intraepithelial carcinomas (STICs) are all implicated in HGSOC. All these lesions share histological and immunohistochemical markers with the HGSOC [15, 16]. In case of LGSOC, some cyst adenomas have been implicated to LGSOC as they are found to co-occur with LGSOC. The mutational analysis to show clonal identity and a continuum in progression has not been shown. Most APSTs and SBTs already have *KRAS, BRAF* or *ERBB2* mutations and thus fall under the category similar to STICs [13, 14]. One lesion reported in the FTs were known as salpingoliths. These are now known as papillary tubal hyperplasia [17]. They are typically epithelial calcifications and small papillae which are very distinct to LGSOC. They are identified by psammonna bodies and may be multi-focal co-occurring in peritoneum, ovaries and FTs [17]. It is hard to determine if they actually originate in the FT or ovaries or can originate in both the sites [18, 19]. Currently only morphological and histological characteristics are determined and no immunohistochemical markers are known. However, these lesions are very distinct and can be identified by their histology alone. Whether these growths have any mutations is not very clear [20]. Are the *KRAS, BRAF* or *ERBB2* mutations the first of the genetic changes or do they need other changes in the gene and protein expression which create a cellular environment conducive for mutagenesis? This question is addressed in our work here. We have micro-dissected papillary tubal hyperplasia and co-occurring endosalpingiosis and subjected them to genomic and transcriptomic analysis.

Results:

Experimental design: The goal of this study was to test if the atypical endosalpingiosis in the peritoneum and the papillary tubal hyperplasia in the Fallopian tubes were originated in the Fallopian tubes or were independent lesions with different trajectories. The goal was also to figure whether driver mutations of LGSOC in *KRAS, BRAF* and *ERBB2* genes occur in the early stages of LGSC evolution. Papillary tubal hyperplasia are the earliest known precursors of LGSOC.

The histology indicating micropapillary growths and psammonna bodies are shown in **Figure1**. The microdissected section has been encircled in dotted black lines.

To undertake this, we performed WES and RNA sequencing of microdissected PTH and AES lesions. The approach also detects germline mutations. No germline mutations were found in this patient. This approach allowed us to identify nonsynonymous and synonymous sequence changes, including single base and small insertion or deletion mutations, as well as copy number alterations and heterozygosity in coding genes. We also found 87-94% correlation between the RNA seq and WES data for each sample, further confirming our data. As the samples were FFPE and microdissected and 2 years old, a lot of care was taken to screen as multiple sections (50 in each sample after which the lesions ran into the normal tissue).

Common tumour associated mutations found between PTH and AES lesions

Pathogenic variants previously implicated to LGSOC were not found in any of the lesions. This may indicate that the lesions are very early in their evolutionary track. But these lesions are morphologically consistent to LGSOCs. However, all three samples showed multiple mutations common between one another and all three. The affected genes have been summarised in **Figure2A** in the circus plot. The pathways with highest number of pathogenic variants found correlation with the RNA Seq data which is indicated in **Figure5D**.

CNV and LOH profiles of PTH and AES

The goal of this study is to characterise molecular markers in rare lesions such as PTH and AES. Although both the lesions have similar morphology and proliferation rates, the AES displayed multitudes of copy number variations. The specific genes are indicated in the Circos plot in **Figure3A.**

The LOH sites common between AES and PTH in FT1 and FT2 are indicated in the circus plots. The unique sites are indicated in **Figure4A, B.** Loss of heterozygosity is a hallmark trait of HGSOC and is not highly prevalent in LGSOC.

Oncogenic pathway activated in AES

The Differentially expressed genes (DEGs) are shown in the Venn diagram. The fold change threshold was set to 1.5 **(Figure5A).** The correlation between samples was determined by the Pearson's coefficient **(Figure5C).** The interesting point is that despite having multitude of mutations in common, the correlation between gene expression of AES and PTH was around 80% which is due to different sites of the lesions. The AES seems to be highly acclimatised to the peritoneal microenvironment. Multiple novel genes and transcripts have been identified which need further probing. As the samples are FFPE and have higher degradation, the samples were very small lesions and no repeats were available, very stringent criteria were set to verify the results. The hierarchical clustering indicated many DEGs common between AES and PTH **(Figure5B).**

The detailed pathway analysis was done through the KEGG method and gene ontology analysis and is illustrated in **Figure6A and B.** The most dysregulated pathways were pathways of cancer, transcriptional misregulation in cancer, ribosomes, phagosomes, lysosomes and the immune response. The top few DEGs between PTH1,2 Vs AES belonging to above mentioned pathways are as follows: RN7SK, RN7SL2, RN7SL1, RNU2-1, RNU105A, OVGP1, ELK2AP, FOS, DONSON, IGFBP5, EEF1A1, FOSB, ZFP36, RPS27, NR4A1, EGR1, JUNB, SCARNA13, TMSB10, COL6A1, FTL and EEF2.

Discussion

Low grade serous ovarian carcinoma is a complex disease with low growth rates, high heterogeneity and are very chemoresistant. Thus, understanding the exact site of origin and mechanisms of early disease occurrence is important. These tumours are more multifocal and present multi-site occurrence than any other form of peritoneal cancer that are mostly diagnosed at stages, I and II.

The most likely precursors which may likely occur prior to even cystadenomas or endosalpingiosis are the papillary tubal hyperplasia. These lesions are the most frequently co-occurring lesions with both LGSC and SBTs [17, 19, 21]. These are the lesions which exhibit all morphological aberrations of LGSOC but lack the proliferative potential and invasiveness of LGSC. Thus, PTH form very compelling precursor lesions for endosalpingiosis, SBTs and LGSC of the ovaries and Fallopian tubes.

So far one study has conducted mutational analysis (although specifically for only the *KRAS* gene) and found no *KRAS* mutations [20]. No other study had investigated the molecular changes leading to the occurrence of PTH. Our study extensively investigated the genomic and transcriptomic changes through WES and RNA sequencing. The results suggest that mutational changes need not be the first step as is the case of *TP53* mutations in high grade serous ovarian carcinoma.

The most important point of contention is the site of origin for LGSC, as it is mostly multifocal [22]. We establish clonal identity between PTH and endosalpingiosis in the peritoneum, as they share multitudes of SNPs and INDELs and same mutations occurring in two different sites independently is a biological non plausibility. We also found a clonal link between the PTH in the two tubes. This indicates that PTH originated in one of the tubes and spread to the other FT and peritoneum.

We also find non-driver abberrations in the KRAS pathway, transcriptional misregulation of cancer, MAPK pathway, estrogen pathway and Calcium signalling pathway **(Figure5D, 6)** [9, 12, 23-25]. All these indicates a need for pre-driver mutational changes at the whole genome and whole transcriptomic levels which create an intra cellular niche responsible for driver mutagenesis in cases of LGSC. It is more of a priming mechanism for mutagenesis. The misregulation of calcium signalling pathway may have lead to mucosal calcifications and generation of psammonna bodies. The *MAPK*

genes are highly regulated by the estrogen signalling in the Fallopian tube epithelium. An estrogen modulator is currently under clinical trials [24]. With the advent of Laser capture microdissection, Insitu mutational hybridization and WES and RNAseq methods on FFPE low yield degraded samples, work such as ours is possible. We need large scale deployment of such resources on precancerous lesion analysis to create next generation detection and prevention strategies. Our work suggests genome wide changes predate driver mutations explaining the low frequency of *KRAS* and *BRAF* mutations in SBT cases [10].

As mentioned earlier, there was a clonal identity between endosalpingiosis and PTH. This means the lesions have spread from the PTH into endosalpingiosis. Whether this is a one-off case or this is more likely the case with all the PTH, needs further investigations. Are all PTH at the same evolutionary scale in terms of acquisition of molecular aberrations needs further large scale investigations. Our work produces first invivo evidence for the same. Such multifocal precancerous lesion analysis was carried out only on HGSC precancerous lesions. This is the first study to investigate early low grade serous carcinogenesis.

