Investigating the molecular mechanisms of FOXM1 and DNA repair in drug resistance

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Background: Forkhead box M1 (FOXM1) is a transcription factor that belongs to the forkhead box family, which share a highly conserved winged helix DNA binding domain[1]. FOXM1 also consists of an N-terminal repressor domain and a transactivation domain[2]. FOXM1 has many cellular roles and is particularly important in genome stability, such as regulating the transcription of DNA repair genes. For instance, FOXM1 has been suggested to transcriptionally target RAD51, which allows strand exchange in the homologous repair pathway[2].

FOXM1 has been associated with carcinogenesis, first evidenced as a downstream target of glioma transcription factor 1 (Gli1) in human basal cell carcinomas, with resulting increased FOXM1 expression and transcriptional activity[1, 3]. Recent evidence suggests the importance of FOXM1 in chemoresistance given its role in DNA repair gene expression, including RAD51 expression by FOXM1 in glioblastomas which caused temozolomide resistance[2]. Due to the known roles of FOXM1 in chemoresistance, studies suggest that this could be the case for Trastuzumab (Herceptin, a drug which targets the extracellular domain of HER2) resistance[4]. FOXM1 has been suggested to be a downstream target of HER2, and HER2 overexpression occurs in 20-25% of all breast cancer patients, known as HER2 positive (HER2+) breast cancer[5]. Herceptin is an FDA approved treatment given in combination with chemotherapy drugs, such as paclitaxel (taxol), for HER2+ breast cancer patients, but 70% of patients become resistant to Herceptin[5]. Figure 1 indicates the possible roles of FOXM1 and DNA repair in drug resistance; however, a better understanding of these concepts remains important for improving treatment options. Based on the role of FOXM1 in regulating DNA repair proteins, we hypothesise that this regulation may be involved in FOXM1-mediated chemoresistance. The aim of this project is to investigate the role of FOXM1 and RAD51 in cell line models of HER2+, Herceptin-resistant breast cancer.

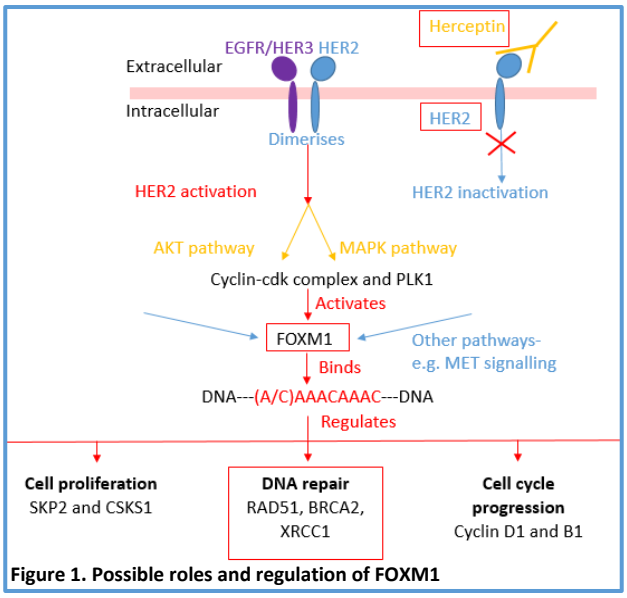

Figure 1. Possible roles and regulation of FOXM1

Methods:

Cell culture and drug treatment: HCC1954 were selected as a model of HER2+ and inherent Herceptin resistance and SKBR-3 TR (TR; Trastuzumab resistant) were selected as a model of HER2+ and acquired Herceptin resistance. HCC1954 cells were cultured in RPMI medium (37°C, 5% CO_2), and SKBR-3 TR cells were cultured in DMEM medium supplemented with 40ug/mL Herceptin, to maintain the acquired resistance (37°C, 5% CO_2). To demonstrate inherent resistance in HCC1954, 3,000 cells/well (96-well plate) were seeded on day 1 and were treated with Herceptin (100, 75, 50, 25, 12.5ug/mL and vehicle (H_2O), in triplicate) on day 2. On day 5 (after ~72hrs in Herceptin), cell viability was analysed using an ATP-based luminescence assay (CellTiterGlo, Promega).

siRNA transfection: FOXM1 expression was silenced using siRNA and the potential role of FOXM1 was investigated by assessing cell viability and/or Herceptin resistance, in HCC1954 and SKBR-3 TR cells. Then, the same was carried out against RAD51 expression. On day 1, 100,000 cells/well (6-well plate) were seeded. On day 2, cells were transfected with 50 nM siRNA, using the transfection reagent Lipofectamine RNAiMAX, according to the manufacturer's instructions. The Allstar siRNA negative control (siCon), siFOXM1 (6; a single siRNA), siRAD51 (7; a single siRNA), siRAD51 pool (a pool of 4 different siRNAs targeting RAD51) and the positive control siPLK1 were transfected. Untransfected cells were also analysed as a further negative control. On day 3, cells were trypsinised and 3000 cells/well were seeded (96-well plate). On day 4, cells were treated with Herceptin (100, 75, 50, 25, 12.5ug/mL and vehicle (H_2O), in triplicate). On day 7, cell viability was analysed (Cell Titre Glo, Promega). On day 4 (~72hrs after transfection), protein was extracted to assess protein silencing.

