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PHOTON IRRADATION AND CISPLATIN ENRICH CANCER STEM CELLS IN OVARIAN CANCER

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PHOTON IRRADATION AND CISPLATIN ENRICH CANCER STEM CELLS IN OVARIAN CANCER

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biology

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by

Ashley Marie Antonissen

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ABSTRACT

High Grade Serous Ovarian Cancer (HGSOC) has a 5-year survival rate of less than 50%. Ovarian cancer is one of the deadliest gynecological diseases and the 7th most common female cancer worldwide. Ovarian cancer patients generally have a poor prognosis despite the relatively successful treatments. When conventional cancer treatments, such as cisplatin chemotherapy and photon irradiation, are administered, residual cancer stem-like cells (CSCs) can survive, leading to CSC enrichment. CSCs are a small population of cancer cells that exhibit stem-like characteristics: quiescence (slowing of the cell cycle), differentiation, proliferation, and self-renewal to regenerate new CSCs. We hypothesized that providing cancer treatments such as irradiation and chemotherapies enriches the CSC population. We aimed to determine the induction of CSC activity after conventional treatment in 2 ovarian cell lines: OVASHO and PDX4 (patient derived xenograft) at a 72-hour time point after treatment. Our project used a GFP reporter system, SORE-6, which fluoresces in the presence of SOX2 and OCT4, core pluripotency factors to determine levels of stemness. Here, we observed a dose-dependent increase in stemness in PDX4 after cisplatin and photon irradiation treatment. In response to photon irradiation, OVASHO exhibited a dose-dependent response and an overall increase in GFP-expression in response to cisplatin. We also observed notable changes in mRNA gene expression of OCT4 and SOX2 in response to cisplatin treatment. These results indicate that cancer cell stemness is enriched after treatment in association to CSC persistence and/or cancer stem cell reprograming.

ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation to Dr. Juli Unternaehrer for this unique and wonderful opportunity to submerge myself in a world of learning. Dr. Unternaehrer's dedication, expertise, and unwavering support has truly made a significant impact on my scientific journey, personal growth and discovery. I would like to also thank my lab members for their dedication and support, Tise Suzuki, David Jung, Jacqueline Coats, Ashlyn Conant, Dr. Nora Badiner, Dr. Julia Ritchie, Salina Singh, Julia Fernandez, Dr. Hanmin Wang, Dr. Evgeny Chirshev and many more. I would like to thank members of my committee Dr. Nicole Bournias-Vardiabasis and Dr. Daniel Nickerson for their mentorship and guidance. I would also like to acknowledge the CSUSB Graduate Equity Fellowship, CIRM Bridges program, and Loma Linda University for their generous funding that allowed this project to be undertaken. I would like the opportunity to thank my mentors at CSUSB: Dr. Douglass Smith, Dr. James Noblet, and Dr. David Rhoads. I would like to express sincerest gratitude to the O'Hara family, Montgomery family, Gini family, and Antonissen family (and Ralph) all of whom have loved and supported me throughout my education. I would like to especially acknowledge the Omary family for their guidance, love, and support throughout my education. Lastly, I would like to thank my partner, Brendan O'Hara, for his constant, unrelenting support and love throughout my education at CSUSB.

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CHAPTER ONE:

STATEMENT OF PURPOSE

Hypothesis:

We hypothesized that providing cancer treatments such as irradiation and chemotherapies enriches the cancer stem cell population.

Specific Aims:

The specific aims of this project are to (1) define stemness induced by therapies (2) define stemness by functional assays after treatment (3) define stemness by measuring the levels of mRNA gene expression after treatment (Figure 1).

Three specific aims will be performed to test this hypothesis:

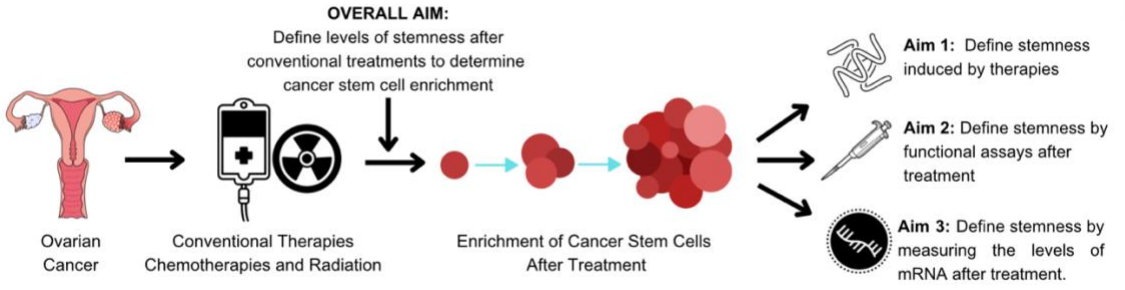


FIGURE 1: OVERVIEW AND SPECIFIC AIMS. Ovarian cancer is treated with conventional therapies such as chemotherapy and radiation which can result in the enrichment of the CSC population after treatment. Here, we aim to define levels of stemness after treatment.

Aim 1: Determine Effects Of Treatments On Stemness As Measured By The Sore-6 Reporter.

Working hypothesis: we hypothesize that providing cancer treatments such as irradiation or chemotherapy increases stemness.

Aim 2: Determine Effects Of Treatments On Stemness By Functional Assays.

Working hypothesis: we hypothesize that providing cancer treatments such as irradiation or chemotherapy increases stem-like functional characteristics.

Aim 3: Determine Effects Of Treatments On Stemness By Determining The Mrna Levels Of Stemness Genes.

Working hypothesis: we hypothesize that providing cancer treatments such as irradiation or chemotherapy will increase expression of stemness genes on mRNA level.

The Overall Impact:

The overall impact of this research will be to determine in vitro the effects of irradiation and cisplatin (cis-dichlorodiammineplatitum (II)) on the cancer stem

cell population as measured by Sore-6, and to test the effect of therapy-induced stemness in ovarian cancer cells. Future directions of this research will be to overexpress let-7i concurrently with cisplatin treatment or irradiation in in vitro and in vivo, to test the effect on inhibiting chemoresistance.

Experimental Approach

Aim 1: Determine Effects Of Treatments On Stemness As Measured By The Sore-6 Reporter.

Working hypothesis: we hypothesize that providing cancer treatments increases stemness.

Rationale: Treatments are known to induce CSC activity. The goal is to determine if there is a dose-dependent response with the Sore-6 reporter system after treatment, indicating the increase of stemness.

AIM 1 Approach:

1.1: Define the half maximal inhibitory concentration, defined as the concentration of a drug or inhibitor needed to inhibit a biological process or response by 50% (IC50) after treatment.

Ovarian cancer cells will be treated with serial dilution range of 1 mM to 0 mM cisplatin to determine the IC50 for each cell line using a viability assay.

1.2: Define the levels of GFP as a surrogate for SOX2 and OCT 4 by the SORE-6 reporter in ovarian cancer cells with varying doses of cisplatin.

Ovarian cancer cells will be treated with cisplatin at the IC50 dose, and after 72-hours flow cytometry will be used to determine percent positive for SORE-6 GFP to determine levels of stemness compared to the untreated sample (IC0).

1.3: Define the levels of GFP as a surrogate for SOX2 and OCT 4 by the SORE-6 Reporter in ovarian cancer cells with varying doses of photon irradiation.

Ovarian cancer cells will be treated with 0 GY, 1 GY, 2 GY, 4 GY, and 8 GY of photon irradiation, and after 72-hours flow cytometry will be used to determine percent positive for SORE-6 GFP to determine levels of stemness.

Aim 2**:** Determine Effects of Treatments On Stemness By Functional Assays.

Working Hypothesis: we hypothesize that providing cancer treatments increases stemness. Determine if there is a dose-dependent response exhibited by functional assays.

Rationale: Treatments are known to induce cancer stem cell activity. The goal is to determine if there is a dose-dependent response in functional assays after treatment, indicating the increase of stemness.

AIM 2 Approach:

2.1: Define functional properties of the cancer cell population after being treated with half maximal inhibitory concentration of cisplatin defined as the concentration of a drug or inhibitor needed to inhibit a biological process or response by 50% (IC50) after treatment.

Perform functional assays to determine the stem-like properties: colony and spheroid formation.

Colony Forming Assay.

Ovarian cancer cells will be treated with cisplatin and then plated 72-hours after treatment to determine the capacity for clonogenic growth, including proliferation and colony formation.

Spheroid Forming Assay.

Ovarian cancer cells will be treated with cisplatin and then plated 72-hours after treatment to determine the ability to self-renew, for growth in anchorage-independent conditions.

2.2: Define functional properties of the cancer cell population after being treated with photon irradiation

Perform functional assays to determine the stem-like properties: colony and spheroid formation.

Colony Forming Assay.

Ovarian cancer cells will be treated with photon irradiation and then plated 72-hours after treatment to determine the capacity for clonogenic growth, including proliferation and colony formation.

Spheroid Forming Assay.

Ovarian cancer cells will be treated with photon irradiation and then plated 72-hours after treatment to determine the ability to self-renew, for growth in anchorage-independent conditions.

Aim 3**:** Determine effects of treatments on stemness by determining the level of mRNA of stemness markers OCT 4 and SOX2.

Working Hypothesis: we hypothesize that providing cancer treatments increases mRNA stemness gene expression.

Rationale: Treatments are known to induce cancer stem cell activity. The goal is to determine if there is an increase in stemness after chemotherapy treatment or photon irradiation treatment.

AIM 2 Approach:

3.1: Define the levels of mRNA for SOX2 and OCT 4 in ovarian cancer cells at the IC50 cisplatin dose compared to the untreated ovarian cancer cells.

Ovarian cancer cells will be treated with cisplatin at the IC50 dose, and after 72-hours will be harvested for reverse transcription- quantitative polymerse chain reaction (RT-qPCR).

3.2: Define the levels of mRNA for SOX2 and OCT 4 in ovarian cancer cells at the IC50 cisplatin dose compared to the untreated ovarian cancer cells

Ovarian cancer cells will be treated with photon irradiation at 0 GY, 2 GY, and 4 GY dose, and after 72-hours will be harvested for RT-qPCR.

