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Iron Addiction In Tumor Initiating Cells: A Novel Therapeutic Target In Ovarian Cancer

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Iron Addiction In Tumor Initiating Cells: A Novel Therapeutic` Target In Ovarian Cancer

Debargha Basuli, M.D., PhD

University of Connecticut, 2016

Ovarian cancer is a highly lethal malignancy that has not seen a major therapeutic advance in over 30 years. We demonstrate that ovarian cancer exhibits a targetable alteration in iron metabolism. Ferroportin (FPN), an iron efflux pump, is decreased and transferrin receptor (TFRC), an iron importer, is increased in ovarian cancer tissue. Expression of FPN and TFRC are strongly associated with patient survival. Ovarian cancer tumor-initiating cells demonstrate a similar profile of iron excess. Iron deprivation induced by desferroxamine, knockout of IRP2, or overexpression of FPN preferentially blocks growth of tumor initiating cells. Iron restriction inhibits invasion, synthesis of MMPs and IL6, and reduces intraperitoneal spread of tumor cells *in vivo*. Growth of ovarian tumors is inhibited by induction of ferroptosis, an iron-dependent form of cell death. Thus, enhanced levels of iron create a metabolic vulnerability that can be exploited therapeutically. We show that this dependence is already evident in the tumor initiating cell and creates a new therapeutic opportunity. Thus, alterations in iron import and export in ovarian cancer result in an iron acquisitive phenotype and an increase in metabolically available iron. A reduction in intracellular iron decreases IL6, which reduces MMPs, invasion and intraperitoneal metastases. Iron dependence can be targeted in two ways: by reducing intracellular iron with chelators, or by treating with agents that induce iron-dependent cell death.

Iron Addiction In Tumor Initiating Cells: A Novel Therapeutic Target In Ovarian Cancer

Debargha Basuli

M.B.B.S, Calcutta University, 2007

A Dissertation

Submitted in Partial Fulfilment of the

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Debargha Basuli

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Doctor of Philosophy Dissertation

Iron Addiction In Tumor Initiating Cells: A Novel Therapeutic Target In Ovarian Cancer

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2016

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I also thank my family especially Malati Basuli, Isha Sen for their support during my PhD.

I dedicate my doctoral thesis to the soil that begets life into nature, the sarapilheira.

Table of Contents _____ Page Number

LIST OF FIGURES _____ **Page Number**

LIST OF ABBREVIATIONS

CN	Copy Number
CSC	Cancer Stem Cell
DMT	Divalent Metal Transporter
EMT	Epithelial-Mesenchymal Transition
FPN	Ferroportin
HGSOC	High Grade Serous Ovarian Cancer
HIF	Hypoxia Inducing Factor
HMWK	High Molecular Weight Kininogen
HRR	Homologous Recombination Repair
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OSE	Ovarian Surface Epithelium
STEAP3	Six-Transmembrane Epithelial Antigen of Prostate 3
STIC	Serous Tubular Intraepithelial Carcinoma
TF	Transferrin
TFR	Transferrin Receptor
TIC	Tumor initiating cells
TNF	Tumor Necrosis Factor

CHAPTER 1

INTRODUCTION

Introduction of thesis

This thesis details my research on iron addiction exhibited by ovarian cancer tumor initiating cells (TICs) (or popularly known Cancer Stem Cells/CSCs) and targeting it to modulate tumor growth. In this introduction, first I will provide a brief overview of the relation between iron and cancer. Next, I will provide a background for ovarian cancer. I will then describe the current concept about tumor initiating cells. Finally I will introduce our concept of how the iron phenotype in the ovarian TICs can be targeted. Altogether, this introduction will underscore the rationale for pursing the studies described in this thesis.

Overview of role of iron in human body

Iron is an essential element for mammalian cells. Iron is critical for cell division, growth and metabolism. It is contained or acts as a cofactor in many vital enzymes. In mammalian cells, iron enables ribonucleotide reductase activity thereby helping in DNA replication and cell growth(1) . Reversible coordination of O_2 and its transport by iron containing metalloproteins (hemoglobin and myoglobin) is crucial for life. Iron-sulphur proteins are best known for their role in oxidation-reduction reactions of the mitochondrial electron transport system. Cytochromes contain a variety of heme cofactors and are involved in electron transfer reactions required for cellular respiration(2). Iron has important role in cellular detoxification through enzymes like peroxidase and catalase (3). Thus iron is beneficial to the basic biological processes to sustain life.

Normal iron homeostasis in human

The main source of iron in human is through diet. The majority of dietary iron is absorbed by the enterocytes in the duodenum. A ferric reductase enzyme, duodenal cytochrome B (DcytB) present of the luminal border of the enterocytes reduces any ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). Divalent metal transporter 1 (DMT1), another protein in the enterocytes help in internalizing Fe^{2+} into the cells and transporting across the cell. On the basolateral side of the enterocyte, Fe^{2+} is again oxidized back to Fe^{3+} by hephaestin. Fe^{3+} is released by the cell into the circulation through the only known iron exporter in mammals called ferroportin (FPN). The body regulates iron absorption mainly by regulating the levels of FPN, via hepcidin, an enzyme produced in liver and binds to FPN to degrade it. Fe^{3+} thus released into the blood binds to Transferrin (TF) and circulates throughout the body and is delivered to peripheral tissues. Iron bound TF is recognized by transferrin receptor (TFR), a cell surface receptor. The Fe^{3+}- TF- TFR complex is endocytosed into the cell. In the acidic environment of the endosome, and through the enzymatic activity of STEAP reductase, Fe^{3+} is reduced to Fe^{2+}. TFR is recycled back to the cell membrane. With the help of DMT1, Fe^{2+} effluxes from endosome into the cytosol into a pool of loosely bound metabolically active iron, termed as labile iron pool (LIP). Iron from this LIP is used for biosynthesis of heme and iron- sulphur clusters, other enzymes and the excess iron is stored in an iron storage protein called ferritin. Iron can also be pumped out of the cell through FPN.

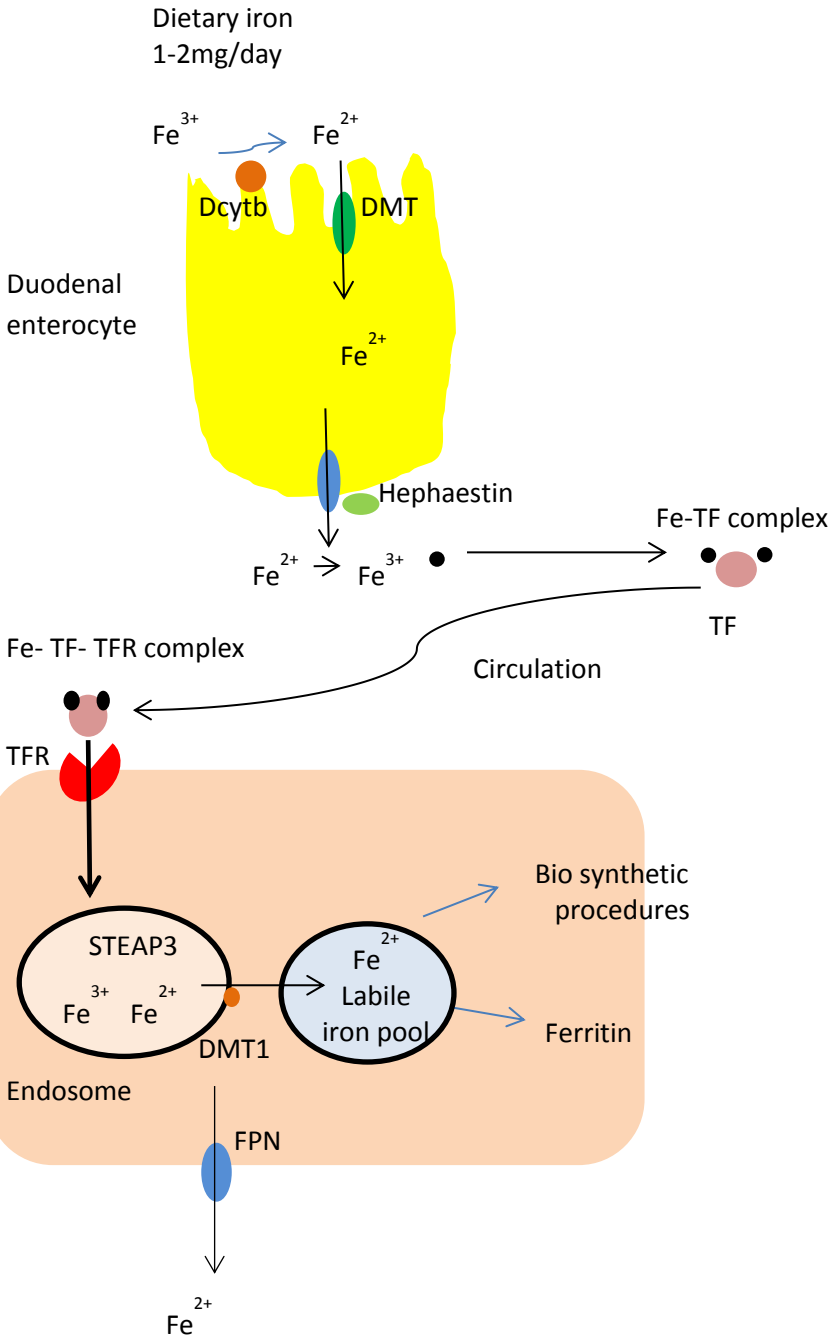


Figure 1. Normal cellular iron homeostasis.

Upper diagram shows absorption of dietary iron through duodenal enterocyte.

Lower diagram shows normal cellular iron metabolism.

Iron regulatory proteins (IRP1 and IRP2) are the master regulators of iron homeostasis and regulate other iron proteins post-transcriptionally **(Figure 2).** When the cell is iron depleted, IRBs bind to the iron response elements (IREs) in the 5' untranslated region of ferritin and FPN mRNAs leading to stalling of translation of these mRNAs. The TFR mRNA also has IRE in the 3' untranslated region. Binding of IRBs to the IRE of TFR mRNA stabilizes the mRNA and increased translation of TFR. When the cell is iron sufficient, IRBs unbind from the IREs of the respective mRNAs leading to increased translation of ferritin and FPN, and decrease in TFR translation.

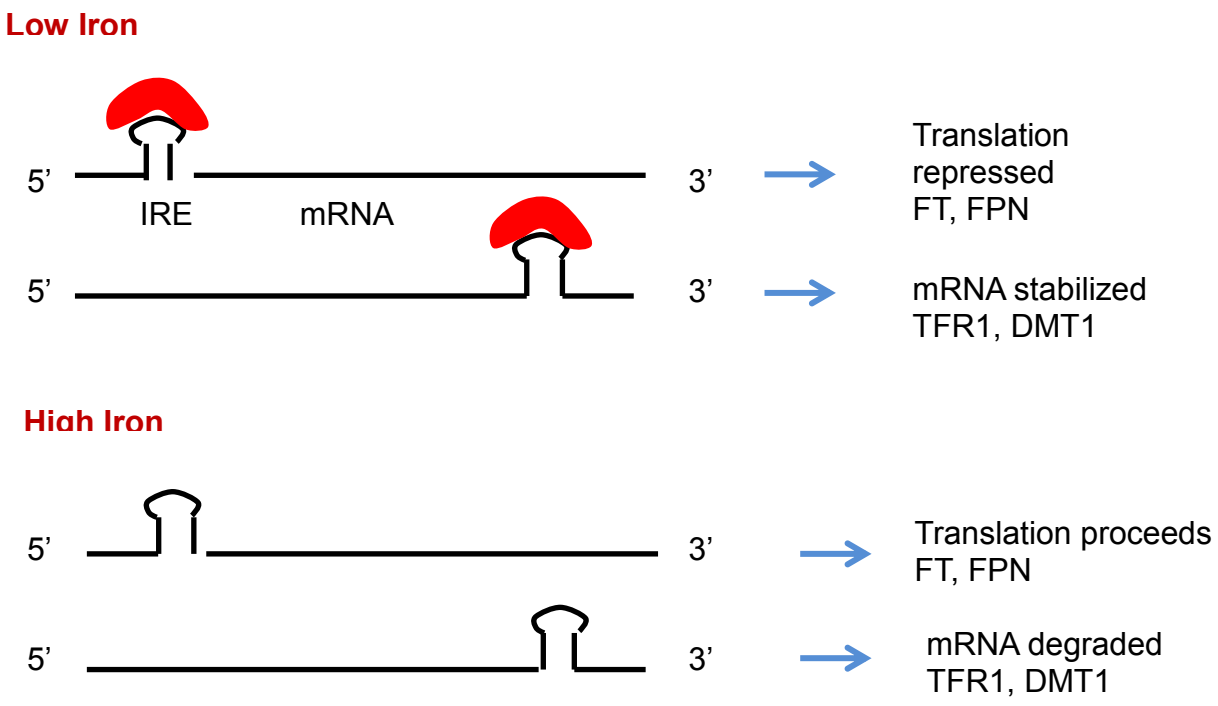


Figure 2. Control of iron metabolism through IRB.

Iron and cancer

Despite its beneficial role of iron, iron is found to the culprit of diverse pathological conditions in human including cancer. There is a growing body of evidence, both epidemiological and biological about the strong association of iron and cancer.

In one of the early observational studies, Stevens et al demonstrated that in a cohort of 14000 human participants, higher TF saturation was associated with increase in risk of cancer in men within a follow up period of 10-13 years(4). Several studies were conducted later, most of which indicated a general trend of increased cancer risk with increased body iron stores. Other studies have shown that in hemochromatosis, a genetic disease (mutation in HFE gene) leading to massive iron accumulation with in the body, the risk of cancer is about 20-200 fold higher than normal people (5, 6). Hemochromatosis patients are at increased risk of mainly liver cancer, including cancers in other organs like pancreas, breasts and colon (7, 8). In an interventional study, it was observed that reduction in body iron through repeated phlebotomy in elderly men was associated with overall reduction in cancer risk (9).

The underlying mechanisms of the strong association of iron and cancer are not understood clearly. One popular hypothesis is iron may promote tumor initiation through its ability of production of reactive oxygen species via Fenton reaction. Iron reacts with hydrogen peroxide to form hydroxyl and hydroperoxyl radicals as follows:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^-$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO\bullet + H^+$$

ROS produced in Fenton reaction can mutate DNA through oxidative dmage potentially leading to tumor initiation(10, 11).

During the last two decades, several studies manifested that iron metabolism is altered in cancer and many iron proteins are found to have important contribution in tumor growth. TFR, the major iron import protein has been shown to be highly expressed in many cancers including breast cancer, lung cancer, bladder cancer, glioma to name a few(3). Studies have shown that targeting TFR can be effective as a therapeutic strategy to halt cancer cell growth in vitro and in vivo(12). Antibodies against TFR have shown promising results in preclinical trails (13). Cytotoxic moieties conjugated to its ligand TF has been used to target highly expressed TFR within the cancer selectively (14). Ferritin, the iron storing protein is found to contribute to tumor growth. In B cell lymphoma and other HRAS expressing cancers, ferritin is found to be downregulated, thereby releasing the stored iron into the metabolically active labile iron pool of the cancer cells to promote tumor growth(15-17). Ferritin is transcriptionally upregulated by TNF(18). TNF induced ferritin upregulation is mediated by NF-κB in the fibroblasts (19). In their review, Torti et al (3), opine that increased ferritin through NF-κB in the fibroblast might help in sequestering iron and thereby preventing oxidative stress within the tumor microenvironment leading to tumor survival. Ferritin may also promote angiogenesis in cancer by binding to high

molecular weight kininogen (HMWK)(20). Thus ferritin can modulate tumor growth both through up and down regulation.

The FPN- hepcidin regulatory axis is the major pathway through which systemic iron levels are regulated with in the body (21). When iron level is high within the body, hepcidin is induced in the liver. Hepcidin binds to the FPN in the enterocytes and downregulates FPN leading to lesser iron absorption (22). FPN and hepcidin are expressed in breast epithelial cells (23). FPN is found to be downregulated in breast cancer cells at protein level, probably through a local autocrine FPN-hepcidin loop in the cancer cells (23). FPN mRNA expression pattern in the tumor tissue of breast cancer patients is significantly associated with patient survival and thus can predict clinical outcome of the disease (23).

IRP2 is overexpressed in breast cancer and plays an important role in iron accumulation in breast cancer (24). IRP2 is shown to upregulate tumor growth and may contribute to the poor outcome of the breast cancer patients (24).

Thus there is substantial evidence, both factual and conceptual that iron plays a pivotal role in cancer initial and progression. Altered iron metabolism has been suggested as a “hallmark of cancer”(25). A subset of iron genes comprising of 16 genes (termed as iron regulatory gene signature, IRGS) has been shown to be superior for risk stratification in breast cancer compared to conventional prognostic factors (26). Iron genes and proteins are being evaluated for prognosis and therapy in several types of cancers (23, 27, 28). Although a lot of studies have been conducted to understand the relation between iron and different cancer types, a little work has been done in respect to ovarian cancer, the 5th most common cancer in women in US and the most lethal gynecological malignancy.

In this thesis, the objective of my research work is to explore the characteristics of iron metabolism in ovarian cancer, particularly high grade serous ovarian cancer (HGSOC).

High grade serous ovarian cancer (HGSOC)

Overview of ovarian cancer

Ovarian cancer is the fifth leading cause of cancer-related deaths among women in the United States(29). In 2014, approximately 22,000 women were diagnosed with ovarian cancer, and approximately 14,000 died of the disease, making it the most lethal of the gynecologic malignancies (29, 30). Ovarian cancer is a heterogeneous disease composed of multiple subtypes that are defined both histologically (31) and molecularly (32-34). High grade serous ovarian carcinoma is the most common histologic subtype, accounting for over 50% of ovarian epithelial malignancies. The 5 year survival rate of patients with HGSOC is a dismal 9-34% (31).

