The Role of Pseudokinase *TRIB1* in Breast Cancer

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Background

The Tribbles Homologue (*TRIB)* pseudokinase family has a wide range of functions in human health and disease and has been implicated in various cancers[1]. *TRIB1* is located on the 8q24 amplicon with oncogene *MYC*. Experimental screening identified *TRIB1* as a potential synthetic lethal interaction partner of *MYC*[2]. Exploring this possible relationship and the role of *TRIB1* in solid tumours requires the use of cell line models. *TRIB1* is also implicated in lipid metabolism, differentiation and cellular stress response suggesting that the role of *TRIB1* spans many pathways[1,3].

Overall Aims

In this project we aim to:

- Identify how changes in *TRIB1* can lead to the dysregulation of cell signaling in breast cancers.
- Evaluate the effect of *TRIB1* knockdown on breast cancer cell line growth
- Identify TRB1 direct interaction partners.
- Investigate the genetic and potential synthetic lethal link between *TRIB1* and *MYC* using *in-silico* and *in-vitro* approaches.

The Model

The investigation of the effects of *TRIB1* knockdown on breast cancer cell growth was studied in three breast cancer cell lines of differing molecular subtypes and levels of *TRIB1* expression. Knockdown was achieved for up to seven days in these cells using siRNA transfection (Figure 1). The affect of knockdown was monitored in real time using confluency tracking with the IncuCyte imaging system and validated using nuclear counting by DAPI staining.

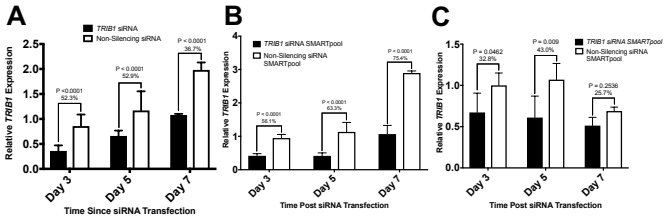

Figure 1: *TRIB1* expression in MCF7, MDA-MB-231 and HCC1419 over seven days after transfection with Dharmacon ON-TARGETplus siRNA SMARTpools. A MCF7 cells were seeded at 2000 cells per well 24hrs prior to RNAiMax mediated transfection with either Dharmacon *TRIB1* or non-silencing ON-TARGETplus siRNA SMARTpools. **B** MDA-MB-231 cells were seeded at 3000 cells per well 24hrs prior to RNAiMax mediated transfection with either Dharmacon *TRIB1* or non-silencing ON-TARGETplus siRNA SMARTpools. **C** HCC1419 cells were seeded at 15000 cells per well 24hrs prior to RNAiMax mediated transfection with either Dharmacon *TRIB1* or non-silencing ON-TARGETplus siRNA SMARTpools. In all cases RNA was extracted from cells using RNAGEM Tissueplus at three, five and seven days post transfection. *TRIB1* expression determined by RT-qPCR relative to the geomean of *FKBP15* and *PUM1* and expression normalised to 72hr non-silencing siRNA treated expression. Error is standard deviation of one to three biological replicates each with three technical replicates. Significance calculated by 2-way ANOVA with FDR correction.

Results

TRIB1 knockdown in MCF7 cells cause a decrease in cell growth rate of approximately 50% when tracked with the IncuCyte live cell imaging system (Figure 2A, B). Despite not observing any significant changes in cell morphology with *TRIB1* knockdown, the change in growth rate was confirmed with end point analysis of cell number through nuclei counting three days after *TRIB1* knockdown which confirmed a 50% decrease in cell number after three days of growth (Figure 2C). Similar results were seen in MDA-MB-231s and HCC1419s with *TRIB1* knockdown being associated with a decrease in cell growth rate and a change in cell number (Figure 3, 4).

