General Overview of Ovarian Cancer

Introduction

Ovarian cancer (OC) is the 9th most common cancer and the 9th most common cause of cancer-related death in women, with an overall 5-year survival of approximately 40%. In 2018, 295,414 new cases of OC were documented worldwide, 184,799 of which resulted in death (1). High-grade serous ovarian carcinoma (HGSOC) is the most common and deadliest subtype of epithelial ovarian cancer (EOC), accounting for 70-80% of OC deaths (2). Originating from premalignant lesions in the epithelium of the fallopian tubes, HGSOC is known for its important genomic instability (3). Aside from universal TP53 mutation, HGSOC has numerous aberrations that vary among different tumours (4). To this day, some murine models with specific mutations have been developed, which serve as representatives of some particular tumours. ID8 cells, derived from mouse ovarian surface epithelial cells, have been used for the creation of these new cell lines (5). Previous work conducted in the lab consisted of targeting mutations characteristic of HGSOC by CRISPR/Cas9 knockout as a means of creating more representative models. These included tumour suppressor Trp53, known to induce growth arrest or apoptosis; Brca2, another tumour suppressor that maintains the genome's stability by regulating the homologous recombination (HR) pathway for double-strand DNA (dsDNA) repair; and Nf1 gene, a negative regulator of the RAS signal transduction pathway (6) (uniprot.org). Mutation of the TP53 gene is found in the majority of HGSOC patients (7), whereas both BRCA2 and NF1 mutations are found in around 20% of patients (8)(9). Results showed that Trp53-/-;Brca2-/- double mutant mice have longer survival following treatment with both platinum and PARP inhibitor compared to Trp53-/-;Brca1-/- and Trp53-/- mutants, but that survival of mice bearing mutations in Trp53 and Nf1 genes is reduced after platinum treatment compared to Trp53-/- mice, along with higher intra-tumoural growth due to prolonged activation of the RAS/RAF/MAPK signalling pathway (6). Surprisingly, 50% of HGSOC patients harbouring BRCA2 mutation also present with a mutation in NF1 (8). Here we create a new murine ID8 Trp53-/-;Brca2-/-;Nf1-/- cell line to understand the consequences of Nf1 loss in ID8 Trp53-/-;Brca2-/- cells by CRISPR/Cas9 Nf1 knockout. We want to study the effect of the combined triple loss in cell doubling time in vitro, in the regulation of cell growth and survival pathways, and of response to platinum chemotherapy and PARP inhibition. We hypothesize that lack of Nf1 in ID8 Trp53-/-;Brca2-/- models will counteract to some extent the effect of Brca2 loss in cell growth and response to chemotherapy.

Material And Methods

Cell lines and cell culture

ID8 Trp53-/-;Brca2-/- 2.14 and ID8 Trp53-/-;Nf1-/- 1.20 clones were previously generated (6). ID8 Trp53-/-;Brca2-/-, ID8 Trp53-/-;Nf1-/-, and newly generated ID8 Trp53-/-;Brca2-/-;Nf1-/- --- clones were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4%(v/v) fetal calf serum (FCS; COMPANY), 2mM?? L-glutamine (COMPANY), 100µg/mL penicillin (COMPANY), 100µg/ml streptomycin (COMPANY), and ITS (5µg/ml insulin, 5µg/ml transferrin, and 5ng/ml sodium selenite (COMPANY)) in a 37ºC, 5% CO2 humidified incubator. Cells were detached from flasks by incubation with trypsin 2x when reaching 70-80% confluency

and split in new medium at a ratio of 1/10.

FBS with 10% DMSO was used to freeze cells. Cells were transferred to cryovials and stored at -80ºC. For later thawing, cells were washed thoroughly to ensure no freezing media was still present.

CRISPR/Cas9 TECHNIQUE/PROTOCOL/KNOCKOUT

Invitrogen TrueGuide sgRNA for Mus musculus Nf1 5'-GUUGUGCUCGGUGCUGACUU-3' (ThermoFisher Scientific, gRNA #A35510) was used for CRISPR/Cas9 knockout.