Origin of HSOC

To more effectively approach HGSOC, efforts have focused on identifying its cell of origin. This subject remains an area of active debate (35). Since early 70s, ovarian surface epithelium (OSE) was proposed to be the origin of HGSOC by Fathala (36). For 30 years, the exact process of how OSE transforms into HGSOC was never precisely understood. In the 90s, following the discovery of BRCA1 and BRCA1 genes to be responsible for high risks of breast and ovarian cancer (37), a new hypothesis about the cell of origin of HGSOC started to emerge. After this, salpingo-oophorectomy became the standard

practice for prevention of ovarian cancer in women who were carriers of germline mutations in BRCA1 and 2. With the availability of surgical samples from preventive salpingo-oophorectomy, the pathologists started to carefully examine the specimen and surprising they didn't find any lesion in the ovaries. Instead, a dysplastic lesion was found in the fimbriated end of the fallopian tube samples. Following this observation, a standard protocol for systematic evaluation of the surgical samples of the fallopian tubes was adopted, called SEE-FIM (referenced in (38)) which led to the identification of an occult noninvasive and invasive carcinomas in the fallopian tubes(38, 39). Subsequently, Peik et al proposed the occult tubal carcinoma might be the precursor lesion for the high grade serous carcinoma in the ovary (40). Several other findings upheld histological, molecular and genetic evidence that pointed towards the fallopian origin of HGSOC. Genetic expression studies demonstrated that the expression profile of HGSOC resembled fallopian tubular epithelium more closely compared to the OSE (41). Tubal intraepithelial carcinoma was found positive in 48% of the women with HGSOC but who were not carriers of BRCA mutations (42). This lesion was only found in patients with serous carcinoma but not in other types of ovarian cancer; hence the term "serous tubular intraepithelial carcinoma (STIC) "was proposed for this lesion (42). The first defining feature of STIC was the "p53 signature", characterized by evidence of DNA damage, TP53 mutations and P53 protein stabilization found within the secretory cells of the fallopian epithelia. (41). Pathological studies of surgical specimens of STICs and HGSOC showed that 92% of the STIC have TP53 mutation and are identical to the TP53 mutation in the concurrent pelvic high grade cancer supporting the evidence of clonal relationship between the two lesions(43).

Pathogenies of HGSOC

The process of transformation of STIC into HGSOC is poorly characterized. Traditionally ovarian serous carcinomas have been graded into low grade and high grade. Recent molecular and genetic studies have shown that the low and high grade serous ovarian cancer have entirely distinct pathogenies pathways (44). Based on the histological features and clinical outcome, epithelial ovarian cancer is grouped into 2 major types: type 1 and type 2 (45). Type 1 includes low grade serous cancer, low grade endometriod cancer, mucinous cancer and some clear cell cancers that arise from a defined precursor called borderline tumors (46).Type 1 cancer evolves in a stepwise fashion and exhibit more chromosomal stability. Common mutations in Type 1 cancer are Type 1are in KRAS, BRAF, PTEN, CTNNB1 and TGFBR2 (46). Type 2 cancer mostly includes the HGSOC. HGSOC progresses rapidly and contain TP53 mutations. TP53 mutation in the fallopian tube fimbria is probably the earliest event in HGSOC pathogenesis since STICs have shortened telomeres compared to HGSOC (47). A TCGA pilot study of HGSOC, showed nearly 100% of the tumors harbor TP53 mutation and catalogued low prevalent but statistically significant recurrent somatic mutations in 9 other genes including NF1, B1 and CDK12 RCA1/2, RB1 and CDK12 (48). c-myc amplification is a common finding in advanced-stage ovarian cancer (49). TCGA also identified widespread DNA copy number variation in the tumors. Based on these findings, a viable model of progression of HGSOC has been proposed where the primary events include p53 loss and BRCA mutation leading to a deficiency of homologs recombination repair (HRR) that initiate chromosomal instability and widespread copy number (CN) change (Figure 3) (46) (50). Thus though

early events in HGSOC are more or less properly defined, HGSOC is characterized by a high level of genomic heterogeneity(51) making it difficult to generalize its genetics.

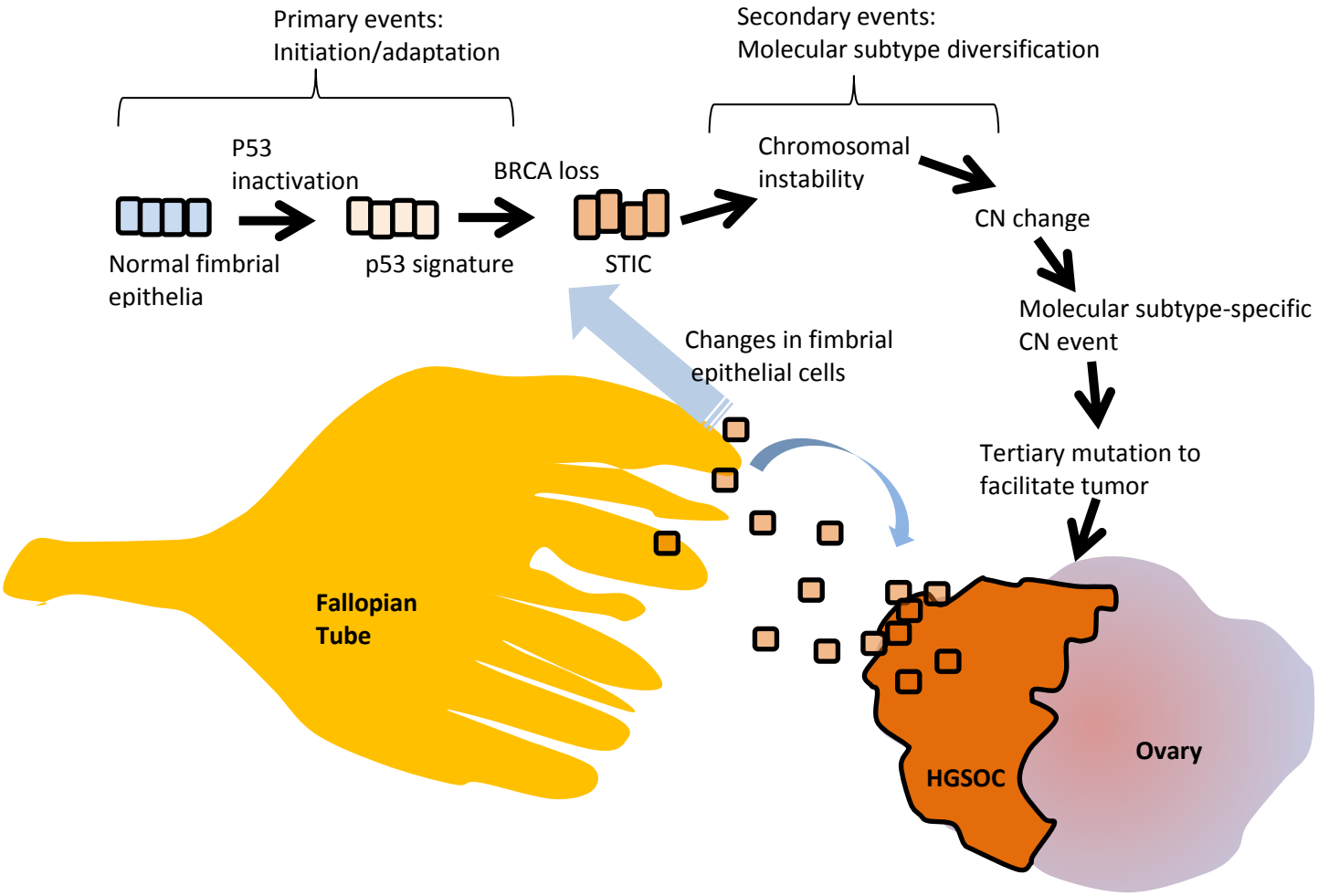


Figure 3. Proposed model for initiation and progression of HGSOC.

Tumor initiating cells (TICs)

Overview of the concept of TIC

One current popular concept of tumorigenesis is that the tumors are composed of heterogeneous cells populations with different biological properties and tumorigenic potentials. A small subset of the tumor cells, called the tumor initiating cells (TICs) is thought to play a crucial role in tumor development, chemoresistance and relapse (52, 53). The recent “cancer cell hypothesis” upholds the concept that tumor growth is not a simple monoclonal expansion of the transformed cells but a decisively driven by minority of these cellular populations (TICs) that display stem like properties (54). The concept of TICs is evolving and there remain several controversies concerning this model. The TICs are defined possess inherent ability for unlimited self-renewal through asymmetric division and differentiation leading to the formation of a tumor mass (55). “Asymmetric division” is defined as the process where a mother cell divides into two daughter cells: one daughter cell being the exact clone of the parental cell while the second daughter cell may not necessarily have the parental traits (56). Thus, tumors arising from TIC consist of TICs and mixed population of cells which gives rise to the full heterogeneity of the tumor (57). The working model of TICs defines the properties of such cells as having the following properties (58):

1) Unlimited proliferative ability
2) Potential to self-renew indefinitely in an undifferentiated form
3) Resistant to chemo and radio therapy

4) High DNA repair capacity
5) Ability to propagate a tumor by expansion of cells that are deregulated at various stages of differentiation

The TICs are reported to be characterized by specific identifying surface markers. Although a distinctive profile of surface markers have been identified and described, the profile of such surface markers have been found to differ from tumor to tumor and probably from patient to patient (54).

TICs in ovarian cancer

The theory origin of a TIC is equivocal. Some suggest that TICS not necessarily arise from an oncogenic transformation from a normal stem cell but can arise from a progenitor or differentiated cell acquiring stem like characteristics through un-differentiation (59). However there is also evidence that TICs can arise from neoplastic transformation of a normal stem cell (60, 61).

The first report of stem cells in ovarian cancer was made in 2005 (62). The description of the stem cell was derived from a single cell isolated from the ascetic fluid of an ovarian cancer patient that could sequentially propagate tumors over several generations. Following that, TICs populations have been identified and studied in primary ovarian tumors and ovarian cancer cell lines. The major drawback in studies of ovarian TICS is the lack of a clearly defined distinct cell surface marker. This makes isolation of a pure population of ovarian TICs very difficult. In recent studies, several attempts have been made to characterize the ovarian TICs based on the known stem cell markers of other types of solid malignancies. CD44, CD24, CD133, CD117, aldehyde hydrogenase

isoform 1 (ALDH1A1) have been proposed to denote the stem cell signature within ovarian tumors and have been used to isolate these cells from entire bulk of the tumor cells (63-66). Ovarian TICs have also been isolated based on their ability to efflux DNA dyes (67) and this population of cells are termed as “side population”. The side populations exhibit properties of tumorigenicity and chemo resistance at par with the properties of a TIC (68) but have shown to have heterogeneity for the known stem cell markers. Ovarian TICs isolated based on the surface markers and side population correlate with drug resistance and worse clinical prognosis (69). Genetic analysis of these cells were enriched in ovarian cancer patients suffering from high recurrence (69). Though the cells isolated by the abovementioned ways correlated with some of the defining features of stem cell properties, these models have always been debated as a true representation of TICs. Some more recent studies have used the functional properties of the stemness rather than isolating them based on cell surface markers. Stem cells are isolated from colonies growing from single cells possessing self-renewal capacity in culture conditions which force the cells to remain in their undifferentiated ground state (70).

Why studying TICs in ovarian cancer so important

The main challenge for ovarian cancer therapy is to overcome the resistance to treatment and recurrence of the disease. Current management strategy of advanced ovarian cancer is cytoreduction or tumor debulking surgery followed by chemotherapy to eradicate residual cancer (71). The chemotherapy consists of platinum (cisplatin or carboplatin) and taxane based agents. Though there is a remission of the cancer in 80% of the patients, majority of the patients suffer from a relapse within 2 years. The low 5 year survival rate

of only 10-30% is mainly because of the recurrence (72). The standard platinum based chemotherapy is very efficient in eradicating the bulk of the tumor mass. But recent studies show that the cisplatin fails to remove a core of cancer stem cells (CSCs) or tumor initiating cells (TICs) (73) and chronic treatment with this drug promotes enhanced damage repair and tumor progression and invasion (74). The TICs are thought to be resistant to traditional cytotoxic drugs due to high expression of ATP-binding cassette drug transporters and anti-apoptotic proteins, and an efficient DNA damage repair mechanism (75). Some current studies have also demonstrated that the residual TICs secrete soluble factors that facilitate the recurrence of the tumor (76). Thus there is accumulating evidence that TICs have crucial role if ovarian cancer progression and relapse.

Because of the above mentioned reasons, the current approach of the anti-cancer research is slowly shifting from conventional chemotherapies that target the bulk tumor cells to molecular targeted therapies against the TICs. This is probably more important in the context of ovarian cancer than any other cancer types. As discussed earlier, the lack of a thorough characterization of the ovarian TICs complicates any chemotherapy research aimed to target the ovarian TICs.

My thesis is a study to characterize these ovarian TICs in addition of what is currently known. We have tried to shed light to iron phenotype of the TICs that may uncover a targetable iron dependence of these cells.

Thesis Outline

As discussed in the introduction, iron seems to play an important role in tumorigenesis and tumor growth and invasiveness of different kinds of cancers. In most of the cancer, the net result of the alteration of the iron proteins is an increase in intra cellular iron levels leading to an increased proliferation. Whether this alteration of iron is acquired at an early stage of TICs is unknown and has not been explored. A little is known about the role of iron in ovarian cancer, particularly HGSOC.

The objective of this thesis is to provide insight in the iron phenotype of the TICs of HGSOC by addressing the following questions:

1) Is there any distinct iron phenotype in the tumor of the HGSOC patients?
2) Do iron gene and proteins predict clinical outcome of these patients?
3) Is iron metabolism altered in the ovarian TICs?
4) Do the ovarian TICs exhibit any dependence to iron metabolism?
5) Can iron metabolism be targeted in TICs chemotherapeutically?

In chapter 2, I will discuss the experimental methods used to address the questions.

Chapter 3 describes the iron phenotype of the tumors in HGSOC patients. Here I will demonstrate that HGSOC express high iron import proteins and low iron export proteins. . In chapter 4 I will further show that the mRNA expression of the iron proteins in tumor correlates with the clinical outcome of the patients. Chapter 5 describes the profile iron genes and proteins in ovarian TICs. We used patient derived ovarian TICs and a genetic model for ovarian TICs to characterize the iron parameters in these cells. Chapter 6 discusses an important finding of enhanced sensitivity of the ovarian TICs to iron

alteration. In chapters 7 and 8 I will show two major ways of how iron can be targeted in the ovarian TICs to modulate tumor growth and invasion.

Chapter 9 will provide elaborate discussion of the observations made in the study. Chapter 10 will provide a list of references.

CHAPTER 2

METHODS AND MATERIALS

Cell isolation from tissues

Patient derived ovarian TICs were isolated from tumor tissues obtained from HGSOC patients who had not received neo-adjuvant therapy and normal fallopian stem cells from unaffected fimbrial tissue of the same patient (70) (Yamamoto Y., et al., in preparation). The tissues were digested in 2mg/ml collagenase A (Roche) at 37°C for 1.5 hour. Disaggregated cells were cultivated onto a feeder layer of lethally irradiated murine 3T3-J2 cells in stem cell culturing media (SCM-6F8) (70). SCM media contains a cocktail of a Notch agonist, a ROCK (Rho Kinase) inhibitor, a Bone Morphogenetic Protein (BMP) antagonist, a Wnt agonist, a mitogenic growth factor, insulin or IGF, a TGFP signaling pathway inhibitor, and nicotinamide. Single cells were isolated from the cell clones growing on the feeder layer and cultured further on a second feeder layer in SCM media. Only the cells that are capable of self-renewal (stem cell property) formed clones. Each of the clones originating from a single cell represented a clonal expansion of the TIC or the normal fallopian stem cell. Clones were then isolated and expanded on a feeder layer with SCM media. The patient-derived TIC clones showed a compact cell morphology and expressed typical markers of HGSOC, such as Ki67 (proliferative marker), Krt7 and E-Cadherin (epithelial marker), p53 (indication of stabilized p53 protein due to mutation), PAX8 (serous cancer marker)(Yamamoto Y., et al., in preparation). Consistent with the properties of TICs, these cells can self-renew, form tumorspheres and are tumorigenic. Xenografts derived from these cells exhibited similar expression profile to the primary tumor (Yamamoto Y., et al., in preparation). The normal fallopian stem cells also exhibited long term renewability in vitro and the ability to differentiate into secretory and ciliated type cells. The cells were PAX8 positive (marker for fallopian tube epithelium stem cells),

E-cadherin positive (marker for epithelial origin), and Ki67 positive (marker for proliferation).

Immortalization and transformation of fallopian stem cells

Adult fallopian stem cells (FT^{stem}) were isolated from fimbrial tissue obtained from discarded surgical specimens of women undergoing benign procedures. Cells were isolated and cultured in a process similar to that of patient derived TICs mentioned above. The FT^{stem} cells thus isolated exhibited long term renewability in vitro and the ability to differentiate into secretory and ciliated type cells. The cells were PAX8 positive (marker for fallopian tube epithelium stem cells), E-cadherin positive (marker for epithelial origin), and Ki67 positive (marker for proliferation). Fallopian stem cells (FT^{stem}) were immortalized by infecting with retrovirus expressing hTERT, and SV40 large T antigen as previously described(77). Immortalized cells were further transformed by *c-Myc* (77). Immortalized cells are referred to here as FT^{i} cells and transformed cells as FT^{t}. Consistent with the expected properties of tumor-initiating cells, these cells can self-renew, form tumorspheres and as few as two thousand FT^{t} cells were sufficient to form palpable tumors in immunodeficient mice in two weeks, and tumor xenografts demonstrated major hallmarks of HGSOC, such as loss of PAX2 and gain of p53, EZH2 and MUC4 expression, and gene expression profiles similar to HGSOC from human patients (77).

