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14. ABSTRACT

Solid cancers are caricatures of normal tissues, and as such represent complex cellular 'ecosystems' in which the pathogenesis and drug response of the tumour is a product of the cellular milieu. However, the tumour microenvironment (TME) of solid cancers is poorly defined, limiting the development of the next generation of diagnostics and therapies.

Triple negative breast cancer (TNBC) is an aggressive disease associated with poor prognosis. Several lines of evidence suggest that anti-tumour immunity can contribute to breast cancer control if appropriately activated. For instance, tumour-infiltrating lymphocyte (TIL) number associates with prognosis, and activating TILs through cell-antibody-mediated therapy can improve outcomes for breast cancer. However, the current regimen of checkpoint immunotherapy is yet to make a substantial impact on the management of breast cancer, suggesting mechanisms of immune evasion other than via the PD1/CTLA4 axes.

This proposal combines the analysis of human clinical specimens with cutting edge spatial transcriptomics, *ex vivo* functional assays, single cell RNA-Sequencing and syngeneic mouse models of disease.

Specific Aims

1) Map the location & interactions of stromal & immune cells in TNBC
2) Define factors essential to the differentiation and survival of stromal cell states
3) Identify the cellular and mechanistic basis of stromal-immune signalling

Achievements

In the first 12 months of this project we have moved quickly and are ahead of schedule in several areas. We have achieved the following:

- Recruited staff as described in the budget.
- Performed spatial transcriptomics on 32 breast cancers (see Major Task 1- Subtask 1). Analysis of this data (Subtask 2) is now underway
- Prepared RNA from 32 TNBC cases, in anticipation of Major Task 2
- - Piloted scATAC-Seq methods (Major Task 3- Subtask 1)
- Made substantial progress towards analysis of scRNA-Seq from cultured stromal cells (Major task 3- Subtask 2)
- Commenced functional screens in vitro (Major Task 4- Subtask 2)

Results and significance

As planned in the proposal the first 12 months saw us recruit staff and students, prepare methods and materials, and to start generating data. This is progressing well. Analysis is currently underway but we do not yet have results with biological significance.

15. SUBJECT TERMS

Breast cancer, TNBC, stromal, immune, tumor microenvironment, triple negative, treatment

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TABLE OF CONTENTS

	Page
1. Introduction	1
2. Keywords	1
3. Accomplishments	1 - 5
4. Impact	5 - 6
5. Changes/Problems	6
6. Products	6 - 7
7. Participants & Other Collaborating Organizations	7 - 8
8. Special Reporting Requirements	8
9. Appendices	8

1. Introduction

Triple negative breast cancer (TNBC) is an aggressive disease associated with poor prognosis. Several lines of evidence suggest that anti-tumour immunity can contribute to breast cancer control if appropriately activated. In this proposal we will identify and deeply characterise novel stromal cell subsets that suppress anti-tumour immunity. We will then identify novel therapeutic strategies to relieve immune evasion. This proposal combines the analysis of human clinical specimens with cutting edge spatial transcriptomics, *ex vivo* functional assays, single cell RNA-Sequencing and syngeneic mouse models of disease.

2. Keywords

Breast cancer, TNBC, stromal, immune, tumor microenvironment, triple negative, treatment

3. Accomplishments

- **What were the major goals of the project?**

We have conducted work against the following Major goals:

1) ***Major Task 1*** *Spatial Analysis of TNBC: perform Spatial Transcriptomics on 32 human TNBC.*
    a. Subtask 1: perform Spatial Transcriptomics on 32 human TNBC. Months 4-9. *100% complete*
    b. Subtask 2: Analysis. Months 10-15. *20% Complete*
2) **Major Task 2:** Bulk RNA-seq of TNBC cases analysed in Major Task 1
    a. Subtask 1 : Conduct Bulk RNA-Sequencing on the 32 TNBC cases. Months 1-3. *20% Complete*
3) **Major Task 3:** Analyse scRNA-Seq and scATAC-Seq to identify factors unique to CAF and PVL
    a. Subtask 1: Generate scATAC-Seq data. Months 10-12. *20% Complete*
    b. Subtask 2 : scRNA-Seq analysis. Months 13-18. *30% Complete*
4) **Major Task 4:** Determine the functional requirement for candidate factors in PVL and CAF cells
    a. Subtask 2: Conduct small molecule and receptor ligand screen in 3 independent imPVL and iCAF cultures. Months 13-18. 50% complete

- **What was accomplished under these goals?**

Major task 1: *Spatial Analysis of TNBC*

The technology used to perform spatial transcriptomics (ST) continues to evolve. Since submitting this proposal, 10X Genomics have released a new Visium solution tailored to FFPE samples. The ability to analyse FFPE specimens expands the sample collections that we can work with and secondly enables analysis of high resolution morphology associated with the ST. We have evaluated the performance of the FFPE solution and find that it generates high quality data from TNBC tissues and is well suited to this project. We have now completed data generation for all cases (Fig 1) and data analysis is currently underway.

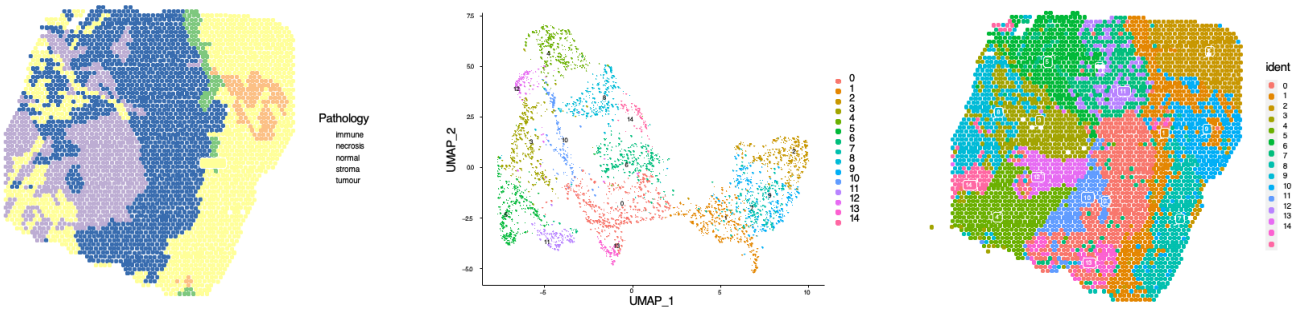

Fig 1: Example of spatial transcriptomics analysis of a TNBC case. (a) Pathological annotation. (b) Spots clustered based on gene expression. (c) Clusters projected onto tissue space. Note the association of gene expression clusters with pathological annotation

Major Task 2: Bulk RNA-seq of TNBC cases analysed in Major Task 1

RNA has been purified and quantitated from all cases, and is ready for RNA-Seq analysis. In addition, we have updated our RNA-Seq data analysis pipeline in anticipation of data arriving. This task has been delayed, mostly as we prioritised other work. We anticipate that data will be generated by May 2023.

Major Task 3: Analyse scRNA-Seq and scATAC-Seq to identify factors unique to CAF and PVL

We have generated scRNA data from 12 patient samples. The raw data have been pre-processed and filtered. We show that these cells express established CAF (eg ACTA2) and PVL (eg THY1) genes (Fig 2). Further analysis is currently underway to identify factors unique to CAF and PVL.

