Identification of breast cancer biomarkers linked with interstitial fluid flow and hypoxia using a spheroid-on-chip perfusion system

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Introduction

Solid tumours are complex and heterogeneous systems, which exist in a dynamic biophysical tumour microenvironment (TME), including low oxygen levels (tumour hypoxia) and the presence of interstitial fluid flow (IFF). Both have been shown to be involved in driving tumour progression, but IFF has largely been overlooked in *in vitro* recapitulations of the TME. Microfluidic approaches are well suited to bridge this gap and incorporate fluid perfusion to model IFF. We have previously developed a spheroid-on-chip model to address this gap (Collins et al 2021). In this study (Pyne et al 2024) we used our model to perform an unbiased analysis of the impact of IFF on cancer biology in experimental (tissue culture) and hypoxic conditions, by conducting transcriptomic analyses to elucidate possible mechanisms by which IFF could be influencing TME progression and identify biomarkers associated with IFF.

Conclusions

- Our study, using a spheroid-on-chip microfluidic system, is the first to systematically explore transcriptional changes linked with tumour IFF-like conditions, particularly in low oxygen conditions relevant to those observed in the hypoxic tumour microenvironment.
- We have also identified and analysed ACTL8 as a candidate biomarker linked with IFF-like conditions and showed high expression of ACTL8 to be associated with poor prognosis in breast cancer.
- Identifying IFF-linked biomarkers would provide an alternative to traditional invasive and imaging-based methods to measure IFF.