Infection and isolation of ferroportin-expressing Ft^t cells

Human FPN cDNA was amplified using Open Biosystem clone 4823308 (primers shown in Suppl. Table 2) and introduced into the lentiviral tetracycline (tet) inducible vector pLVX-TetOne-Puro (Takara-Clontech, Mountain View, CA). Lentivirus particles were produced by transient cotransfection of the FPN tet-on expression vector and packaging vectors (VSVG, pMDLG, and RSV-REV) into 293T cells (78, 79). Viral particles containing control empty vector were prepared similarly.

Knock down of IRB2 in Ft^t cells

IRP2 knockdown was performed using lentiviral shRNAs as described (24).

Cell culture

Stem cells and tumor progenitor cells (FT^{stem}, CP2-OC, CP2-N, CP34-OC, CP34-N) were cultured on δ- irradiated 3T3 fibroblast feeder cells as described. Immortalized and transformed stem cells (FT^i, FT^t) were cultured in DMEM (GIBCO) containing 10% FBS (BenchMark). All cells were maintained at 37 °C in a humidified incubator at 7.5% CO2.

Real-time RTPCR

Briefly, 200 – 400 ng of RNA was reverse transcribed in a total volume of 50 µl with a reverse transcription reagents kit (Applied Biosystems). To make a standard curve, serial dilutions of RNA from one sample were added to the RT reaction. Aliquots (2 µl) of cDNA were added to a 18 µl reaction mixture containing 10 µl of 2× SYBR® Green PCR Master Mix (BioRad) and 400 nm primers. Absence of DNA contamination was confirmed by

performing PCR from cDNA without reverse transcriptase. Primers used for each gene are provided in Supplemental Table 2.

Western blot

Cells were lysed with NP-40 lysis buffer (25 mM tris (pH 7.4), 1% Triton X-100, 1% SDS, 1% sodium deoxy-cholate, 150 mM NaCl, aprotinin (2 µg/ml), 1 mM phenylmethylsulfonyl fluoride) containing complete protease inhibitor cocktail (Roche Diagnostics). Samples were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes before being incubated with primary and secondary antibodies. Bands were detected using chemiluminescence (Thermo Scientific). Membranes were probed with antibodies to β-actin (Sigma), ferroportin (Novus Biologicals), transferrin receptor1 (Invitrogen), IRP2 (Santa Cruz), ferritin H (24), p-STAT3 (cell Signaling) and STAT3 (cell Signaling). Western blots were quantified using ImageJ software.

IL6 ELISA

Cells were seeded in a six well plated in growth media for 24 hours. Conditioned media was collected and viable cells counted. IL6 secretion was measured using a Quantikine IL6 ELISA kit from R & D systems. ELISA results were normalized to number of viable cells at the time of collection of media.

Cell Invasion assay

Cell invasion was conducted by Cultrex® BME Cell Invasion Assay kit by Trevigen in a 96 well plate with or without basement membrane extract (BME) coating on the inserts as per manufacturer's instructions. Because FPN affects cell proliferation, we corrected for cell number by comparing the number of cells that invaded through basement membrane protein to those that migrated through an uncoated 8 micron porous membrane.

Labile iron pool (LIP Assay)

Calcein acetoxymethyl ester (CA-AM) was obtained from Molecular Probes. The iron chelator, isonicotinoyl salicylaldehyde hydrazone (SIH) (a gift from Dr. P. Ponka, Lady Davis Institute for Medical Research, Montreal, Canada) was prepared as a 50 mM stock solution in dimethyl sulfoxide (DMSO). Briefly, 25,000 – 50,000 cells were cultured in 96-well plates (Black with Clear Bottom purchased from Coring) overnight. Cells were loaded with 2 μM CA-AM for 15 to 30 minutes at 37°C, and then washed with PBS. 100 μM starch-conjugated desferrioxamine (DFO; a generous gift of Biomedical Frontiers, Inc., Minneapolis, MN) was added to cells to remove extracellular iron. Fluorescence was measured at 485 nm excitation and 535 nm emission with a fluorescence plate reader (BioTek Synergy 2). After the fluorescence signal was stabilized, SIH was added at a final concentration of 10 μM to remove iron from calcein, causing dequenching. The change in fluorescence following the addition of SIH (ΔF) was used as an indirect measure of the labile iron pool.

Cell viability

3×10^3 cells were seeded in 96 well plates and treated with either deferoxamine mesylate (Sigma), erastin (Selleckchem), or ferrostatin-1 (Selleckchem). Cell viability was assessed at 24-72 hours post-treatment using Cell Titer 96 Aqueous (Promega, Madison, WI, USA).

Immunohistochemistry

Paraffin-embedded formalin-fixed (PEFF) slides of de-identified human tissues obtained from HGSOC (10 subjects), low grade serous ovarian cancer (10 subjects), STIC (6 subjects), normal oviduct (10 subjects), and normal ovary (10 subjects) were collected from the biorepository of UCHC (IRB IE-08-310-1). Tissues were immunostained with antibodies to human Transferrin Receptor1 (Invitrogen), Ferroportin (Amgen), IRP2 (LSBio), ferritin H (24), and Ferritin L (Abcam), Cytokeratin 7 (abcam) and Pax 8 (proteintech). Relevant IgGs were used as negative controls. Images were acquired using a Zeiss Axio Scan Z1. Images of three to four random fields per slide were taken with 40X objectives using Zeiss Axio Imager A.2 and quantified using the DAB application in the open source Fiji software (ImageJ) was used. Reciprocal intensity was measured by subtracting the mean intensity of the stained area of interest from the maximum intensity (80).

Immunofluorescent staining of cells

Cells were plated in 8-chamber slides (BD Falcon) and incubated with anti-human Ferroportin 38C8 (Amgen), anti-human Ferritin H (24), anti-Human Ferritin L (Abcam), anti-human IRP2 (LSBio), or anti-human Transferrin Receptor (Invitrogen). Rodamine-Red (Jackson ImmunoResearch), Alexa fluor 488, and Alexa Fluor 555-conjugated secondary antibodies were used. Slides were mounted with ProLong Gold anti-fade reagent (Invitrogen). Images were acquired using inverted microscopy (Zeiss Axio Vert.A1).

Animal Ethics Statement

All animal studies were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The experiment protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut Health Center (protocol # 100881).

Animal Experiments

Female NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice (NSG; ~ 6 weeks of age) were obtained from Jackson Laboratory. 10^5 FT^t cells containing either the FPN tet-on vector or empty vector were injected intraperitoneally (i.p) (n=10/group). FPN was induced in xenografts by adding 2 mg/ml doxycycline to the drinking water and mice were euthanized after 4 weeks. For erastin treatment, mice were treated with 20 mg/kg Erastin in 2% DMSO/PBS or vehicle alone beginning one day after tumor cell injection. Treatment was continued

at five doses per week for 18 days before mice were sacrificed. Tumors were counted and the weight of all tumors found within the abdomen of each mouse was measured.

Statistical analysis of cell culture experiments

Statistical analyses were performed using Excel or Prism 6 (Graphpad software). All experiments were performed at least three times using a minimum of three replicates/condition in each experiment. Comparison tests were performed between two groups and statistical significance assessed using two-tailed unpaired Student's t tests. Statistics are reported as the mean ± standard deviation.

Publicly available datasets

For gene expression studies involving publicly available data, three datasets were employed: GSE9891 (n = 285) (34); GSE27651 (n=42) (81); and GSE69428 (n=10 paired samples). All expression datasets were obtained using an Affymetrix U133 Plus 2 microarray platform, and datasets were downloaded from the NCBI Gene Expression Omnibus (GEO) database (82) as raw CEL files. Data were processed using robust microarray average methods as implemented in the Bioconductor software package *affy* (83, 84). Clinical metadata associated with GSE9891 were obtained from the original study (34).

Statistical analyses of publicly available datasets

All hypothesis tests comparing mean expression levels for genes of interest between groups were run as two-sided t-tests (or paired t-tests for the paired samples) with significance level = 0.05. All data presented are from probesets 223044_at (FPN) and 237214_s_at (TFRC). Association between FPN and TFRC gene expression and patient survival using progression free and overall survival outcomes reported in the GSE9891 data was assessed using Cox proportional hazards regression implemented in the *survival* package in R (85, 86). Significance for regression coefficients was taken at the 0.05 level. To examine differences in survival for FPN using Kaplan Meier methods for high and low gene expression groups, expression groups were binned by locating an optimal risk stratification cutoff point based on unadjusted p values from log-rank tests. Testing for differences in gene expression amongst molecular subtypes defined for the GSE9891 dataset was done using overall F-tests for across-group comparisons, followed by multiple testing adjusted pairwise t-tests for the subgroups. All pairwise tests were two-tailed with significance level = 0.05 and multiple testing adjustments were done using Benjamini Hochberg FDR adjustments implemented in the software R. Subgroup analyses were carried out in the GSE9891 dataset using all subgroups (including the set of individuals (n=34) not assigned to a subgroup) for the purposes of pooled variance estimation.

Chapter 3

Iron phenotype is altered in tumors of patients with HGOSC and is correlated with the outcome of the patients

Chapter 3

3.1 Chapter Introduction

As discussed in the introduction, iron metabolism is found to be altered in several types of cancer. However, it has not been studied extensively in ovarian cancer, particularly HGSOC. Our first step was therefore to study the expression pattern for the iron genes and proteins (especially TFR and FPN) within the tumor tissues of HGSOC patients. We also explored if expression of TFR/FPN correlates with patient survival.

3.2 Results

3.2.1 HGSOC is characterized by consistent alterations in expression of iron-related genes and proteins

We began by testing whether iron metabolism was altered in HGSOC by assessing levels of transferrin receptor 1 (TFRC) and ferroportin (FPN), proteins that play a central role in iron metabolism by regulating the uptake and efflux of iron, respectively. As seen in **Fig. 4,** immunohistochemistry of normal ovarian epithelium versus tissue from patients with HGSOC revealed that the iron importer TFRC was strongly expressed ($p = 2x10^{-7}$) and the iron efflux protein FPN was weakly expressed ($p = 2x10^{-11}$) in HGSOC when compared to normal ovarian epithelia. Because of a growing body of evidence that serous tubal intra-epithelial carcinoma (STIC) is a precursor lesion of HGSOC within the fimbrial region of the fallopian tubes (87-89), we also compared the staining of normal ovary to STIC. It revealed the same trend: TFRC was elevated ($p = 3x10^{-7}$) and FPN was decreased ($p = 5x10^{-6}$) in STIC relative to the normal ovary. Since HGSOC is suspected to originate in fimbria, we also performed IHC analysis of normal fimbria compared to HGSOC. Staining

of these cells similarly revealed an increase in TFRC ($p = 0.0001$) and decrease in FPN ($p = 7x10^{-7}$) when compared to HGSOC.

IRP2 is a post-transcriptional regulator of TFRC and FPN that is overexpressed in some cancers (24). Since overexpression of IRP2 would lead to an increase in TFRC and decrease in FPN, we also assessed levels of IRP2 in our samples of normal and malignant ovary (**Fig. 4**). Although IRP2 was substantially increased in STIC ($p = 3x10^{-8}$) and was modestly elevated in HGSOC ($p = 0.0008$) when compared to normal fimbria, it was not statistically different in HGSOC versus normal ovary, suggesting that differences in levels of IRP2 alone cannot account for all of the observed changes in TFRC and FPN. Collectively, these results suggest a phenotype of increased iron retention in ovarian cancer cells when compared to epithelial cells of the non-malignant ovary and oviduct fimbria.

We tested whether this profile of iron retention was also characteristic of low grade serous ovarian cancer (LGSOC), a less aggressive form of ovarian cancer with a more favorable prognosis that is believed to have a different pathogenesis than HGSOC (90, 91). As shown in **Fig. 5**, IHC staining for TFRC, FPN and IRP2 revealed that malignant tissue from patients with LGSOC exhibited a staining profile that was quite distinct from HGSOC and more reminiscent of normal tissue, with decreased TFRC ($p = 0.001$), increased FPN ($p = 0.0001$)and decreased IRP2 ($p = 5.9x10^{-6}$) relative to HGSOC. We also performed IHC staining for ferritin, an iron storage protein composed of H and L subunits that is translationally induced by iron and frequently used as a marker of elevated intracellular iron (92). Consistent with a picture of excess iron acquisition and retention in HGSOC, staining for either ferritin H or ferritin L demonstrated an increase in HG vs LG ovarian

cancer ($p = 1.6 \times 10^{-8}$) (**Fig. 6**), as well as in HGSOC vs normal ovary or fallopian tube (**Fig. 5**). Thus, a profile of iron retention characterizes HGSOC, an ovarian cancer subtype with an especially poor prognosis.

To determine whether changes in TFRC and FPN were observed at the RNA as well as protein level, we examined several publicly available gene expression datasets from ovarian cancer patients. In a study (GSE69428) comparing normal oviduct to HGSOC within the same patient, we observed a decrease in FPN mRNA in tumor tissue ($p = 0.0001$, paired t test) (**Fig. 7)**. Results for TFRC were inconsistent, with some probes showing an increase and some showing a decrease between normal and tumor. Comparison of low grade to high grade ovarian cancer (GSE27651) showed a significant decrease in FPN ($p = 0.05$, two-tailed t-test) and a similar inconsistency in TFRC (**Fig. 8**).

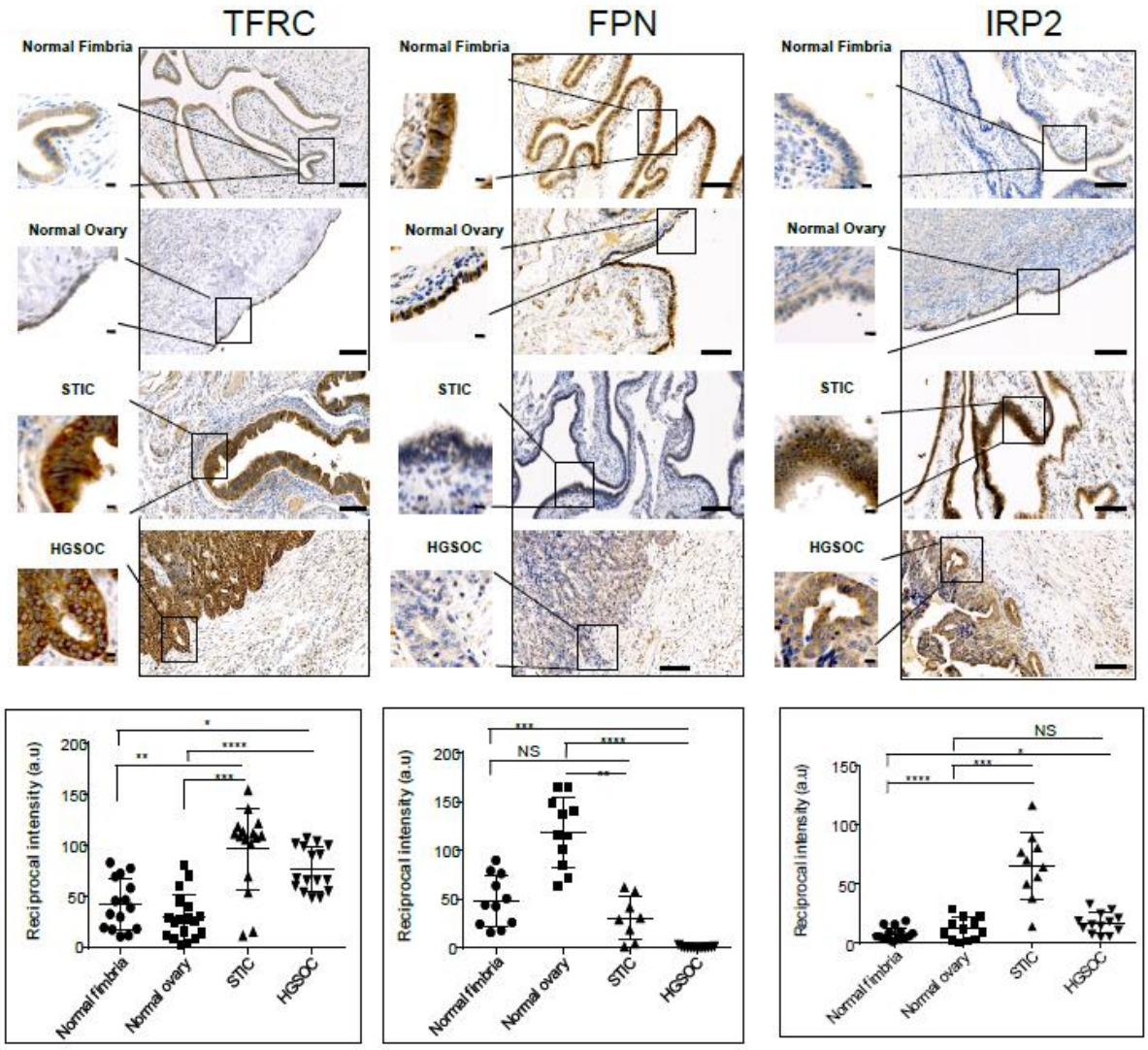


Fig. 4. Proteins that control intracellular iron are altered high grade serous ovarian cancer (HGSOC). Representative images of immunohistochemical staining of normal fimbria, normal ovary surface epithelia, serous tubal intra-epithelial carcinoma (STIC) and HGSOC stained with antibodies against transferrin receptor 1 (TFRC), ferroportin (FPN) and iron regulatory protein 2 (IRP2). Dot plots represent quantification of staining of tissues collected from 8 patients with HGSOC and 5 with STIC compared to 8 subjects

with normal fimbria and 6 individuals with normal ovarian surface epithelium. Images of three to four random fields per slide were quantified. Differences in TRFC ($p<0.0001$), FPN ($p<0.0001$ and IRP2 ($p<0.01$) were statistically significant (one way ANOVA). *$p<0.001$, **$p<5x10^{-5}$, *** $p<5x10^{-6}$, ****$p<5x10^{-7}$, one tailed t test for individual comparisons. Scale bar 1 mm; inset scale bar 10 µm.