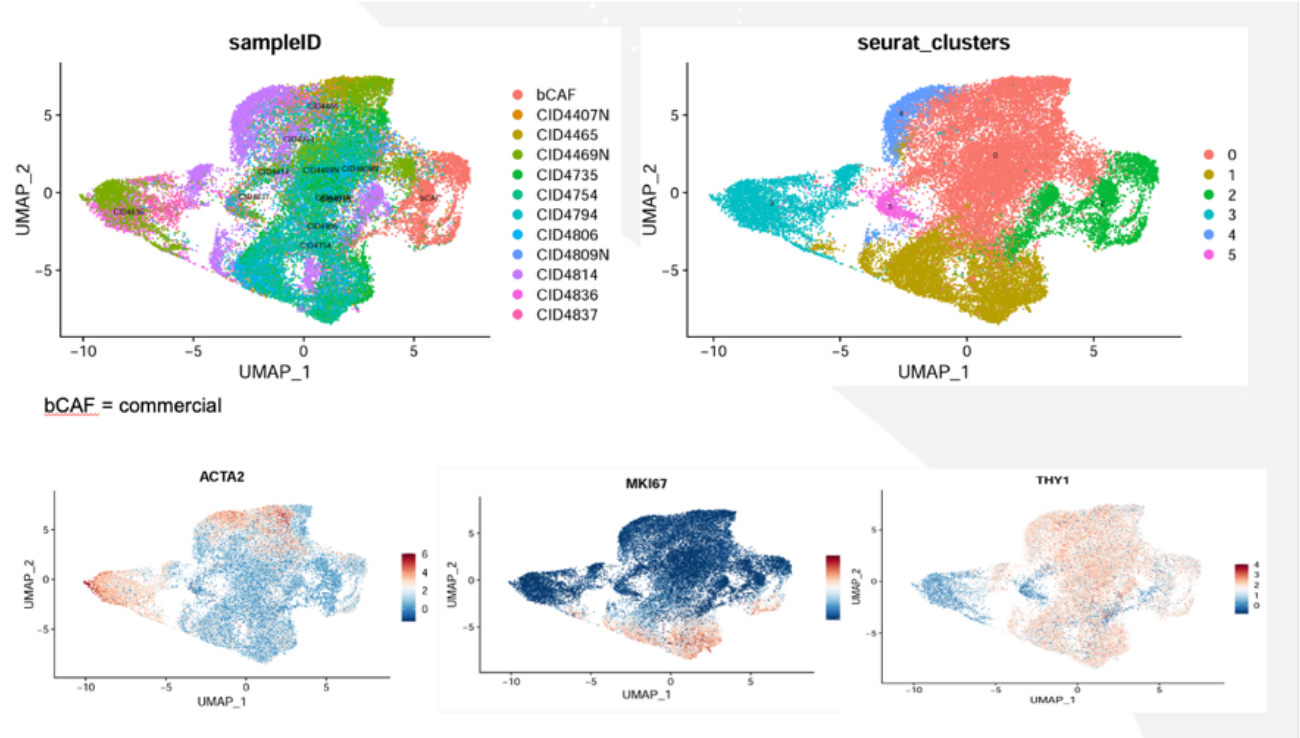

Fig 2: single cell RNA-Seq clustering of data derived from 12 patient samples. Top left panel shows clusters annotated by sample. Top right shows gene expression clusters. At bottom are feature plots showing example expression of classical CAF and PVL genes

Major Task 4: Determine the functional requirement for candidate factors in PVL and CAF cells

This task has progressed well. We have performed a preliminary screen of ~350 FDA approved compounds in 2 TNBC patient derived stromal cell cultures (Fig. 4&5). The immunofluorescent markers chosen for the screen were based on established literature and our in-house scRNA dataset, which included PDGFRA (marking CAF cultures), aSMA (marking myCAF and dPVL cultures) and RGS5 (marking imPVL cultures) (Fig 3). We also included Hoechst (marking cell nuclei) and Phalloidin in our immunofluorescence panel to examine the overall cellular morphology upon compound treatment. The effect of compound treatment was assessed at 2 timepoints (72hrs and 144 hrs) and in 3 dose concentrations (5, 1 and 0.1μM) to additionally enable the assessment of time and dose effect on stromal cell biology.

