Identifying the functional role of breast cancer associated fibroblasts in promoting disease progression.

by

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Montréal, Québec, Canada
June 2022

A thesis submitted to McGill University in partial fulfillment of the requirements of Doctor of Philosophy.

Abstract

Cancer associated fibroblasts (CAFs) play a key role in dynamically modulating the tumour microenvironment; they form fibrotic foci through contraction, secretion and crosslinking of the extracellular matrix. These CAF-dense fibrotic foci activate and recruit surrounding fibroblasts, leading to larger-scale tissue stiffening and enhanced cancer aggression. While the presence of collagen and CAF-dense fibrotic foci in the breast and corresponding changes in mechanical stiffness have been correlated with human breast cancer aggression, the impact of this evolving microenvironment on the ability of CAFs to promote metastasis remains largely unknown. Here, CAFs from 4 human triple-negative breast cancer patients presenting with (2) or without (2) lymph node metastasis were isolated and expanded to further interrogate their behavior under mechanical stress and the implications of this on disease progression.

In Chapter 2.1, we demonstrate using 3D physiologically relevant *in vitro* assays, that CAFs isolated from primary tumours of patients with and without lymph node metastasis, retain functional characteristics representative of disease state. Using an engineered, stiffness tunable, culture model system, we deconstruct the mechanical cues presented to cancer-associated fibroblasts during the process of fibrotic foci formation and extracellular matrix remodelling; and probe the effects of local mechanical stiffness arising from the dynamic process of remodelling on CAF invasion into the surrounding tissue. With this, we demonstrate CAFs derived from patients presenting with non-metastatic disease require substrate stiffness cues to prompt invasion, while CAFs from patients presenting with lymph node metastasis are no longer mechanoresponsive.

In Chapter 2.2, we explore the link between CAF invasion and substrate stiffness. By examining the transcriptome of CAFs derived from patients with and without lymph node metastasis using RNA-sequencing, we confirm the transcriptomic profile of each CAF subset is distinct, where CAFs derived from non-metastatic patients have significantly more genes elevated in response to changes in fibrotic foci stiffness. The transcription factor Aryl Hydrocarbon Receptor (AHR – protein, AhR – gene) previously correlated with fibrosis and migration in fibroblasts, is found to

be specifically elevated both at RNA and protein level in CAFs derived from metastatic patients, suggesting a potential role in facilitating invasion.

In Chapter 2.3, this work demonstrates the role of AhR in CAF invasion. Through chemical and genetic inhibition of AhR, we confirm in human CAFs, for the first time, a dependence on AhR for CAF invasion. In CAFs derived from non-metastatic patients, we find that overexpression of AhR is sufficient to enhance CAF invasion. Interestingly, those CAFs derived from patients presenting with non-metastatic disease exhibit a substrate stiffness dependent increase in AhR expression, whereby culturing them on stiffnesses representative of late-stage disease increases AhR expression and concomitant CAF invasion.

As CAF invasion has previously been demonstrated to be a key mechanism promoting cancer metastasis from the primary tumor, these findings indicate that mechanobiological screens of patient CAFs may be used to understand the current state of tumor progression, stratify patients, and ultimately contribute towards the selection of precision medicine therapies. This work identifies a novel role for transcription factor AhR in facilitating CAF invasion and finds a link with overall patient survival in highly fibrotic tumours. The increased protein levels of AhR suggests a mechanobiological mechanism responsible for clinical failure of drugs aimed at targeting CAF activation.

Abrege

Les fibroblastes associés au cancer (cancer associated fibroblasts, CAF) jouent un rôle essentiel dans la régulation du microenvironnement tumoral, par le remodelage dynamique de la matrice extracellulaire (MEC). Une MEC riche en collagène contribue à une rigidification tissulaire, la fibrose tumorale, qui est associée à l'agressivité des cancers du sein. Toutefois, les conséquences du remodelage de la MEC sur la capacité des CAF à influencer le potentiel métastatique du cancer reste encore méconnu. Dans cette étude, nous avons isolé les CAF de quatre patients atteints d'un cancer du sein triple-négatif métastatique (2) ou non-métastatique (2), afin d'étudier leur réponse à la rigidification tissulaire, ou mécanotransduction, et les conséquences de ces réponses sur la progression métastatique du cancer.