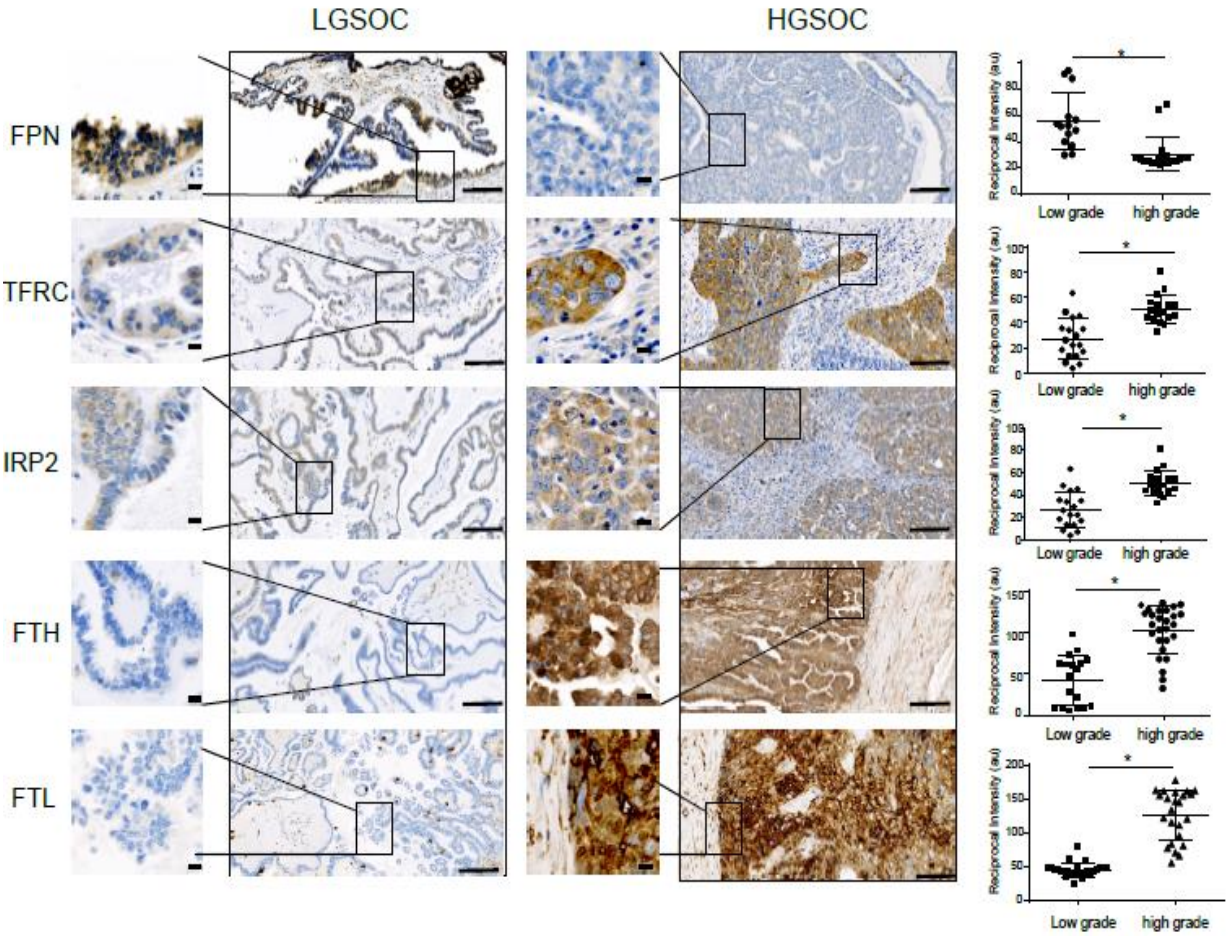


Fig 5. Proteins that control intracellular iron differ in high grade and low grade serous ovarian cancer. Representative images of immunohistochemical staining of tumor tissues from patients with low grade serous ovarian cancer (LGSOC) and high grade serous ovarian cancer (HGSOC). Proteins stained are ferroportin (FPN), transferrin receptor (TFRC), Iron Regulatory Protein 2 (IRP2), ferritin heavy chain (FTH) and ferritin light chain (FTL). Dot plots show quantification of staining of tissues from 5 patients with LGSOC and 8 patients with HGSOC (3 to 4 random fields from each patient tissue slide). *p<0.002. Scale bar 1 mm; inset scale bar 10 μm.

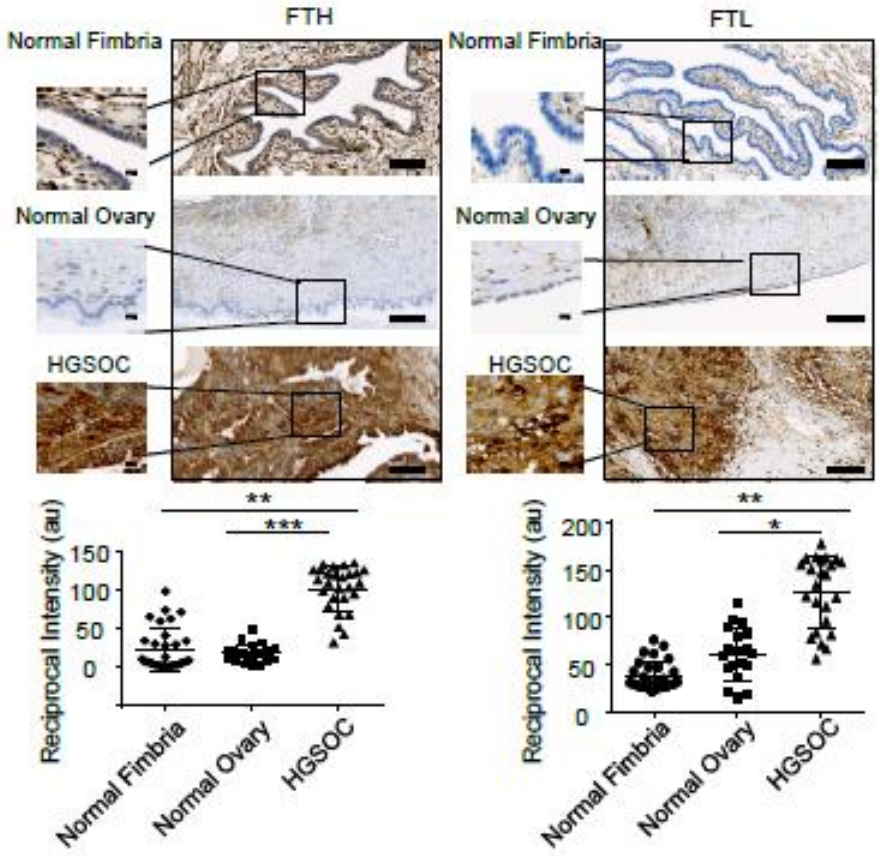


Fig 6. Increased expression of ferritin heavy (FTH) and ferritin light chain (FTL) in HGSOC. Representative images of immunohistochemical staining using antibodies to ferritin heavy chain (FTH) and ferritin light chain (FTL) in normal fimbria, normal ovary surface epithelia, and high grade serous ovarian cancer (HGSOC). Dot plots represent quantification of staining from multiple patients with HGSOC (8 patients) compared to normal fimbria (8 subjects) and normal ovarian surface epithelium (6 subjects) (3 to 4 random fields from each slide). *$p<0.1x10^{-7}$; **$p<1x10^{-14}$; ***$p<1x10^{-16}$. Scale bar 1 mm; inset scale bar 10 μm.

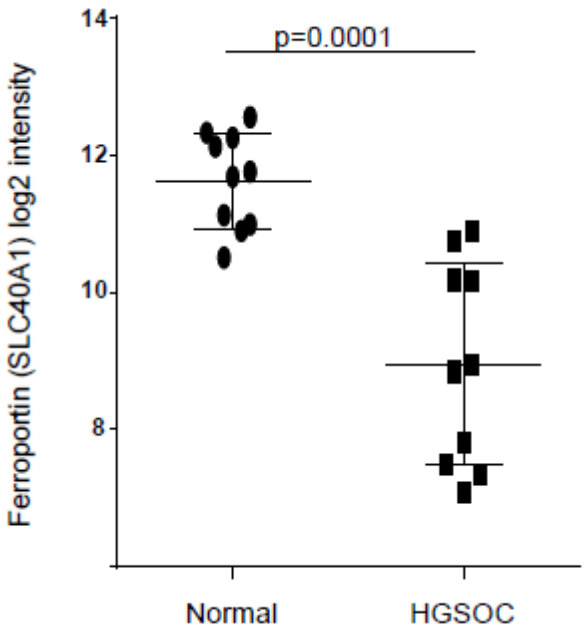


Fig 7. Ferroportin expression is decreased in HGSOC compared to normal oviduct. Log 2-transformed microarray signal intensity values for ferroportin (SLC40A1) in paired samples from 10 normal oviduct and 10 high grade serous ovarian cancer (HGSOC) samples (GSE69428). P=0.0001, paired t-test.

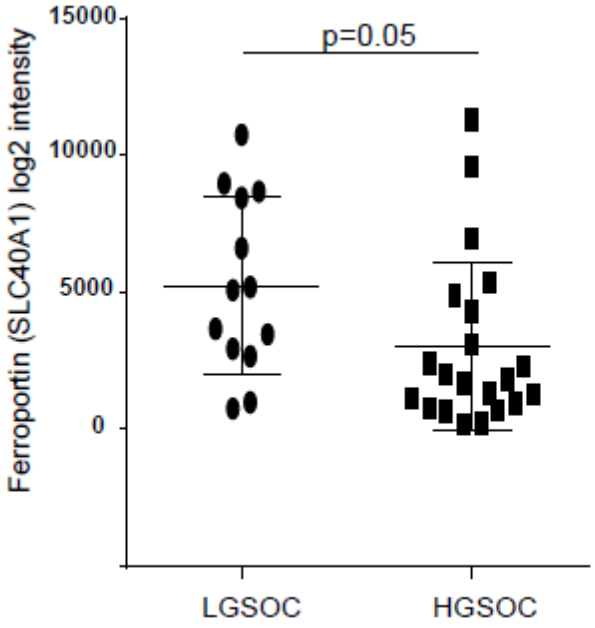


Fig 8. Ferroportin expression is decreased in high grade serous ovarian cancer (HGSOC) compared to low grade serous ovarian cancer (LGSOC). Log 2-transformed microarray signal intensity values for ferroportin (SLC40A1) in samples from 13 patients with LGSOC and 28 patients with HGSOC (GSE27651). P=0.05, unpaired t-test.

3.2.2. Expression of FPN and TFRC are associated with ovarian cancer patient prognosis

We next explored whether the changes in FPN and TFRC were associated with survival in ovarian cancer patients. Using a dataset of 285 patients (GSE9891(34)), we examined the relationship between gene expression and overall survival using a Cox proportional hazards model. There was a significant association between FPN and patient survival ($p = 0.049$ and 0.013, respectively, for overall and progression free survival). We also used Kaplan Meier analysis to examine the association between FPN expression and survival, and observed a significant increase in both overall and progression-free survival in high FPN expressors ($p = 6.5 \times 10^{-5}$, log-rank test (**Fig. 9 A,B**). Cox PH analysis did not reveal a significant association between TFRC and survival.

We reasoned that heterogeneity in the patient population contained in the GSE9891 database might have obscured associations between TFRC and patient outcome. We therefore performed two additional analyses of the association between FPN, TFRC and patient outcome. We first assessed the association between FPN and TFRC and molecular subtype. In their study, Tothill *et al*. (34) identified and validated six molecular subtypes of ovarian cancer that had different clinical outcomes (termed C1-C6). Of these, C3, representing predominantly serous ovarian cancers of low malignant potential (LMP), and C6, early stage endometriod tumors, had the most favorable outcome. Ferroportin expression differed significantly among subgroups ($p < 2 \times 10^{-16}$, overall F-test), and was increased in the good prognosis C3 subgroup in multiple-testing adjusted pairwise comparisons to all other subgroups (**Fig. 9C).** Similarly, TFRC expression varied significantly among subgroups ($p=5.6 \times 10^{-5}$, overall F-test) and was reduced in the good

prognosis C3 subgroup in multiple-testing adjusted pairwise comparisons to C1, C2, and C4 subgroups (and marginally to C5) (**Fig. 9D**). We also examined the association between FPN and TFRC and grade, a known indicator of prognosis. When patients were subdivided according to grade, FPN was significantly different across the grade groups ($p = 0.029$), and showed a decrease in mean expression between the lowest and higher grades (**Fig. 9E**). TFRC was also significantly different across grade groups ($p = 0.009$, overall F-test), and showed increased mean expression level with increasing grade (**Fig. 9F**). Thus decreased expression of FPN and increased expression of TFRC characterize ovarian cancer patients with poorer prognosis.

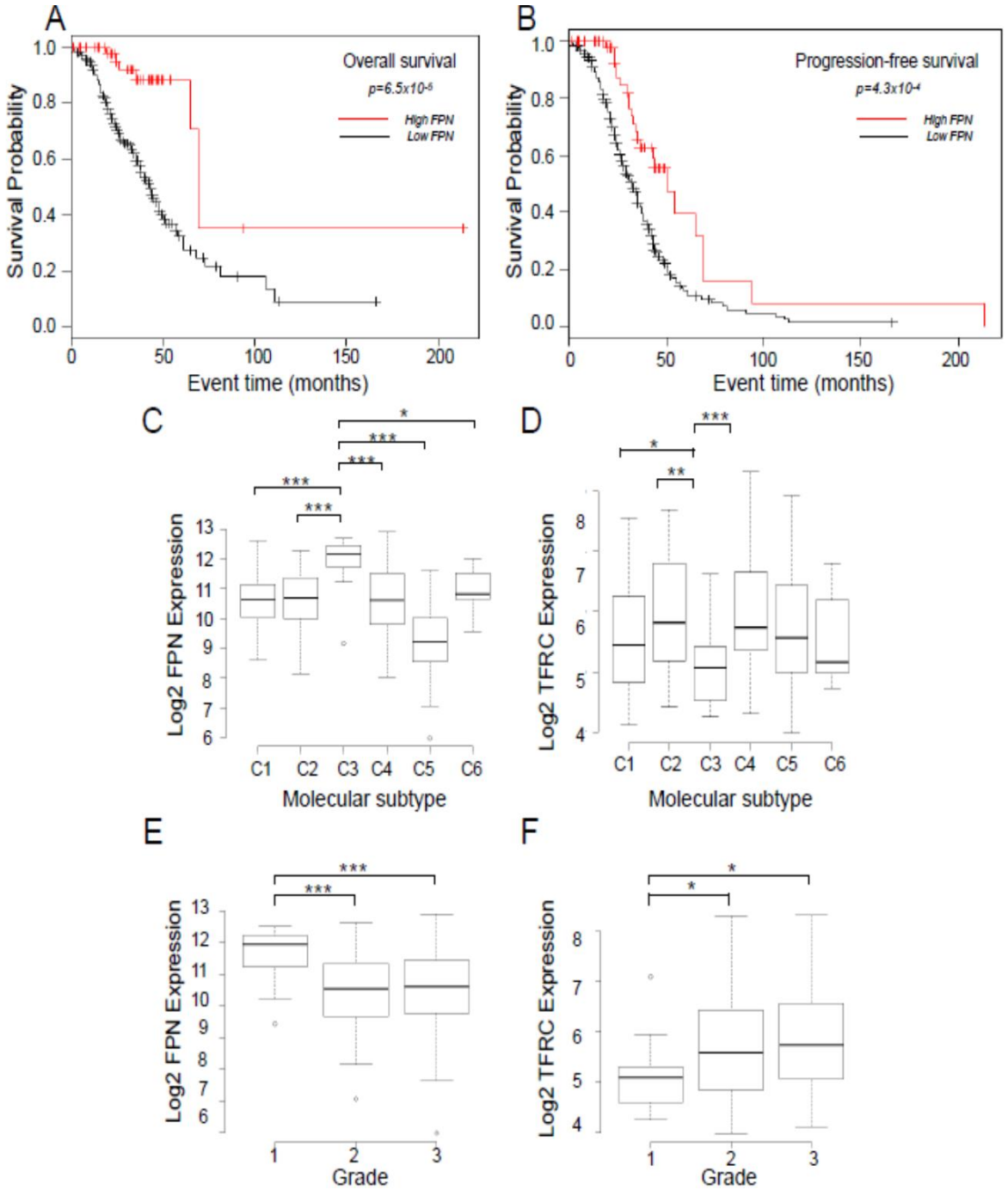

A
Overall survival
p=6.5x10-6
High FPN
Low FPN
Survival Probability
Event time (months)
B
Progression-free survival
p=4.3x10-4
High FPN
Low FPN
Survival Probability
Event time (months)
C
Log2 FPN Expression
C1 C2 C3 C4 C5 C6
Molecular subtype
D
Log2 TFRC Expression
C1 C2 C3 C4 C5 C6
Molecular subtype
E
Log2 FPN Expression
Grade
F
Log2 TFRC Expression
Grade

Fig. 9. Low ferroportin and high transferrin receptor expression are associated with poor prognosis in patients with HGSOC. (a,b) Kaplan Meier survival curves for FPN expression groups based on optimal risk stratification for GSE9891 dataset (p based on the log-rank test). (b,c) Boxplots of log2 gene expression levels for FPN and TFRC across molecular subtype groups (data from GSE9891). Shown are significant differences between subgroup C3 and other subgroups: *$p < 0.05$; **$p < 0.005$; *** $p < 0.0005$. Individuals who were not assigned a molecular subtype are not shown in this Fig. (e,f) Boxplots of log2 gene expression levels for FPN and TFRC across tumor grades.