1. Whole transcriptome analysis reveals impact of IFF-like perfusion on cancer spheroid biology

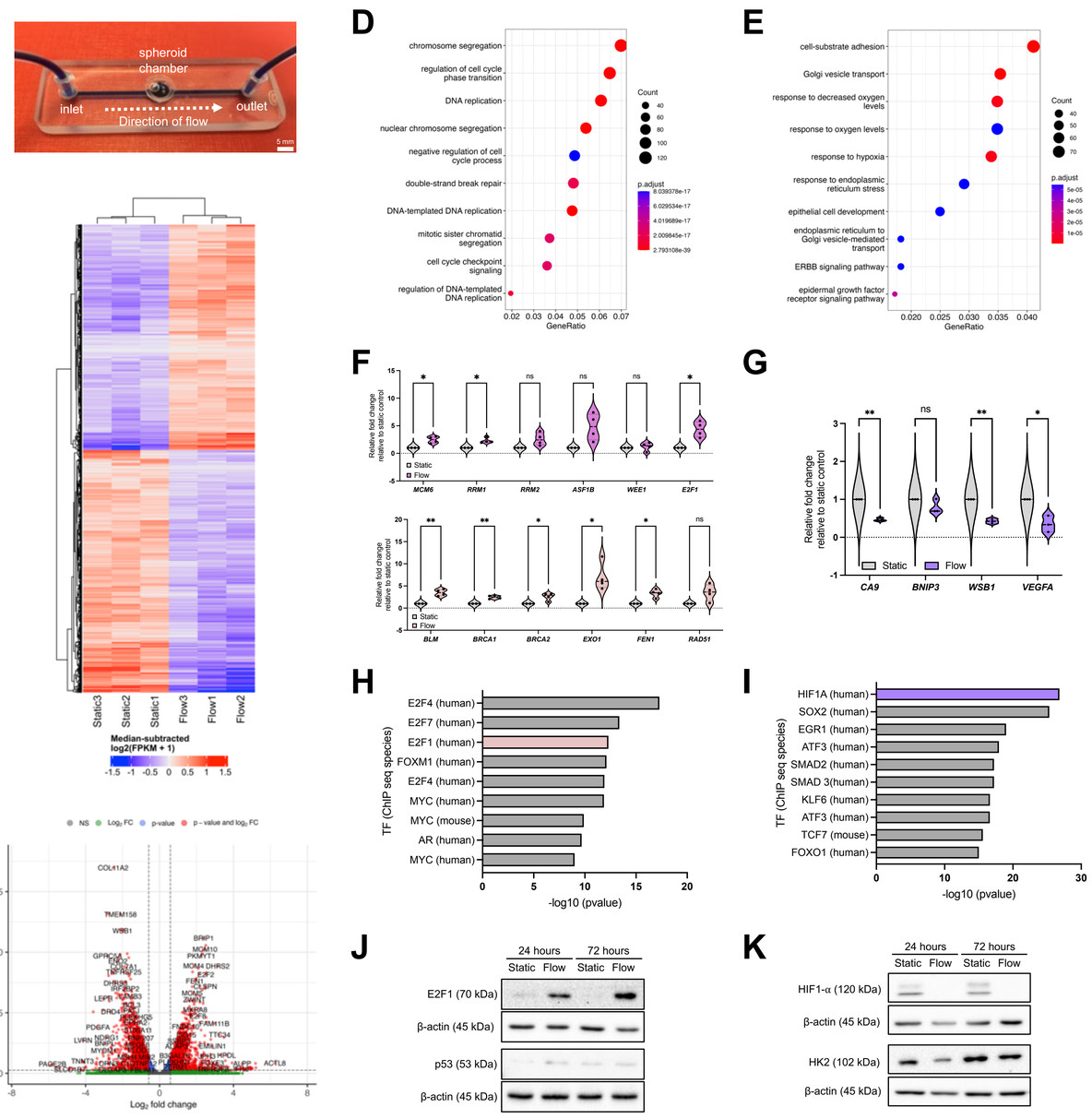

Figure 1. To determine the whole transcriptome impact of IFF-like flow, MCF7 spheroids were exposed to in static or flow conditions for 24 h in the presence of Matrigel using our spheroid-on-chip device **(A)** and RNA samples extracted. Bulk RNA-sequencing was performed (n=3), with Heatmap **(B)** and volcano plot **(C)** represent significantly differentially expressed genes (DEGs), with p.adjust<0.05 and fold change > log2(1.5). **(D-E)** Pathway enrichment analysis using GO (gene ontology) categories was performed for upregulated **(C)** and downregulated **(D)** genes, with key factors validated using qPCR **(F-G)**. Transcription factor enrichment was performed for the upregulated **(H)** and downregulated **(I)** DEGs. Immunoblotting **(J-K)** was used to analyse the protein expression of HIF-1a, E2F1 and p53. Blots are representative of n = 3 experiments. Unpaired student's t-test was performed to test for statistical significance between samples. ns = nonsignificant; * p<0.05; ** p<0.01