CHAPTER TWO:

INTRODUCTION

Overview of Ovarian Cancer

High Grade Serous Ovarian Cancer (HGSOC) has a 5-year survival rate of less than 50% (Wu et al., 2018). Ovarian cancer is one of the deadliest gynecological diseases and the 7th most common female cancer worldwide (Torre et al., 2015). Ovarian cancer patients generally have a poor prognosis despite the relatively successful treatments. Treatments for ovarian cancer are generally limited to surgical debulking and chemotherapy, but despite treatment, ovarian cancer has a 70-80% chance of recurrence (Pignata et al., 2017). “Ovarian cancer” is used as umbrella terminology to define and denote various heterogeneous groups of malignancies that arise in or involve the ovary; generally a large proportion of ovarian cancers are derived from the fallopian tube (Karnezis et al., 2017) due to malignant forms of the epithelial secretory cells located within the fallopian tube (Coleman et al., 2013). When undetected, ovarian cancer may disseminate and invade to local regions such as the pelvic and abdominal cavities (Pignata et al., 2017). Further understanding of tumor reoccurrence in ovarian cancer is important to develop better strategies to enhance treatment options. Tumor reoccurrence may be caused by cancer stem cells (CSCs) (Steg et al., 2012). CSCs are a small population within the tumor that exhibit stem cell like properties which promote cancer growth and metastasis and abate the response to treatments such as irradiation and chemotherapy.

Epithelial-Mesenchymal Transition (EMT)

Epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) are dynamic, reversible mechanisms in embryonic development that cause cells to undergo phenotypic changes to establish tissue formation (Dongre and Weinberg, 2019). In combination, both mechanisms, EMT and MET, enable growth, development, and wound healing. During development, cells undergo several rounds of EMT and MET to develop specialized cell types as well as acquire the complex three-dimensional structure of organs (Thiery et al., 2009). In cancer, tumor cells can de-differentiate into cancer stem cells and spontaneously undergo EMT (Kim et al., 2017). The de-differentiated cells express cancer stem cell markers such as cell surface markers and have been shown to acquire the capacity to invade and metastasize in mice (Kim et al., 2017).

Epithelial cells can be characterized as a two-dimensional structure with apical-basal cell polarity bound to the basement membrane, with cell-cell adhesion mediated by proteins such as integrins and cadherins (Ikenouchi et al., 2003). Mesenchymal cells can be described as having back-front polarity (which is a characteristic of migrating cells), with increased levels of proteins such as N-cadherin and vimentin (Yang et al., 2020). EMT plays an integral role in embryo development during processes such as gastrulation and tissue morphogenesis, induced by a distinctive set of transcription factors, such as SNAI1, to initiate

differentiation into specific tissue types (Dongre and Weinberg, 2019). Epithelial and mesenchymal states are endpoints of the EMT/ MET spectrum while the hybrid state shares traits of both the epithelial and mesenchymal phenotypes.

Snai1/ Let-7 Axis

SNAI1 (snail) is a zinc finger protein and transcription factor that is crucial for EMT in both cancer and in embryonic processes such as primitive streak formation during gastrulation. When Snail is expressed it promotes differentiation during gastrulation, initiates EMT, and promotes mesoderm commitment while repressing primitive ectoderm and endoderm fates (Gill et al., 2011). Snail is a master regulator in embryonic and cancer development: it induces EMT by repressing epithelial gene expression, including cell to cell adhesion proteins such as E-Cadherin (Acloque et al., 2009; Batlle et al., 2000). Snail repression of E-Cadherin is sufficient to initiate EMT. Research has shown that Snail represses genes that might be involved in promoting the stem cell state. Snail downregulates tumor suppressors such as microRNA (miRNA) let-7 by directly binding to their promoters, and thereby indirectly induces stemness in cancer cells (Wang et al., 2021).

The let-7 family are small non-coding miRNA molecules made up of 22 nucleotides. Let-7 family miRNAs, similar to all miRNAs, function by binding to 3' untranslated regions (3' UTR) of messenger RNA (mRNA) to promote mRNA

degradation and prevent translation (Ma et al., 2021). Let-7 is known to keep cells differentiated and repress oncogenes (Chirshev et al., 2019). Let-7i has also been shown to repress cancer cell stemness, inhibit migration and invasion, and may reduce cancer progression, relapse and metastasis (Chirshev et al., 2021).

The Snail/let-7 axis is an intriguing pathway because Snail binds to several let-7 family member promoters and represses their transcription. Studies have shown that knockdown of Snail increased let-7 and conversely, overexpression of Snail decreased let-7i. Overexpression of Snail resulted in an upregulation of Lin28, a stem cell marker, which may indicate a stem-like state (Unternaehrer et al., 2014). Studies have also demonstrated let-7i decreases cancer stem cell markers Lin28 and HGMA2 reducing stemness in HGSOC, and let-7 overexpression was shown to decrease the population of CSCs in-vitro (Chirshev et al., 2021).

The Snail/let-7 pathway is an attractive mechanism to use as a potential therapeutic target simultaneously with treatments such as chemotherapy and irradiation. We aim to identify treatment-induced CSC enrichment after conventional treatments to provide sufficient evidence for pursuing strategies such as let-7 overexpression concurrently with chemotherapy or irradiation to reduce the effects of treatment-induced stemness.

Cancer Stem Cells

Cancer stem cells are a small subpopulation within a tumor that can initiate tumorigenic growth as well as sustain tumor development. CSCs exhibit many stem-like characteristics: quiescence (slowing of the cell cycle), differentiation, proliferation, and self-renewal to generate new stem cells while regenerating a new cancer cell (Figure 2).

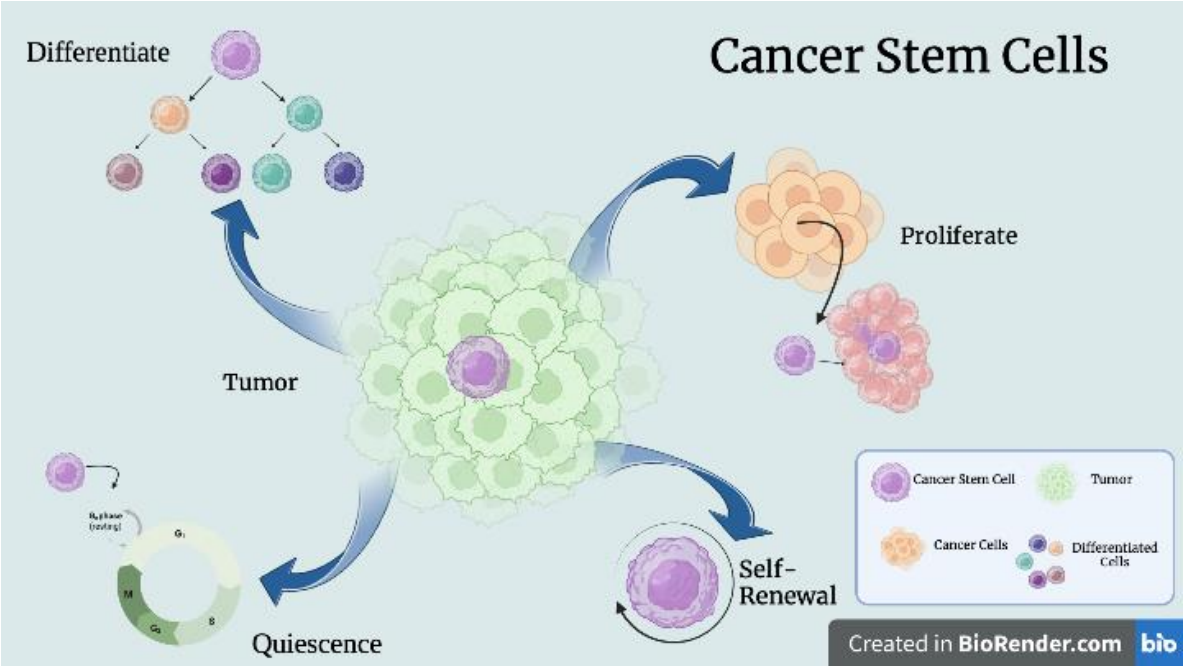


FIGURE 2: CANCER STEM CELLS. Cancer stem cells have the ability to (1) differentiate into different cell types, (2) proliferate, (3) self-renew, and (4) remain quiescent by slowing the cell cycle.

Resembling stem cells, CSCs may express core embryonic stem-cell transcription factors(Ni and Huang, 2013) associated with stemness, and upregulate genes involved in pluripotency and self-renewal such as OCT4 and SOX2, as well as inhibit genes that drive differentiation (Young, 2011). SOX2

and OCT4 are stem cell markers that correlate to the CSC ability to self-renew, proliferate, and differentiate into different cell types (Cruz et al., 2022). SOX2 has been shown to a have a critical role in tumor initiation and chemoresistance in ovarian cancer (Robinson et al., 2021). OCT4 has been associated with increased cell viability, tumorigenesis and drug resistance (Ruan et al., 2018). CSCs are also primarily responsible for tumor maintenance and proliferation of the tumor as well as the promotion of radiation resistance(Shen et al., 2014) and chemotherapy resistance (Pieterse et al., 2019). CSCs have the ability to self-renew and generate heterogeneous lineages of cancer cells that make-up the tumor (Clarke et al., 2006). Stemness factors such as SOX2 and OCT4 induce pluripotency and reduce the effectiveness of treatments such as chemotherapy (Zhu et al., 2021) and radiation (Shen et al., 2014).