3.3 Chapter summary

In this chapter we show that iron trait is altered within the tumor tissues of HGSOC patients. TFR, the major iron import protein is increased and FPN, the iron exporter protein is decreased in tumor tissues when compared to normal fallopian epithelia or normal ovary surface epithelia. STIC, which is thought to be the precursor lesion of HGSIC shows same trend of increased iron import and decreased export when compared to normal fallopian tube epithelia. There is only a modest or no increase in IRP2 in tumor tissue. Ferritin, which is an iron storage protein and is a surrogate marker for iron retention is increased in tumor. This comparative iron profile is also demonstrated between low and high grade ovarian malignant tissues. FPN mRNA expression in tumor tissue correlates with survival of patients. TFR mRNA expression is lower in favorable prognosis subtypes of ovarian cancer patients.

Chapter 4

Iron phenotype is altered in ovarian TICs

Chapter 4

4.1. Chapter introduction

In chapter 3, we have reported an altered iron phenotype in tumor tissues of HGSOC patients. An important clinical problem in the management of ovarian cancer is late stage diagnosis: most patients present with advanced disease that has spread. Patients who have been initially treated with successful response suffer from recurrence and chemoresistance of the recurrent tumor. Since the recurrence and drug refractoriness of cancer is attributed to the remaining TICs, we sought to determine whether alterations in iron metabolism could be detected (and thus ultimately potentially targeted) in the TICs resident in the tumor tissue.

4.2. Results

4.2.1. Iron metabolism is altered in patient-derived ovarian cancer TICs

To confirm the early appearance of changes in iron homeostasis in TICs, we used a second approach. We isolated tumor-initiating cells directly from HGSOC patients who had not received neo-adjuvant therapy and compared them to normal fallopian stem cells from the same patient (70)(Yamamoto et al., in preparation). Patient-derived tumor initiating cell clones showed a compact cell morphology and expressed typical markers of high-grade ovarian cancer, such as Ki67 (proliferative marker), Krt7 and E-Cadherin (epithelial marker), p53 (indication of stabilized p53 protein due to mutation), PAX8 (serous cancer marker)(Yamamoto Y., et al., in preparation). Cells were tumorigenic in immunodeficient mice and xenografts derived from these cells exhibited a similar expression profile to the primary tumor (Yamamoto Y., et al., in preparation).

Analysis revealed a profile of iron acquisition and retention in patient-derived TICs with a decrease in FPN, an increase in TFRC (**Fig. 10A-F**), and an increase in ferritin **(Fig. 10G,H**). The net effect of increased iron import and decreased iron export should be increase in the labile iron pool (LIP). We found that the LIP (**Fig. 10I**) is increased with the TICs relative to normal fallopian tube stem cells. Cells derived from a second, independent, patient donor yielded similar results at mRNA levels (**Fig. 11**). Thus ovarian cancer TICs exhibit a profile of increased iron retention.

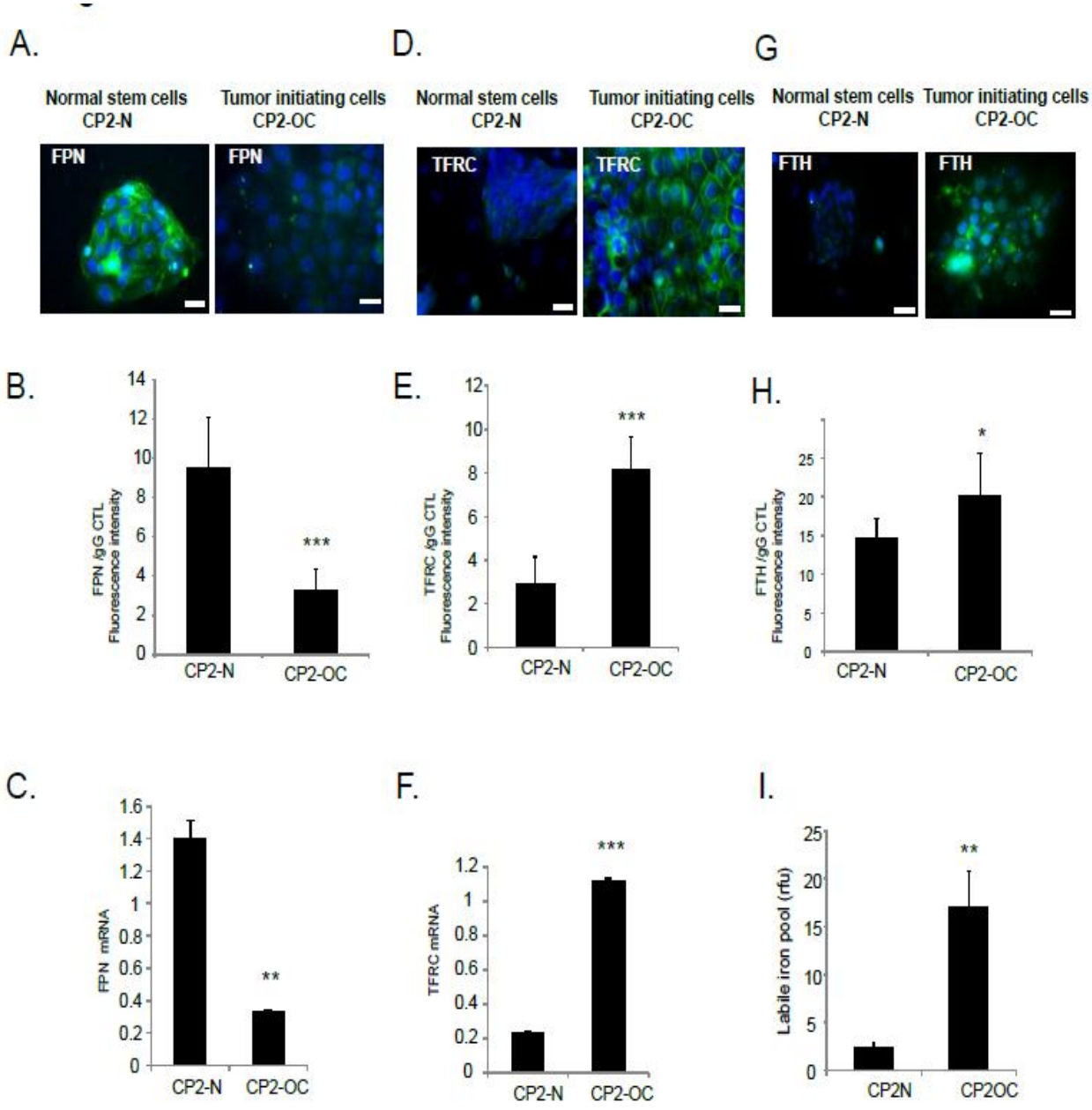


Fig. 10. **Iron metabolism is altered in patient-derived normal stem cells and tumor initiating cells.** (A) Representative images of immunofluorescent staining of ferroportin (FPN) in normal stem cells (CP2-N) and tumor-initiating cells (CP2-OC) derived from the same patient. Green, FPN; blue, nuclei. (B) Quantification of FPN fluorescence intensity;

(C) qRT-PCR of FPN mRNA; (D) Immunofluorescent staining of transferrin receptor (TFRC); (E) quantification of TFRC staining intensity: (F) qRT-PCR of TFRC mRNA; (G) Immunofluorescent staining of ferritin H; (H) Quantification of FTH fluorescence intensity; (I) Labile iron pool. *p<0.02; **p<0.0002; ***p<$2x10^{-5}$. Scale bar 20 µm.

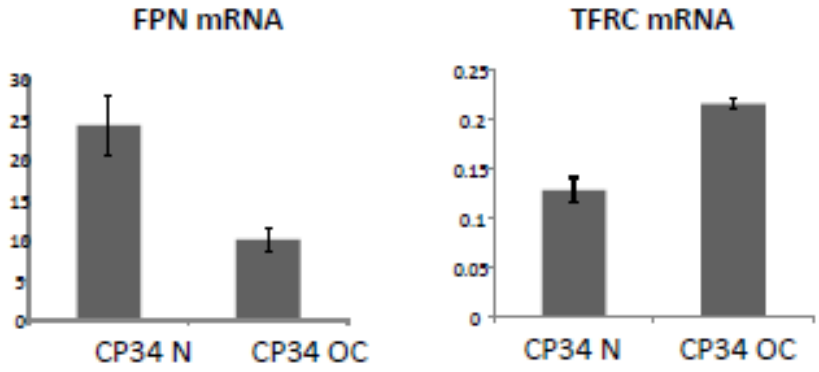


Fig 11. Normal stem cells and tumor initiating cells differ in their iron phenotype. Normal and tumor-initiating cells were isolated from a patient with HGSOC (CP34-N and CP34-TIC) and expression of ferroportin (FPN) and transferrin receptor 1 measured using qRT-PCR with normalization to β-actin.

4.2.2. Iron metabolism is altered in a genetic model of ovarian cancer TICs

HGSOC is characterized by a high level of genomic heterogeneity(51). Although patient-derived TICs most closely represent this heterogeneity of the human disease, a model of ovarian TIC with limited and defined genetic alterations can help identify specific genetic changes that drive alterations in iron metabolism. We therefore built a genetic model of TICs in HGSOC by transducing the normal stem cells with SV40TAg, hTERT and c-Myc (77), an approach that has been successfully used previously used to model high grade serous ovarian cancer (41, 93-95). Beginning with the normal stem-like cells derived from human fimbria, which we term FT^{stem} cells, we first introduced SV40TAg and hTERT by retroviral infection to create immortalized but non-tumorigenic FT^{i} cells. We then we fully transformed them by infecting with c-Myc to create FT^{t} cells. Consistent with the expected properties of tumor-initiating cells, as few as two thousand FT^{t} cells were sufficient to form palpable tumors in immunodeficient mice in two weeks, and tumor xenografts demonstrated major hallmarks of HGSOC, such as loss of PAX2 and gain of p53, EZH2 and MUC4 expression, and gene expression profiles similar to HGSOC from human patients (77).

We tested whether changes in iron metabolism occurred during the transition of normal FT^{stem} cells to malignant FT^{t} cells. As shown in **Fig. 12A-C,** there was a progressive decrease in FPN expression at both the mRNA and protein levels as cells transitioned from normal to immortalized to fully transformed. Similar to what we observed in the tumor tissues of patients with HGSOC, this was accompanied by a progressive increase in TFRC (**Fig. 12D-F**). Since the increase in TFRC would lead to an increase in iron uptake, and a decrease in ferroportin would lead to a concomitant decrease in iron efflux,

these changes would be anticipated to result in an increase in the metabolically active labile iron pool (LIP) as stem cells transition to malignancy. We tested this prediction by measuring the LIP in FT^{stem}, FT^{i} and FT^{t} cells. As shown in **Fig. 12J**, the LIP progressively increased in these cells. Consistent with the increase in LIP, which increases the translation of ferritin mRNA, there was an increase in ferritin protein without a corresponding increase in ferritin H mRNA (**Fig. 12G,H, I**). These data suggest that perturbations in iron metabolism occur early in the genesis of HGSOC from TICs , and that key elements of the changes in iron metabolism can be recapitulated by genetically manipulating three defined genes.

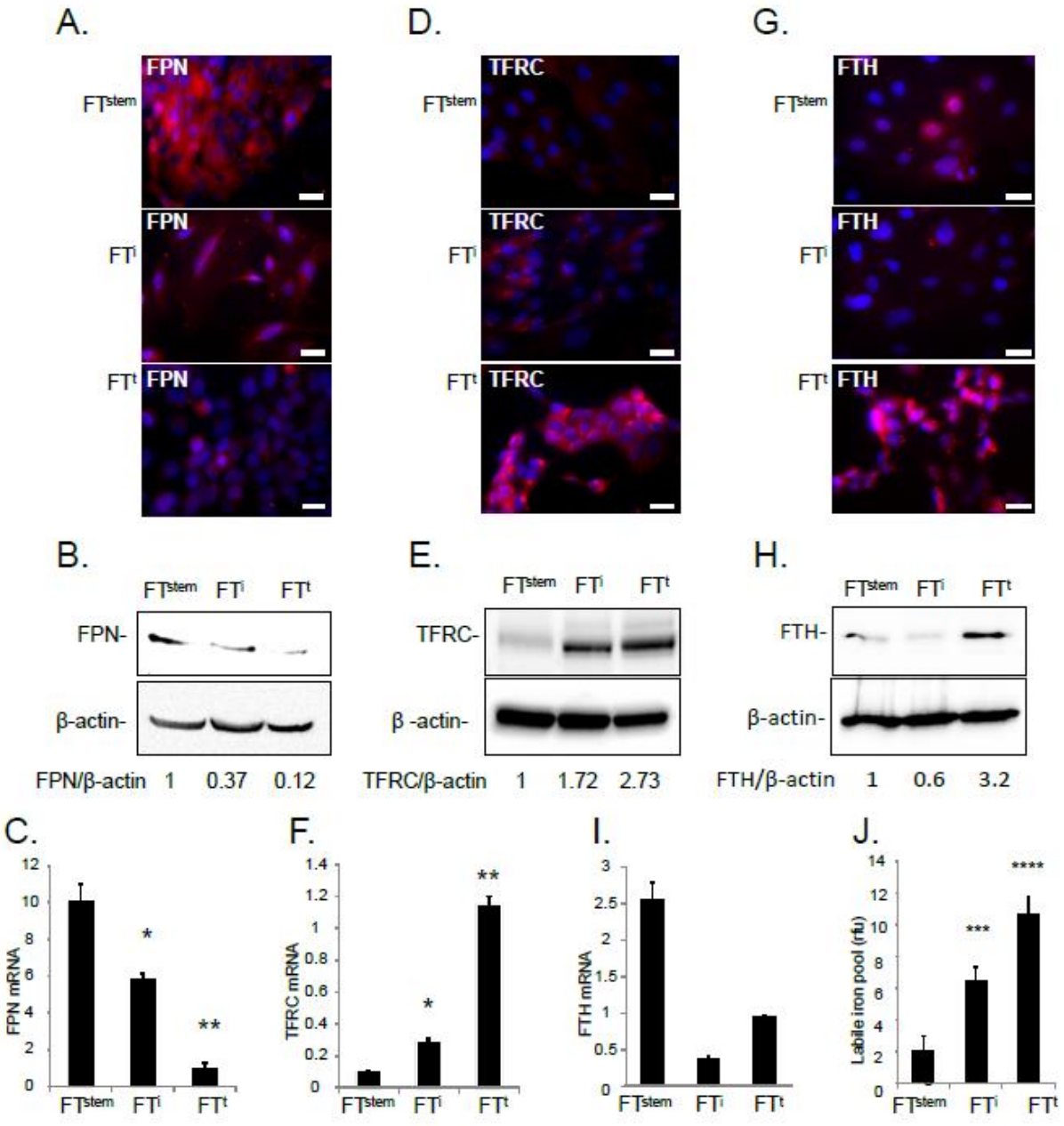


Fig. 12. Iron metabolism is modified in a genetic model of ovarian cancer tumor initiating cells. (A) Immunofluorescent staining of ferroportin (FPN), in normal fallopian tube stem cells (FT^{stem}), immortalized fallopian tube stem cells (FT^{i}) and transformed fallopian tube stem cells (FT^{t}). Red, ferroportin; blue, nuclei. (B) Western blot of

ferroportin and β-actin, with Image J quantification of ferroportin/β-actin shown below; (C) qRT-PCR of ferroportin mRNA; (D)Immunofluorescent staining of transferrin receptor 1 (TFRC); (E) TFRC western blot; (F) qRT-PCR of TFRC mRNA; (G) Immunofluorescent staining of ferritin heavy chain; (H) Ferritin H western blot; (I) qRT-PCR of ferritin H mRNA; (J) labile iron pool.*$p<0.02$, **$p<0.0.0007$, ***$p<5x10^{-8}$; ****$p<5x10^{-11}$. Scale bar 20 µm.

4. 3. Chapter summary

In this chapter we studied the expression of iron proteins in patient derived ovarian TICs. Consistent with what we had seen in tumor tissues of HGSOC patients, we see a similar iron trait of increased TFR and decreased FPN in ovarian TICs compared to when the fallopian stem cells. We also created a genetic model of HGSOC TICs by immortalizing fallopian stem cells with hTERT and SV40 and then transforming with c-Myc. In this model, we observed that the iron seeking phenotype progressively develops as we go from normal to immortalized to fully transformed. This implies that the 3 target genes (hTERT, p53, and c-Myc) may have some role (at least in part) in the development of the observed iron phenotype in ovarian TICs. As it is expected, the changes in TFR and FPN led to increased ferritin and LIP in the ovarian TICs.

