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TITLE: Mechanisms of Vascular Mimicry Impacting Tumor Progression and Response to Therapy in Breast Cancer

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13. SUPPLEMENTARY NOTES					
14. ABSTRACT Vascular mimicry (VM) describes the formation of pseudo blood vessels constructed of tumor cells that have acquired endothelial-like properties. VM channels endow the tumor with an alternative vascular system that directly connects to host blood vessels whose presence is associated with poor prognosis. However, our molecular understanding of how tumor cells acquire endothelial-like characteristics is relatively poor. Here we show that the transcription factor, Foxc2, promotes VM in breast cancer mouse models by driving ectopic expression of endothelial genes in tumor cells, a process which is stimulated by hypoxia. VM-proficient tumors are resistant to anti-angiogenic therapy and suppression of Foxc2 augments response thus motivating the search for VM-inhibitory agents that could form the basis of combination therapies with anti-angiogenics.					
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1. Introduction

Vascular mimicry (VM) describes the formation of pseudo blood vessels lined by tumor cells that have acquired endothelial-like properties. VM is associated with poor prognosis in breast cancer patients and has been postulated to be a mechanism of resistance to cancer therapies that target the host blood vessels, so called anti-angiogenic therapies (AATs). Using emerging technologies coupled with advanced mouse models of human cancer our work seeks to understand the molecular underpinnings of VM and whether we can target VM therapeutically to circumvent resistance to AATs.

2. Keywords

Vascular mimicry (VM), anti-angiogenic therapy, endothelium, patient-derived xenografts (PDX), single cell RNA-Seq (scRNA-Seq).

3. Accomplishments

Our accomplishments to date are as follows:

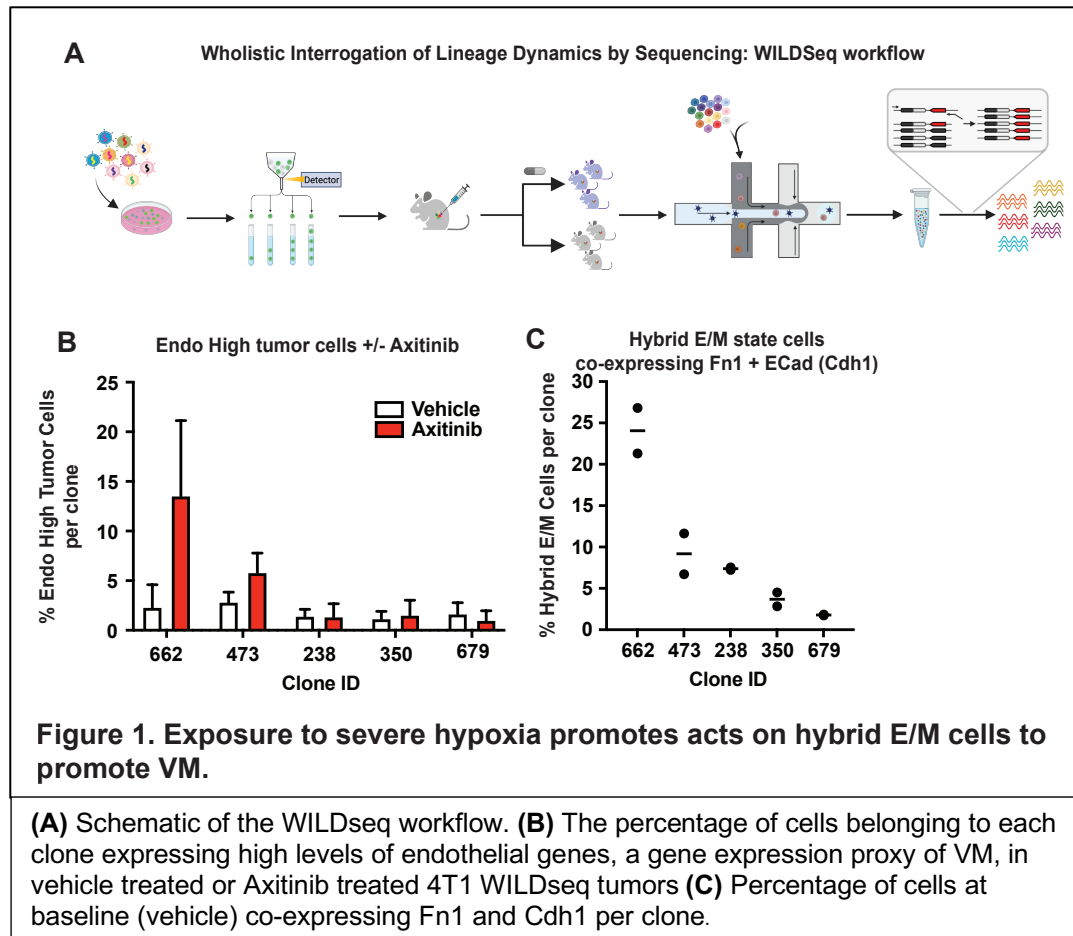
- 1) We have shown that loss of FOXC2 *in vivo* leads to a suppression of VM-derived vessels and an increase in host-derived vessels.
- 2) We have demonstrated a conserved role for FOXC2 in cell culture models of VM across cancer types including small cell lung cancer, renal carcinoma and glioblastoma.
- 3) We have identified a role for FOXC2 in the regulation of hypoxia genes and in promoting survival under hypoxic conditions in cell culture.
- 4) Using single cell RNA-Seq we have shown that exposure to hypoxia *in vivo*, through the inhibition of angiogenesis, promotes endothelial gene expression in tumor cells and activation of a FOXC2 transcriptional program.
- 5) **Under Aim 2.1 of the SOW we have generated 4T1-T Foxc2 knockdown RNA-Seq data in vitro which we have overlaid with our human data from MDA-MB-231 cells to identify genes that are regulated by FOXC2 in a conserved manner across species.**
- 6) **We have shown, using a newly developed single cell clonal tracking system developed by us, that hypoxia likely acts upon tumor cells in a hybrid epithelial-mesenchymal state characterized by co-expression of E-Cadherin (an epithelial marker) and Fibronectin (a mesenchymal marker) that then acquire endothelial gene expression upon anti-angiogenic treatment. This replaces the parental CellTag approach originally outlined in Aim 2.2 of the SOW.**
- 7) **Ovarian cancer patients from the ICON7 trial can be stratified based on the tumour expression of Foxc2-target genes and hybrid E/M genes, with those showing low levels of both signatures benefitting long-term from anti-angiogenic therapy. This was not included in the SOW as this analysis was performed using CRUK funding but is extremely relevant to the BCRP project so is included here.**
- 8) **We have developed of a new genome-wide dual guide RNA library utilizing a tRNA backbone to facilitate equal processing of both guides. This replaces and improves upon the shRNA library under SOW Aim 2.3.**
- 9) **Development and validation of acetylated low-density lipoprotein (acLDL) uptake as a surrogate assay for endothelial character of tumor cells and VM-proclivity. This replaces and improves upon the tagged FOXC2-target genes approach listed in Aim 2.3 of the SOW as a read-out of VM propensity.**