Materials and methods

Clinicopathological features and histology

This study is approved by the Institutional Human Research Ethics Committee at the University of Newcastle. The sample was procured from the Hunter Cancer Biobank (HCB). A woman with Endometrial adenocarcinoma with a nodule in the uterus underwent removal of uterus, bilateral ovaries, bilateral Fallopian tubes and the cervix. Endosalpingiosis was also found in the peritoneum, which were removed. Extensive histological examination showed nests of serosal growths with morphological abnormalities in the uterus, left ovary and showed psammomatous calcifications similar to the papillary tubal hyperplasia in both the Fallopian tube mucosa.

Tissue microdissection: FFPE tissue blocks were collected for the two FT mucosa and the endosalpingiosis. Approximately fifty 10-micron sections were taken for each sample and areas of interest were micro-dissected manually. A stereoscope was used for the purpose of microdissection, as it provides ample distance between the objective and the slides. A black sheet was used for a dark background which provided good contrast to distinguish stroma and epithelia. A sterile needle was used in case of FT epithelia, for low surface areas and the sharp edge of the scalpel for the tumour. Hematoxylin and Eosin (H&E) staining was carried out using a standard protocol. Stained slides were imaged at high resolution with the Olympus DP72 microscope or the Aperio Scanscope slide scanner. The gain and exposure time were set constant across tissue samples.

The DNA and RNA isolation, WES and RNA sequencing were performed by BGI genomics, Hongkong.

DNA and RNA isolation: Deparaffinisation was carried out using the Qiagen deparaffinisation solution (ID: 19093). DNA and RNA were isolated using the QIAamp DNA FFPE Tissue Kit (ID: 56404) and RNeasy FFPE Kit (ID: 73504) respectively and were subjected to the sequencing pipeline.

Whole exome sequencing:

The quantified genomic DNA sample was randomly fragmented by Covaris technology with the size of library fragments averaging 150bp and 250bp (specifically as FFPE samples). DNA fragment ends were repaired, and an "A" base was added at the 3'-ends. Adapters were then ligated to both ends of the tailed DNA fragments for amplification and sequencing. DNA fragments were amplified by ligation-mediated PCR (LM- PCR), purified, and hybridized to the exome array for enrichment. Non-hybridized fragments were then washed out. Captured products were then circularized. The rolling circle amplification (RCA) was performed to produce DNA Nanoballs (DNBs). The captured library was loaded on BGISEQ-500 sequencing platforms, and high-throughput sequencing for each captured library was performed.

Bioinformatics pipeline: Briefly: BGISEQ-500 base calling Software was used for base-calling and data was generated in FASTQ format. Data was filtered to generate clean data. Burrows-Wheeler Aligner software was used to do the alignment. Local realignment around InDels and base quality score recalibration were performed using Genome Analysis Toolkit (GATK, https://www.broadinstitute.org/gatk/guide/bestpractices) and duplicate reads were removed by Picard tools. SNPs and InDels were detected by inhouse software, HaplotypeCaller of GATK(v3.6). The hard-filtering method was applied to get high-confident variant calls. Then the SnpEff tool (http://snpeff.sourceforge.net/SnpEff_manual.html) was applied to perform a series of annotations for variants. Data was filtered to remove adapters and low-quality base ratio. Clean reads were mapped by BWA-MEM method. The HaplotypeCaller of GATK(v3.6) was used to call SNPs and InDels simultaneously through a de-novo assembly of haplotypes, outputted into the VCF files. After high-confident SNPs and InDels were identified, the SnpEff tool (http://snpeff.sourceforge.net/SnpEff_manual.html) was used for gene based and platform based annotations.

RNA sequencing:

The poly-A containing mRNA molecules were separated using poly-T oligo-attached magnetic beads. Following purification, the mRNA were fragmented into smaller pieces using divalent cations under high temperature. The cleaved RNA fragments were copied into first strand cDNA using reverse

transcriptase and random primers. Following this second strand cDNA was synthesized using DNA Polymerase I and RNase H. A single 'A' base was added and adapters were ligated. The resultant fragments were purified and enriched with PCR amplification. The final library was a single strand DNA circle. DNA nanoballs (DNBs) were generated with the ssDNA circle by rolling circle replication (RCR) to enhance fluorescent signals at the sequencing process. The DNBs were loaded into the patterned nanoarrays and pair-end reads of 100 bp were read through the BGISEQ-500 platform using Combinational Probe-Anchor Synthesis Sequencing Method.

Bioinformatics pipeline: Sequencing reads were filtered for low base ratio and adapters and clean data was generated. HISAT (Hierarchical Indexing for Spliced Alignment of Transcripts) was used to do genome mapping. StringTie was used to reconstruct transcripts, and Cuffcompare to compare transcripts to reference annotation. CPC tool was used to predict coding potential of novel transcripts and coding novel transcripts were merged with reference transcripts to get a complete reference. SNP and INDEL were called using GATK tool. Clean reads were mapped to reference using Bowtie2 and gene expression levels were calculated with RSEM tool. Pearson correlation between all samples were calculated using cor, perform hierarchical clustering between all samples using hclust, and plots were generated by ggplot2 on R.

Statistics and visualisation: For identifying differentially expressed genes, Poisson distributions were fitted on distributions of gene expression for each group and test statistics were calculated from mean and standard deviation of fitted Poisson distributions. Test statistics were then compared to a cut-off value (i.e. 1.45) to determine the significance of the genes among clinical groups. GO, KEGG, STRING tools were used for GO, KEGG and protein-protein interaction analysis, respectively. R version 3.4.0 was used for all analysis. Circos plot was generated using https://github.com/venyao/shinyCircos. p-values were set at either 0.01 or 0.05, varying upon number of genes detected. Statistical data was verified on Graphpad prism 8.

Figure legends:

Figure1: Histology of patient samples. Enclosed within black dotted lines comprises the papillary tubal hyperplasia lesions in the FTs, **B, D** and the endosalpingiosis lesions of the peritoneum in **C**. The circular blue large structures are the psammonna bodies.

Figure2: The mutational profile of atypical endosalpingiosis in comparison to papillary tubal dysplasia. The circos plots represent SNP and INDELs. Some pathological variants which lead to differentially expressed genes are named in the outer most rings.

Figure3: The LOH and CNV profiles of atypical endosalpingiosis lesion. A. The circus plot shows the LOH variations for Left and right FT and the outermost circle depicts changes in genes due to LOH in the AES lesion. **B.** The copy number changes plot showing specific chromosomal positions which are either amplified or lost copies in AES lesion.

Figure4: The copy number changes plot. The FT samples, FT1(**A.**) and FT2(**B.**) showing specific chromosomal positions which are either amplified or have lost copies.

Figure5: The transcriptomic profile of PTH in the FTs Vs AES in peritoneum. A. The venn diagram summarizes the differences in gene expression between PTH in the FT1 and FT2 Vs AES in peritoneum Cut off in fold change set at 1.45. **B.** The hierarchical clustering between FT1, AES and FT2. The colour scale represents log2 transformed fold change. **C.** The heatmap shows the Pearson's coefficient plot showing FT1 and 2 are closer genomically, almost identical lesions although separated through laterality in comparison to the papillary tubal hyperplasia. The intensity of the colour indicates the Pearson value or correlation depth. **D.** The barplot summarises RNA sequencing results comprising total genes and transcripts identified which showed p value of <0.05. The dfferentially expressed genes are well correlated as FT samples skew together and more changes are found between OT Vs FT. **E.** The mutational pathway analysis shows the top pathways with pathological variants belonged to DNA damage repair pathway, Ras family of genes, RNA processing and Fox family of genes

Figure6: The pathway analysis. A. The dotplots indicate the KEGG pathway analysis for the three comparisons. The gene count is indicated by the size of the dot and the colour scale indicates the p-values. **B.** The barplots indicate number of DEGs under different cellular compartments and functions. The colour scheme indicates the p-values.