Dans le chapitre 2.1, nous avons développé un modèle de culture cellulaire physiologique en trois dimensions dont la rigidité matricielle est flexible et contrôlée, afin d'évaluer les propriétés mécano-biologiques des CAF en réponse à la rigidification tissulaire. Grâce à ce système, nous avons démontré que les CAF issus de tumeurs non-métastatiques deviennent invasifs lorsque soumis à une matrice rigide, contrairement aux CAF provenant de patients présentant un cancer métastatique, suggérant une désensibilisation des ces derniers à la mécanotransduction.

Au cours du chapitre 2.2, nous explorons la relation entre le potentiel invasif des CAF et la rigidité matricielle. Des analyses de l'expression des gènes par séquençage de l'ARN démontrent que les CAF issus de patients présentant un cancer non-métastatique sont caractérisés par un profil moléculaire particulier, davantage influencé par la rigidité matricielle. L'expression du facteur de transcription Aryl Hydrocarbon Receptor (AhR), associé à la fibrose et au potentiel migratoire des fibroblastes, est significativement plus élevée chez les CAF provenant de patients présentant un cancer métastatique, ce qui suggère son implication dans le processus invasif de la maladie.

Dans le chapitre 2.3, nous nous attardons au rôle du facteur AhR dans le potentiel invasif des CAF. Par des approches génétique et pharmacologique ciblant le facteur AhR, nous avons démontré pour la première fois que le potentiel invasif des CAF de patients atteints de cancer du

sein est régulé par le facteur AhR. En effet, la surexpression de ce facteur augmente significativement la capacité invasive des CAF provenant de patients présentant un cancer non-métastatique. De plus, la rigidification tissulaire augmente l'expression du facteur AhR suggérant que les CAF issus de tumeurs non-métastatiques sont particulièrement sensibles aux propriétés mécaniques du microenvironnement.

Les CAF sont des acteurs majeurs de la progression métastatique en facilitant l'invasion et la dissémination des cellules cancéreuses. Or, les propriétés mécano-biologiques des CAF provenant de patients peuvent aussi informer sur la progression de la maladie et faciliter la stratification des patients pour la médecine de précision. Ces travaux ont permis d'identifier le facteur de transcription AhR en tant que modulateur du potentiel invasif des CAF et facteur pronostique de tumeurs fibrotiques, et supporte l'importance de la mécanotransduction dans la réponse aux nouvelles approches thérapeutiques ciblant les CAF.

Acknowledgements

I feel incredibly grateful to have such a long list of people to thank for their contributions and help both scientifically and emotionally throughout my graduate studies. These intelligent, creative, and passionate people have greatly influenced my love of science and have helped me become the researcher I am today.

Firstly, I would like to thank my two co-supervisors, Dr. Morag Park and Dr. Christopher Moraes, for their support and guidance along the way. Morag, thank you for your love of science, providing guidance, questioning me on each minute detail shown on a slide, and always encouraging (and supplying the wine for!) lab socialization. Chris, thank you for accepting me into the lab after being dropped on your doorstep so many years ago. I'm grateful for your support, your dedication to helping with manuscripts and scholarship applications, and for always having your door open. It has been a wonderful experience learning from you two, both inside and outside the lab. I know the skills I have gained from working with you will be formative to my own career.

Thank you to all the members of the Park lab that I've had the opportunity to work with. It's been amazing to be a part of such a fast-paced, committed lab with a work-hard, play-hard mentality. Tina, thank you for your guidance, support, and dedication during my first years of graduate school. You were exactly the mentor I needed. Anne-Marie, thank you for your valuable insight, love of science, and continual assistance throughout graduate school; I don't know anyone as dedicated to science as you and I'm so thankful for your help along the way. I look forward to catching up over wine in the future. Hellen, thank you for kindness and always being available to help, especially in the most stressful of moments. Abdul, thanks for so patiently re-teaching me how to run Western Blots, and always being available to help with troubleshooting. Dongmei, thank you for your friendship and love of fashion.