- 10) **We have now performed the screen in multiple cell lines by using fluorescently labelled acLDL and sorting for cells that lose their ability to uptake this molecule. This replaces and improves upon the RNAi screen outlined in Aim 2.3.**
- 11) **Data analysis for the screens is underway. We hope to be able to identify new regulators of VM that may be accessible to pharmacological intervention.**
- 12) Identification of VM as a resistance mechanism to anti-angiogenic therapies. Non-VM murine 4T1 tumors respond to therapy whereas VM-proficient 4T1 tumors do not.
- 13) FOXC2 regulates gene expression signatures associated with resistance to AAT in patients.
- 14) Genetic suppression of Foxc2 in VM-proficient 4T1 tumors renders these previously resistant tumors sensitive to AAT.
- 15) The Caldas lab have pioneered an approach of bulk gene expression profiling by RNA-Seq followed by alignment to a combined human-mouse genome which allows deconvolution of human/tumor derived gene expression and that derived from the mouse/host.
- 16) Using this approach, we have shown that FOXC2-target genes and endothelial genes are specifically upregulated in tumor cells of the aggressive Basal/Claudin-low subtypes of breast cancer which largely consist of triple negative tumors.
- 17) By correlating FOXC2-target genes and endothelial genes in tumor cells (human expression) we have identified VM-high and VM-low PDTX models which we have validated by CD31/PAS staining.
- 18) **Now we have validated VM-high PDTXs we are utilizing these to trial combination therapies targeting VM and angiogenesis, an approach we plan to continue to pursue in the expansion award. We have implanted these tumours and are waiting for them to establish as some of these models have a long latency. Under Aim 3.1 of the SOW.**

We will address relevant progress in each of the areas that are new since our last report specifically below.