Cells were trypsinized, counted with Cellometer (COMPANY), and seeded in 24-well plates at different concentrations: 5000 cells/ml, 10000 cells/ml, and 20000 cells/ml. After overnight incubation, cell growth was analyzed with the microscope and the 20000 cells/ml plate was chosen for transfection. DMEM medium was replaced with 50µl Cas9nuclease/gRNA solution (5pmol/µl), Cas9 Plus Reagent and Opti-MEM medium mix for CRISPR/Cas9 transfection according to manufactures instructions (PROTOCOL SUP FIGURES?). For the 2 non-targeting controls (NT), Nf1 gRNA was substituted for H2O, and for the 2 negative controls (ctrl -) OptiMEM Medium alone was used for transfection. After 45 minutes, 500µl of OptiMEM Medium alone were added to each well. Following overnight incubation, OptiMEM Medium was replaced with for 500µl of DMEM, and after 48 hours, half of the cells were frozen at -80ºC and the other half were used for subsequent analyses.

Mismatch cleavage assay

GeneArt Genomic Cleavage Detection Kit (Life Technologies, Catalog #A24372) was used for PCR amplification and mismatch cleavage assay. 50µl of Cell Lysis Buffer and Protein Degrader mix was added to ID8 Trp53-/-;Brca2-/- 2.14 cells transfected with Nf1, and incubated in a thermal cycler (BIO-RAD, DNA Engine, Peltier Thermal Cycler) at 68ºC for 15 minutes and 95ºC for 10 minutes.

PCR primers were designed using Primer3 Software (http://primer3.ut.ee) to expand exon 2, where Cas9 is expected to cut (F: 5'-gggacatagctctggttcct-3'; R: 5'-acatgctctccaaacttcca-3'). 1µl of 10µM (IN MOLS!) F/R primer mix was added in PCR tubes with 2µl of cell lysate, 25µl of AmpliTaq Gold 360 Master Mix, and water to a final volume of 50µl. For the PCR control 1µl of Control Template & Primers, 25µl of AmpliTaq Gold 360 Master Mix, and 24µl of water were used. Amplified samples were run in a 2% agarose gel and imaged in GelDoc ultraviolet (UV) light transilluminator (COMPANY). After PCR validation, samples were denatured and re-annealed and 1µl of Detection Enzyme was added. After 1 hour incubation at 37ºC, samples were run on a 2% agarose gel with 10µl of water for 40 minutes at 80V. The gel was imaged in the UV transilluminator, band intensities were calculated using Fiji Software, and cleavage efficiency was calculated using the following equations: Cleavage Efficiency = 1-[(1-Fraction Cleaved)1/2], where Fraction Cleaved = sum of cleaved band intensities/(sum of the cleaved and parental band intensities). Background noise was ----------.

PROTOCOL SUP FIGURES? And less explanation here

Limiting dilution cloning assay

0.5 cells/well from Brca2 2.14 Nf1.1 Mix were seeded in 5 96-well plates and incubated at 37ºC for 7 days.

1. Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries
2. Rethinking ovarian cancer II: reducing mortality from high-grade serous ovarian cancer.
3. High-Grade Serous Ovarian Cancer: Basic Sciences, Clinical and Therapeutic Standpoints.
4. Driver mutations in TP53 are ubiquitous in high-grade serous carcinoma of the ovary
5. Development of a syngeneic mouse model for events related to ovarian cancer.
6. CRISPR/Cas9-derived models of ovarian high-grade serous carcinoma targeting Brca1, Pten and Nf1, and correlation with platinum sensitivity
7. Integrated genomic analyses of ovarian carcinoma
8. Copy-number signatures and mutational processes in ovarian carcinoma. MACINTYRE 2018
9. Neurofibromin 1 (NF1) defects are common in human ovarian serous carcinomas and co-occur with TP53 mutations