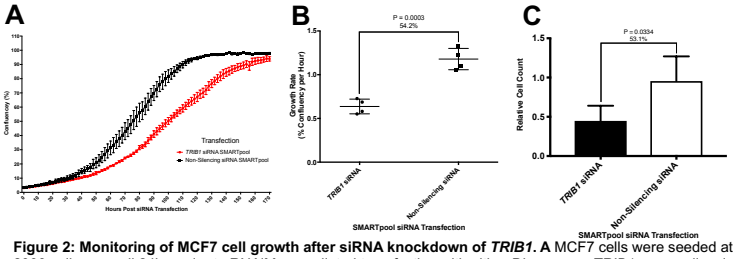

Figure 2: Monitoring of MCF7 cell growth after siRNA knockdown of *TRIB1*. A MCF7 cells were seeded at 2000 cells per well 24hrs prior to RNAiMax mediated transfection with either Dharmacon *TRIB1* or non-silencing ON-TARGETplus siRNA SMARTpools. Cell growth was tracked immediately post-transfection for seven days using the IncuCyte live cell imaging system. Error is standard deviation of three technical replicates. **B** Change in rate of growth calculated from the exponential growth phase of four independent knockdown experiments tracked with the IncuCyte live cell imaging system. Error is standard deviation of biological replicates. Significance calculated by unpaired T-test. **C** Nuclear counts 72hrs post-transfection, cells seeded at 1000cells per well. Cells were fixed with 4% paraformaldehyde and the nuclei stained with 1mg/ml Hoechst stain to allow nuclei counting with the Cytation 5 imaging system. All nuclei counts were normalised to untansfected cell counts for each biological replicate. Error bars are standard deviation of four biological replicates each containing three technical replicates. Significance calculated by unparied T-test.

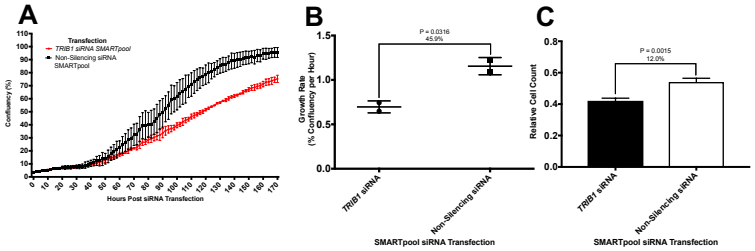

Figure 3: Monitoring of MDA-MB231 cell growth after siRNA knockdown of *TRIB1*. A MDA-MB-231 cells were seeded at 3000 cells per well 24hrs prior to RNAiMax mediated transfection with either Dharmacon *TRIB1* or non-silencing ON-TARGETplus siRNA SMARTpools. Cell growth was tracked immediately post-transfection for seven days using the IncuCyte live cell imaging system. Error is standard deviation of three technical replicates. **B** Change in rate of growth calculated from the exponential growth phase of two independent knockdown experiments tracked with the IncuCyte live cell imaging system. Error is standard deviation of biological replicates. Significance calculated by unpaired T-test. **C** Nuclear counts 72hrs post-transfection, cells seeded at 2000cells per well. Cells were fixed with 4% paraformaldehyde and the nuclei stained with 1mg/ml Hoechst stain to allow nuclei counting with the Cytation 5 imaging system. All nuclei counts were normalised to untansfected cell counts for each biological replicate. Error bars are standard deviation of one biological replicates each containing three technical replicates. Significance calculated by unparied T-test.

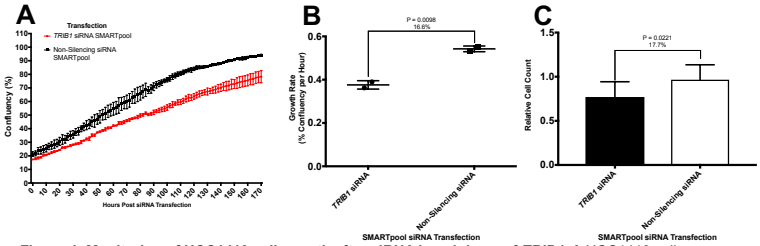

Figure 4: Monitoring of HCC1419 cell growth after siRNA knockdown of *TRIB1*. A HCC1419 cells were seeded at 15000 cells per well 24hrs prior to RNAiMax mediated transfection with either Dharmacon *TRIB1* or non-silencing ON-TARGETplus siRNA SMARTpools. Cell growth was tracked immediately post-transfection for seven days using the IncuCyte live cell imaging system. Error is standard deviation of three technical replicates. **B** Change in rate of growth calculated from the exponential growth phase of two independent knockdown experiments tracked with the IncuCyte live cell imaging system. Error is standard deviation of biological replicates. Significance calculated by unpaired T-test. **C** Nuclear counts 72hrs post-transfection, cells seeded at 15000cells per well. Cells were fixed with 4% paraformaldehyde and the nuclei stained with 1mg/ml Hoechst stain to allow nuclei counting with the Cytation 5 imaging system. All nuclei counts were normalised to untansfected cell counts for each biological replicate. Error bars are standard deviation of three biological replicates each containing three technical replicates. Significance calculated by unparied T-test.