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Figure 1

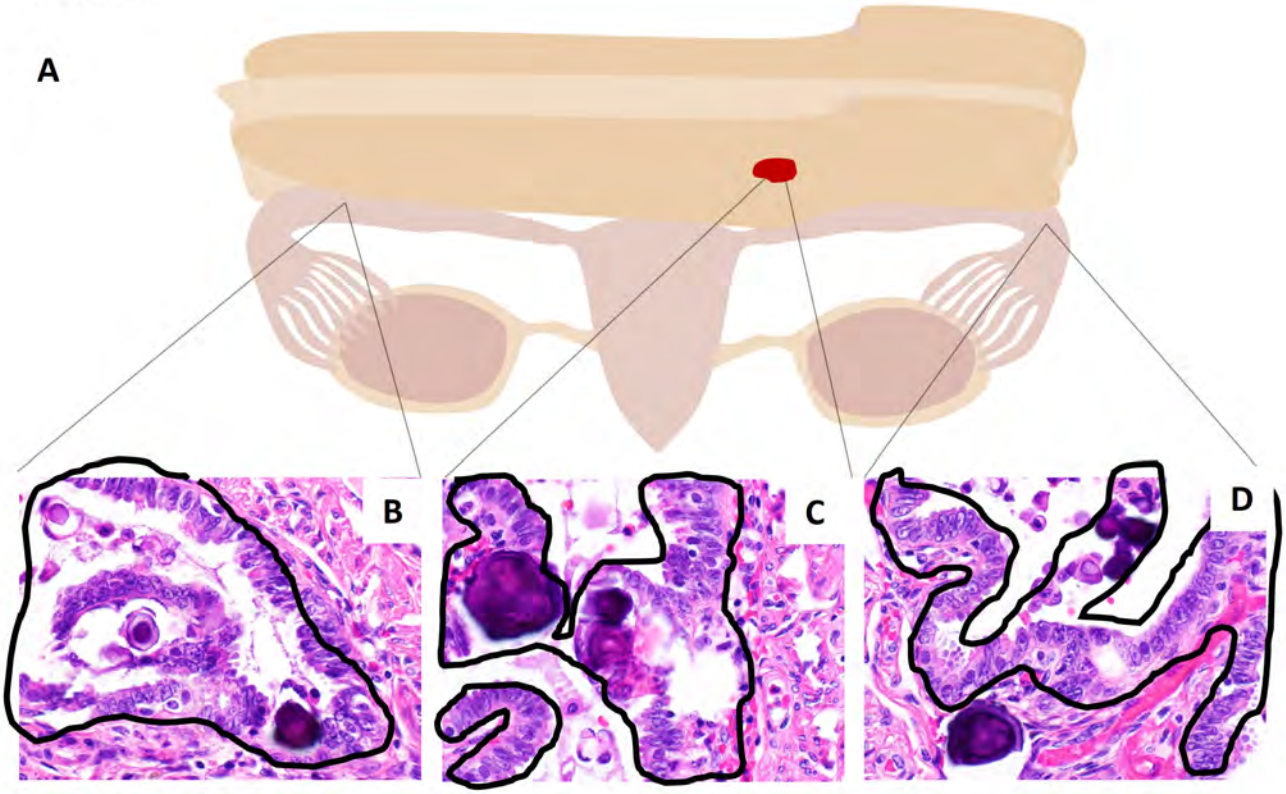
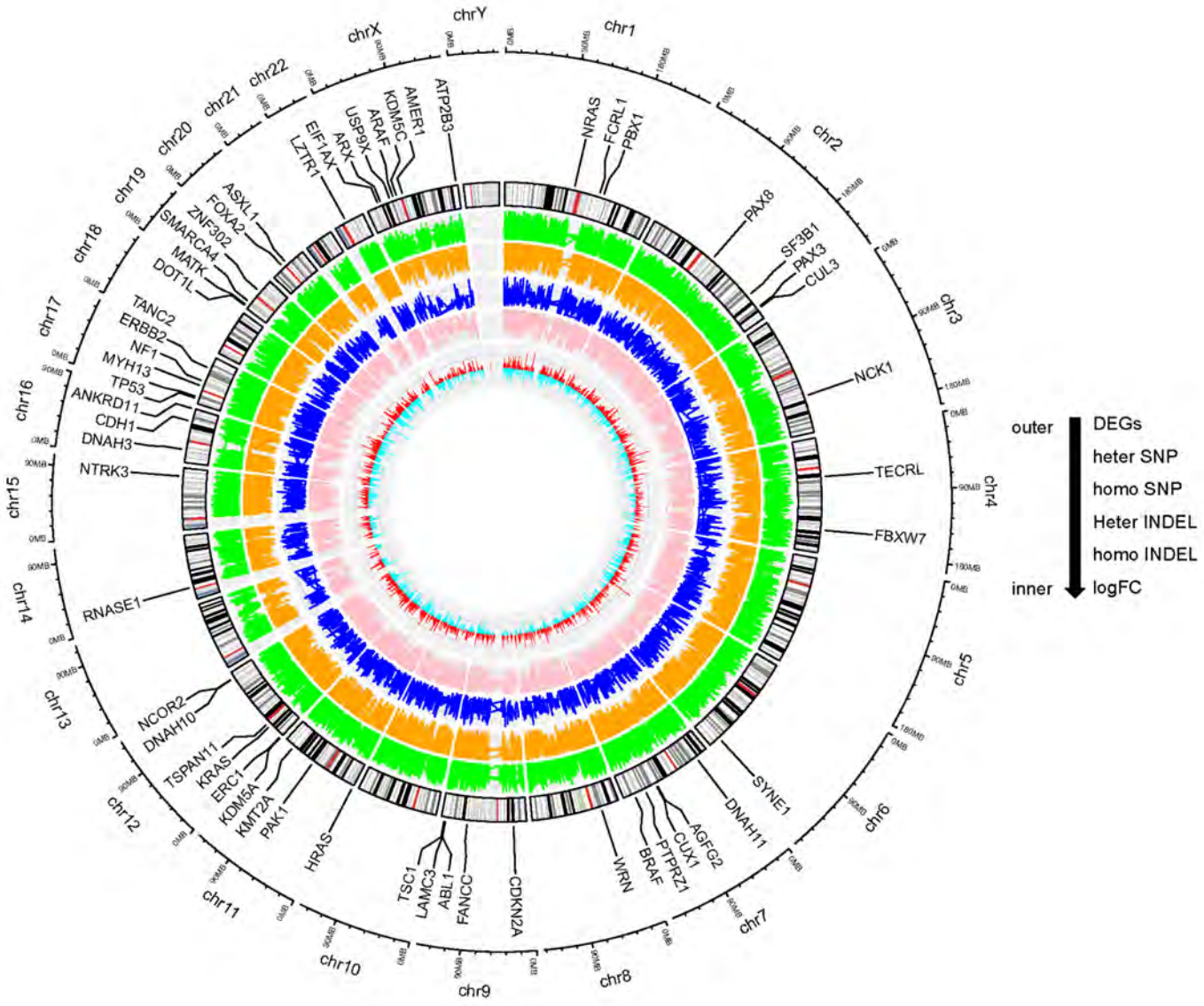