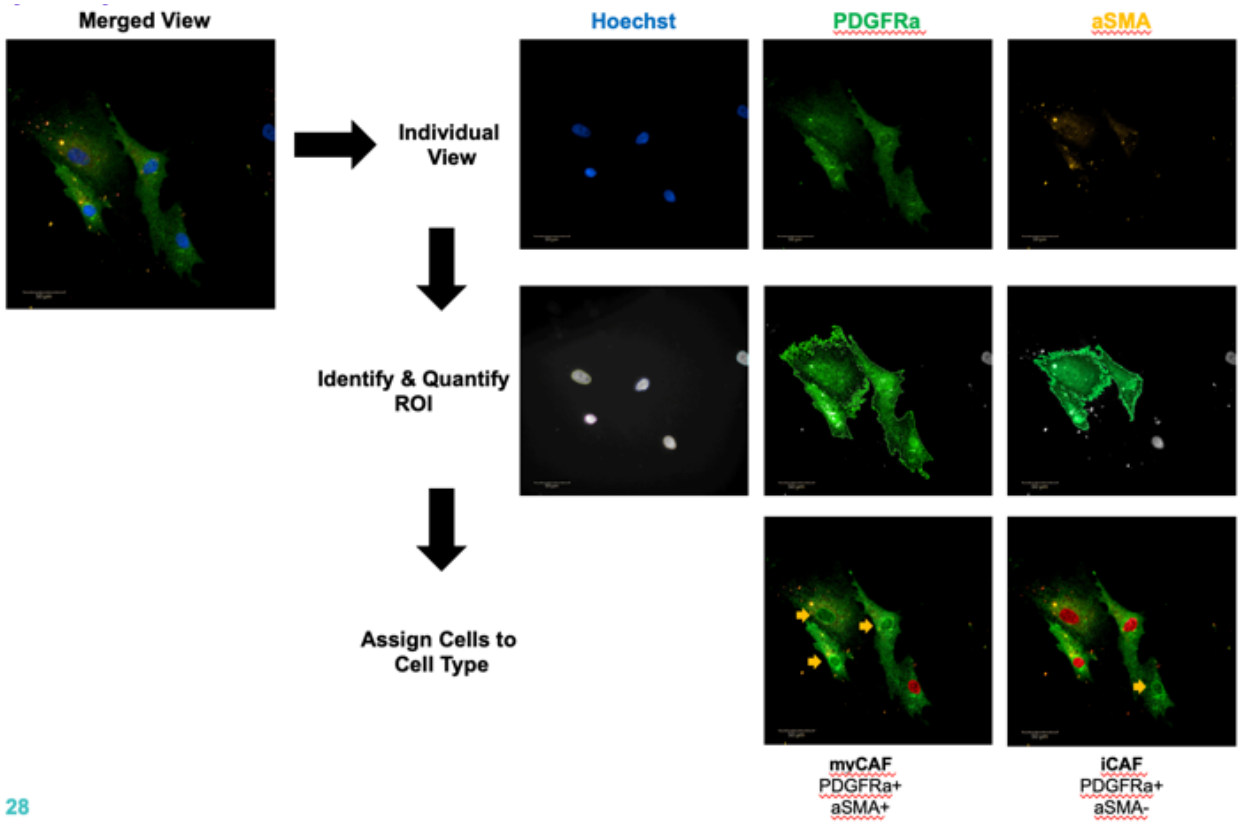

Fig. 3, Example image analysis pipeline using the Opera Phenix™ Plus High-Content Screening System. First, input image is split into individual view (top row). Second, a threshold filter is applied to remove background signal and positive regions are highlighted in white (Hoechst) or green (PDGFRA and aSMA) (middle row). Last, cells were assigned an identity based on marker expression as indicated by the yellow arrow (bottom row).