Conclusions

- siRNA knockdown of *TRIB1* in breast cancer cell lines slows cell growth irrespective initial *TRIB1* expression and across subtype
- *TRIB1* association with *MYC* may be important in *TRIB1* regulation of cell proliferation
- Identification of TRB1 interaction partners is need to determine how *TRIB1* is able to regulate proliferation
- RNAseq analysis of knockdown model could provide insight into global changes caused by *TRIB1* knockdown

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Acknowledgements

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Combination of aromatase inhibitor and anti-inflammatory treatments in a model of oestrogen receptor positive breast cancer

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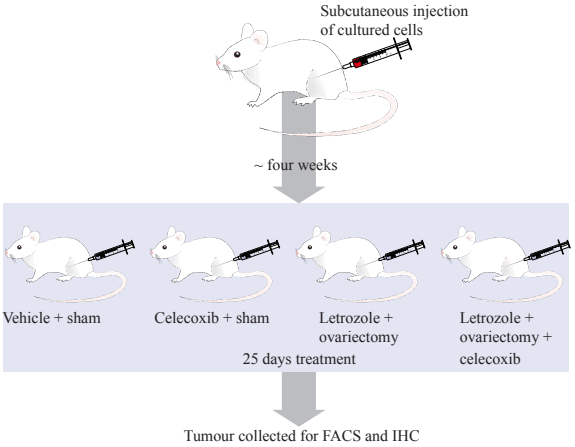
Background

- Breast cancer is the most common malignancy in women, with 1 in 8 NZ women developing breast cancer in their lifetime.
- The majority of breast carcinomas express oestrogen receptor-α (ER) and the most effective form of anti-oestrogen therapy for post-menopausal patients is the use of aromatase inhibitors (AIs).
- Poor response to AI treatment is associated with lymphocytic infiltration and this may be a potential mechanism of resistance to therapy.
- Immune cells could contribute to a tumour microenvironment favouring growth, irrespective of oestrogen deprivation.
- ER+ve breast cancer is not traditionally thought of as an immunogenic cancer, and the majority of mouse models are in immune deficient animals.
- We have demonstrated an ER+ model with a fully intact immune system which responds to AI treatment and shows alterations in tumour infiltrating lymphocytes (TILs) as a result of a combination of AI (letrozole) and anti-inflammatory (celecoxib) treatment.

Tumour model

Adult female mice were injected with $5x10^5$ cells of a mouse oestrogen receptor positive cell line (SSM3 cells, kindly donated by Schreiber Lab) into the mammary gland. When the tumour reached $49mm^2$, mice received either an ovariectomy or sham operation, and the tumour was biopsied with a 18G needle. Treatment of letrozole (1mg/kg), celecoxib (25mg/kg) or the combination, commenced on the same day. Mice were weighed and the tumour measured daily. After 25 days, the mice were sacrificed, the majority of the tumour processed for FACS analysis and part of the tumour kept for IHC. Experiments were approved by the Animal Ethics Committee of the University of Otago



IHC

Tumour biopsies were preserved in 10% NBF and stained with antibodies to ER, CD3, CD11c, CD19, Ki67. The percentage of TILs was assessed in accordance with guidelines from Salgado *et al* (2014). Briefly, stromal areas were identified and the percentage of TILs estimated to the nearest 10%. For CD3 evaluation, areas of stroma were assessed and the number of CD3+ cells in as many 40× fields of view as possible were counted and the average number calculated for each tumour.

FACS

To examine the tumour immune infiltrate, tumours were crushed between gauze in media and cells passed through a 70µM filter. Cells were washed in media and frozen until staining. Cells were stained with the following mouse anti-human antibodies; CD3, CD4, CD8, CD11b, CD11c, CD14, CD19, CD45, and Texas Red live/dead fixable cell stain at optimised concentrations. Cells were then washed with FACS buffer (PBS +1% FCS + 0.1% sodium azide, pH 7.5) and fixed with 1% PFA. Samples were run on a BD Fortessa FACS machine and analysed with Flo Jo (version 9.5.2).

Results

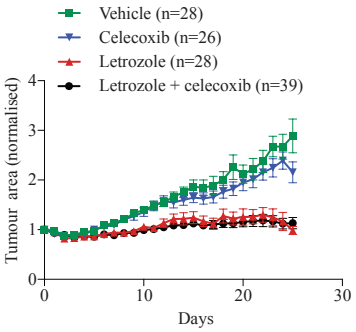


Figure 1. Orthotopic ER+ breast cancer model in mice responds to aromatase inhibitor letrozole.
Mice received daily treatment of a combination of letrozole and celecoxib, or the individual drugs for 25 days. Letrozole significantly reduced the tumour growth compared to controls, both alone or in combination with the anti-inflammatory drug celecoxib ($p < 0.0001$ for both).