A
B
C
D
Left FT-PTH
Peritoneal atypical endosalpingiosis
Right FT-PTH

Figure 2


chrY
chrX
chr1
chr2
chr3
chr4
chr5
chr6
chr7
chr8
chr9
chr10
chr11
chr12
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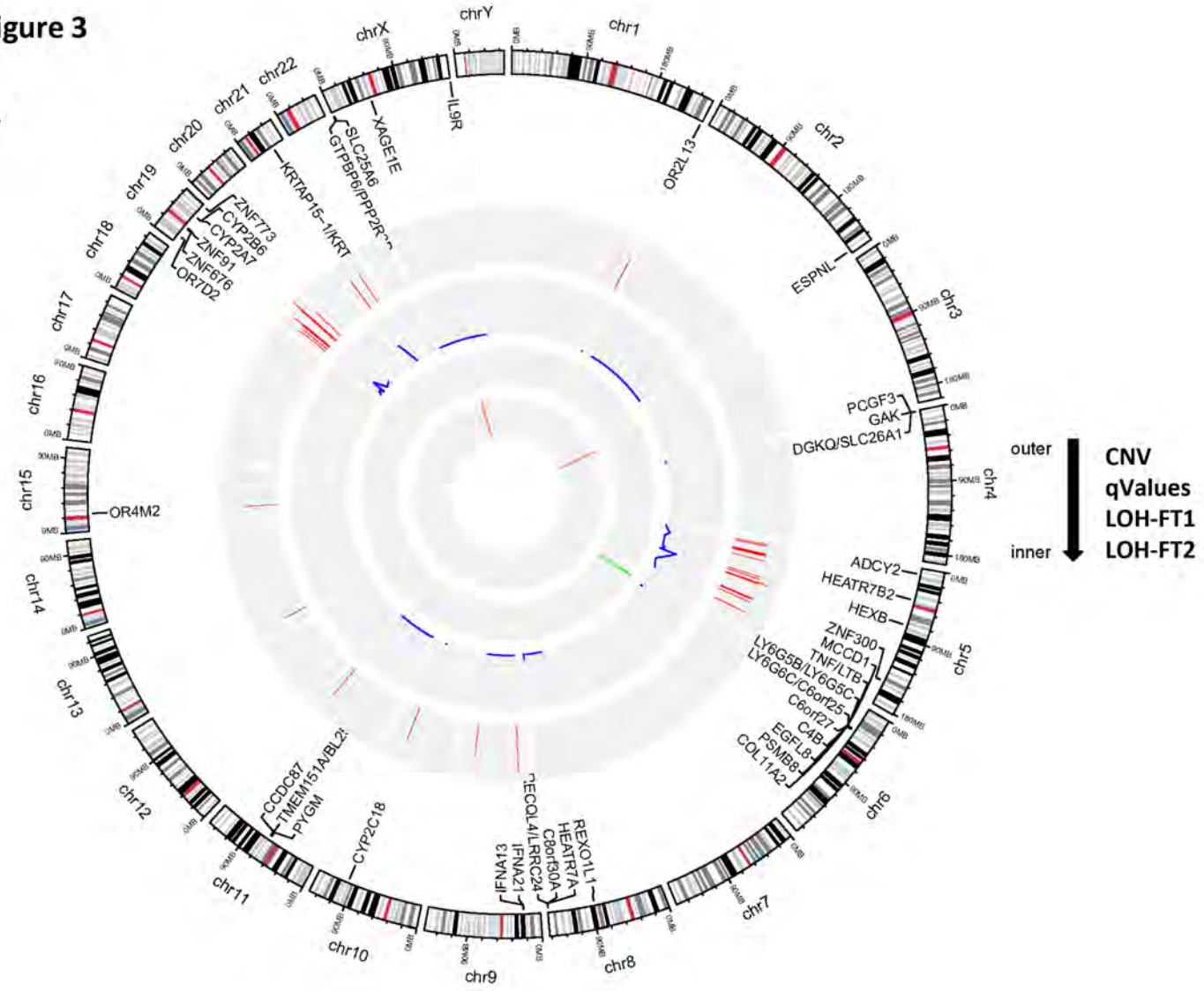
chr14
chr15
chr16
chr17
chr18
chr19
chr20
chr21
chr22
ATP2B3
AMER1
KDM5C
ARAF
USP9X
ARX
EIF1AX
LZTR1
NRAS
FCRL1
PBX1
PAX8
SF3B1
PAX3
CUL3
NCK1
TECRL
FBXW7
SYNE1
DNAH11
AGFG2
CUX1
PTPRZ1
BRAF
WRN
CDKN2A
FANCC
ABL1
LAMC3
TSC1
HRAS
PAK1
KMT2A
KDM5A
ERC1
KRAS
TSPAN11
DNAH10
NCOR2
RNASE1
NTRK3
DNAH3
CDH1
ANKRD11
TP53
MYH13
NF1
ERBB2
TANC2
DOT1L
MATK
SMARCA4
ZNF302
FOXA2
ASXL1
outer
DEGs
heter SNP
homo SNP
Heter INDEL
homo INDEL
inner
logFC

Figure 3

A



B

AES

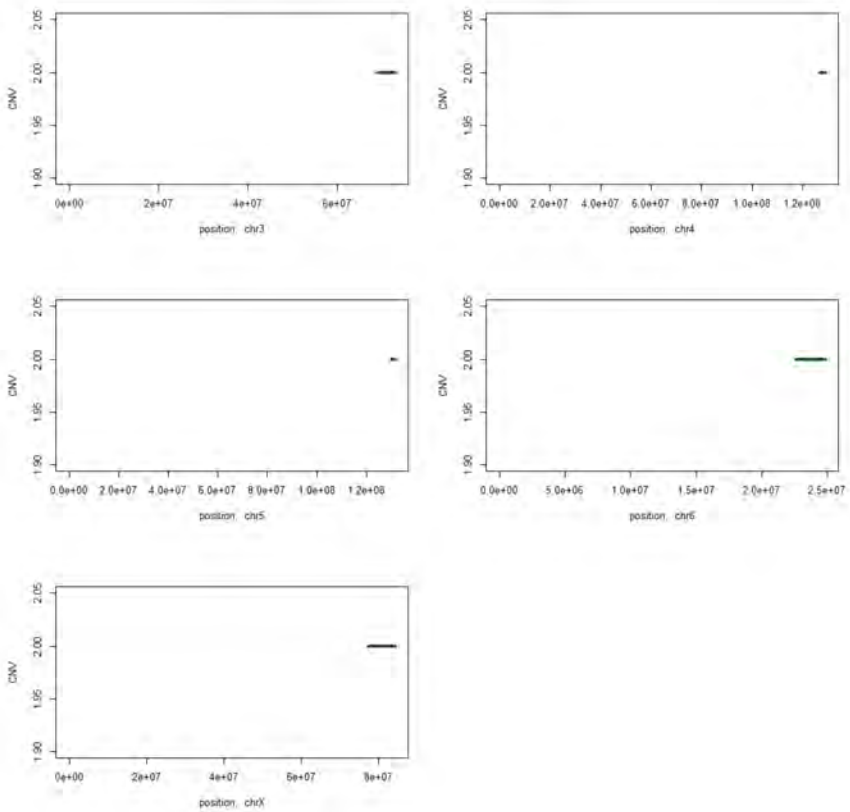
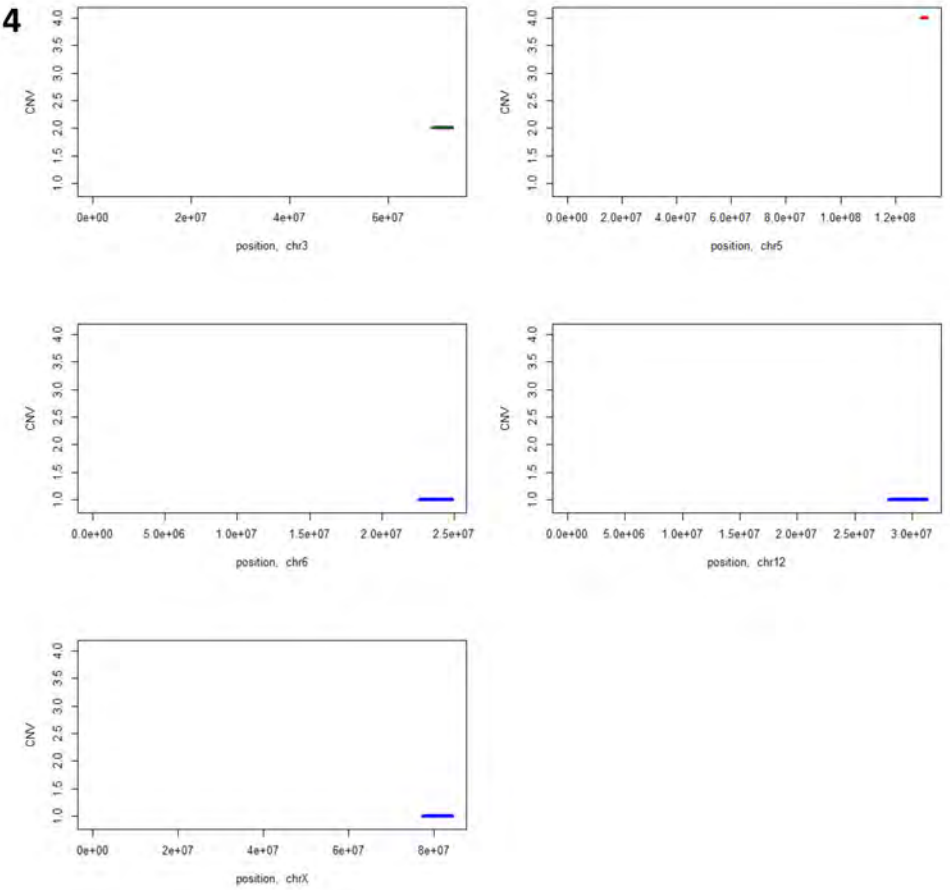