Western blot analysis: Protein lysates were obtained using NP-40 lysis buffer (20mM Tris [pH8.0], 200 mM NaCl, 0.5% NP-40, 10% glycerol, 1mM EDTA) with protease inhibitors cocktail (Roche). The protein was quantified using the Bradford (Abs. 630nm) assay, and 10 ug protein was loaded onto a NuPAGE Novex 4-12% Bis-Tris protein gel. The gel was electrophoresed at 120V for 90 mins in 1x MOPS buffer. The gel was transferred to a nitrocellulose membrane, which was then blocked in 5% milk in TBS-Tween (TBS-T) for ~1 hr. The blot was cut and placed in primary antibody solutions, accordingly. RAD51 and vinculin antibodies were used at 1:500 and 1:1000 dilutions, respectively (5% milk/TBS-T, 4°C overnight). Blots were washed in TBS-T (3x10 mins) and incubated in secondary antibody (1:4000 anti-mouse antibody in 5% milk/TBS-T) at room temperature for 1 hr. Blots were then washed in the same manner as before, and incubated in SuperSignal West Pico Chemiluminsecent substrate (5 mins). The blots were developed on x-ray film.

Results:

HCC1954 have innate resistance to Herceptin: The HCC1954 cells were treated with increasing concentrations of Herceptin (Figure 2A) and no reduction in surviving fraction was observed. These results demonstrate that HCC1954 cells are resistant across a range of Herceptin concentrations, up to 100ug/mL (Figure 2a).

FOXM1 influences cell viability but not resensitisation: The HCC1954 and SKBR-TR cells were transfected with siControl and siFOXM1 and treated with increasing concentrations of Herceptin. Our results suggest that there is a reduction in cell viability, due to FOXM1 silencing, which is not significantly increased upon Herceptin treatment (Figure 2b-d). In 100 ug/mL Herceptin, there is a significant reduction in cell viability (~25%) upon FOXM1 silencing, relative to siControl in HCC1954 cells (Figure 2b). For SKBR-3 TR in 100 ug/mL Herceptin, there is a significant reduction in cell viability (~30%) upon FOXM1 silencing, relative to siControl

(Figure 2c-d). However, there is no significant reduction in cell viability with those not treated, to those treated with 100ug/mL Herceptin in FOXM1 silenced cell lines, suggesting no significant resensitisation (relative to siCon H_2O) (Figure 2b-d). The reduction in cell viability can be explained by the many biological roles which FOXM1 regulates but our results suggest that FOXM1 does not influence resensitisation at the 100 ug/mL dose. Across the range of concentrations tested, siCon and siFOXM1 (6) in HCC1954 demonstrates a slight decrease in surviving fraction at 75 and 100ug/mL Herceptin, compared to lower doses (Figure 2e). This suggests that analysis at a higher concentration range could be done to see if significant resensitisation occurs.

RAD51 may influence cell viability but not resensitisation: Similar to the FOXM1 analysis above, the HCC1954 and SKBR-TR cells were transfected with siControl, siRAD51(7) and siRAD51(pool) and treated with increasing concentrations of Herceptin. Our results suggest that similar to FOXM1, silencing of RAD51 alone reduce the surviving fraction of both the HCC1954 and SKBR-3 TR cell lines, regardless of Herceptin treatment. The siRAD51 pool reduces the surviving fraction greater than siRAD51 (7), and this could be related to the fact that the pool has four siRAD51 oligonucleotides, as opposed to siRAD51 (7)'s single oligonucleotide (Figure 2f-h). The results suggest that RAD51 contributes to cell viability, which could be explained by its main role in double strand repair to prevent senescence or apoptosis. Like FOXM1, RAD51 does not seem to influence resensitisation at 100ug/mL Herceptin due to no significant surviving fraction reduction with those not treated with the drug to those that have been. Across the range of doses tested, siCon and siRAD51 (7) in HCC1954 shows a slight decrease in surviving fraction at the two highest concentrations compared to the lower concentrations, suggesting also that optimisation of the dose range is required before further conclusions are made (Figure 2i).

RAD51 silencing was achieved: Figure 2j shows a decrease in RAD51 expression in HCC1954 and SKBR-3 TR cells transfected with siRAD51 (7) and siRAD51 pool, where the pool oligonucleotide performed better. SKBR3-TR (TR+) appears to have better silencing than HCC1954, but slightly less loading in the siRAD51 pool transfected HCC1954 makes it hard to fully conclude. Further transfection optimisations may be required but it shows silencing was achieved in relation to the cell viability results.