Figure 2. Combination treatment alters the amount of CD11+ cells measured by FACS in whole tumours.
The combination of letrozole and celecoxib significantly reduces the percentage of CD11+ cells compared to vehicle only control (Student t-test, $p = 0.015$, line shows median).

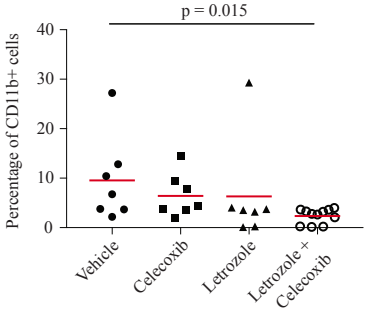
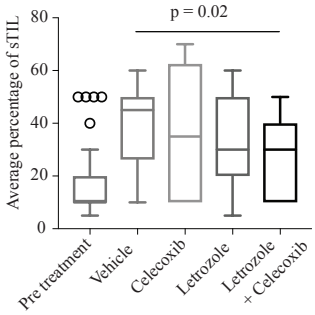


Figure 3. Combination treatment alters the immune infiltrate as determined by immunohistochemistry.



A. Letrozole + celecoxib reduces the average percentage of stromal TILs in mammary tumours.
All tumours show a significant increase in sTILs compared to the pretreatment biopsy (Student t-test, $p < 0.0001$). Tumours treated with combination therapy of letrozole + celecoxib had significantly less sTILs than the vehicle only control ($p = 0.02$). Line shows median.

Conclusions

- We have established that the ER+ SSM3 mouse model, with a fully intact immune system, responds to the aromatase inhibitor, Letrozole.
- The combination of letrozole and celecoxib is able to reduce the infiltration of CD11b+ cells within whole tumours and decrease the percentage of sTILs.
- The recruitment of immune cells may provide a mechanism by which breast cancer cells can develop resistance to oestrogen deprivation treatment.

References

Salgado *et al.*, *Annals of Oncology* 2015, **26** (2) 259 - 271

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centre for translational cancer research

The establishment and characterisation of gastric organoids as a model for hereditary diffuse gastric cancer.

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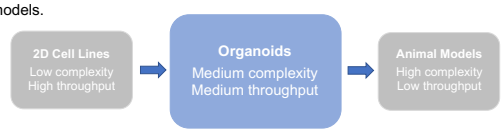
Background

Hereditary diffuse gastric cancer (HDGC) is a familial form of gastric cancer caused by germline mutations in the cell adhesion gene *CDH1* (E-cadherin)[1–3]. Those born with a heterozygous loss of *CDH1* have a 70% chance of developing diffuse-type gastric cancer in their lifetime, and females have an additional 40% chance of lobular breast cancer [4,5].

Currently, the primary treatment for HDGC is total prophylactic gastrectomy. Our goal is to develop a chemopreventative treatment to stop or delay the onset of the HDGC phenotype. *CDH1* is a tumour suppressor gene, consequently loss-of-function mutations result in cancer-causing abnormal cell activity[3], meaning that there is no protein to target with conventional drug treatments. To overcome this, we are taking a synthetic lethal approach. Synthetic lethality is a strategy for targeting tumour suppressor gene mutations in which cellular vulnerabilities are exploited to render the cell inviable.

Organoids are 3D structures comprised of both stem cells and differentiated cells that recapitulate the organisation and function of selected organs. Using gastric organoids as a model for HDGC will allow for a closer study of disease mechanism *in vitro*, as well as provide a platform for drug screening. Organoids are more complex than cell lines, but have higher throughput than animal models in drug testing. As a result, they fill an important niche in between cell lines and animal models.

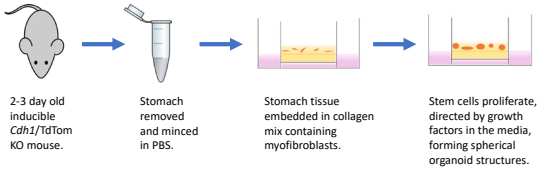


Aims

- To establish and characterise murine-derived gastric organoids as a model for HDGC cancer progression.
- To screen drugs that have been identified as synthetic lethal targets for *CDH1* in 2D cell lines to validate their effect in a more complex system.

Methods

Organoids are cultured using an air-liquid interface method to promote the development of epithelial/mesenchymal structures.