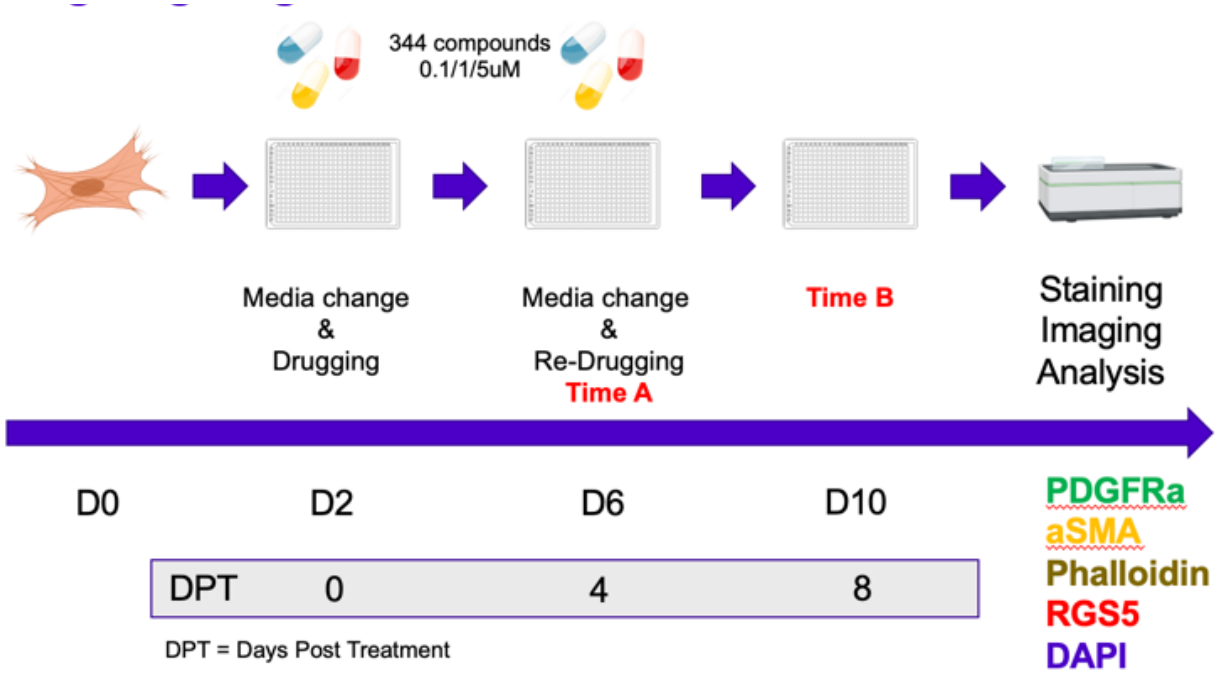

Fig. 4, Overview of the high throughput drug screen experimental design

Compound treated stromal cells were imaged and we developed an image analysis pipeline to accurately segment and identify different stromal subtypes based on their expression of the markers described above. The cells were assigned a stromal subtype identity based on their marker gene expression (Fig. 2). Cells will be further analysed based on a number of morphological features (E.g., marker intensity, size etc.) to determine the effect of compound treatment (in progress).

We observed a dose dependent response in both stromal cell cultures at 72 hours post treatment (Fig. 5). An increase in compound concentration resulted in reduced cell proliferation compared to untreated control (0.1% DMSO, green column), as measured by relative confluence We are currently analysing the data to identify candidate compounds for in-depth validation.