We have shown, using a newly developed single cell clonal tracking system developed by us, that hypoxia likely acts upon tumor cells in a hybrid epithelial-mesenchymal state characterized by co-expression of E-Cadherin (an epithelial marker) and Fibronectin (a mesenchymal marker) that then acquire endothelial gene expression upon anti-angiogenic treatment.

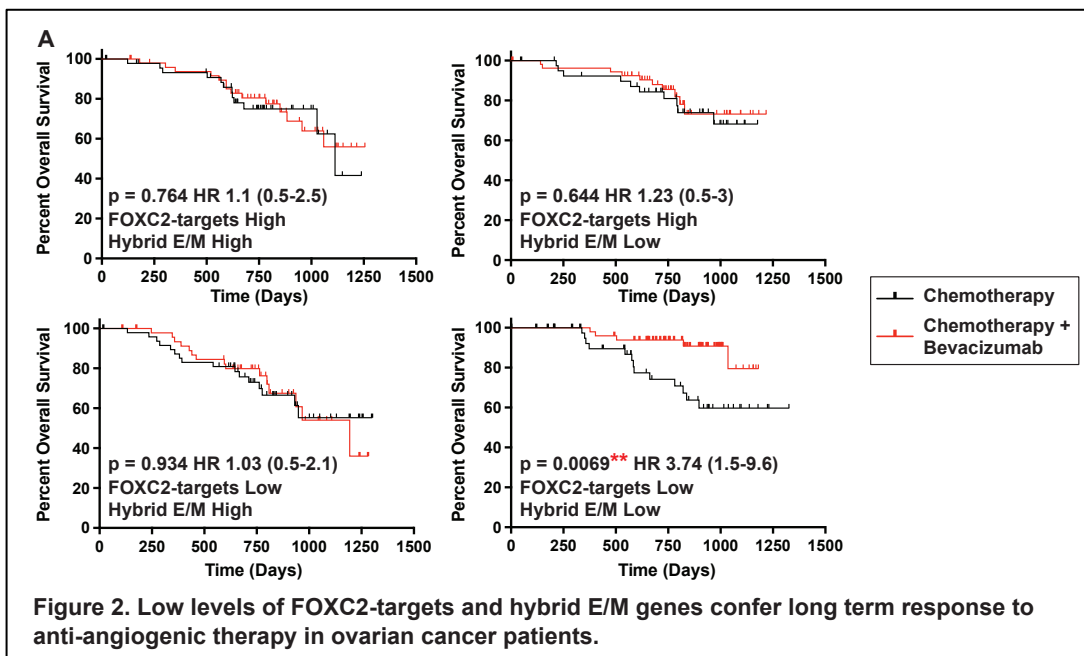
We had previously shown that treatment with anti-angiogenic therapy (AAT) in mouse models that do not normally have high levels of VM promotes VM and using single cell RNA-Seq that tumor cells turn on endothelial genes upon AAT. However, this did not allow us to understand what it is about those cells in the absence of treatment that allows them to turn on endothelial genes upon AAT. To address this problem, we turned to a technology that we recently developed in the lab called WILD-Seq (wholistic interrogation of lineage dynamics by sequencing) in which individual tumor cells are labelled with an exogenous transcript that has a unique barcode within its 3' UTR positioned such that it is easily captured and read-out by standard single cell RNA-Seq workflows (Figure 1A). This approach allows the identification of the same population of cells in vehicle/untreated animals and treated animals and allows us to infer baseline characteristics of cells that change



upon treatment. We generated a WILD-Seq 4T1 clonal pool and treated the resulting tumors with Axitinib. This replaces the parental CellTag single cell RNA-Seq listed in Aim 2.2 of the statement of work. As we have done previously, we identified tumor cells with high levels of endothelial gene expression but this time on a per clone basis. As shown in Figure 1B clones 662 and 473 both displayed an increased frequency of endo high cells with Axitinib treatment. To determine features of these cells that prime them to undergo VM in response to hypoxia we compared the gene expression of clones 662 and 473 to all other major clones in baseline (vehicle) tumors revealing

an enrichment of cells that co-express both the epithelial marker E-Cadherin and the mesenchymal marker fibronectin (Fn1) (Figure 1C). Cells that co-express epithelial and mesenchymal markers are often referred to as hybrid E/M cells that lie along a continuum between purely epithelial and purely mesenchymal, and it is these hybrid cells that are thought to exhibit the most plasticity, making them the ideal seed for VM given a conducive hypoxic environment.

Ovarian cancer patients from the ICON7 trial can be stratified based on the tumour expression of Foxc2-target genes and hybrid E/M genes, with those showing low levels of both signatures benefitting long-term from anti-angiogenic therapy.