- Organoids are generated using stem cells from inducible knockout mice with a cre-lox system controlling both *Cdh1* and the fluorescent marker protein TdTomato under the *CD44* promotor (*CD44*-cre/*Cdh1*$^{-/-}$/TdTomato). Knockout of *Cdh1*, and activation of TdTomato, is induced with the addition of endoxifen to the media (metabolite of tamoxifen).
- The entire stomach is removed and washed in ice-cold PBS containing gentamycin. Stomach tissue is then minced into <0.5mm^3 pieces.
- Minced tissue is resuspended in ice-cold collagen mix containing myofibroblast cells (5x10^5 MFB cells per 1.2ml collagen layer). Myofibroblast co-culture aids in maintaining the stem cell niche within the organoids.
- Two 1.2ml layers of collagen (one containing myofibroblasts only, one containing stomach tissue and myofibroblasts) are set in a permeable scaffold insert.
- The insert is then placed in a dish containing 3ml of growth media (F12 + 20% FBS). Additionally, endoxifen and/or any drugs being screened are administered to organoids through the media.

Results

Characterisation

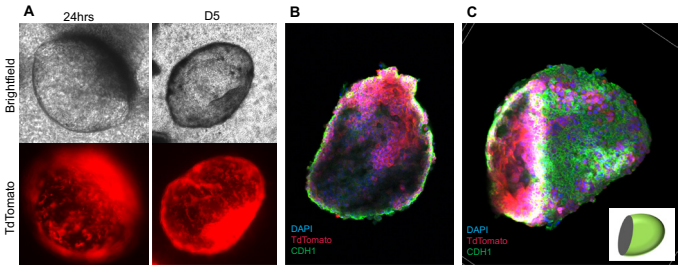


Figure 1. A) Induction of *Cdh1* KO and TdTomato expression using endoxifen. Organoids are induced with the addition of endoxifen to the media (5uM) at 0hr post seeding. Endoxifen is removed with a media change at 48hrs post seeding. TdTomato expression is visible <24hrs post seeding and continues to increase. Fluorescence plateaus approximately Day 6. **B) Confocal image showing a cross-section through the centre of an organoid (Day 5).** Immunofluorescence staining using a *Cdh1* antibody (Alexa Fluor 488) shows the outer epithelial layer of the organoid is enriched with *Cdh1+* cells. *Cdh1-* cells expressing TdTomato are infiltrating the organoid lumen. **C) 3D reconstruction of an organoid created using confocal z-stack images.** Outer epithelial layer shows patchy TdTomato expression. Cross-section exposing organoid lumen shows *Cdh1-* cells expressing TdTomato lining the inner side of the epithelium.

Drug Screening

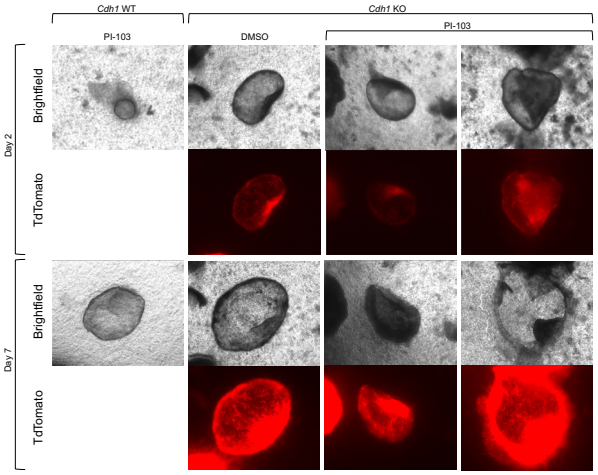


Figure 2. PI-103 induces death in knockout organoids. PI-103, a PI3K inhibitor, causes organoid death when administered at 10uM 48hrs post seeding. Death can be identified through the darkening, flattening and disintegration of the organoids. TdTomato expression becomes disorganised, but does not reduce in intensity. DMSO control displays a small amount of damage due to the toxicity of DMSO, enhanced by the compromised epithelium of the *Cdh1* knockout. The morphology of non-induced organoids (*Cdh1*-WT) is unaffected by PI-103.

Discussion and future directions

- Gastric organoids can be successfully cultured into cystic, epithelial structures using the ALI system. *Cdh1* knockout and TdTomato expression can be induced with the addition of endoxifen to the growth media and validated using immunofluorescence with an E-cadherin antibody.
- *Cdh1*-ve cells accumulate in the lumen of the organoid, possibly due to their impaired cell-cell adhesion ability relative to the *Cdh1*+ve cells.
- PI-103, a PI3K inhibitor, induces death in the organoids, characterised by inhibition of growth, flattened and dark appearance, disintegration, and disorganisation.
- Once a method of quantifying death has been established, organoids will be used for medium-throughput drug screening.

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