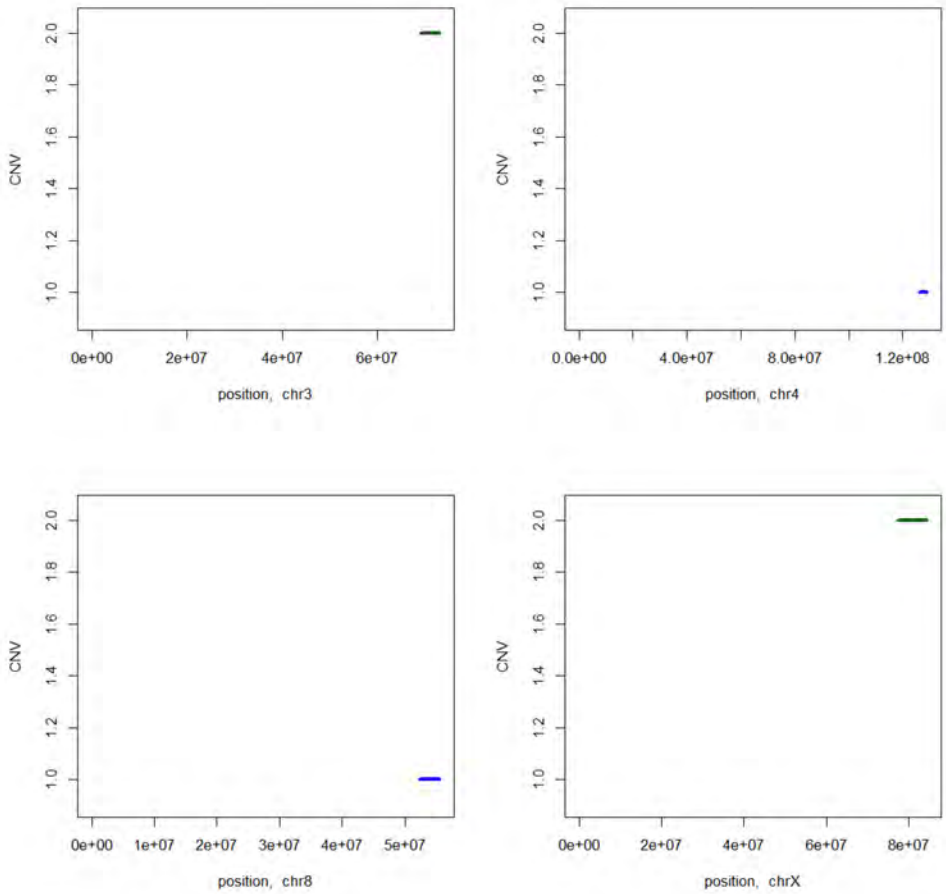
Figure 4

A



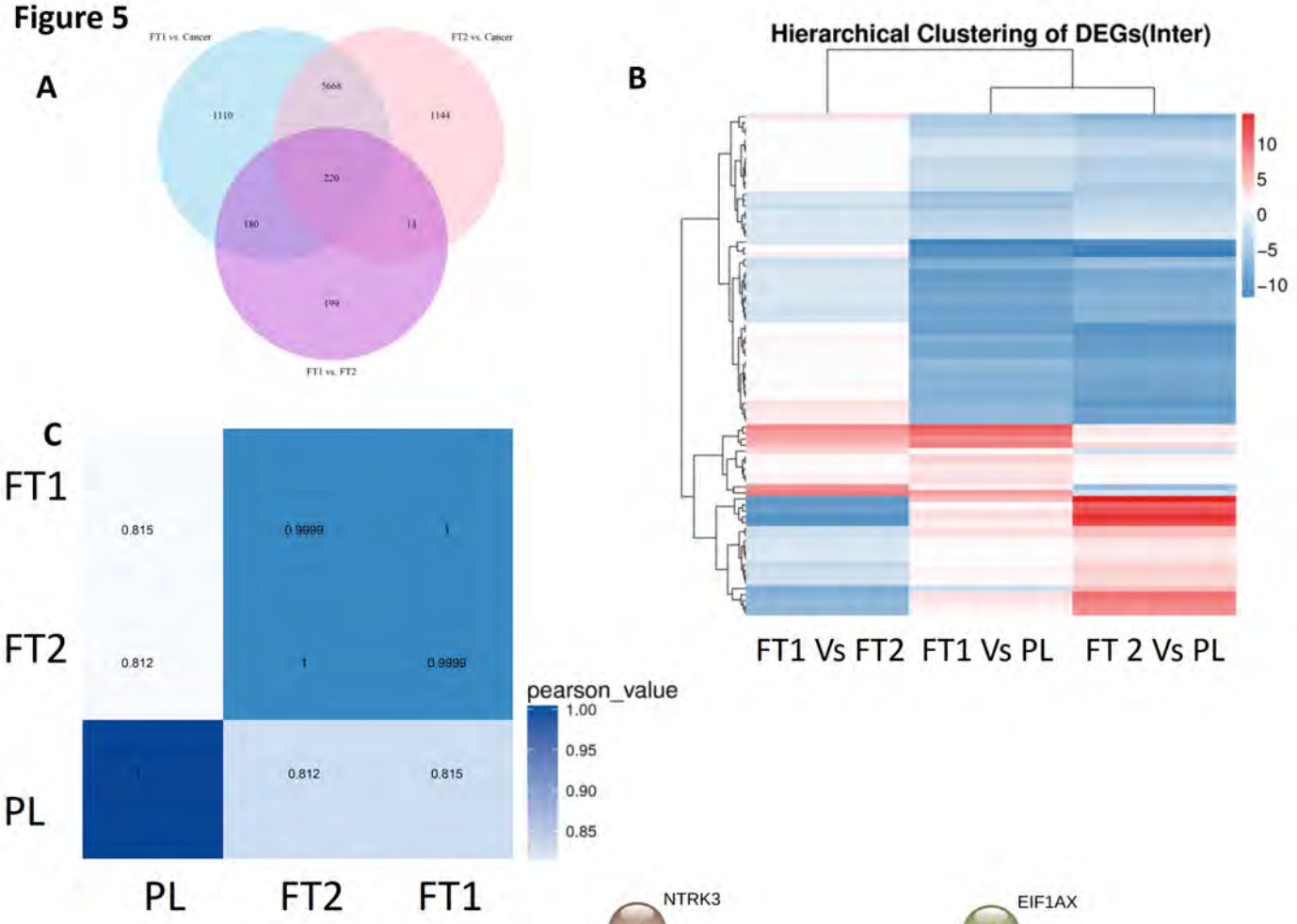
FT1

B



FT2

Figure 5


A
FT1 vs. Cancer
FT2 vs. Cancer
FT1 vs. FT2
5668
1110
1144
220
180
11
199
B
Hierarchical Clustering of DEGs(Inter)
10
5
0
−5
−10
FT1 Vs FT2
FT1 Vs PL
FT 2 Vs PL
C
FT1
FT2
PL
0.815
0.9999
1
0.812
1
0.9999
1
0.812
0.815
pearson_value
1.00
0.95
0.90
0.85
PL
FT2
FT1