Radiation and Chemotherapy Treatment for Ovarian Cancer

Treatments such as irradiation and chemotherapy have been shown to induce CSCs (Ghisolfi et al., 2012; Phi et al., 2018; Wang et al., 2017). CSCs may be the cause of tumor recurrence (Luero et al., 2014), treatment resistance (Phi et al., 2018), dedifferentiation, migration, and invasion (Suster and Virant-Klun, 2019) (Figure 3). Historically, irradiation has not been a cornerstone of primary treatment for ovarian cancer. Irradiation treatment for ovarian cancer is not generally recommended because irradiation can be highly toxic to the surrounding tissues and organs. Additionally, ovarian cancer is often highly

disseminated at diagnosis, making it difficult to target with radiation. Despite toxicity, irradiation of ovarian cancer is an option in specific ovarian cancer cases, most often in advanced disease (Fields et al., 2017). One type of irradiation, photon therapy, utilizes X-rays to kill cancer cells by causing DNA damage. Conversely, it enriches the cancer stem cell population after treatment (Bütof et al., 2013; Pieterse et al., 2019). Irradiation has been shown to make cancer cells more aggressive, leading to proliferation, self-renewal, radio-resistance, and increased invasiveness.

Chemotherapies are the mainstay of ovarian cancer treatment. Cisplatin (cis-dichlorodiammineplatitum (II) forms non-specific bonds with DNA bases to form DNA adducts (Dasari and Bernard Tchounwou, 2014; Rocha et al., 2018). The adducts force DNA cross-links that arrest the cell cycle during S, G1, or G2 and induce apoptosis (Aldossary, 2019). Research has shown that in ovarian cancer cells cisplatin treatment induces stemness in cisplatin-naive cells in-vitro (Wiechert et al., 2016). Cisplatin treatment in osteosarcoma is also associated with increased EMT showing higher expression of Snail, SNAI2, and other EMT factors such as increased N-Cadherin, and promoted increased migratory and invasive capacity (Fang et al., 2016).

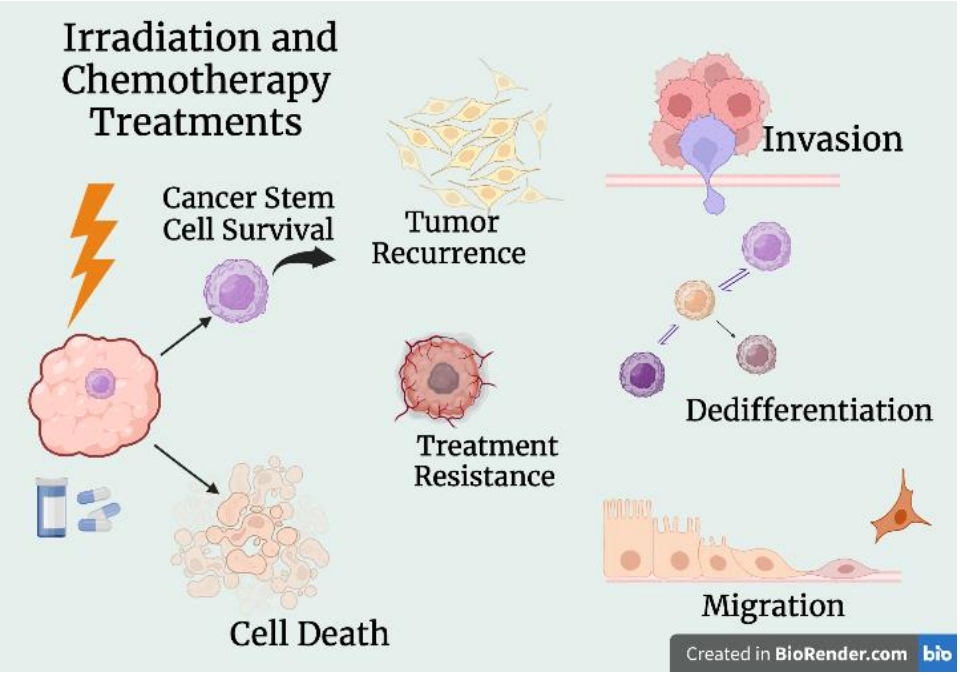


FIGURE 3: IRRADIATION AND CHEMOTHERAPY TREATMENTS. Irradiation and chemotherapies induce cell death in tumor cells, but cancer stem cells can persist. Stemness induced by treatment can cause tumor recurrence, treatment resistance, invasion, dedifferentiation, and migration.

CHAPTER THREE:

MATERIALS AND METHODS

Cell Culture

PDX 4 was isolated from a patient with HGSOC in accordance with institutional research board (IRB) protocols. Cells were cultured in 75% Ham's F12 (all media from Fisher Scientific, Waltham, MA, USA), 25% DMEM with 5% fetal bovine serum (FBS; Omega Scientific, Tarzana, CA, USA), 10 μM insulin (chemicals are from Millipore), 0.4 μM hydrocortisone, 2 μg/mL isoprenaline, 24 μg/mL adenine, and 100 U/mL penicillin, 10 μg/mL streptomycin (pen/strep). OVSAHO were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 2 mM of L-Glutamine, 100 U/mL of penicillin, and 10 μg/mL of streptomycin.

Irradiation Treatment

Cells were seeded at a density of 8.5x104 in 6-well plate and incubated overnight. Cell irradiations with photons (x-rays) was completed at the James M. Slater Proton Treatment and Research Center, Loma Linda California. Photons (x-rays) irradiations, a 22 MeV TrueBeam linear accelerator (Varian Medical System, Palo Alto, CA) was employed to expose cells with single doses of 0, 1, 2, 4, and 8 Gy with a dose rate of 3 GY/min at room temperature. The x-ray field

size employed for the irradiation of the ovarian cancer cell lines was square beam spot of 20 x 20 cm. The cells were then incubated for 72-hours after treatment, and then harvested for flow cytometry. FlowJo Version 10 (FlowJo LLC, Ashland, OR USA) was utilized for data analysis. *(Statistical tests are noted in figure caption).*

Cisplatin Treatment

Cells were seeded at a density of 1.0x105 in a 6-well plate and incubated overnight. The cells were treated with increasing cisplatin concentrations (0, .14, .28, .42, .56, 1.3, 46 µM). The cells were then incubated for 72-hours, and then harvested for flow cytometry. FlowJo Version 10 (FlowJo LLC, Ashland, OR USA) was utilized for data analysis. *(Statistical tests are noted in figure caption),*

Flow Cytometry (Cancer Stem Cells)

Cells were detached using 0.05% trypsin and resuspended in FACS stain (phosphate buffered saline (PBS), 1% FBS, 0.1% Sodium Azide and 2mM EDTA), and 7-AAD (viability dye) at 5µL per 100µL of cell suspension. Cells were analyzed using MACSQuant Analyzer 10 (Miltenyi Biotec, Auburn, CA, USA) for flow cytometry. FlowJo Version 10 (FlowJo LLC, Ashland, OR USA) was utilized for data analysis. *Flow cytometry average GFP expression data analysis was*

conducted with a Welch's One-way ANOVA on GraphPad Prism Version 7.0 (GraphPad Software, La Jolla, CA, USA).

Viability Assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) assay was used to determine cell viability. Cells were seeded at a density of 1 × 103 cells/well in 96-well plates and incubated overnight. The cells were then treated with increasing concentrations of Cisplatin (0, 3.9 mM, 7.8 µM, 15.6 µM, 31.3 µM, 62.5 µM, 125 µM, 250 µM, 500 µM and 1mM) for 3 days. After drug treatment, 10 µl of MTT solution was added to each well, and the plates were incubated for 3 h at 37 °C. The formed formazan crystals were dissolved in dimethyl sulfoxide (DMSO), and the absorbance was measured at 570 nm using a SpectraMax i3x microplate reader (Molecular Devices, Sunnyvale, CA, USA). The half-maximal inhibitory concentration (IC50) of cisplatin was analyzed using GraphPad Prism Version 7.0 (GraphPad Software, La Jolla, CA, USA). *(Statistical tests are noted in figure caption),*

Spheroid Formation Assay

Cells were plated at a density of 1 cell per well in 96-well non-tissue culture coated plates and maintained in serum-free medium (DMEM/ F12 50/50, supplemented with 0.4% bovine serum album, 10 ng/mL FGF, 20 ng/mL EGF,

6.7 ng/mL selenium, 5.5 µL/mL transferrin, 10 µL/ mL insulin and 1% knockout serum replacement) for 7 days. The number of wells containing a spheroid was counted and statistically analyzed *(Statistical tests are noted in figure caption).*

Colony Formation Assay

Cells were plated at a density of 1x103 cells per well in 6-well plates for 14 days. At day 7, 1 mL of media was added to each well and maintained in complete media for 14 days. At day 14, the colonies washed with 1x PBS, fixed with 3:1 acetic acid and methanol solution, stained with 0.5% crystal violet solution dissolved in methanol, and rinsed with 1x PBS to remove the excess stain. Colonies were inverted and air dried overnight and imaged with an EPSON Perfection V500 scanner at 1200 dpi. Colonies were analyzed using Fiji(ImageJ((Schindelin et al., 2012) Plugin:ColonyArea (Guzmán et al. 2014) *(Statistical tests are noted in figure caption).*

CHAPTER FOUR:

RESULTS

Aim 1: Determine Effects Of Treatments On Stemness As Measured By The Sore-6 Reporter.

Aim 1.1: Define the half maximal inhibitory concentration, defined as the concentration of a drug or inhibitor needed to inhibit a biological process or response by 50% (IC50) after treatment.

Ovarian cancer cells will be treated with 1 mM to 0 mM of cisplatin to determine the IC50 for each cell line using a viability assay.

Defining The IC50 Of PDX4 And OVASHO. Viability assay was used to define the half maximal inhibitory concentration, defined as the concentration of a drug or inhibitor needed to inhibit a biological process or response by 50% (IC50) after treatment in two cells lines. OVASHO is an HGSOC cell-line commercially available, and PDX4 is a HGSOC patient derived xenograft maintained in our lab. OVASHO results yielded an average cisplatin-induced IC50 of 2.99 µM (Figure 4A.) and PDX4 yielded a cisplatin induced IC50 of .59 µM (Figure 4B.) Figure 4C shows a visual comparison of IC50 between the PDX4 and OVASHO.