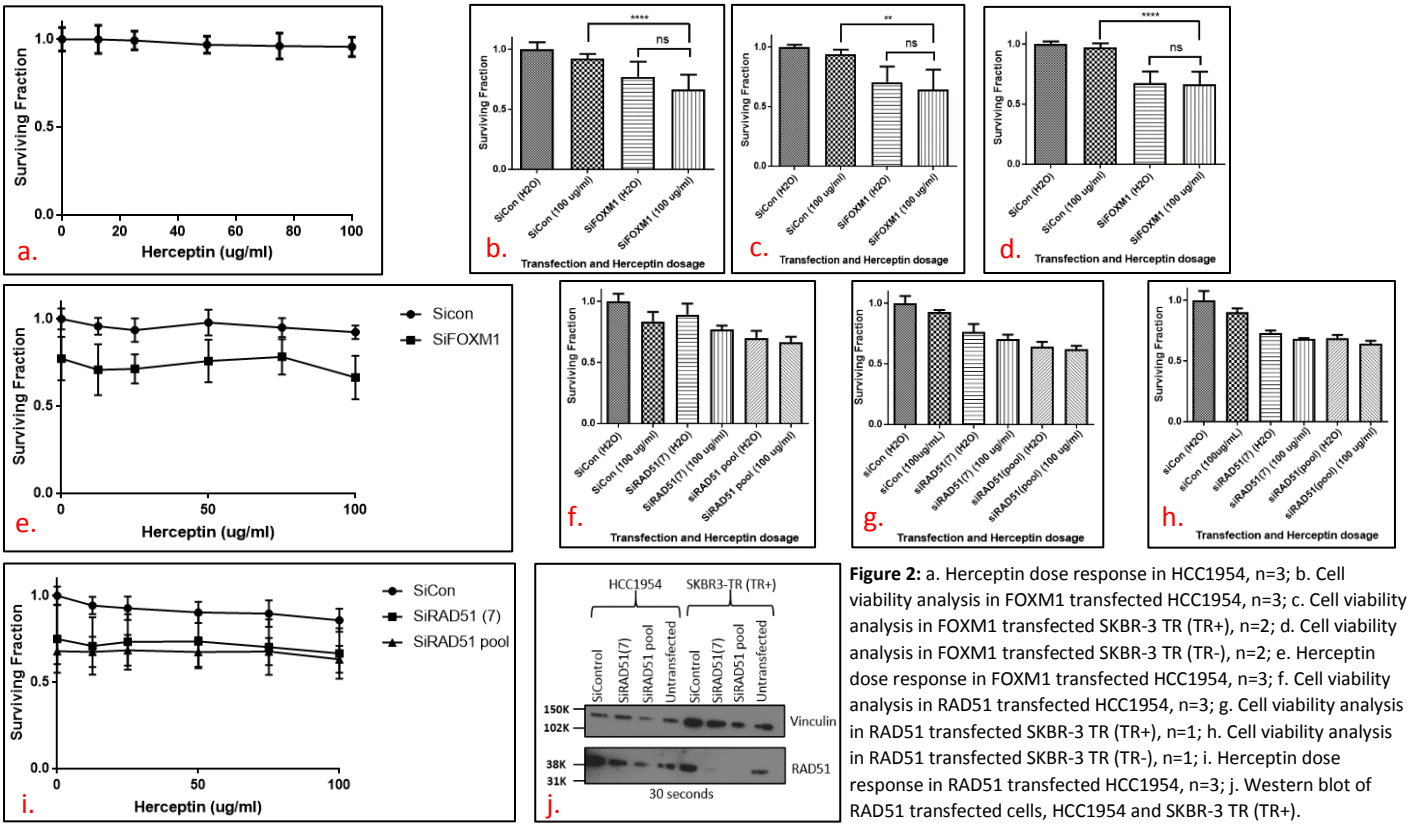

Figure 2: a. Herceptin dose response in HCC1954, n=3; b. Cell viability analysis in FOXM1 transfected HCC1954, n=3; c. Cell viability analysis in FOXM1 transfected SKBR-3 TR (TR+), n=2; d. Cell viability analysis in FOXM1 transfected SKBR-3 TR (TR-), n=2; e. Herceptin dose response in FOXM1 transfected HCC1954, n=3; f. Cell viability analysis in RAD51 transfected HCC1954, n=3; g. Cell viability analysis in RAD51 transfected SKBR-3 TR (TR+), n=1; h. Cell viability analysis in RAD51 transfected SKBR-3 TR (TR-), n=1; i. Herceptin dose response in RAD51 transfected HCC1954, n=3; j. Western blot of RAD51 transfected cells, HCC1954 and SKBR-3 TR (TR+).

Future directions: Following on from my placement, optimisation of the Herceptin treatment range in the cell lines will be done and a FOXM1 antibody will be optimised for the blots. Further experiments investigating the requirement for FOXM1 and RAD51 in a range of different cell lines, with varying expression levels of the proteins will be carried out.

Value of studentship: The studentship has provided me an invaluable experience in the laboratory. It has confirmed to me about my goal of pursuing further education and going into research. As I worked with Rebecca, I helped to produce data and validated her results. I would like to thank the Martin lab, particularly Rebecca for teaching me in the lab and Sarah for offering me this opportunity. Thanks to the Biochemical society also for funding my placement.

Any departures from the original proposal: The project was carried out as originally proposed.

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