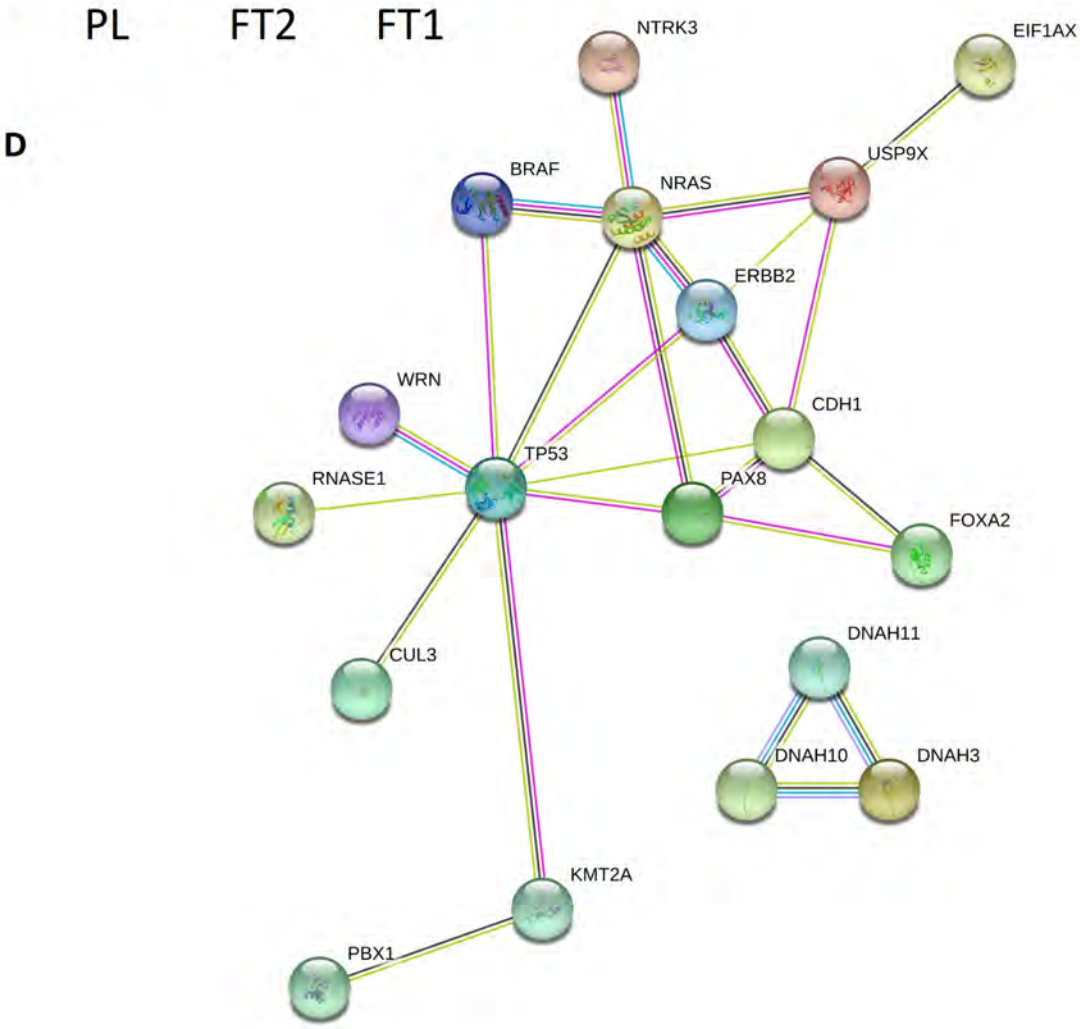
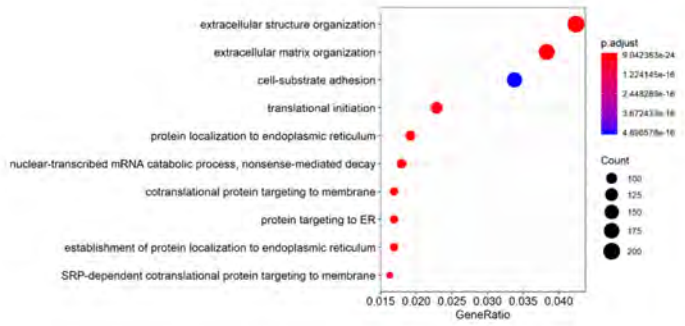

D
NTRK3
EIF1AX
BRAF
NRAS
USP9X
ERBB2
WRN
CDH1
TP53
RNASE1
PAX8
FOXA2
CUL3
DNAH11
DNAH10
DNAH3
KMT2A
PBX1

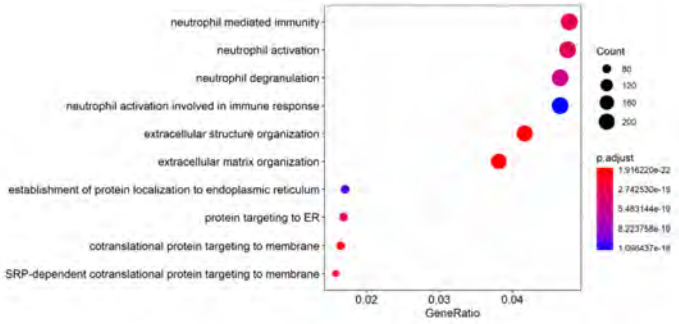
Figure 6

A

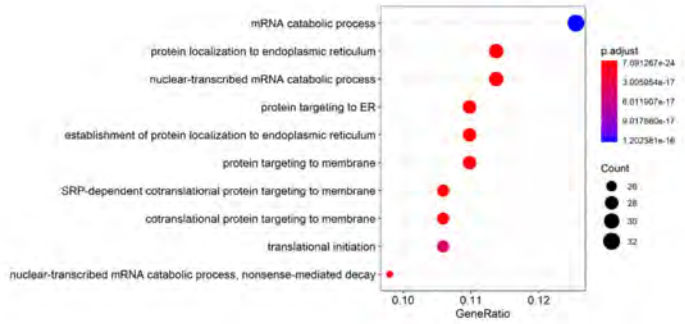
F1 Vs OC



F2 Vs OC

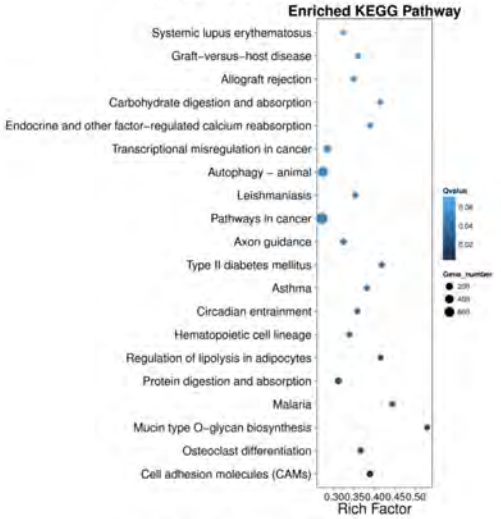


F1 Vs F2

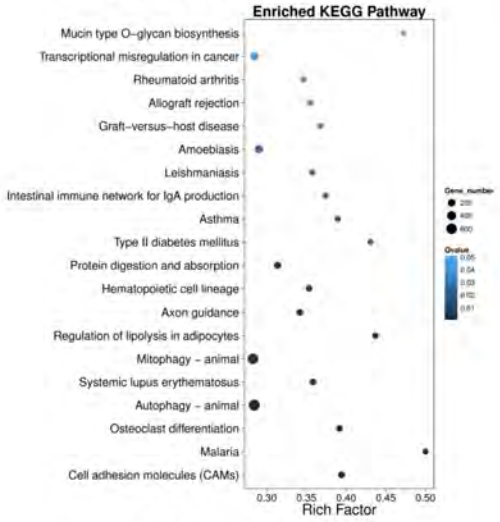


B

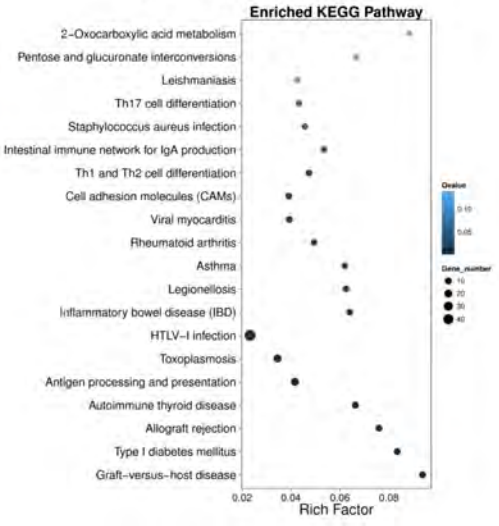
F1 Vs OC



F2 Vs OC



F1 Vs F2



Discussion and conclusions

Ovarian cancer mortality has remained stagnant since early 1980s, since the advent of neoadjuvant chemothetrapy. The 5 year survival rate is at 42%, lowest in comparison to any other women's cancers (90% in case of breast cancer). The treatment strategy has remained the same in the last 100 years, a combination of debulking surgery and chemotherapy. There is an urgent need for alternative disease management strategies. Less than 10% of the cases are detected in stages, I and II.