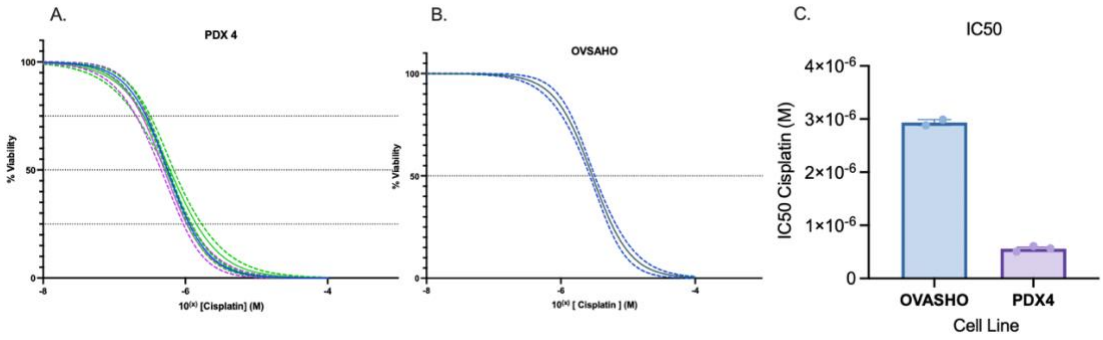


FIGURE 4: DEFINED HALF MAXIMAL INHIBITORY CONCENTRATION OF CISPLATIN IN OVASHO AND PDX4. A. OVASHO (N=2) B. PDX4 (N=3) C. Comparison of OVASHO (2.99 µM) and PDX4 (.56 µM) Cisplatin IC50. Error bars: Standard Deviation (SD).

Aim 1.2: Define the levels of GFP as a surrogate for SOX2 and OCT 4 by the SORE-6 reporter in ovarian cancer cells with varying doses of cisplatin. Ovarian cancer cells will be treated with cisplatin at the IC50 dose, and after 72-hours flow cytometry will be used to determine percent positive for SORE-6 GFP to determine levels of stemness compared to the untreated sample (IC0).

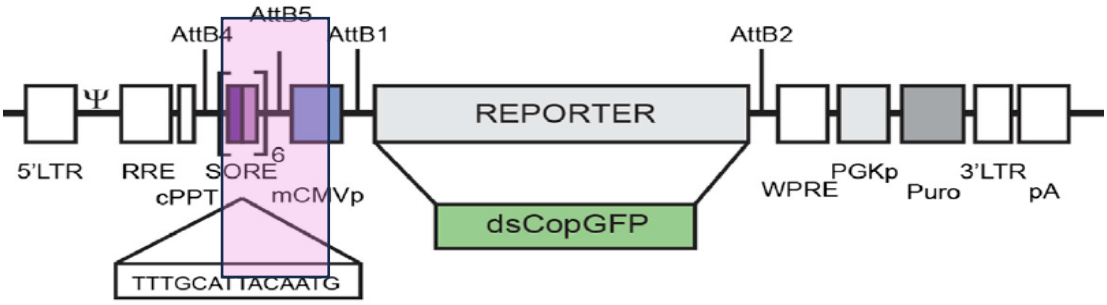


FIGURE 5: THE SORE-6 REPORTER SYSTEM. The SORE-6 Reporter system was designed with six concatenated repeats of composite SOX2/OCT4 response element (shown in pink box). The short DNA sequences within the promoter enable the binding of transcription factors to regulate the transcription of genes enabling the activation of the response element to stimulate transcription of GFP expression as a surrogate of stemness. (Tang et al. 2015)

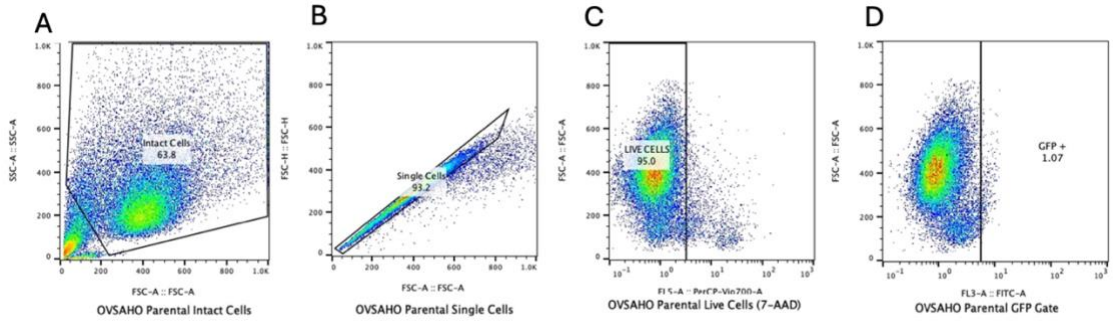


FIGURE 6: GATING SCHEMATIC FROM FLOWJO VER.10 OF OVASHO CELLS.A. OVSAHO gates for intact cells within the population in parental cells (cells that do not have the SORE-6 GFP reporter) B. OVASHO parental cells gated for single cells. C. Parental cells gated for alive cells. D. The alive gate was applied to the GFP + cells (cells with the SORE-6 reporter system) to define GFP baseline levels within the population. Gating schematic is the same of PDX4.

AVERAGE SORE-6 BASELINE EXPRESSION

CELL LINE	GFP Baseline Percentage (%)
PDX 4	17%
OVSAHO	3%

TABLE 1: AVERAGE SORE-6 BASELINE EXPRESSION. PDX4 had an average GFP expression of 17% compared to OVASHO with a 3% GFP baseline GFP expression.

SORE-6 GFP Expression 72-Hours After Cisplatin Treatment. We wanted to determine the effects on cisplatin induced stemness by the SORE-6 GFP reporter system (Figure 5) 72-hours after cisplatin treatment (Tang et al. 2015). We used flow cytometry to define average GFP expression within the sample populations. To analyze the GFP response to treatment, we combined the relative average GFP expression compared to the control, 0µM (gating schematic shown in Figure 6). Cisplatin was used at concentrations determined in viability assay (Figure 4). PDX-4-SORE6 GFP baseline average was 17% compared to cells without the GFP reporter system (Table 1). The baseline GFP average level indicates the level of GFP present with the SORE-6 reporter compared to cells without the Sore-6 reporter (parental) line (Figure 6D). The gate was drawn consistently at ~1% GFP positive cells (GFP+) then compared to cells with the SORE6 reporter system. The PDX4 cell line was treated with a dilution of cisplatin 0 µM, .14 µM, .28 µM, .42 µM, .56µM, 1.3 µM, and 46 µM. The cisplatin induced a dose-dependent increase in GFP expression to 1.3 µM and a significant decrease of GFP expression at 46 µM (Figure 7A). OVASHO-SORE6 GFP baseline average at 0µM was 3% (Table 1) before treatment compared to

cells without the GFP reporter system. OVASHO was treated with 0µM, .18 µM, 0.86 µM, 1.6 µM, 2.5 µM, 7.7 µM, 8.7 µM cisplatin. OVASHO Sore-6 shows an overall GFP increase in expression but is not dose-dependent (Figure 7B).

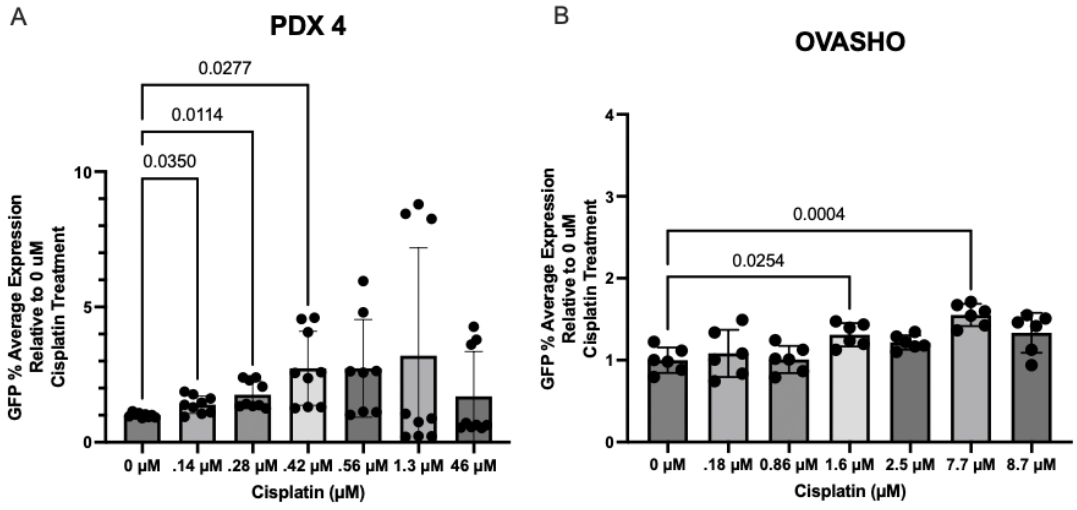


FIGURE 6: SORE6 GFP EXPRESSION AFTER CISPLATIN. A. PDX4 (N=9) GFP Expression resulting in a dose-dependent response to cisplatin. B. OVASHO (N=6) GFP Expression had an increase in GFP expression as cisplatin dose increases. Data for cisplatin induced GFP expression. Significance calculated via Welch's one-way ANOVA with *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001. Error Bars: SD (Standard Deviation). All graphs are scaled to the individual cell line.

Aim 1.3: Define the levels of GFP as a surrogate for SOX2 and OCT 4 by the SORE-6 Reporter in ovarian cancer cells with varying doses of photon irradiation. Ovarian cancer cells will be treated with 0 GY, 1 GY, 2 GY, 4 GY, and 8 GY of photon irradiation, and after 72-hours flow cytometry will be used to determine percent positive for SORE-6 GFP to determine levels of stemness.

SORE-6 GFP Expression 72-Hours After Photon Irradiation. We wanted to determine the effects on stemness by the SORE6 GFP reporter system 72-hours after photon treatment. We used flow cytometry to define average GFP expression within the sample populations. We combined the relative average GFP expression compared to the control, 0GY (gating schematic shown in Figure 6). PDX4-SORE6 GFP baseline average was 17% compared to cells without the reporter system. Compared to the control, 0 GY, PDX 4 results show a dose-dependent response to photon irradiation (Figure 8A). The maximum fold difference was 3.0-fold at 8 GY compared to 0 GY. OVASHO-SORE6 had an average GFP expression baseline of 3.7% compared to cells without the reporter system. The OVSAHO-SORE6 expression showed a dose dependent response relative to the control (Figure 8B). The maximum fold change was 1.7-fold at 8 GY compared to 0 GY.