(A) Overall survival curves of ovarian patients in the ICON7 trial stratified by expression of FOXC2-target genes and co-expression of Fn1 and Cdh1 as proxies of VM and Hybrid E/M respectively. Patients received either chemotherapy or chemotherapy plus anti-VEGF Bevacizumab

Analysis of publicly available data from ovarian cancer patients' tumours according to levels of VM cells (using FOXC2-target genes) and levels of hybrid E/M cells (using E-Cadherin/Fibronectin co-expression), has revealed that patients who have low levels of both VM and hybrid E/M cells are those who receive long-term benefit from anti-angiogenic therapy in the form of Bevacizumab (Figure 2). These data suggest that we may be able to identify patients at diagnosis who are likely to benefit from anti-angiogenic therapy

alone and those who may benefit from a combination of VM inhibition in conjunction with anti-angiogenic therapy.

Loss of function screens for VM regulators.

We have made huge strides screening for new regulators of VM. This was originally intended to be in the form of an RNAi screen but recent developments in the lab of next generation CRISPR guide RNA libraries in which critical exons of target genes are flanked by 2 guide RNAs co-expressed from the same viral vector, prompted a shift in our approach as this new strategy greatly improves loss of function of genes of interest. Our accomplishments in this area include:

- 1) As mentioned above, development of a new genome-wide dual guide RNA library utilizing a tRNA backbone to facilitate equal processing of both guides.
- 2) Development and validation of acetylated low-density lipoprotein (acLDL) uptake as a surrogate assay for endothelial character of tumor cells and VM-proclivity.
- 3) We have now performed the screen in multiple cell lines by using fluorescently labelled acLDL and sorting for cells that lose their ability to uptake this molecule.
- 4) Data analysis for the screens is underway. We hope to be able to identify new regulators of VM that may be accessible to pharmacological intervention.

We have validated VM-high PDTXs we are utilizing these to trial combination therapies targeting VM and angiogenesis and further assess the tumor perfusion status via intravenous administration of fluorescent lectins. We have implanted these tumors and are waiting for them to establish as some of these models have a long latency.

4. Impact

Together the work outlined in this report has furthered our understanding of how hypoxia triggers VM to promote acquired resistance to AATs and potentially identify avenues to prevent its emergence. We have performed several loss-of-function screens for VM regulators which makes significant strides towards identifying drug-like molecules that can suppress VM in tumors that have high pre-existing levels of VM. We believe we can now identify cancer patients that are likely to benefit from AAT alone, or combinations that target pre-existing VM and/or its emergence upon therapy, thus our work has the potential to reinvigorate how these well-tolerated potent AATs are used clinically in breast cancer.

5. Changes/Problems

There have been some delays due to the COVID-19 pandemic and a change in approach from an RNAi screen to a CRISPR/Cas9 screen with acLDL as the read-out as we have newly developed extremely efficient tools available. We have changed our single cell RNA-Seq experiments listed in Aim 2.2 of the SOW to use WILD-Seq as this allows us to identify the same cells before and after treatment and this has allowed us to glean new information that would not be possible with the previous approach.

6. Products

We have recently submitted a manuscript to *Cancer Cell* on our FOXC2 work entitled “FOXC2 promotes vasculogenic mimicry and resistance to anti-angiogenic therapy”. In terms of technologies, we have developed WILDseq which we plan to publish in the next 6 months thus disseminating the technology to the broader scientific community.

7. Participants & Other Collaborating Organizations

Name:	Gregory Hannon
Project role:	Principal investigator
Research Identifier:	
Nearest person month worked:	1
Contribution to project:	Prof Hannon has overseen all aspects of the project
Funding Support:	CRUK core grant pays Prof Hannon's salary

Name:	Ian Cannell
Project role:	co-Principal investigator
Research Identifier:	https://orcid.org/0000-0001-5832-9210
Nearest person month worked:	12
Contribution to project:	Dr Cannell has overseen all aspects of the project
Funding support:	This award

Name:	Fatime Oasaj
Project role:	PhD student
Research Identifier:	
Nearest person month worked:	12
Contribution to project:	Ms Oasaj has performed many of the experiments outlined in the progress report in conjunction with Dr Cannell.
Funding support:	This award

Name:	Kirsty Sawicka
Project role:	Post-doc
Research Identifier:	
Nearest person month worked:	6
Contribution to project:	Dr Sawicka has performed many of the computational analyses outlined in the progress report.
Funding support:	This award

8. Special Reporting Requirements

None

9. Appendices

None