To all the graduate students of the Park lab, thank you for making the student room a refuge from the stresses of grad school and a place of comradery. I'll miss joking around in there the

most. I have been incredibly fortunate to learn from each of you and look forward to seeing where life takes you. Colin, thank you for always asking "what's the question?", Yaakov, for your scientific excitement and insight, Tunde, for your kinship, and Vanessa, for demonstrating the importance of extracurricular involvement. Paula, thank you for your invaluable scientific knowledge, willingness to help and being a great friend and co-worker overall. Promita and Annika, I know we haven't overlapped as students in the lab for long but I'm grateful to have met you both. Promita, thanks for your kindness and willingness to help. Annika, thank you for your sense of humour and the level of fun you bring to the lab. I wish we got to work together longer. Marina I'm so thankful we had the opportunity to be co-presidents together; it facilitated a close friendship that I'm very grateful for. You are one of the hardest workers I know, and I'm excited to see where that takes you. Thank you, Bruce for always sharing your controversial views, dedication to science and the secret to your multi-billion-dollar discovery. I look forward to sharing the profits with you. Thank you, Irina for always being there to laugh, eat chips, or cry with me; the lab wasn't the same for me after you graduated. Lastly, Laura, although you weren't officially a Park lab member, I'm so grateful for your time as a visiting student. Thank you for your friendship and all the memories we made while you were living in Montreal.

Thank you to all the members of the Moraes lab that I've had the opportunity to work with. The lab is certainly a place for fun and creativity, and because of that I have so enjoyed all the trials that have gone on in our lab. I could mostly sum up my gratitude for you all with "thanks for always finding ways to laugh". It's been a pleasure. Thank you, Ray, Steph and Sanya, for creating such a welcoming office space and lab group when I first joined the group. Thank you, Ray, for always being there to listen or laugh depending on my experimental predicament and your engineering insights. Steph, thank you for being a great desk-mate, your understanding of cell biology and the general comradery I felt with you in this lab. Sanya, thank you for your team spirit and the fun you brought inside and outside the lab. Nikita, thanks for unique perspectives on the lab and readiness to help. Lisa, thanks for always being a pal and for always having a fun party-girl energy. Sonya and Carley, thank you for continually welcoming me into your office, serving me coffee and making me laugh (Sorry Julia for our disruptions!). Sonya, thank you for quite

literally *always* finding a way to laugh about things, sharing loud and embarrassing conversations in public spaces and navigating the fart box with me. Carley, thank you for your kindness and listening ear, and general enthusiasm both inside and outside the lab. Christina, thank you for being a fantastic lab-mate, your diligence and leadership in the lab has made you a pleasure to work with. James, I haven't known you for long but I'm glad our time in the Moraes lab has overlapped. Thank you for your friendship and entertainment during mundane lab tasks, as well as your initiative in the lab and being a great office mate.

Thank you to my friends outside the lab who provided extracurricular fun and excitement in my life that made the sometimes-overwhelming amount of work and experiments feel surmountable. To Grant and Kate, I don't know what I would have done without you guys. Thanks for your constant support, love and friendship, and for always being a WhatsApp message away. Thank you, OSSHHHHHHHHH for aaaalways being up for activities to distract me from the pains of graduate school; I'm thankful to have been apart of this crew. Thank you to my SJAM girls for the comradery, support and distraction you have all provided.

I would like to express my gratitude to my family. Mom, Dad and Blair, thank you for your endless support in my endeavours, for always sharing my accomplishments and being there (even from afar) every step of the way. Your support has enabled my education and ability to pursue my interests. I'm grateful for the privilege you have afforded me through your own hard work and nurturing.

Finally, thank you to my Nicky for being my ever-present support system, listening ear and provider of back-rubs in stressful situations. I'm not sure what I would have done without your patience and unwavering belief in me.

I'm grateful for the financial support I have received over the course of my graduate education from the CURE Foundation, Défi Canderel, Department of Biochemistry, Faculty of Medicine and Natural Sciences and Engineering Research Council of Canada – Post Graduate Scholarship –

Doctoral (NSERC-PGS-D). Thank you to Standup2Cancer, Canadian Breast Cancer Foundation, CIBC, Oncopole and Canadian Cancer Society for funding the Park and Moraes laboratories and research I conducted as a student.

Lastly, thank you to the women who have generously participated in tissue donation for this project. Thank you for giving our work a deeper sense of purpose. You have been the fuel for this research.