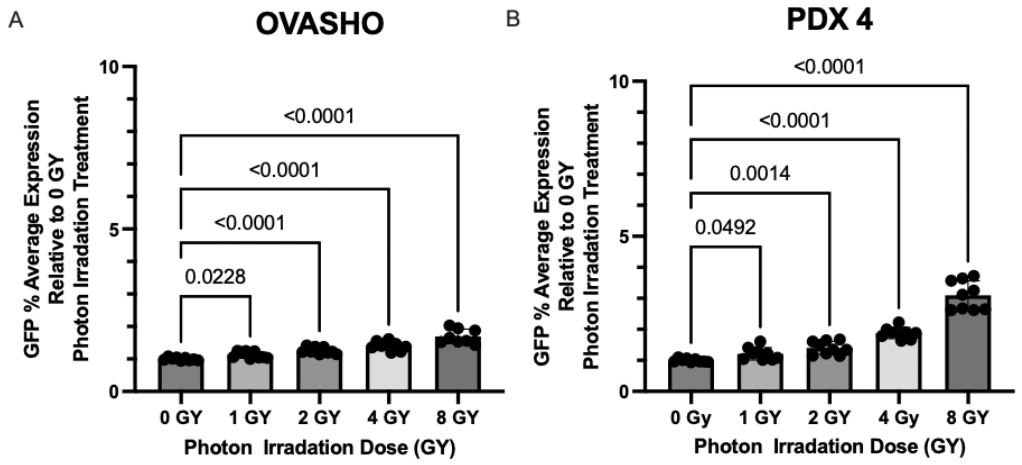


FIGURE 7: SORE6-GFP EXPRESSION AFTER PHOTON IRRADIATION. A. PDX4 (N=9) GFP Expression resulted in a dose-dependent increase in response to photon irradiation. B. OVASHO (N=9) GFP Expression resulted in a dose-

dependent increase in response to photon irradiation. Significance calculated via Welch's one-way ANOVA with *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001. Error Bars: SD.

Aim 2: Determine Effects Of Treatments On Stemness By Functional Assays.

Aim 2.1**:** Define functional properties of the cancer cell population after treatment with half maximal inhibitory concentration of cisplatin defined as the concentration of a drug or inhibitor needed to inhibit a biological process or response by 50% (IC50) after treatment.
Perform functional assays to determine the stem-like properties: colony and spheroid formation.

Colony Forming Assay.Ovarian cancer cells will be treated with cisplatin and then plated 72-hours after treatment to determine the capacity to proliferate and form colonies.

Spheroid Forming Assay. Ovarian cancer cells will be treated with cisplatin and then plated 72-hours after treatment to determine the ability to self-renew grow in anchorage-independent conditions.

Assessing The Capacity To Form Colonies After Cisplatin Treatment. Colony forming assay was used to determine the ability to proliferate in adherent 2-D

conditions in a tissue culture plate. The cells were plated 72-hours after cisplatin treatment and incubated for 14 days. After 14 days of incubation, PDX4 demonstrated a significantly decreased number of colonies (Figure 9A) compared to the control (IC0). PDX4 resulted in an overall significant decrease in percent area the colonies occupied in the well when treated with .56µM cisplatin (Figure 9C). However, PDX4 formed larger colonies at the IC50 dose compared to the control. OVASHO resulted in a significant decrease in colony formation after 14 days (Figure 9D), decreased average size of colonies, (Figure 9E) and of decreased area coverage in the wells (Figure 9F).

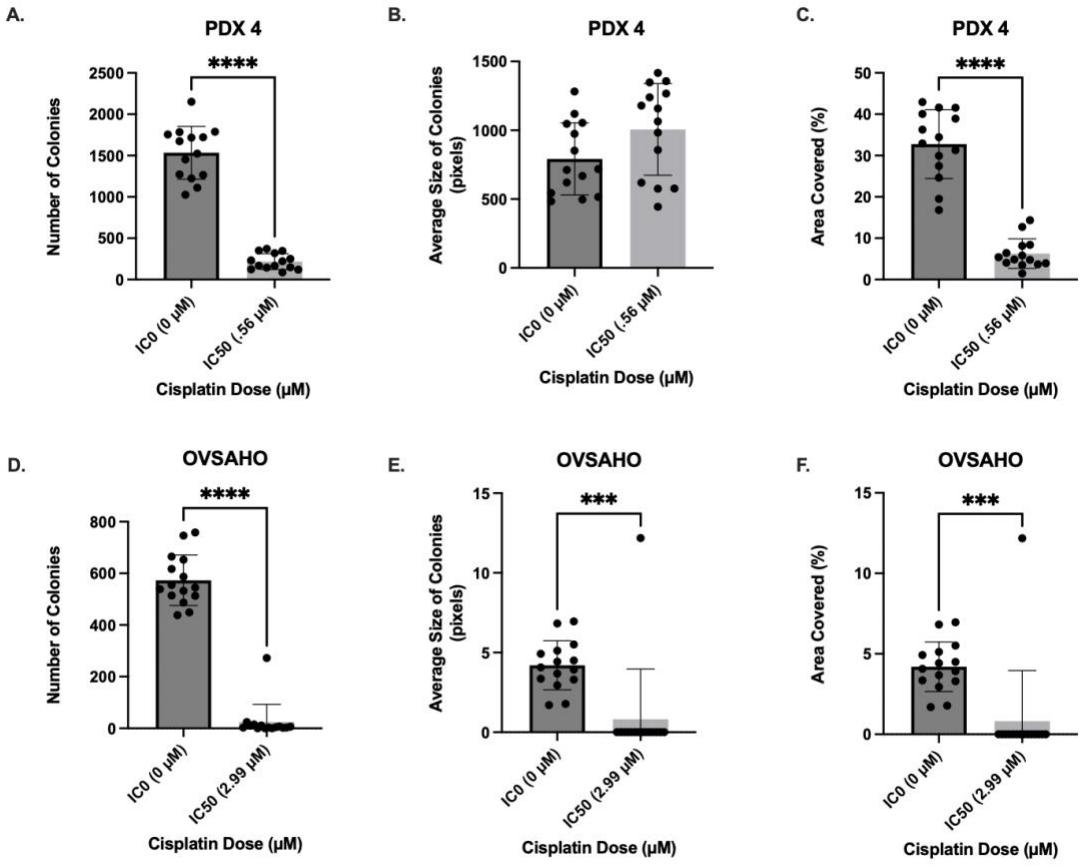


FIGURE 8: ASSESSING THE CAPACITY TO FORM COLOIES AFTER CISPLATIN TREATMENT. A. When treated with cisplatin at the IC50 dose,

PDX4 resulted in a decrease in number of colonies. B. PDX4 had increased average size of colonies when treated with cisplatin. C. PDX4 had a decreased area covered in percentage. D. Cisplatin decreased number of OVASHO colonies. E. Cisplatin decreased the average size of OVASHO colonies. F. When treated with cisplatin, OVASHO resulted in decreased percent area coverage. Significance calculated via Student's T-Test with *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001. Each dot represents an individual sample. Error bars: SEM.

Assessing The Capacity To Form Colonies After Photon Irradiation Treatment. Colony forming assay was used to determine the ability proliferate in adherent 2-D conditions in a tissue culture plate. The cells were plated after 72-hours after photon irradiation treatment and incubated for 14 days. After 14 days of incubation, PDX4 showed a significant decrease in the number of colonies at 2 GY and 4 GY compared to the control 0 GY (Figure 10A), and a significant increase in area coverage percentage in the well at 4 GY compared to the control (Figure 10C). PDX4, however, did not have much of a difference between the size of the colonies at 2 GY and 4 GY compared to the control (Figure 10C). OVASHO colony formation significantly increased at 2 GY compared to a reduction in colony formation at 4 GY (Figure 10D). OVASHO indicated the same significant increase for the average size of colonies (Figure 10E) as well as the area of the well covered (Figure 10F).

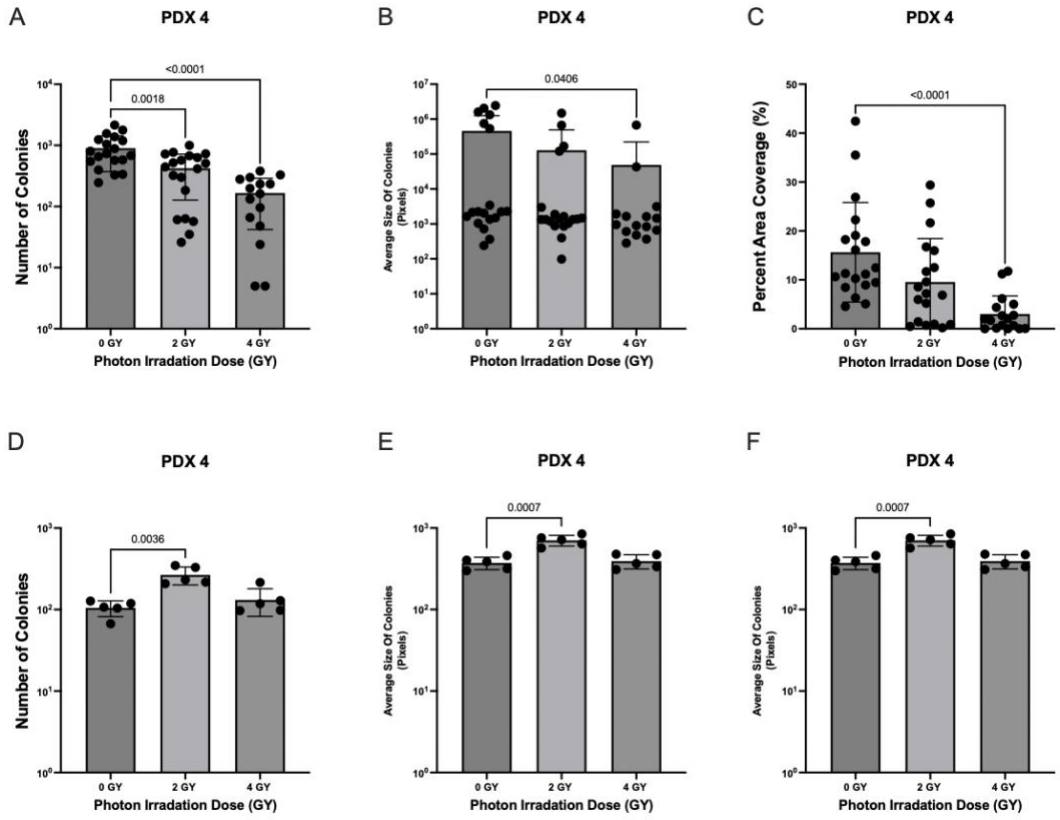


FIGURE 9: ASSESSING THE CAPACITY TO FORM COLONIES AFTER PHOTON IRRADIATION TREATMENT. A. when treated with photon irradiation, PDX4 had decreased number of colonies. B. PDX4 decreased the average size of colonies. C. PDX4 decreased in area covered in percentage when treated with photon irradiation. D.OVSAHO colonies increased at 2 GY and decreased at 4 GY. E. OVASHO average size of colonies increased at 2 GY and decreased 4 GY. F. OVASHO percent area coverage of colonies increased at 2 GY and decreased at 4 GY. Significance calculated via Welch's one-way ANOVA with *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001. Error Bars: SD.