The available modes of early detection are transvaginal ultrasound, CA-125 blood typing and *BRCA1/2* germline mutation screening for genetic susceptibility. The CA-125 antigen is common in multiple peritoneal cancers and thus not very sensitive specifically in early stages. Ultrasounds are not scalable to the general population due to cost burdens. Germline mutation screening is currently accessed by 3-17% of the population depending on the nationality.

Women who are found susceptible to the disease due to germline mutations undergo RRSO. RRSO leads to surgical menopause, loss of child bearing ability and multiple morbidities. There is a need for alternative prevention strategies. With the multitude of evidences implicating Fallopian tube epithelium as the site of origin in the last decade, salpingectomy and tubal ligation show promise as prevention strategies for previvors. Epidemiological evidences have suggested benefits of pregnancy, lactation and combined contraceptive formulations in reducing risk of ovarian cancer. The right combination of these approaches would greatly benefit high risk populations.

Barring a few homogenous cancers which respond very well to targeted therapies (such as Herceptin for Her2+ breast cancers), most of currently untreatable cancers are heterogenous. For these tumours, the current single targeted agent approach is not successful. Cancer has long been treated as an outcome rather than a process. Cancer is an ever-evolving process starting off as an aberration in a single cell or a clone of few hundred cells and evolving into a complex mass with specialized microenvironment, vasculature, multiple clones of cancer cells coexisting and transforming to accommodate change in microenvironment due to metastasis or changes in the immediate molecular cues. These ever-adapting cell fate decisions are random in many cases but can also be predicted through cellular memory.

Every cell passes through specific molecular cues from within the cell and outside the cell when it traverses from zygotic state to a differentiated adult cell. Each of these cues are stored in the cell's molecular memory. The timing, quantity, origin, immediate microenvironment, length of exposure and neighbouring cell fates, all these factors collectively determine the cell fate of a cell. Depending on these, cells are capable of differentiation, dedifferentiation and transdifferentiation to maintain homeostasis. Changes in one or more of the aforementioned factors may lead to carcinogenesis. The

aim of this thesis was to study the first of the molecular changes in the serous ovarian carcinomas. The approach is summarised in **Figure1**.

Just as is the case of normal cell differentiation, the transformation of a differentiated cell to a carcinoma also follows common patterns. These patterns specifically depend on the genomic content and genomic context. These patterns can be traced as footprints in dysplasia or preneoplasia. These patterns show commonality, repeatability and often take long stretches of time to reach the carcinoma states. Understanding the patterns needs studying molecular mechanisms from the embryonic states to the adult stages and then their aberrations as cancers. Dysplastic transformation to neoplasia is a point of no return and interventions are needed in diagnosis of preneoplasia and preventing the progression of preneoplasia to neoplasia. This thesis has investigated preneoplasia of serous ovarian cancer in the Fallopian tube. A summary of the key findings is stated below.

Key outcomes

The study of preneoplasia is necessary in establishing the timing and sequence of molecular changes in the process of serous ovarian carcinogenesis.

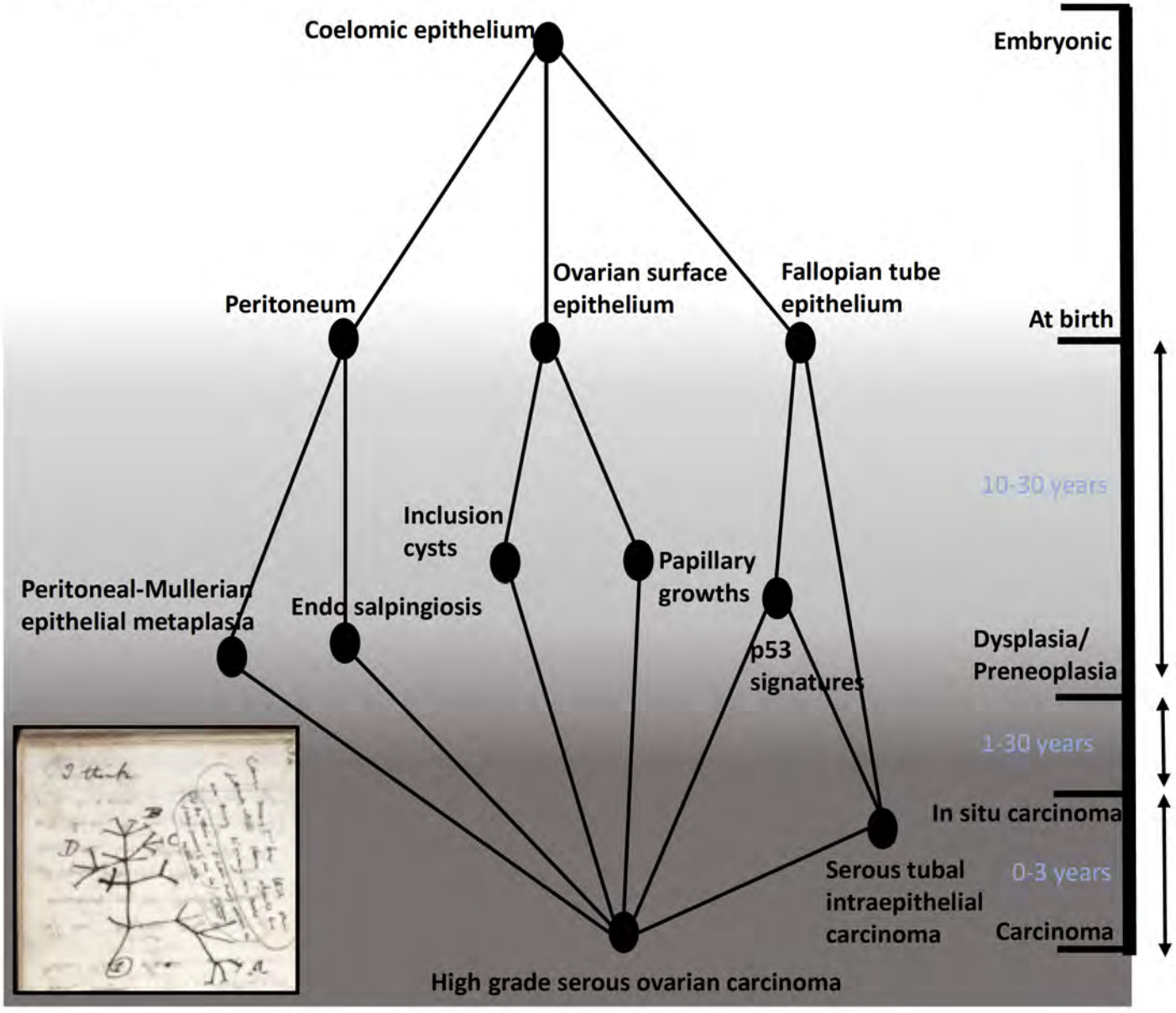
1. **Activation of Wnt signalling in Fallopian tube secretory epithelial cells leads to secretory cell expansions and/or secretory cell outgrowths**

Over the years several mouse models have been developed which demonstrate the neoplastic transformation potential of Fallopian tube epithelium into high grade serous ovarian carcinoma. These mouse models establish the FTSECs as the cells of origin [1-4]. However, all of these models use 2 or more induced mutations generating "frank carcinomas" and demonstrate metastatic abilities. These models fail to incorporate the timing and sequence of these mutations. There were no models to demonstrate the intermediary preneoplastic transformations. Our mouse model with constitutive activation of β-Catenin specifically in PAX8 expressing FTSECs leads to secretory cell expansions and secretory cell outgrowths. These lesions mimic the morphological and immunohistochemical signatures of SCOUTS and SCEs, thus establishing activation of canonical Wnt pathway as a driver of SCOUTS/SCEs and secretory cell fate.