Chapter 5

Ovarian cancer TICs exhibit enhanced iron dependence

Chapter 5

5.1. Chapter introduction

In previous chapter we see that ovarian TICs exhibit an iron retention phenotype. We tested whether the profile of iron acquisition and retention seen in ovarian cancer tumor-initiating cells is reflective of an enhanced dependence on iron ("iron addiction")(96). The impetus behind this question is if the TICs possess an iron addiction, it would be a potential node to target the TICs through drugs.

5.2. Results

Ovarian cancer TICs exhibit enhanced iron dependence

We treated FT^{stem}, FT^{i} and FT^{t} cells with desferroxamine (DFO), an iron chelator, and measured effects on viability. As shown in **Fig. 13A**, TICs were remarkably more sensitive to iron chelation than normal stem cells; immortalized cells exhibited an intermediate sensitivity. Similarly, patient-derived cancer TICs exhibited an enhanced sensitivity to iron depletion when compared to their normal counterparts (**Fig. 13B**). To confirm the sensitivity of ovarian cancer TICs to iron depletion using a genetic rather than pharmacologic approach, we knocked down IRP2 in FT^{t} cells. As expected, knockdown of IRP2 decreased TFRC and increased ferritin, which depletes intracellular iron (**Fig. 13C,D**). Proliferation FT^{t} cells was dramatically inhibited following this genetically-induced reduction in intracellular iron (**Fig. 13E**), consistent with the sensitivity of these cells to iron depletion by DFO.

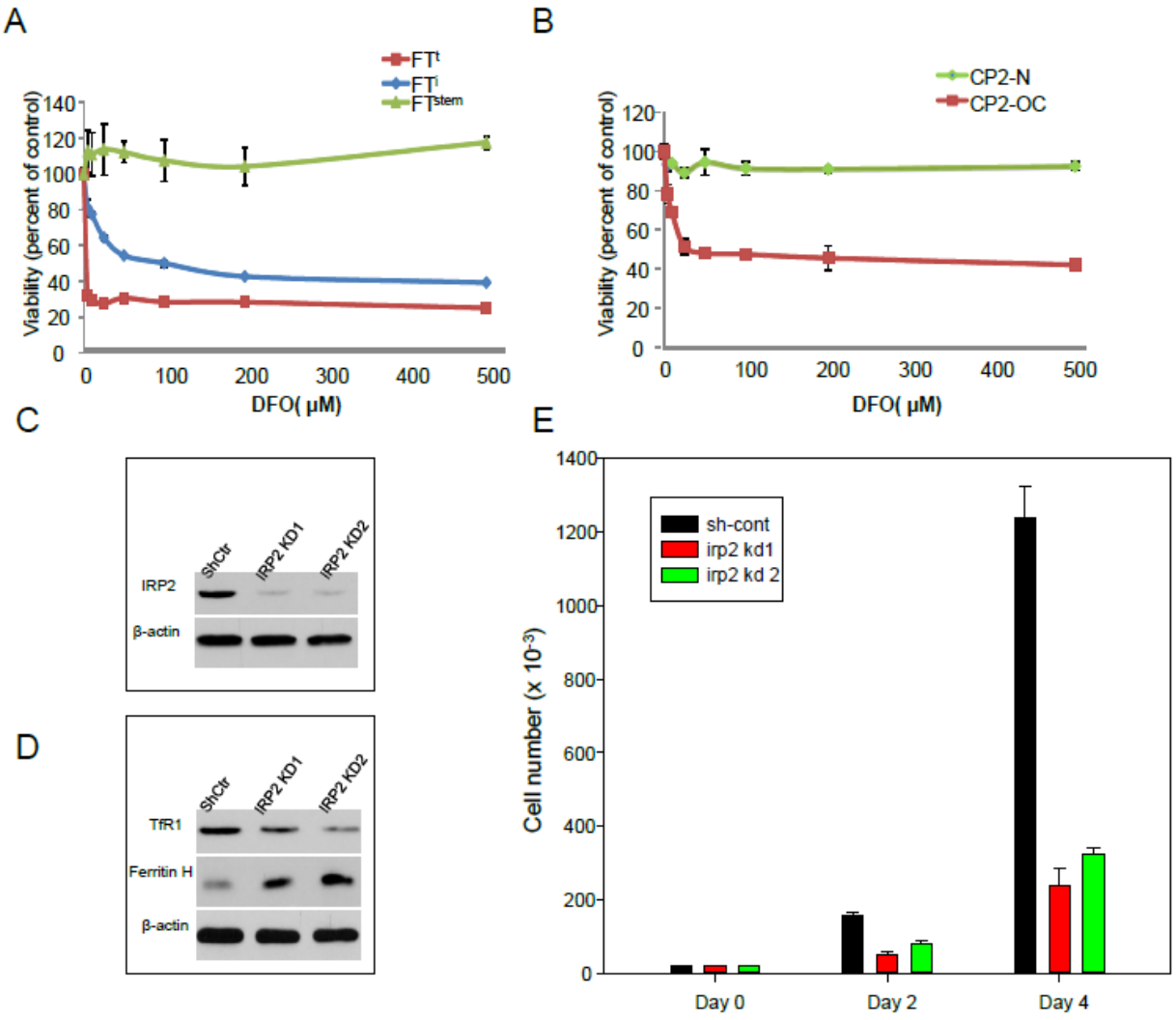


Fig. 13. Tumor-initiating cells and patient derived cancer stem cells exhibit increased iron dependence. (a, b) Cells were treated for 72 hrs. with the indicated concentrations of deferoxamine (DFO) and viability was assessed using an MTS assay. (c) Western blot analysis of IRP2, transferrin receptor, (TFRC) and ferritin heavy chain (FTH) in IRP2 knock-down in FT^t cells (IRP2 KD1, IRP2KD2) and control cells. (d) Colony formation in FT^t cells treated with control shRNA or IRP2 knockdown vectors.

5.3. Chapter summary

In results shown in this chapter denote that ovarian TICs are sensitive to iron deprivation. When iron levels in these cells are decreased (either through treatment of iron chelating drugs or by disrupting iron metabolism through IRP2 knockdown), the proliferation is inhibited. Interestingly this effect was more prominent in the TICs when compared to normal stem cells. This finding opens a new avenue of targeting TICs selectively without affecting the normal stem cells.

Chapter 6

Tumorigenicity of ovarian TICs is impacted by targeting their iron addiction

Chapter 6

6.1. Chapter introduction

In this chapter we explored if the tumorigenicity of ovarian TICs can be modulated by targeting the iron addiction in these cells. To study this, we adopted two different ways: by increasing the iron efflux and by inducing an iron dependent cell death (ferroptosis).

Previously we have observed that iron genes and proteins are altered in ovarian TICs. We asked the question if the altered iron metabolism is driver or just passenger in malignant dissemination of ovarian cancer. To study that we chose to manipulate FPN, a protein that was consistently reduced in ovarian cancer tissues (**Fig. 4**) and tumor-initiating cells (**Fig. 10-12**), and assessed the effect of increasing its expression in ovarian cancer tumor-initiating cells.

In the second approach we induced an iron-dependent form of cell death, called ferroptosis in the TICs. The rationale behind this was to take advantage of the increased iron levels in the ovarian TICs and target it to modulate tumor formation. Ferroptosis is a mechanism of cell death first identified during a screen for novel cancer therapeutics (97, 98). Ferroptosis is a morphologically and mechanistically distinct form of cell death that is distinguished by its dependence on iron. Ferroptosis causes cell death through iron-mediated accumulation of lipid ROS (97, 99), and can be induced by small molecules such as erastin (98), and inhibited by specifically designed molecules such as ferrostatin (Fer-1)(97). Despite its route of discovery, erastin and other ferroptosis-inducers do not require oncogenic *HRAS* for activity (100). We reasoned that the excess iron retained by ovarian cancer tumor-initiating cells might render them more susceptible to agents that

induce ferroptosis. To test this hypothesis, we measured the effect of treating ovarian cancer tumor-initiating cells and non-cancer stem cells with erastin.

6.2. Results

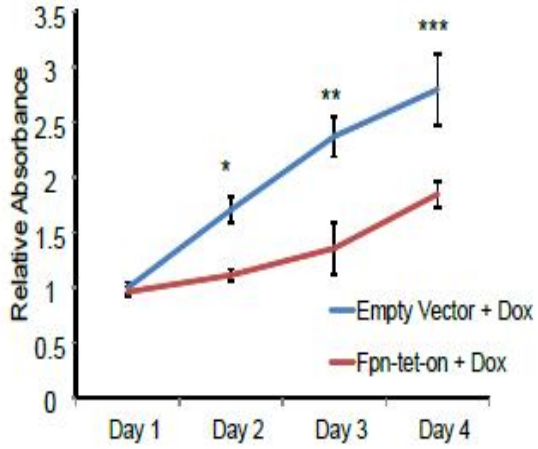
6.2.1. An increase in iron efflux decreases the malignancy of ovarian cancer tumor-initiating cells

We overexpressed FPN in the TICs by a conditional doxycycline-driven promoter (FPN-tet-on). Overexpression of FPN reduces intracellular iron by enhancing iron efflux, thus enabling us to test the consequences of altered intracellular iron on the proliferation and tumor-forming ability of ovarian cancer tumor-initiating cells. As shown in **Fig. 15**, following induction of FPN with doxycycline, cells exhibited the predicted increase in FPN and decrease in intracellular iron. This was accompanied by a significant reduction in cell number (**Fig. 14A**), indicating that a change in the level of FPN is in itself sufficient to modulate tumor-initiating cell proliferation in vitro.

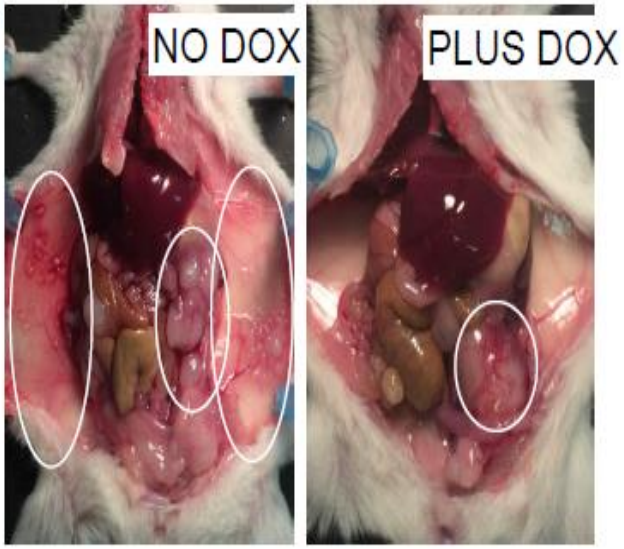
A defining characteristic of tumor-initiating cells is their high degree of tumorigenicity *in vivo* (101); this was also observed in our FT^t cells. To assess whether high intracellular iron contributes to tumor growth *in vivo*, we injected NSG mice intraperitoneally with FT^t cells containing doxycycline-inducible FPN or control empty vector. Mice were treated with vehicle or with doxycycline, and tumors were allowed to grow for 4 weeks before sacrificing the mice. IHC analysis confirmed the induction of FPN in tumors of mice treated with doxycycline **(Fig. 16).** As illustrated in **Fig. 14B**, necropsy revealed widely disseminated tumors in control mice, reminiscent of tumors observed in ovarian cancer patients with advanced disease. FPN-overexpressing cells also gave rise to tumors, but

the tumor burden and area of metastasis were markedly reduced relative to controls. Mice injected with tumor cells that did not overexpress FPN also exhibited large blood vessels supplying small and multiple metastases; these were not evident in mice with FPN-overexpressing tumors. Quantification revealed a significant decrease in tumor number ($p=0.003$) as well as a significant decrease in tumor mass ($p=4.6x10^{-7}$) in mice whose tumors over-expressed FPN (**Fig. 14C,D).**

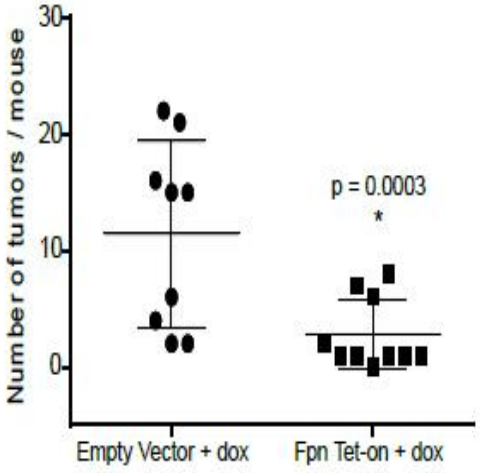
A.


Relative Absorbance
3.5
3
2.5
2
1.5
1
0.5
0
*
**
***
Empty Vector + Dox
Fpn-tet-on + Dox
Day 1
Day 2
Day 3
Day 4

B.


NO DOX
PLUS DOX

C.


Number of tumors / mouse
30
20
10
0
p = 0.0003
*
Empty Vector + dox
Fpn Tet-on + dox

D.

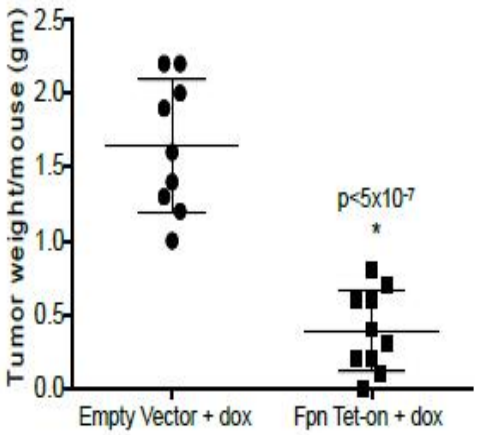

Tumor weight/mouse (gm)
2.5
2.0
1.5
1.0
0.5
0.0
p<5x10-7
*
Empty Vector + dox
Fpn Tet-on + dox

Fig. 14. **Increased iron efflux decreases cell viability and tumorigenicity of ovarian cancer tumor-initiating cells**. (A) Cell viability of FT^t cells as assayed by MTS assay at indicated time points with and without ferroportin overexpression. Blue line represents control (empty vector with doxycycline), and red line represents $FPN^{IND}FT^t$ cells (ferroportin tet on with doxycycline). *$p<0.0002$, one tail t test. (B) Representative images of mice inoculated IP with FPN^{IND} FT^t cells and left untreated (left) or treated with doxycycline (right) for four weeks to induce expression of ferroportin. Untreated mice have a greater tumor burden and wider area of metastasis (circled in white). (C) Quantification of tumor number/mouse and (D) tumor mass/mouse following implantation of tumor cells containing empty vector (n=9) or tet-on FPN (n=10) following 4 weeks of doxycycline treatment.

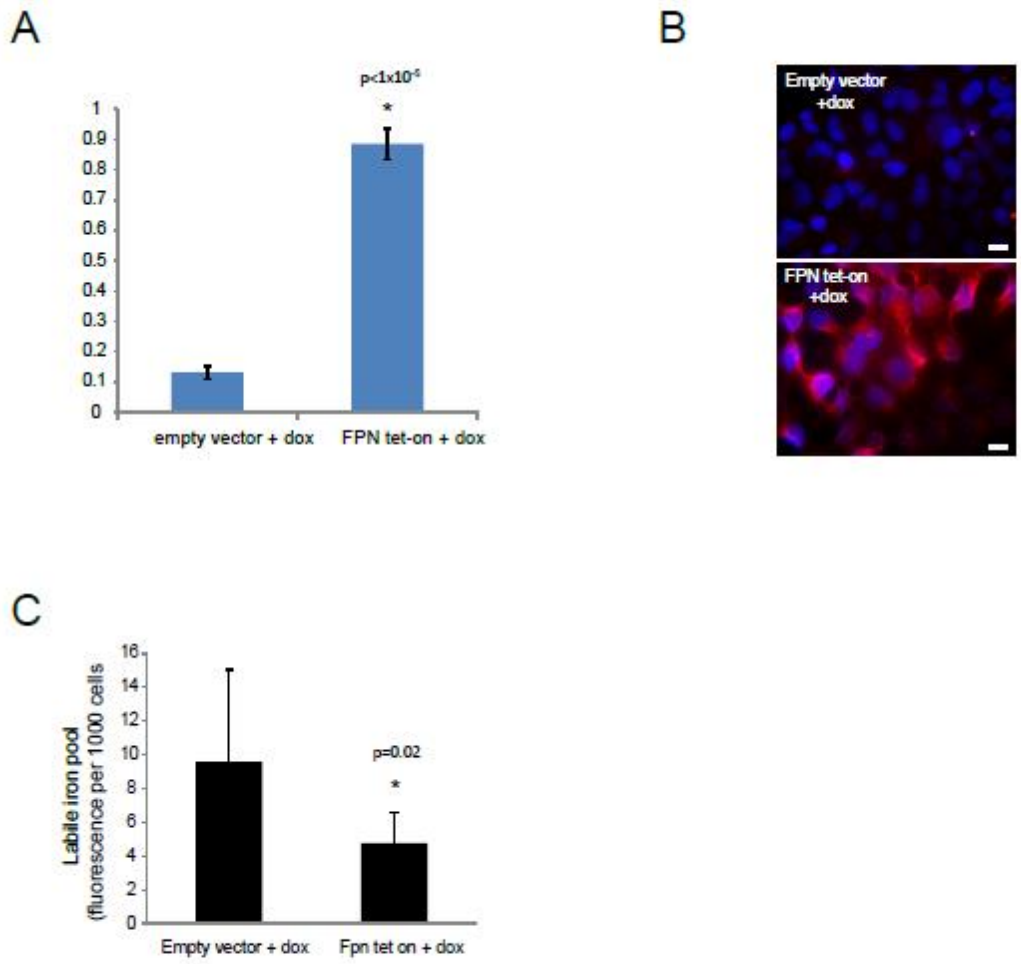


Fig 15. Ferroportin is induced and the labile iron pool is decreased following treatment of conditional ferroportin-overexpressing FT^{t} cells with doxycycline. (A) qRT-PCR measurement of ferroportin (FPN) mRNA in cells infected with empty vector or a tet-on FPN vector following treatment with doxycycline. (B) immunofluorescent staining of ferroportin in cells infected with control or tet-on ferroportin vector following treatment with doxycycline. Red, ferroportin; blue, nuclei. (D) Labile iron pool in control or tet-on

ferroportin cells. Means and standard deviations of sextuplicate cultures. Scale bar 20 µm.