Assessing The Capacity To Form Spheroids After 72-Hours. To assess the stemness, we wanted to determine the ability for a single cell to self-renew in suspension. The cells were replated into suspension culture 72-hours after treatments, incubated for 7 days then counted. Our spheroid assay was limited to PDX4 because OVASHO has low ability to form spheroids (Hojo et al. 2018). PDX4 was treated with cisplatin at 0 µM and the IC50, .56 µM. The results yielded 0.66-fold decrease in the capacity to form spheroids when .56 µM cisplatin is administered compared to the control, 0 µM (Figure 11A). PDX4 was treated with photon irradiation at 0 GY, 2 GY and 4 GY. PDX4 resulted in a 1.7-fold increase in spheroid formation between 0GY and 2 GY, but there was no significant difference (Figure 11B). There was a 0.5-fold decrease between the 0 GY and 4 GY in spheroid formation.

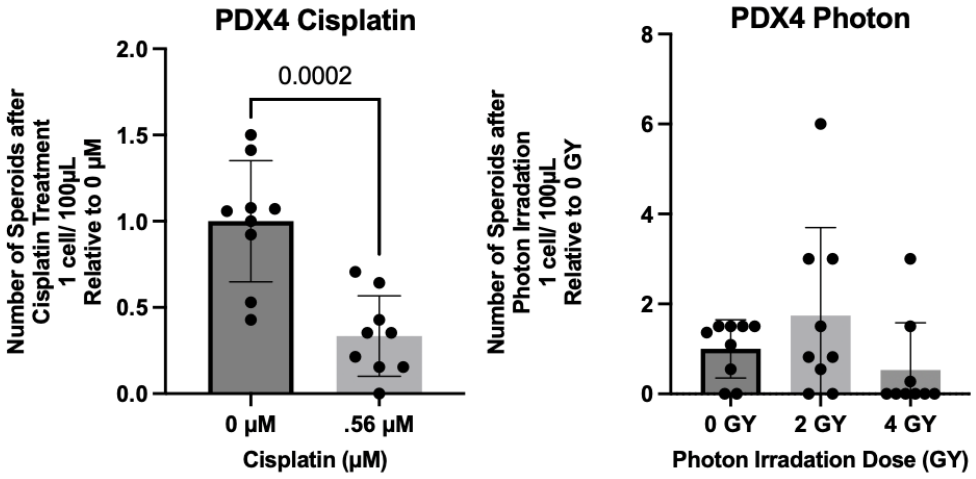


FIGURE 10: ASSESSING THE CAPACITY TO FORM SPHEROIDS AFTER TREATMENT.A. Spheroid count after cisplatin decreased compared to the control. Significance calculated via Student's T-Test with *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001. Each dot represents an individual sample. Error bars: SD. B. Spheroid count after photon treatment resulted in increased spheroid formation at 2 GY and decrease at 4 GY. Significance calculated via Welch's one-way ANOVA with *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001. Error Bars: SD.

Aim 3: Determine Effects Of Treatments On Stemness By Determining The Level Of MRNA Of Stemness Markers OCT 4 And SOX2.

Aim 3.1**:** Define the levels of mRNA for SOX2 and OCT 4 in ovarian cancer cells at the IC50 cisplatin dose compared to the untreated ovarian cancer cells. Ovarian cancer cells were treated with cisplatin at the IC50 dose at 72-hours will be harvested for rt-qPCR.

Assessing SOX2 And OCT4 MRNA Levels After Cisplatin Treatment. To assess the levels of stemness after 72-hours of cisplatin treatment, we wanted to define the relative levels of mRNA SOX2 and OCT4 after treatment in both cell lines, PDX4 and OVASHO, compared to the relative levels its respective actin. PDX4 after the cisplatin treatment resulted in an overall increase in OCT4 and SOX2 (Figure 12). OCT4 resulted in an increase of SOX2 relative to its own actin (Figure 12). OVASHO shows a similar effect in mRNA abundance after cisplatin treatment to PDX4 (Figure 13). After cisplatin treatment, OVASHO resulted in an increase of OCT4 and SOX2 relative to its own actin. The results are preliminary due to large error margins; however, the results indicate an overall trend increase in stemness markers, OCT4 and SOX2, for both cell lines, after 72-hours when treated with cisplatin.

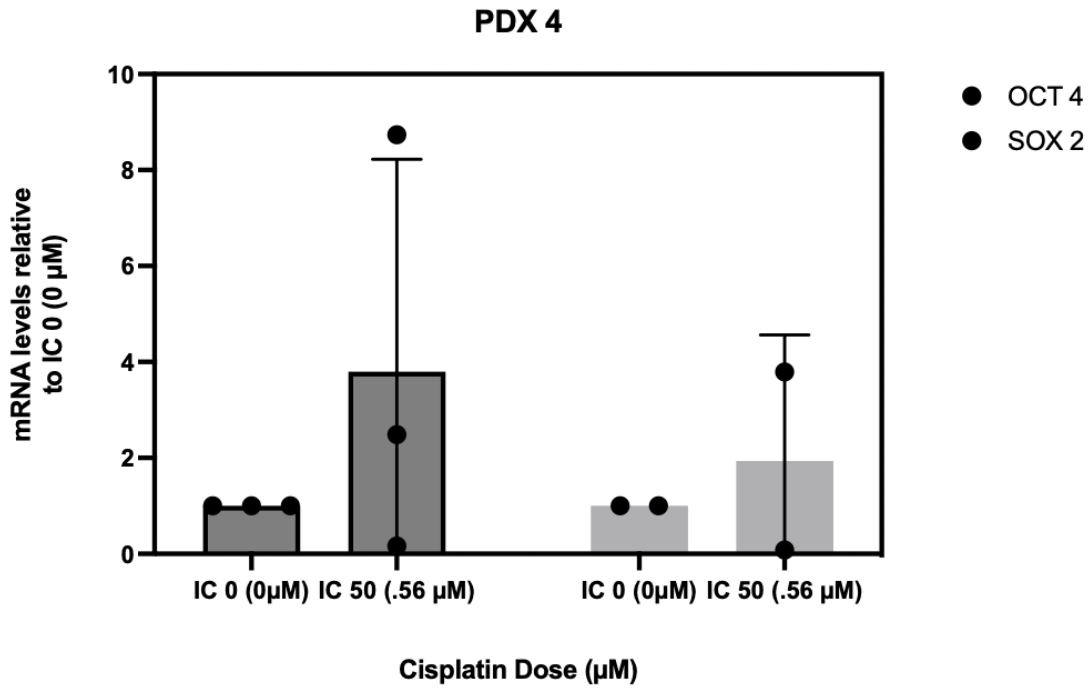


FIGURE 11: PDX4 OCT4 AND SOX2 MRNA LEVELS 72 HOURS AFTER CISPLATIN TREATMENT. After 72-hours resulted in increased OCT4 and SOX2 mRNA levels at the IC50 dose (.56 µM) compared to the control (0µM). The mRNA levels are relative to its own actin and then compared the control 0 GY. Each dot represents an individual sample. Error bars: SD.

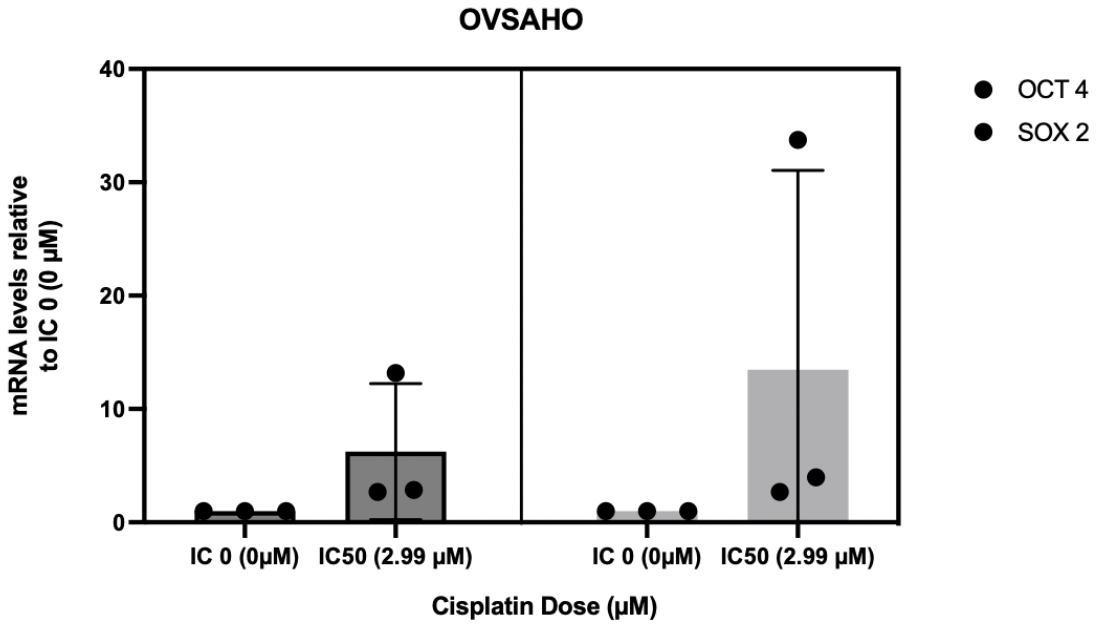


FIGURE 12: OVASHO OCT4 AND SOX2 mRNA LEVELS 72 HOURS AFTER CISPLATIN TREATMENT. Cells treated with cisplatin at the IC50 concentration (2.99µM) had higher levels of OCT 4 and SOX2 compared to the control (0 µM). The mRNA levels are relative to its own actin and then compared the control 0 GY. Each dot represents an individual sample. Error bars: SD.