2. **Oestrogen promotes and progesterone suppresses the growth of secretory cell outgrowths**

There have been multiple epidemiological studies in support of the incessant ovulation hypothesis. Some studies with mouse models have also demonstrated metastatic spread from the Fallopian tube to the ovaries and peritoneum [3, 4]. These studies fail to incorporate the effect of the ovarian hormonal milieu. Our mouse model due to its specificity in cell type (secretory cells) and single genetic modification (β-Catenin overactivation) gave us a unique opportunity to study preneoplastic to carcinoma transformation. The lesions in our mouse model specifically overexpressed oestrogen and

Figure 1


Coelomic epithelium
Embryonic
Peritoneum
Ovarian surface epithelium
Fallopian tube epithelium
At birth
10-30 years
Inclusion cysts
Papillary growths
Peritoneal-Mullerian epithelial metaplasia
Endo salpingiosis
p53 signatures
Dysplasia/ Preneoplasia
1-30 years
In situ carcinoma
Serous tubal intraepithelial carcinoma
0-3 years
Carcinoma
High grade serous ovarian carcinoma

progesterone receptors, ER and PR. Upon treatment of oestrogen, the lesions became invasive and spread to serosal and muscle layers. But no metastatic spread was found. This suggests that additional driver changes are needed for metastasis. Upon supplementation of oestrogen with progesterone, the growth of these lesions was stunted.

3. **Precursors escape Fallopian tubes at early stages**

Previous studies had shown a continuum between preneoplasia such as p53 signatures and STIC in the Fallopian tubes to the high grade serous carcinomas in the ovaries and peritoneum of same patients [5-7]. The preneoplasia and the tumours both shared identical TP53 mutations, thus establishing clonal identity. Atleast 30% of high grade serous carcinomas display a p53 null phenotype (complete loss of *p53* expression). Such cases make it hard to screen for p53 signatures if patients develop loss of function *TP53* mutations in histologically normal Fallopian tubes. Our retrospective analysis of RRSO Fallopian tube samples conclusively demonstrates clonal relation between histologically normal Fallopian tube epithelium and omental high grade serous carcinoma acquired post RRSO. Both share identical loss of function *p53* mutations. This establishes a probability that early preneoplasia may be exfoliated and transported to other sites. This conclusively demonstrates early precursor escape in HGSOC. This also questions the notion that presence of preneoplasia are mandatory evidence to establish clonal origins of tumours.

4. **Atypical endosalpingiosis arises from the papillary tubal hyperplasia in the Fallopian tube**

Low grade serous carcinomas are very rare tumours, with low growth rates but are extremely chemoresistant. Unlike high grade serous carcinomas, a well charted preneoplasia to carcinoma transition is not established. The only implicated preneoplasia of low-grade serous carcinomas are papillary tubal hyperplasia [8], which are further a rarity (0.1% incidence rate). These lesions also originate in the Fallopian tube secretory epithelial cells. No immunohistochemical markers are available and these lesions were not subjected to genetic and transcriptomic analysis so far. We found a clonal link between papillary tubal hyperplasia and atypical endosalpingiosis. We did not find any driver mutations in the Ras pathway in the papillary tubal hyperplasia or the endosalpingiosis, however we found differentially expressed genes in the Ras pathway, transcriptional regulation pathway and RNA splicing suggesting genomewide changes predate driver mutations and may be necessary to create an environment of mutagenesis.

Study of preneoplastic lesions is necessary to better understand the evolution of the cancer. Later stage markers may not hold true in preneoplastic stages. Tumours are ever evolving entities with abilities to lose and gain molecular cues to their advantage. The work in this thesis hopefully is a pilot to more large-scale intercontinental studies such as pre cancer genome atlas (PCGA). There is a need to see cancer as a process, rather than an outcome. Fallopian tube epithelium displays a specific

propensity towards serous ovarian carcinogenesis. Salpingectomy and use of combined contraceptive formulations may highly benefit in reducing ovarian cancer risk.

Figure legend

The evolution of high grade serous ovarian carcinoma: HGSOC is known to commonly occur in three sites: peritoneum, Fallopian tube epithelium and ovarian surface epithelium. All three sites have common cell of origin in coelomic epithelium. Each of these sites display established preneoplasia, which represent stopped carcinomas. These lesions have specific molecular aberrations in comparison to their normal counterparts. This normal to preneoplastic transformation is a long and slow process often spanning 2-3 decades. The preneoplasia to neoplastic transformation in contrast is a relatively faster process. The normal differentiation of cells and their dedifferentiation to carcinomas are evolutionarily conserved. Inset: The famous Charles Darwin, "I think" evolution chart. The three grand ideas of evolution are: species evolve due to specific environmental cues, are closely related to one another and can be distinguished by specific heritable traits. These are perfectly applicable to the process of carcinogenesis.

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8. Kurman, R.J., et al., *Papillary tubal hyperplasia: the putative precursor of ovarian atypical proliferative (borderline) serous tumors, noninvasive implants, and endosalpingiosis.* Am J Surg Pathol, 2011. **35**(11): p. 1605-14.

LIST OF PUBLICATIONS INCLUDED AS A PART OF THIS THESIS

Contained in:

CHAPTER 1

Nagendra PB and Tanwar PS. Role of Fallopian tube in pelvic serous ovarian carcinogenesis Gynaecologic Oncology. (Submitted)

CHAPTER 2

Nagendra PB, Goad J, Nielsen S, Rassam L, Lombard JM, Nahar P, et al. Ovarian hormones through Wnt signalling regulate the growth of human and mouse ovarian cancer initiating lesions. Oncotarget. 2016;7(40):64836-53.

CHAPTER 3

Nagendra PB, Scurry J and Tanwar PS. Evidence of early Fallopian tube precursor escape in omental serous ovarian carcinoma. Cancer Research. (Submitted)

CHAPTER 4

Nagendra PB, Scurry J and Tanwar PS. Molecular characterisation of papillary tubal hyperplasia: the putative precursors of low grade serous carcinoma. Gynaecologic Oncology. (submitted)

ADDENDUM

1. Bajwa P, Nagendra PB, Nielsen S, Sahoo SS, Bielanowicz A, Lombard JM, et al. Age related increase in mTOR activity contributes to the pathological changes in ovarian surface epithelium. Oncotarget. 2016;7(15):19214-27.

2. Jamaluddin MFB, Ko YA, Kumar M, Brown Y, Bajwa P, Nagendra PB, et al. Proteomic Profiling of Human Uterine Fibroids Reveals Upregulation of the Extracellular Matrix Protein Periostin. Endocrinology. 2018;159(2):1106-18.

3. Jamaluddin MFB, Nagendra PB, Nahar P, Oldmeadow C, Tanwar PS. Proteomic Analysis Identifies Tenascin-C Expression Is Upregulated in Uterine Fibroids. Reproductive sciences (Thousand Oaks, Calif). 2019;26(4):476-86.

LIST OF AWARDS

1. **Department of Biotechnology, Government of India Fellowship:** Research Assistantship, 2012-14

2. **Postgraduate Research Fellowship: International Tuition Fees Scholarship,** The University of Newcastle
   UNRS Central 25:75 Scholarship
   The University of Newcastle, 2015-19

3. **Publication of the month, School of Biomedical Sciences and Pharmacy,** University of Newcastle, August 2016

POSTER PRESENTATIONS

Serous ovarian cancer: Hormones, early detection and contraceptive prevention

PB. Nagendra, J.Goad, S.Nielsen, L. Rassam, JM.Lombard, P.Nahar and PS. Tanwar; ANZGOG- ASM, 2016

Serous ovarian cancer: A synchrony of mutations and hormones

PB. Nagendra, J.Goad, S.Nielsen, L. Rassam, JM.Lombard, P.Nahar and PS. Tanwar; Sydney Cancer Conference, 2016

Serous ovarian Cancer: An interplay of mutations And hormones

PB. Nagendra, J.Goad, S.Nielsen, L. Rassam, JM.Lombard, P.Nahar and PS. Tanwar; HCRA Symposium, 2016

Defining the molecular footsteps of High grade serous ovarian cancer

PB. Nagendra, JM. Lombard, MF B. Jamaluddin and PS. Tanwar; HCRA Symposium, 2017

Splicing inhibition as a chemo-resensitisation mechanism in ovarian cancer

PB. Nagendra, JM. Lombard, MF B. Jamaluddin and PS. Tanwar; HCRA Symposium, 2018

Talks

Ovarian Cancer: Initiation, progression and prevention

PB. Nagendra and PS. Tanwar, Consumer review, HCRA and Cancer council NSW, 2017

Splicing away the chemoresistance in ovarian cancer

PB. Nagendra and PS. Tanwar, HEAPS seminar, HCRA, 2017