2. Hypoxia-IFF interface drives different transcriptomic changes

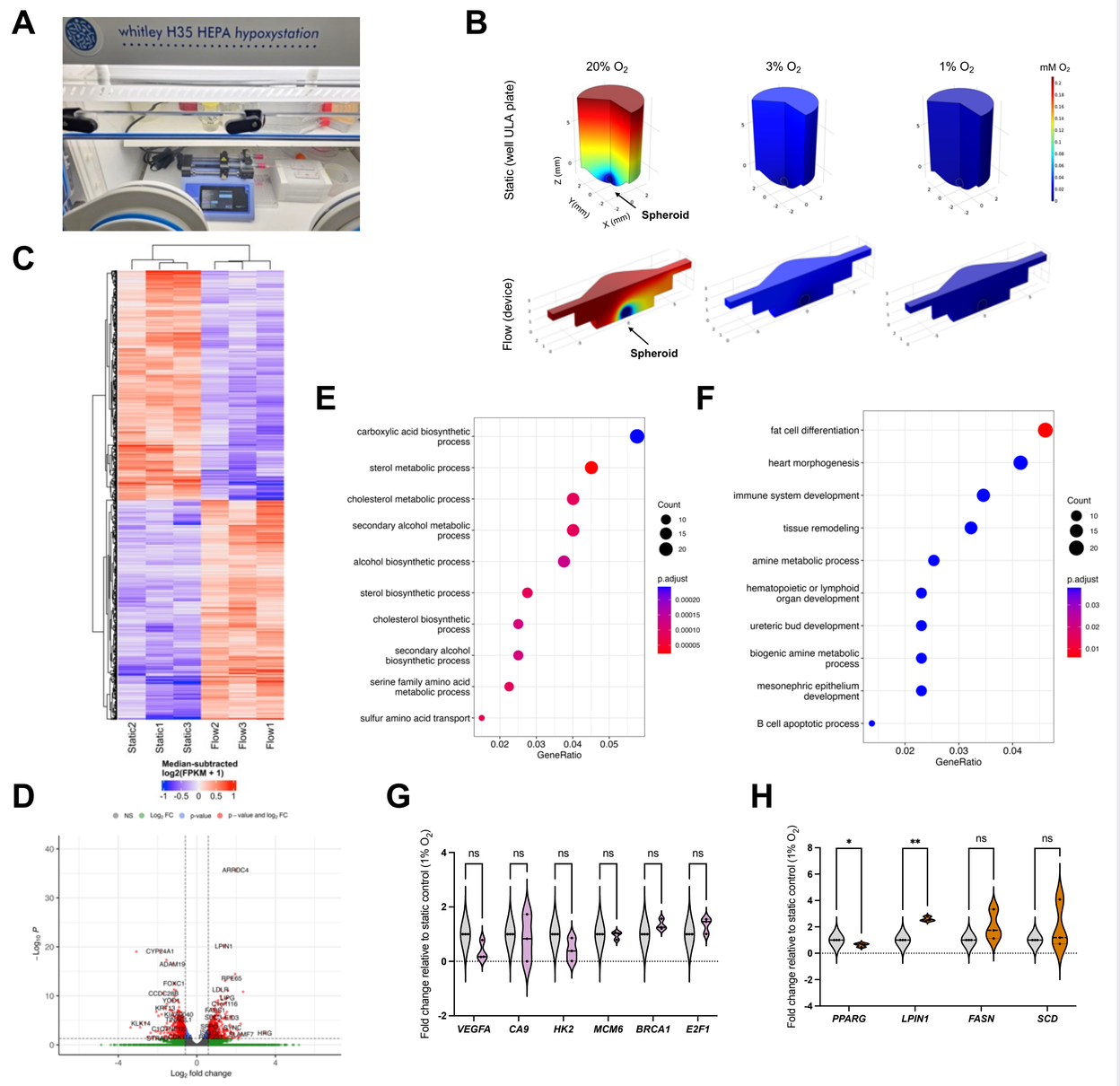

Figure 2. To determine the whole transcriptome impact of IFF-like flow in hypoxic conditions, MCF7 spheroids were exposed to in static or flow conditions for 24 h in the presence of Matrigel using our spheroid-on-chip device in a hypoxia chamber at 1% O_2 **(A).** **(B)** Oxygen consumption modelling of spheroids in static and flow conditions was performed, with the spheroid perfused with air saturated medium (no matrigel) at three different oxygen tensions (20%, 3%, and 1% O_2 levels) in static conditions or at a flow rate of 3 µL min^{-1}. RNA samples extracted, and bulk RNA-sequencing was performed (n=3), with Heatmap **(C)** and volcano plot **(D)** represent significantly differentially expressed genes (DEGs), with p.adjust<0.05 and fold change > log2(1.5). **(E-F)** Pathway enrichment analysis using GO (gene ontology) categories was performed for upregulated **(E)** and downregulated **(F)** genes, with key factors validated using qPCR **(G-H)**. Unpaired student's t-test was performed to test for statistical significance between samples. ns = nonsignificant; * p<0.05; ** p<0.01.