Aim 3.2: Define the levels of mRNA for SOX2 and OCT 4 in ovarian cancer cells at the IC50 cisplatin compared to the untreated ovarian cancer cells.

Ovarian cancer cells treated with photon irradiation at 0 GY, 2 GY, and 4GY dose at 72-hours and harvested for rt-qPCR.

Assessing SOX2 And OCT4 mRNA Levels After Photon Treatment. To assess the levels of stemness 72-hours after photon treatment, we wanted to define the relative levels of SOX2 and OCT4 after treatment in both cell lines, PDX4 and

OVASHO compared to the relative levels its own respective actin. PDX 4 resulted in decreased OCT 4 mRNA levels at 2 GY and 4 GY (Figure 14). Conversely, SOX2 resulted in increased SOX2 levels at 2 GY and 4 GY compared to the control (Figure 14). OVASHO indicated inconsistent trends in mRNA levels OCT4 and SOX2 (Figure 15). At 2 GY, OCT4 and SOX2 levels resulted in a decrease. When OVASHO was treated with 4 GY, OCT4 levels increased but SOX2 levels were decreased (Figure 15).

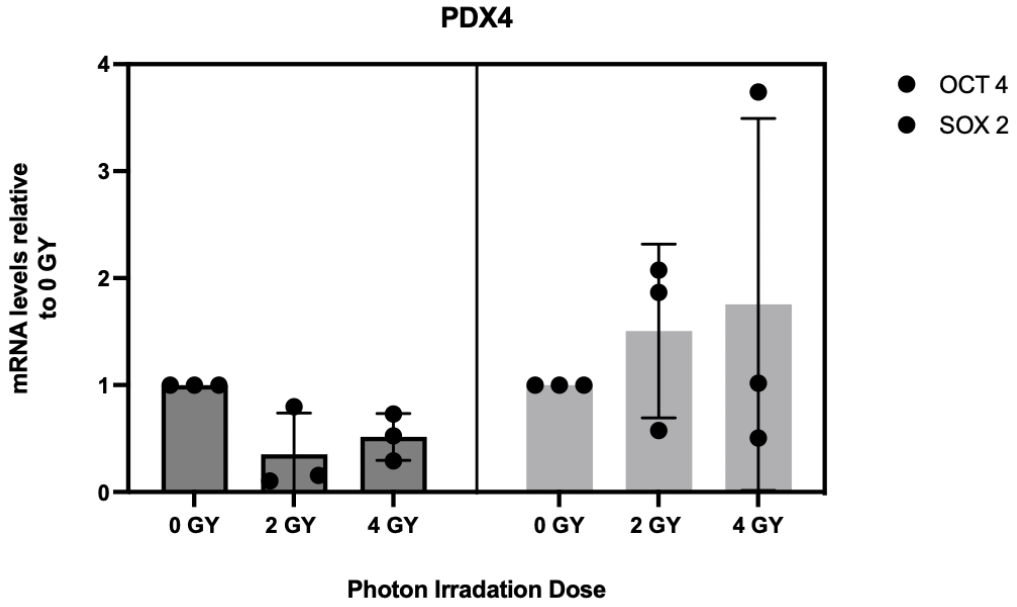


FIGURE 13: PDX4 OCT4 AND SOX2 mRNA LEVELS 72 HOURS AFTER PHOTON IRRADATION TREATMENT. Cells treated with photon irradiation at the 0 GY, 2 GY and 4 GY and incubated for 72-hours. After photon irradiation, OCT4 mRNA decreased compared to the control. SOX2 increased on average compared to the control. The mRNA levels are relative to its own actin. The mRNA levels are relative to its own actin and then compared the control 0 GY. Each dot represents an individual sample. Error bars: SD.

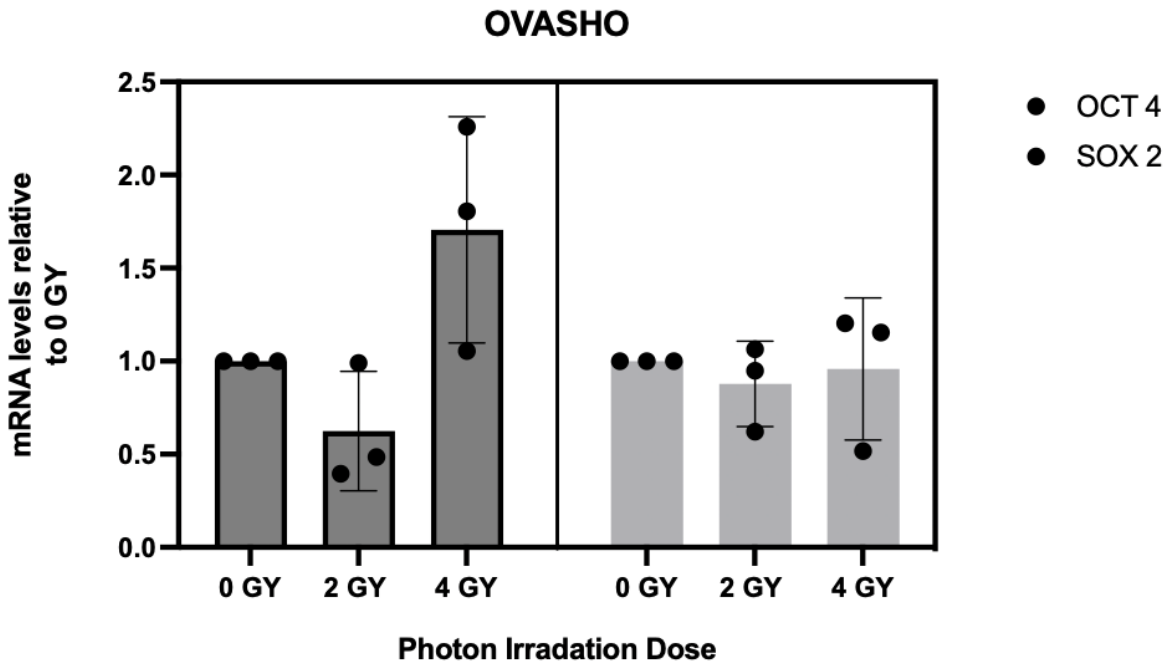


FIGURE 14: OVASHO OCT 4 AND SOX2 mRNA LEVELS AFTER PHOTON IRRADATIONTREATMENT. Cells treated with photon irradiation at the 0 GY, 2 GY and 4 GY and incubated for 72-hours. The mRNA levels are relative to its own actin. OVASHO when treated with photon irradiation resulted in a decrease OCT 4 expression at 2 GY and an increase OCT 4 expression at 4 GY. OVASHO resulted in a decrease of SOX2 expression at 2 GY and 4 GY. The mRNA levels are relative to its own actin and then compared the control 0 GY. Each dot represents an individual sample. Error bars: SD.

CHAPTER FIVE:

DISCUSSION

Conventional Therapies Enrich the Cancer Stem Cell Population

Treatments such as chemotherapy and irradiation have been documented to enhance the stemness in Cancer Stem Cells (CSCs) in glioblastoma, hepatocellular carcinoma, and ovarian cancer.(Arnold et al. 2020; Ghisolfi et al. 2012; Wiechert et al. 2016). While conventional therapies effectively shrink tumor size by targeting therapy-sensitive cancer cells, a notable consequence is the persistence of CSCs or treatment-resistant cells (Pieterse et al. 2019). CSCs exhibit stem cell-like characteristics, including the ability to self-renew, undergo asymmetric and symmetric divisions, proliferate, and engage in multi-lineage differentiation (Ghisolfi et al. 2012; Jordan, Guzman, and Noble 2006; Visvader and Lindeman 2008) These features contribute to the potential for metastasis, proliferation, relapse and recurrence, and disease progression (Pignata et al. 2017). Therefore, it is important to examine treatment-induced stemness. Here, we found that cisplatin and irradiation enrich the CSC population as measured by the SORE-6 GFP reporter system and mRNA gene expression.

Functional Assays Yielded Inconsistent Results

The two stemness factors, OCT4 and SOX2, are key regulators in CSCs maintenance. In ovarian cancer, SOX2 contributes to therapy resistance, tumor

aggressiveness, and self-renewal (Bareiss et al. 2013; Robinson et al. 2021). OCT4 in ovarian cancer enhances tumor cell growth, survival, metastasis and chemo-resistance in-vitro and in-vivo models (Ruan, Yang, and Cheng 2018). Both stemness factors induce pluripotency and reduce the effectiveness of treatments such as chemotherapy (Zhu et al. 2021) and irradiation (Shen et al. 2014), which are aligned with our SORE-6 GFP flow cytometry results. PDX 4 resulted in an increased dose-dependent response after cisplatin treatment with a maximum of 3.7-fold change when treated with 1.3 µM cisplatin. OVASHO displayed an upward trend in GFP expression with a maximum 1.7-fold change at 8.7 µM of cisplatin. When treated with photon irradiation, PDX4 and OVASHO exhibited a dose-dependent increase response. PDX4 had maximum 3.0-fold change at 8 GY, and OVASHO resulted in maximum 1.7-fold change at 8 GY after irradiation. The SORE-6 GFP expression, a surrogate for OCT4 and SOX2, resulted in an average increase in stemness genes within the sample population after treatment. The increase of GFP expression suggests that treatments such as cisplatin and irradiation therapies enrich the CSC population. These results suggest that chemotherapy and photon irradiation induce cell death in non-CSC population (therapy-sensitive cells) consequently, promoting CSC persistence (therapy-resistant cells) (Pieterse et al. 2019). Furthermore, the increased levels of GFP expression compared to the control may suggest that the residual population after treatment may be undergoing cellular reprogramming and may acquire more stem-like characteristics thereby promoting CSC enrichment

(Ghisolfi et al. 2012; Ghisolfi et al. 2012; Lagadec et al. 2012; L. Wang et al. 2017).