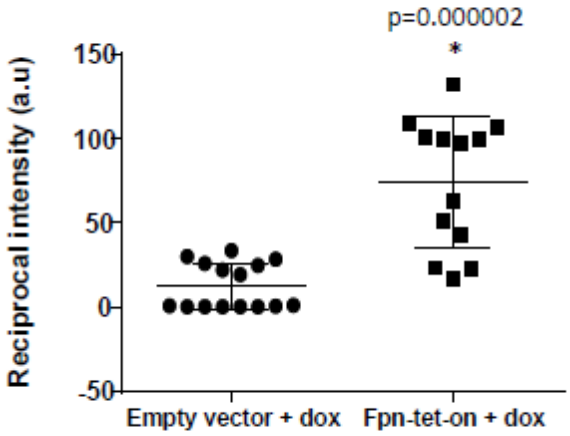


Fig 16. Tumors isolated from mice injected with FT^t tet-on FPN cells over-express ferroportin. Ferroportin was quantified by IHC staining of the tumor xenografts.

6.2.2. Iron flux through ferroportin affects invasion of ovarian cancer tumor-initiating cells

Ovarian cancer is characterized by a propensity towards intraperitoneal tumor spread. This is believed to arise from tumor cells that originate in the primary tumor and invade locally to colonize the peritoneal cavity. Because overexpressing FPN markedly reduced the number of tumors after intraperitoneal injection, we asked whether iron levels might directly affect tumor cell invasion. We tested the invasion of FT^t cells following induction of FPN. Because FPN affects cell proliferation (**Fig. 14A**), we corrected for cell number by comparing the number of cells that migrated through a layer of basement membrane protein to those that migrated through an uncoated porous membrane (**Fig. 17A).** Viable cells expressing FPN showed substantially reduced invasion when compared to controls. Consistent with these results, expression of matrix metalloproteases MMP2, MMP9, MMP14 and uPA, enzymes that degrade the extracellular matrix, were all reduced in cancer tumor-initiating cells following induction of FPN **(Fig. 18**). Thus, iron augments not only proliferation, but invasion and spread of ovarian cancer tumor-initiating cells.

To understand the mechanism linking iron to invasion, we examined IL6, since this cytokine is elevated in ovarian cancer, promotes invasion, and contributes to poor patient outcome (102, 103). Because IL6 is induced via a STAT-3-mediated pathway, we first tested whether induction of FPN affected STAT-3 signaling in ovarian cancer tumor initiating cells. As shown in **Fig. 17B**, induction of FPN decreased STAT-3 signaling in

FT^t cells. This was accompanied by a decrease in both IL-6 mRNA and protein in these cells (**Fig. 17C,D**). Thus, these data suggest that iron accumulation promotes invasion of ovarian cancer tumor-initiating cells at least in part through induction of IL-6.

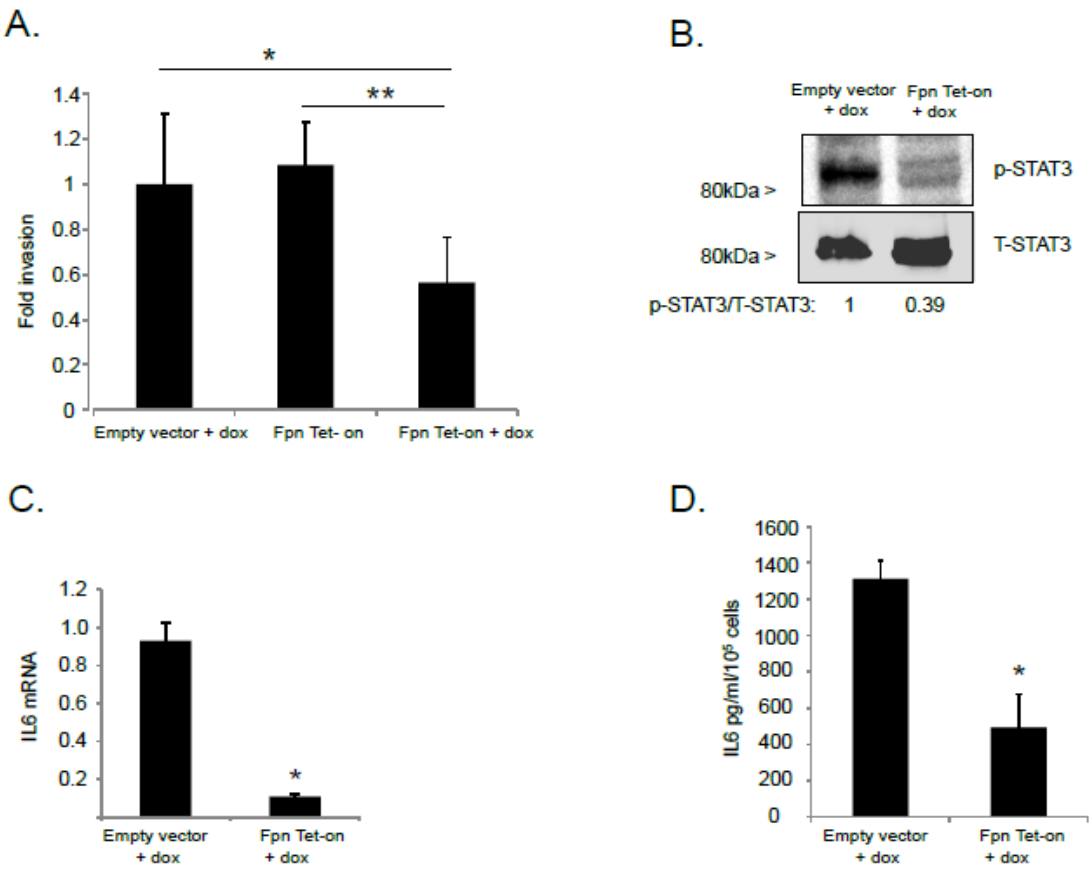


Fig. 17. Ferroportin modulates invasion of FT^t cells through STAT3 and IL6.(A) In vitro invasion was measured in a simplified Boyden chamber consisting of two chambers separated by a 8 micron porous filter coated with or without basement membrane extract (BME). Invasion was normalized to migration of the cells in the inserts without the BME coating. *p=0.002; **p=5x10^{-5}. (B) Western blot analysis of p-STAT3 and total STAT-3 in FT^t cells transduced with empty vector (EV) or FPN-Tet-following 48 hrs. exposure to doxycycline. Band intensities were quantified using ImageJ. (C) IL6 mRNA was assessed using qRT-PCR; (D) IL6 was measured in culture supernates following 48 hrs.' exposure to doxycycline. *p≤0.0002.

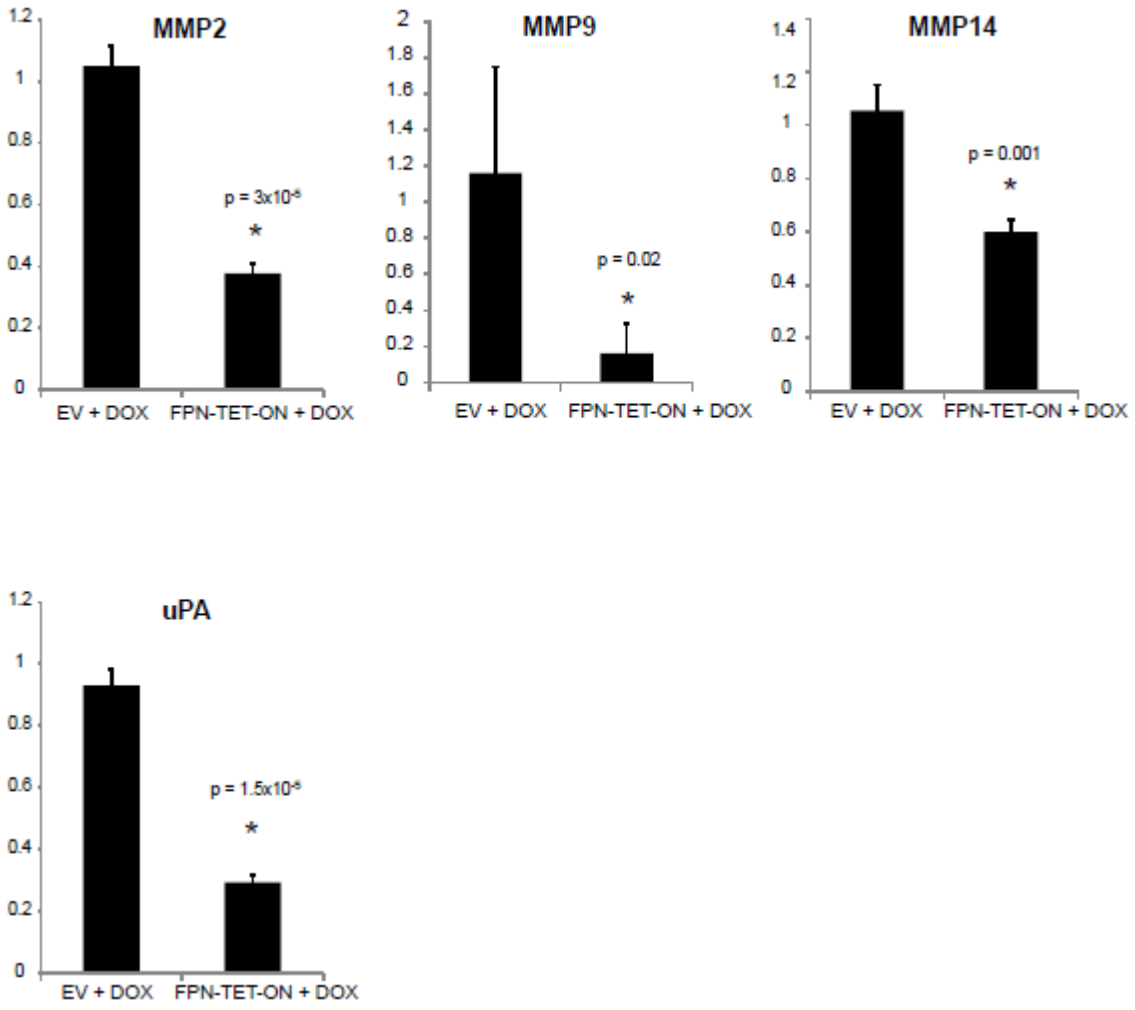


Fig 18. Overexpression of ferroportin reduces expression of invasion markers in FT^{t} cells. FTt cells containing the FPN-Tet-on or control vector were induced with doxycycline and the indicated mRNAs measured using qRT-PCR. MMP2, matrix metalloprotease 2; MMP9, matrix metalloprotease 9; MMP14, matrix metalloprotease 14; uPA, urokinase plasminogen activator. Means and standard deviations of triplicate determinations.

6.2.3. Targeting the iron addiction of ovarian cancer tumor-initiating cells by inducing ferroptosis

We induced ferroptosis in the ovarian TICs by treating them with erastin. To confirm that cell death was proceeding via ferroptosis, we also treated with the combination of erastin and the ferroptosis inhibitor, Fer-1. As shown in **Fig. 19A**, erastin decreased cancer tumor-initiating cell viability, and effects of erastin were completely blocked in cells treated with the combination of erastin and Fer-1 (**Fig. 19B,D**). As predicted, cancer tumor-initiating cells were significantly more susceptible to erastin treatment than non-cancer stem cells (**Fig. 19A,C**).

To test whether erastin was similarly effective *in vivo*, we inoculated NSG mice with FT^t cells and treated them with either vehicle or 20 mg/kg erastin daily for 18 days. Mice were sacrificed and tumor burden measured by both counting tumor number and measuring total tumor mass per mouse. As seen in **Fig. 19E,F**, erastin treatment produced a marked reduction in both tumor number and mass, indicating that ferroptosis-inducing agents have the potential to successfully target ovarian tumors.

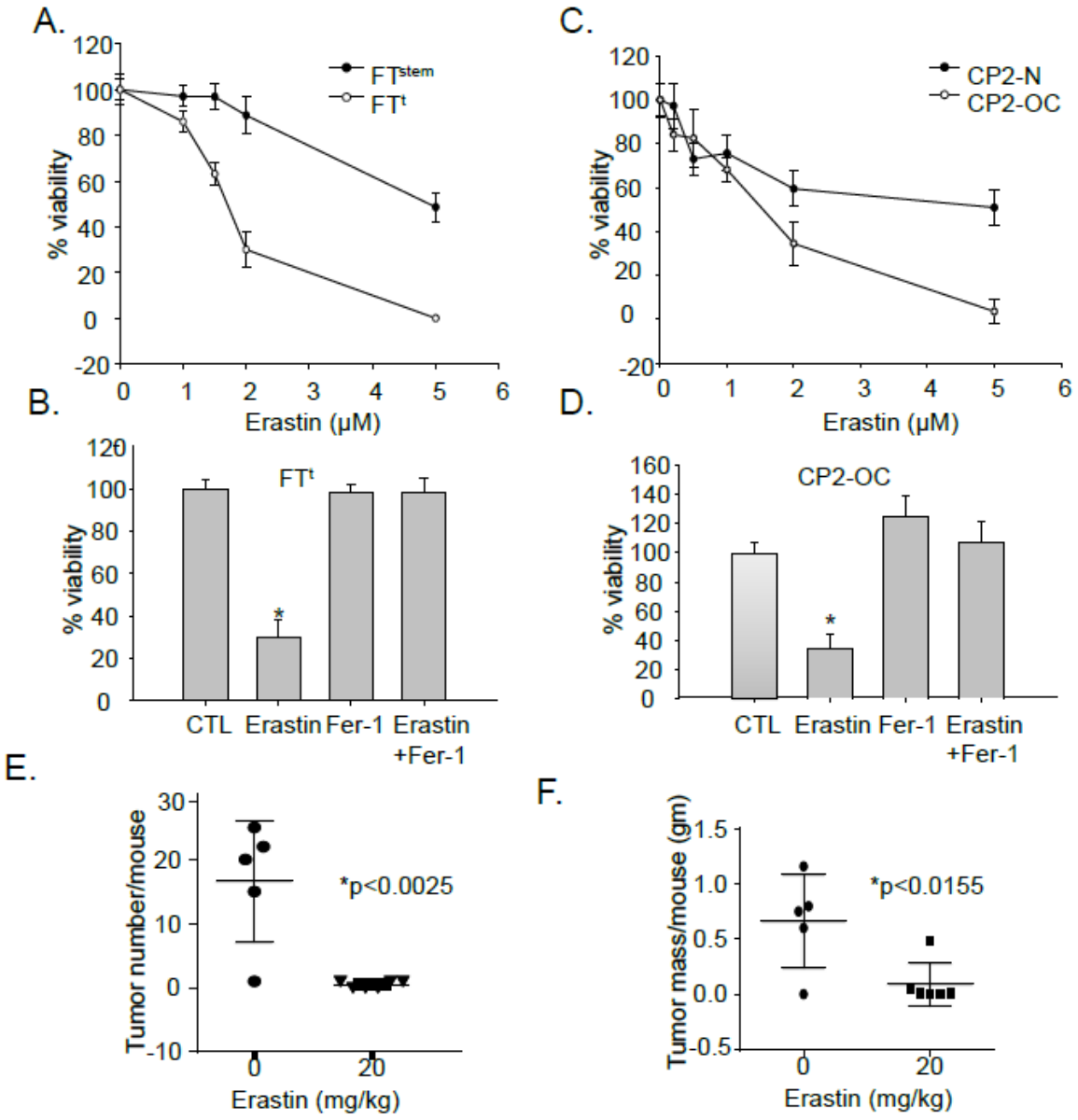


Fig. 19. Erastin induces ferroptosis in tumor-initiating cells and reduces tumor number and mass *in vivo*. (A) 3000 patient-derived normal stem cells (CP2-N) and tumor-initiating cells (CP2-TIC) or (c) FT^{stem} and FT^{t} cells were treated with indicated doses of erastin and cell viability assessed 48 hours later. (B, D) CP2-TIC cells or FT^{t} cells were treated with 2 μM of erastin alone or in combination with 2 μM of ferrostatin-1 for 48 hrs. and cell viability assessed. *p<0.00005. (E, F) Mice were injected

intraperitoneally with FT^t cells and treated for 18 days with either vehicle or 20 mg/kg erastin. The number of tumors/mouse and total tumor mass/mouse were measured in the vehicle-treated group (n=5) and in the group treated with erastin (n=6).

6.3. Chapter summary

In this chapter, we show an important finding that iron proteins (at least FPN) in TICs are drivers of tumor progression in HGSOC. Induction of FPN in TICs slows the tumor growth in mice. In addition, FPN induction also limits the invasion and metastasis of tumors, a phenomenon which is a major clinical obstacle for HGSOC management. We also show that inhibition of invasiveness of the TICs upon FPN induction may work through downregulation of IL-6 p-STAT3 signaling pathway.