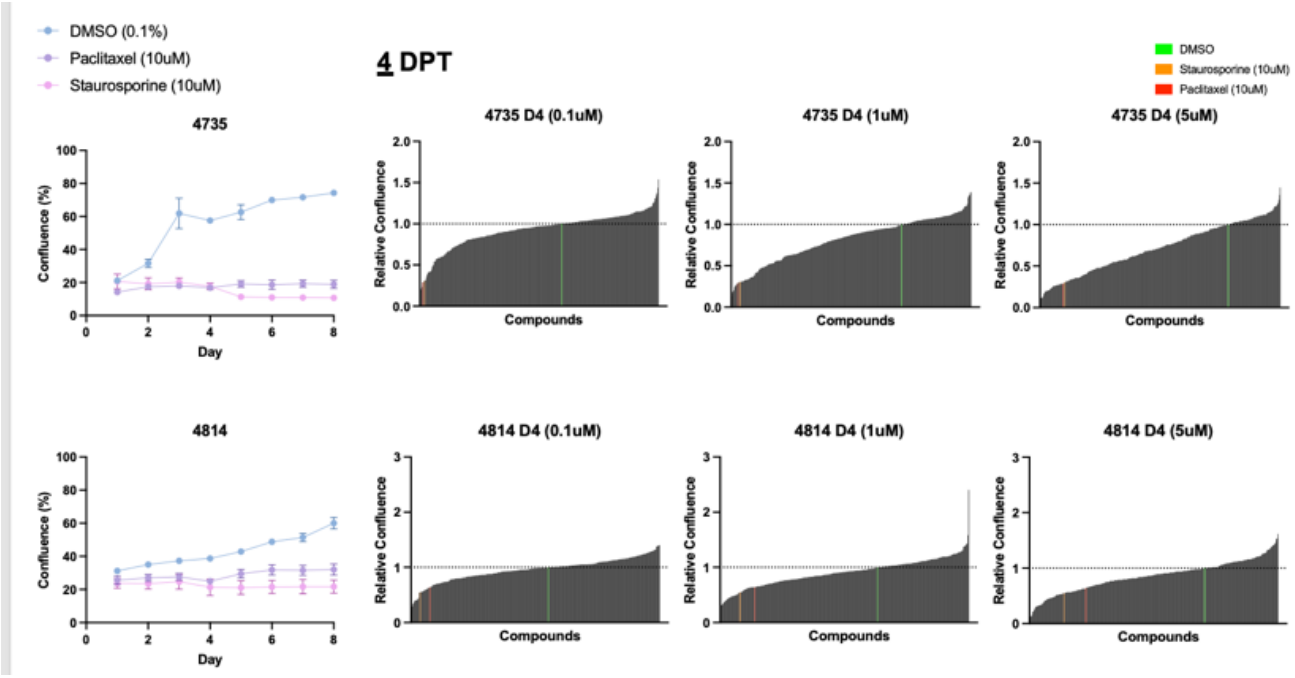

Fig. 5, Overview of the screen result at 72 hours post compound treatment show that a subset of drugs potently reduce stromal cell proliferation or survival.

What opportunities for training and professional development has the project provided?

Postdoctoral fellow Travis Ruan has undergone training in computation biology with his mentor, Prof Swarbrick as well as with other more experienced postdoctoral fellows in the laboratory. Dr Ruan and the wet lab research assistant/technician both attended a conference entitled "Oz Single Cell' where they learned about the use of single cell methods and analysis, which will be important for their work on data analysis in years 2 & 3. Dr Ruan spent 2 weeks at the Victorian Centre for Functional genomics, based at the Peter McCallum Cancer Centre, Melbourne. There he received advanced training in functional screens.

- **How were the results disseminated to communities of interest?**

Preliminary results were presented at the following conferences:

- 2022 9th Annual Metastatic Breast Cancer Research Conference, Utah USA (online). Invited Conference presentation "*Parsing breast cancer ecosystems*"
- 2022 Global Breast Cancer Conference, Korea *(Online).* Invited Conference presentation "Exploring breast cancer ecosystems with cellular genomics"
- 2022 EMBO Conference: The Many Faces of Cancer Evolution, May2022, Italy. Invited Conference presentation "Exploring breast cancer ecosystems with cellular genomics"
- 2021 Sydney Cancer Conference, September 2021, Sydney. Invited Conference presentation "Stromal-immonology of breast cancer"