A fundamental characteristic of CSCs is the ability to self-renew. Spheroid formation is used as a measure of the capacity of a single cell to self-renew, and thus are seen as an indicator of a CSC's ability to contribute to oncogenesis, malignancy as well as tumor initiation (Jordan, Guzman, and Noble 2006). We observed after irradiation PDX4 resulted in an increase in spheroid formation at 2 GY from 0 GY, although this was not maintained at 4 GY. These results were similar to those in a hepatoma cancer study which shows an increase in spheroid formation at 2 GY 7 days after irradiation (Ghisolfi et al. 2012). We did not detect a statistical change in spheroid formation after irradiation. In response to cisplatin treatment at the IC50 dose, spheroid formation significantly decreased compared to the control (IC0) by 0.33-fold. Our assessment of spheroid-forming ability was incomplete at 3 days after cisplatin treatment and 3 days post-irradiation time point at which cells may still have been recovering from acute effects of these treatments and thus unable to self-renew and proliferate as expected. We hypothesized that spheroid-forming ability would be enhanced after these treatments based on research showing enhanced self-renewal and proliferation in treated cells (Abubaker et al. 2013; Chen et al. 2016) and increased SORE-6 GFP expression results demonstrated increased frequency of CSC in treated cells. Experimental timelines were based on a single 72-hour time point to determine if GFP expression agrees with spheroid formation. Results may not capture self-renewal potential due to the limited 7-day spheroid

formation period.

CSCs have extensive proliferation potential and are highly tumorigenic. The colony formation assay is a qualitative measure of CSCs stemness by demonstrating the ability of a single cell to form a colony (Esquer et al. 2020). Here, we used the colony forming assay 3 days after cisplatin treatment and 3 days post photon irradiation treatment to determine CSC enrichment and incubated the assay for 14 days. Photon irradiation resulted in a significant decrease in the number of colonies at 2 GY and 4 GY in PDX4. We observed a percent well coverage decrease at 2 GY; this was significantly decreased at 4 GY in PDX4. The average size of colonies of PDX4 decreased at 2 GY and 4 GY, however we did not detect a significant change in colony size. OVASHO colony formation resulted in significant increased number of colonies, average size of colonies and precent area coverage in the well at 2 GY compared to the control, 0 GY, however, the significance was not maintained at 4 GY. PDX4 results were similar to clonogenic capacity determined by colony formation in a lung cancer study after irradiation (Ghisolfi et al. 2012; Zhang et al. 2013). In both cell lines, PDX4 and OVASHO, after cisplatin treatment, we observed a significant decrease in number of colonies and percent area coverage at the IC50 dose compared to the control. The average size of colonies differed between OVASHO and PDX4: in OVASHO, cisplatin treatment resulted in a significant decrease in size, however, we observed an increase in average colony size in PDX 4. We hypothesized that colony-forming ability would be enhanced after treatment based on previous research suggesting increased

clonogenicity after cisplatin treatment(H. Wang et al. 2021). Overall, in the PDX4 cell line, we observed a reduction in the colony number and size, while in OVASHO an increase in colony size at 2 GY was seen.

Conventional Treatments Increase Stemness Gene Expression

Treatments have been shown to increase mRNA gene expression of OCT4 and SOX2 after treatment (Wiechert et al. 2016; Zhu et al. 2021). Here mRNA measured by quantitative PCR was analyzed 3 days after cisplatin treatment and 3 days post-photon irradiation treatment to determine CSC enrichment by OCT4 and SOX2 gene expression. The mRNA gene expression after cisplatin treatment at the IC50 in both PDX4 and OVASHO show an increase of OCT4 and SOX2 mRNA gene expression. After photon irradiation, PDX4 and OVASHO results had varying levels of OCT4 and SOX2. PDX4 resulted in SOX 2 resulted an increase (1.5-fold) at 2 GY and (1.75-fold) at 4 GY and OCT4 exhibited a decrease in gene expression at 2 GY (.35-fold) and 4 GY (.52-fold) compared to the control. OVASHO resulted in a decrease of SOX2 gene expression at 2 GY (0.87-fold) and 4 GY (.96-fold) and OCT4 decreased at 2 GY (0.62-fold) and increased at 4 GY (1.71). Research has shown that cisplatin induces stemness gene expression after cisplatin treatment in liver cells treatment (Lee et al. 2020). Irradiation results have been shown to have varying gene expression levels of OCT4 and SOX2 between irradiation doses and varying time intervals in various types of cancer (Ghisolfi et al. 2012; Lagadec et al. 2012). Overall, the induction of SOX2 and/or OCT4 results suggest cisplatin and photon irradiation may

induce the reprogramming of differentiated cancer cells to a more stem-like phenotype, which agrees with the SORE-6 GFP reporter flow cytometry results (Figure 7 and Figure 8).

This study aimed to define CSC-like characteristics in ovarian cancer at a 72-hour time point by flow cytometry, functional assays, and mRNA gene expression. We hypothesized that providing cancer treatments such as irradiation and chemotherapy enriches the cancer stem cell population. Our data showed when irradiation is administered, there is a dose-depended response demonstrated by SORE-6 GFP expression. Cisplatin also induced a dose-dependent response in PDX4 and an overall GFP increase in OVASHO after treatment. Cisplatin treatment also resulted in an increase in stemness genes, OCT4 or SOX2 mRNA expression in both cell lines. Irradiation induced an increase in SOX2 mRNA expression in PDX4 and OCT4 levels increase at 4 GY for OVASHO. Despite the molecular measured increased levels of in stemness, functionally the results were less conclusive. It is possible that 3 days after cisplatin treatment and 3 days post-irradiation are insufficient time points at which cells may still have been recovering from acute effects of these treatments and thus unable to self-renew and proliferate as expected.

Rationale for Investigating Stemness After Cancer Treatments

Future directions: In continued research, it may be beneficial to optimize time intervals and treatment dose for functional assays to determine if CSC can

induce repair mechanisms and show increased functional properties such as self-renewal capacity and proliferation. To further analyze the epithelial mesenchymal transition (EMT) dynamic that primes cancer stem cell enrichment or reprogramming, it may be advantageous to investigate EMT markers such as snail, E-cadherin and N-cadherin. Additionally, it would be beneficial to define in-vitro levels of miRNA let-7 after treatment.

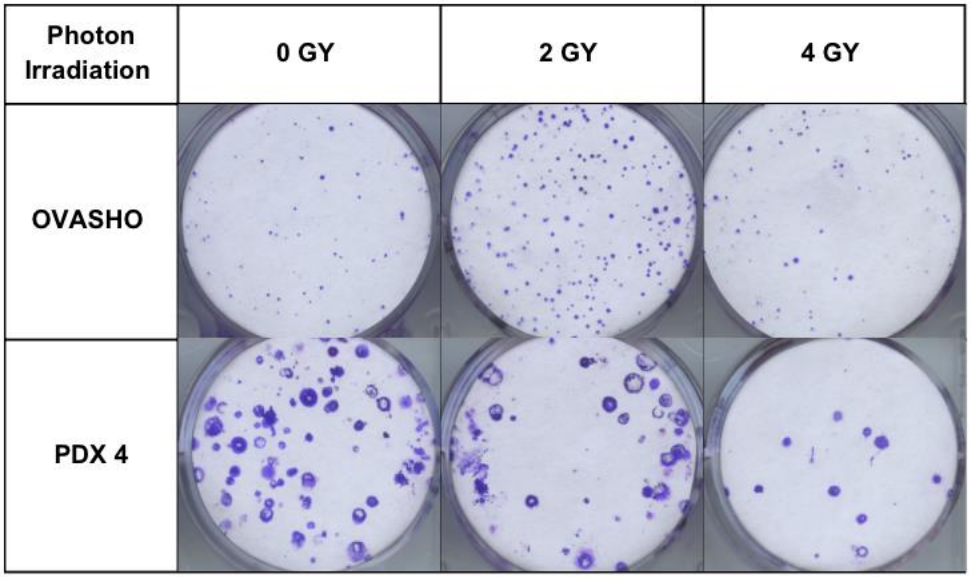
Future Directions

In the future, we plan to define levels of treatment induced stemness as well as determine if stemness can be mitigated with the use of miRNA let-7 to reduce treatment induced stemness in-vitro and in-vivo models. We aim to determine if let-7 could be a viable option in conjunction with traditional treatments to make ovarian cancer treatments more effective.

APPENDIX:

SUPPLEMENTRY DATA

SUPPLEMENTRY DATA



SUPPLEMENTRARY FIGURE 1: COLONY FORMATION AFTER PHOTON IRRADATION AT 0 GY, 2 GY AND 4 GY.

Primer Sequences for RT-qPCR

Gene	Forward Primer Sequence (5' -3')	Reverse Primer Sequence (3' -5')
ACTB (151 bp)	TGA AGT GTG ACG TGG ACA TC	GGA GGA GCA ATG ATC TTG AT
POU5F1 (163 bp)	AAG CGA TCA AGC AGC GAC TAT	GGA AAG GGA CCG AGG AGT ACA
SOX2 (140 bp)	GCG CCC TGC AGT ACA ACT C	GCT GGC CTC GGA CTT GAC

SUPPLEMENTARY TABLE 1: Primer Sequences for RT-qPCR

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