Ferroptosis can be induced in the HGSOC TICs. On account of increased iron levels within the TICs compared to normal stem cells, ferroptosis can be used aa a chemotherapeutic tool to eradicate the TICs selectively in patients with HGSOC.

Chapter 7

Discussion

Chapter 7

7.1. Summary

Ovarian cancer is a highly lethal malignancy that has not seen a major therapeutic advance in over 30 years. We demonstrate that ovarian cancer exhibits a targetable alteration in iron metabolism. Ferroportin (FPN), an iron efflux pump, is decreased, and transferrin receptor (TFRC), an iron importer, is increased in ovarian cancer tissue. Expression of FPN and TFRC are strongly associated with patient survival. Ovarian cancer tumor initiating cells (TICs) demonstrate a similar profile of iron excess and an exquisite dependence on iron for proliferation. Alterations in iron metabolism in TICs can be mimicked by manipulating p53 and c-myc. Iron facilitates invasion, synthesis of IL6, and intraperitoneal spread of tumor cells in vivo. Iron dependence in TIC can be targeted in two ways: by reducing intracellular iron with chelators, or by treating with agents that induce iron-dependent cell death (ferroptosis). Thus, enhanced levels of iron in ovarian cancer TICs create a metabolic vulnerability that can be exploited therapeutically.

7.2. Discussion

Here we have demonstrated that iron metabolism is perturbed in tumors derived from patients with HGSOC. HGSOC tumor tissue exhibits an increase in the iron importer TFRC, a decrease in the iron efflux pump FPN **(Fig 4).** Increased import and decreased export of iron leads to increased iron retention within the tumor tissue. This is evidenced by an increase in the iron storage protein ferritin relative to non-tumor tissue (**Fig. 6**). The transition to an “iron-seeking” phenotype appears to be an early event in the development of HGSOC, as evidenced by perturbations in patient-derived tumor initiating cells and in genetically transformed fallopian tube stem cells (**Figs. 10-12**).

The key objective of cancer therapy is to selectively target the genetic lesions that initiate and maintain tumor growth. Most of the cancers harbor multiple genetic alterations, but recent preclinical and clinical data supports that cancer cells exhibit dependence on a single oncogenic pathway or a protein for its sustained proliferation and survival. The term "oncogene addiction" was coined to connote the dependence of cancer cells on the single pathway or protein and that switching off this crucial pathway will have devastating effect on the cancer growth (104). Our results suggest that ovarian tumor initiating cells may similarly develop an enhanced dependence on iron, or "iron addiction" that can potentially be targeted both by agents that induce iron depletion **(Fig. 13)** and by agents that depend on iron for their activity, such as the ferroptosis inducer erastin (**Fig. 19**). This is particularly relevant for ovarian cancer because of its high genomic heterogeneity and such a "moving target" is difficult to destroy.

Further, we show that changes in iron metabolism in ovarian cancer are not merely incidental events or simple bystanders that accompany the transition to malignancy. They make important contributions to the growth and metastasis of ovarian cancer cells that is reflected in patient prognosis. Thus, reduction of intratumoral iron through upregulation of the iron efflux pump ferroportin reduced tumor burden and spread of ovarian cancer in the peritoneal cavity of mice (**Fig. 16**). In humans, a "high iron" phenotype, particularly low ferroportin, was associated with high tumor grade and poor survival (**Fig. 9**). Although the association of TFRC mRNA with overall survival was less robust than ferroportin, high transferrin receptor was also associated with higher grade and molecular subtypes of ovarian cancer with poorer survival (**Fig. 9**). This perhaps implicates that iron efflux as a

more prevalent contributor to outcome than iron import, or a predominantly post-transcriptional mode of TFRC regulation in HGSOC.

A phenotype of iron acquisition has now been reported in a number of cancers: glioblastoma (105), breast(23, 26), prostate(106), and others (3). Why do cancer cells require excess iron? Iron is required for proliferation and cell cycle progression, and upregulation of these processes in cancer cells underpins at least some of their enhanced dependence on iron. Our results suggest that iron may play an additional, unexplored role in the malignant process, fostering tumor cell invasion (**Fig. 17**). The establishment of tumor cells in a metastatic niche requires the acquisition of invasive properties, including the ability to degrade the extracellular matrix through the secretion of matrix metalloproteases(107). Elevated levels of intracellular iron appear critical to this process, since increasing iron efflux through upregulation of ferroportin led to a decrease in expression of several matrix degrading enzymes, including MMPs and uPA (**Fig.18**).

How does iron affect expression of MMPs? We demonstrate that intracellular iron upregulates IL6 (**Fig. 17**), a key cytokine in the initiation and progression of ovarian cancer (103, 108). Elevated levels of IL6 are found in ovarian cancer tissue, and affect tumor cell growth, proliferation, and angiogenesis as well as contributing to tumor invasion by upregulation of matrix metalloproteases (109). Iron-mediated upregulation of IL6 is consistent with previous work demonstrating that iron chelators inhibit synthesis of IL6 in pancreatic cancer cells (110), and that exogenous iron induces IL6 in hepatocytes (111). Although details of the pathway linking iron to IL6 are yet to be elucidated, STAT3 may play an important role, since a decrease in STAT3 phosphorylation occurred subsequent

to iron depletion by ferroportin induction (**Fig. 17**), and was similarly decreased by iron chelation (110).

Ferroptosis is a recently discovered iron-dependent mode of cell death (98). Agents that trigger ferroptosis represent a potential new class of anti-cancer agents, and are being vigorously explored in both preclinical and clinical studies (112). Iron is required to generate the oxidative stress that characterizes ferroptosis, although the precise role played by iron remains speculative (100, 113). We suggest that the enhanced levels of iron required for the growth and invasion of ovarian (and other) cancer cells may represent an Achilles heel that can be exploited in cancer therapy.

Our evidence of the vulnerability of tumor-initiating cells to ferroptosis may provide not only a new class of compounds that are effective in ovarian cancer, but also an important target at which this therapy can be directed: the tumor-initiating cell. We observed that ovarian cancer tumor-initiating cells exhibited decreased FPN, increased TFRC, and decreased LIP relative to non-cancer stem cells (**Figs. 9-10**). This was accompanied by an enhanced sensitivity to erastin, a ferroptosis-inducing agent (**Fig. 19**). Erastin was also able to profoundly inhibit tumor growth in vivo (**Fig. 19**). Since tumor-initiating cells are believed to represent treatment-refractory cells that contribute to ovarian cancer drug resistance and recurrence (101), the use of ferroptosis-inducing agents in the treatment of ovarian cancer, either alone or in combination with conventional therapies, has the potential to address a significant clinical problem.

7.3. Some limitations of the study

Ovarian cancer is a disease with high genomic heterogeneity. Cancer phenotype of this disease differs from tumor to tumor, from patient to patient. It is therefore important to study a big sample size of patients to achieve a statistically significant conclusion about ovarian cancer. Given the time frame of my thesis, technical challenges of isolation of stem cells, we based our results derived the TICS isolated from 2 patients with HGSOC. Though the iron phenotype observed in the patient derived TICs derived from 2 patients strongly correlates the findings obtained from the transformed cells used a genetic model for the TICs, the small sample size was a big obstacle to make any statistically significant conclusion. Our group is in the process of including more HGSOC patients to isolate the TICs and reproduce the results we have obtained so far.

The survival data on ovarian cancer patients are based on the dataset GSE9891. This consists of a cohort of 285 patients with different types of ovarian cancer and not just only HGSOC. As discussed in the introduction, the origin and pathogenesis of different types of ovarian cancer are distinct leading to diversification of the disease histologically, genetically and clinically. Our findings of altered iron metabolism are based on tumor tissues from HGSOC and TICs isolated from HGSOC patients. Generation of the survival data using a cohort consisting of a pure population of HGSOC patients only, will better conform our objective.

In my thesis, we have used a genetic model of HGSOC TICs by transformation of normal fallopian stem cells with hTERT, SV40 and c-myc. The concept of origin of cancer stem cell or tumor initiating cell is highly debated. While some believe that TICs originate from

an oncogenic transformation of normal stem cells (60, 61), there exists a different school of thought where it is argued that a TIC may originate “un-differentiation” of a transformed non-stem cell (59) .

The inivo studies in this research study are based on inoculation of transformed fallopian stem cells. A more bonafide model of HGSOC animal model would be using the patient derived TICs to inoculate the animals. Though we have confirmed that patient derived TICs are tumorigenic, but we argue that given the high degree of genomic heterogeneity of HGSOC in patients the study of modulating some of the desired iron genes in tumorigenicity can be complex. Though there are disadvantages of using a genetic model of ovarian TICs for animal studies rather than genuine patient derived TICs, the advantages of using transformed cells are that it enables us to dissect out the effects of manipulation of certain genes within these cells with a known genetic background.

Modulating intratumoral iron by increasing iron efflux through FPN decreases tumor growth in our animal model. Based on this finding, we claim that iron chelation can be a potential chemotherapeutic technique to treat HGSOC. However, it is an over-simplified statement in context of treating cancer in human. Cancer patients suffer from associated anemia of chronic disease and systemic iron chelation may deteriorate anemia. However our findings certainly shed some light in understanding the role of iron in ovarian TICs. We believe that our finding will likely help HGSOC treatment with the discovery of tumor selective discovery in future.

We show that FPN induction inhibits invasiveness of ovarian TICs. FPN induction also decreases expression of MMPs. We hypothesize that FPN overexpression may

downregulate invasion by inhibiting IL-6 signaling since most of the MMPs are induced by IL-6. We therefore studied IL-6 signaling and found downregulation of IL-6 expression and phosphorylation of STAT-3. These experiments show an association of FPN induction, and downregulation of IL-6 and invasion. We do not know if FPN drives reduction of IL-6, and thereby causing a change in the invasion.

We propose ferroptosis inducers to be an efficient chemotherapeutic agent to treat and prevent relapses in patients with HGSOC based on our findings that erastin kill TICs and halt tumor growth. However the current study does not include any experiments to show that erastin is effective in preventing tumor replace when compared to the standard chemotherapy used to treat HGSOC.

7.4. Future directions

This thesis leads to a number of intriguing questions worthy of future investigation. The immediate question that comes to my mind and I believe worth exploring is discussed below:

Is there any mechanistic role of iron in maintaining or regulating the TICs in HGOSC?

Altered iron metabolism is an early phenomenon in the tumorigenesis in HGSOC since we observe the trait at the stage of TICs. It is indeed an important finding, but it raises a further interesting question: does iron have any role in regulating the ovarian TICs? In the following discussion, I have summarized the pathways and genes that are known to regulate ovarian TICs and where I think there could be potential role of iron. I am also going to discuss some experiments which I believe can be designed to find the role of iron in regulating such pathways.

Several pathways like Notch, Hedgehog, Wnt and transforming growth factor-β (TGF-β), are found to be crucial for the regulation of the self-renewal and maintenance of CSC/TICs (114-116). Blocking these pathways have been suggested to be a promising strategy to target TICs and thus treating recurrent malignancies.

Notch3 gene is often found overexpressed and amplified in ovarian cancer (117). Moreover, Notch3 pathway is found to reprogram HGSOC tumor cells assume an array of embryonic stem cell markers and is related to the recurrence of ovarian cancer and is responsible for resistance to carboplatin (118). Epigenetic alterations (DNA methylation and miRNAs) in multiple Notch target genes are shown clinically significant and correlates with HGSOC patient survival (119). Some recent studies have shown that Notch3 pathway may have important role in maintaining the TICs in HGSOC (117, 120). Another study showed that hypoxia induces maintenance of undifferentiated cell state or stem cell state via notch signaling pathway (121, 122). Hypoxia leads to recruitment of HIF-1α to a Notch-responsive promoter and activation of Notch downstream genes and thus blocks differentiation of stem cells (121). Iron levels can regulate HIF-1α proteins and transcription factors. High iron levels can activate HIF (123). HIF1 is also shown to induce iron uptake by increasing TFR expression (124, 125). Clearly there is evidence that suggests that hypoxia that maintains stem cell state through notch signaling, can also induce higher iron retention within progenitor cells and can explain the iron addiction exhibited by the ovarian TICs.

Hedgehog signaling pathway has been well implicated in cancer stem cells of several cancers including ovarian cancer (114, 116). Hedgehog pathway is recently reported to regulate growth of ovarian cancer spheroid forming cells, suggesting that this pathway be

directly involved in maintenance of ovarian TICs (126). Though not in cancer cells, but a recent study reported that iron can decrease mRNA levels of HHIPL-2, an inhibitor of hedgehog signaling pathway. Thus iron has the ability to increase hedgehog pathway and may regulate ovarian TICs. This hypothesis can be tested a simple experiment of manipulating iron within our ovarian TICs and measuring the activation of the pathway through GLI reporter assay (increased transcriptional activity of GL1 indicates activation of hedgehog pathway).

EMT (Epithelial-mesenchymal transition) is an important cellular reorganization process by which epithelial cells loose cell polarity and adhesion and transit to a mesenchymal stem cells phenotype with more migratory and invasive properties and occurs during tumorigenesis (127). There is evidence that TICs can be generated through EMT (128, 129). TGF-β signaling plays an important role in EMT and is indicated in induction of ovarian TICs (130). Some recent studies have demonstrated that iron chelators (DFO and thiosemicarbazone) inhibit TGF-β-induced EMT in certain cancer cells (131, 132). These findings make it tempting to hypothesize that iron may have a role in the induction or maintenance of ovarian TICs through EMT pathway. Assay of EMT marker proteins like E-cadherin, vimentin, in the ovarian TICs upon alteration of cellular iron may give us a better insight in this context.

Other pathway that is implicated in regulation of TICs is Wnt pathway which is very rare in epithelial ovarian cancer (114). Recently it has been shown that iron augments Wnt signaling in cells with aberrant APC (133).

Besides these signaling pathways, there are many cancer-associated genes that have been reported to play functional roles in development and maintenance of TICs. The most important gene is probably the TP53. As discussed in the introduction, loss of function pf p53, a tumor suppressor protein is indicated in the pathogenesis of HGSOC (50) . A recent TCGA study revealed that TP53 mutation is present almost in all HGSOC (96%) (48) . A recent study showed that ovarian TICs can be generated by depletion of p53 expression (134). This demonstrates that p53 dysfunction can enhance the self-renewal ability of ovarian stem-like tumor cells. On the other hand, iron is found to regulate p53 signaling through direct heme-p53 Interaction and Modulation of p53 Localization, Stability, and Function (135). Clearly iron may have a crucial role in generation of ovarian TICs through p53 in-stabilization.

Other genes that have been shown to have important roles in regulation and proliferation of ovarian TICs are c-kit, TWIST-1, LIN28, Nanog (reviewed in (136)). miRNAs (miR-199a, miR-214) have also been found to be important regulators of ovarian TICs (136). Though all of these regulators have no clear connection with iron, but some of them like c-kit and miRNAs seem to be affected by iron in both stem cells and non-stem cells (137, 138).

The first step to find the association of iron proteins with the genes and pathways that are responsible for regulation of ovarian TICs, would be a microarray analysis of our ovarian TICs with either knockdown or overexpression of iron genes like TFR, FPN or ferritin. The array information will likely bring up the genes (and the pathways) that are significantly affected due to alteration of iron metabolism. Further invitro and invivo experiments would be necessary to confirm any results.

1) Can targeting iron in ovarian TICs prevent relapse of HGSOC? Or, Can ferroptosis inducers like erastin overcome the failure of platinum to prevent recurrence?

As discussed earlier, ovarian cancer is notorious for recurrence and resistance to chemotherapy. It has been found that the more aggressive and refractory cancers contain more TICs (139, 140). Though the standard surgery and chemotherapy can effectively reduce tumor mass in ovarian cancer, most patients with residual ovarian TICs acquire replaces and chemoresistance (141, 142). Several mechanisms have been suggested so as to how ovarian TICs acquire resistance to drugs: high expression like drug efflux through ABC transporters by TICs (143), enrichment of TICs activation of anti-apoptotic factors by TICs (57), efficient DNA repair (144-146). Currently a lot of attention is being focused at targeting the ovarian TICs. In thesis we have shown that manipulating iron or inducing ferroptosis kills ovarian TICs and inhibit tumor progression and invasion. Based on the current knowledge that TICs are responsible for cancer recurrence and chemoresistance, we anticipate that iron signature in the TICs have some role in those two phenomena. It is therefore interesting to explore the iron phenotype in cisplatin resistant ovarian cancer cells. The first step in this approach would be to do a comparative microarray analysis between cisplatin sensitive and resistant ovarian cancer cells and to look for the iron related genes that may be differentially expressed in the two groups of cells. While lot of studies are being conducted in identifying genes responsible for chemoresistance in ovarian cancer patients, focus has always been biased to the known genes involved the regulation of TICs/CSCs. Provided some iron related genes turn out to be associated with cisplatin resistance, further experiments with cell lines upon

manipulating those specific genes will need to be conducted to expose the specific signaling pathways or downstream events.

7.5. Closing thoughts

In my thesis, we show that HGSOC is characterized by an altered iron phenotype (High TFT, low FPN). This trait is also present within the ovarian TICs. Ovarian TICs exhibit an enhanced dependence on iron. This increased sensitivity of the TICs on iron can be exploited and potentially utilized to treat HGSOC progression, invasion and perhaps recurrence and chemo-resistance. The findings raise some intriguing questions that may be helpful in better understanding of HGSOC and in developing tools to fight this dreadful disease.

Chapter 8

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