- **What do you plan to do during the next reporting period to accomplish the goals?**
    - *Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives*

We intend to adhere to the statement of work. Therefore the next 12 months will see us work towards the following goals and timelines:

Major Task 1 - Subtask 2: Analysis of Spatial Transcriptomic data . *Complete all subtasks*

Major Task 2: Bulk RNA-seq of TNBC cases. *Complete all subtasks*

Major Task 3: Analyse scRNA-Seq and scATAC-Seq. *Complete all subtasks*

Major Task 4: Determine the functional requirement for candidate factors in PVL and CAF cells.

Complete Subtasks 1, 2, 4

4. Impact

- **What was the impact on the development of the principal discipline(s) of the project?**

Nothing to report, as the project is too early in its progress

- **What was the impact on other disciplines?**

Nothing to report, as the project is too early in its progress

- **What was the impact on technology transfer?**

Nothing to report, as the project is too early in its progress

- **What was the impact on society beyond science and technology?**

Nothing to report, as the project is too early in its progress

5. Changes/Problems

- **Changes in approach and reasons for change**

No changes

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Progress against major tasks 2 has been delayed by ~ 6 months. However, this does not reflect an issue with our ability to execute this aim, rather a modest delay caused by the Omicron outbreak in 2022 and our investment of time in other aims. This is a relatively routine parcel of work and we anticipate it will be completed by May 2023.

- **Changes that had a significant impact on expenditures**

NA

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

NA

- **Significant changes in use or care of human subjects**

NA

- **Significant changes in use or care of vertebrate animals.**

NA

- **Significant changes in use of biohazards and/or select agents**

NA

6. Products

- **Publications, conference papers, and presentations**
  Nothing to Report
    - **Books or other non-periodical, one-time publications.**
    - Nothing to Report
    - **Other publications, conference papers, and presentations.**
- 2022 9th Annual Metastatic Breast Cancer Research Conference, Utah USA (online). Invited Conference presentation "*Parsing breast cancer ecosystems*"

- 2022 Global Breast Cancer Conference, Korea *(Online).* Invited Conference presentation “Exploring breast cancer ecosystems with cellular genomics”

- 2022 EMBO Conference: The Many Faces of Cancer Evolution, May2022, Italy. Invited Conference presentation “Exploring breast cancer ecosystems with cellular genomics”

- 2021 Sydney Cancer Conference, September 2021, Sydney. Invited Conference presentation “Stromal-immunology of breast cancer”

- **Website(s) or other Internet site(s)**
  Nothing to Report

- **Technologies or techniques**
  Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Nothing to Report

- **Other Products**

  Nothing to Report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Example:

Name:	*Prof Alexander Swarbrick*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*ORCID* **0000-0002-3051-5676**
Nearest person month worked:	*2*
Contribution to Project:	*Dr Swarbrick leads this project. He has supervised staff and students and managed the project.*
Funding Support:	*A Research fellowship from The National Health & Medical Research Council of Australia*

Name:	*Ms Laura Rachel Nementzik*
Project Role:	*Research assistant*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*12*

Contribution to Project:	*Ms Nementzik works as a research assistant, conducting experiments under the supervision of Drs Swarbrick and Ruan*
Funding Support:	This project

Name:	*Dr Travis Ruan*
Project Role:	*Postdoctoral fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Dr Ruan is the postdoctoral fellow leading experimentation for this project.*
Funding Support:	This project

Name:	*Dr Mun Hui*
Project Role:	*Clinical fellow*
Researcher Identifier (e.g. ORCID ID):	*ORCID* **0000-0003-3238-0119**
Nearest person month worked:	*2*
Contribution to Project:	*Dr Hui is a clinical fellow focussing on tissue cohorts and clinical data for this project*
Funding Support:	This project

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. Special Reporting Requirements

N/A

9. Appendices

None