3. Identification of potential IFF-associated clinically relevant biomarkers – *ACTL8* as a proof of principle analysis in breast cancer

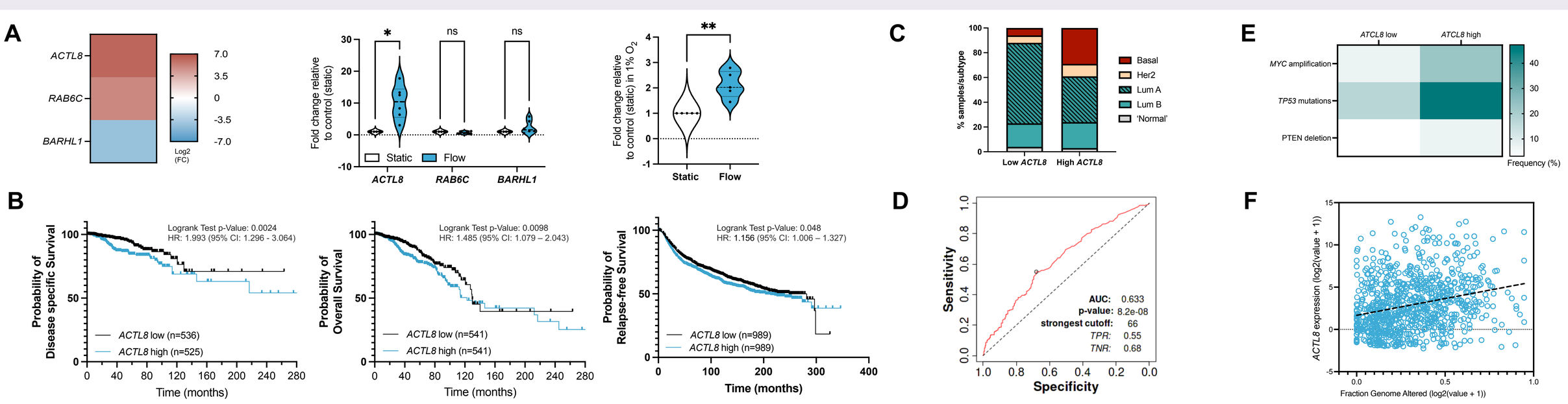

Figure 3. (A) Panel of genes uniquely linked with either static or flow conditions were selected from the normoxic (20% O_2) RNA-sequencing dataset, with Heatmap (A) representing log2 fold change for these genes. MCF7 spheroids were formed as before and exposed to static or flow conditions for 24 hours in the presence of Matrigel in normoxic conditions (20% O_2), and gene expression for the gene panel validated through qPCR as described before, with plots (B) representing n=6 (24 h) independent experiments. Expression of *ACTL8* was also evaluated in static or flow conditions for 24 h in the presence of Matrigel in hypoxic conditions (1% O_2). **(B)** Prognostic value for *ACTL8* expression in breast invasive carcinoma patient samples was determined using the TCGA pan cancer Atlas RNA-seq dataset (n=1084) for Disease-free Survival (n=1061) and Overall Survival (n=1082) and Metabric for Relapse-free Survival (n=1978) plots compare high and low *ACTL8* expression, determined by median *ACTL8* expression. This dataset was also analysed for proportion of breast cancer subtypes **(C)**. **(D)** AUC (Area Under The Curve) ROC (Receiver Operating Characteristics) curve analysis was performed to evaluate the predictive value of ACTL8 gene expression vs responsiveness to any kind of chemotherapeutic treatment to determine predictive biomarker potential in breast cancer patients , n= 426 (non-responders = 197; responders = 229). For Survival plot analysis p value, a Logrank test was used; for ROC p value determination, a Mann-Whitney test was used. Significance is considered if p<0.05. **(E-F)** Analyses of relationship between ACTL8 mRNA expression and genomic instability markers. *ACTL8* expression in patients (n = 994) from the TCGA PanCancer Atlas Breast Invasive Carcinoma study were analysed for links with genomic instability markers. **E** - Correlation analysis of *ACTL8* mRNA levels vs Genomic instability marker 'Fraction Altered Genome'. **F** - Frequency of genomic alterations linked with increased genomic instability (*MYC* amplification, *TP53* mutations, and *PTEN* deletions) comparing patient samples with low of high *ACTL8* mRNA expression.

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