Copyright Acknowledgements

I further acknowledge the following publishing company for allowing me to use the full text and figures of my published works in the following chapter:

- Chapter 1: **Future Science,** Future Drug Discovery, 2022 [1]

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Preface

This thesis is presented in the traditional format. It contains one published review article incorporated into Chapter 1, and one manuscript in submission which has been incorporated into Chapter 2.

Publications Arising from this Thesis

Chapter 1 is included in the following review:

1. Gabrielle Brewer, Anne-Marie Fortier, Morag Park & Christopher Moraes. **The case for cancer-associated fibroblasts: essential elements in cancer drug discovery?** *Future Drug Discovery*, 2022.

Chapter 2 is included in the following manuscript:

1. Gabrielle Brewer, Paul Savage, Anne-Marie Fortier, Hong Zhao, Alain Pacis, Yu-Change Wang, Dongmei Zuo, Monyse Nobrega, Annika Pedersen, Camille Cassel de Camps, Margarita Souleimanova, Valentina Muñoz Ramos, Jiannis Ragoussis, Morag Park, and Christopher Moraes. **Pro-invasive mechanoresponses of patient derived triple negative breast cancer associated fibroblasts are AhR-dependent and correlate with disease state.** *Submit at Nature Cancer.*

Original Contributions to Knowledge

1) This work demonstrates that **contraction and invasion of CAFs from human patient samples increases with disease state**. While prior work has shown this in activated mouse fibroblasts, my work is the first report of *ex vivo* CAF activation correlating with patient disease state. The data presented here therefore adds an understanding that CAFs that become mechano-insensitive retain this phenotype in culture, possibly reflecting an irreversible transition during *in vivo* disease progression.
2) I fabricated stiffness-tunable polyacrylamide hydrogel cores to represent fibrotic foci of varying stiffnesses. Using this I **deconstruct the mechanical cues presented to cancer-associated fibroblasts during the process of fibrotic foci formation and extracellular matrix remodelling**; and probe the effects of local mechanical stiffness arising from the dynamic process of remodelling on CAF invasion into the surrounding tissue. Uniquely, these retrospective studies of cells isolated from human patients, allow us to triangulate local microenvironmental mechanics, functional CAF behaviour, and disease progressive state, with long-term patient outcome.
3) The results of our study demonstrates that **CAF invasion is mechanosensitive only when isolated from early disease stages**. In contrast, CAFs are consistently invasive when isolated from patients with later stage disease and are not additionally activated by local microenvironmental rigidities. As CAF invasion has previously been demonstrated to be a key mechanism promoting cancer metastasis from the primary tumor, these findings indicate that mechanobiological screens of CAF behaviour may be used to understand the

current state of tumor progression, stratify patients, and ultimately contributed towards selection of precision medicine therapies.

4) Using RNA sequencing, I identify differentially expressed genes that contribute to the phenotypic variations in invasion and highlight **a role for the transcription factor aryl hydrocarbon receptor (AhR) in promoting CAF invasion**. Our findings support that CAFs associated with non-invasive breast cancer have low AhR protein levels that can be increased with microenvironmental stiffness, correlating with increased CAF invasion, while CAFs associated with later stage breast cancer are highly invasive and have high AhR protein levels independent of stiffness.
5) For the first time, I **demonstrate the role of AhR in the invasion and mechanical stiffness sensing of breast cancer derived CAFs**; a finding that has potential for broad significance to researchers engaged in targeting microenvironmental support of tumour progression.

Contributions of Authors

In order to achieve the data presented in this thesis, several collaborations were established with experts inside and outside of my field (clinicians, pathologists and bioinformaticians). Author contributions are listed below. I performed all experiments and coordinate any that I did not conduct myself. I analyzed the data generated in these experiments and together with my supervisors, Dr. Morag Park and Dr. Christopher Moraes, made the figures and wrote the manuscripts that arose from this thesis.

Oncologists Dr. Jamil Asselah, Dr. Nathaniel Bouganim and Dr. Kevin Petrecca, Pathologist Dr. Atilla Omeroglu, and surgeons Dr. Mark Basik and Dr. Sarkis Meterissian provided access to patient material. Valentina Munoz-Ramos, Margarita Souleimanova, Adriana Aguilar and Nicholas Bertos assisted with collection of patient tumours and retrieving clinical information. Paul Savage generated CAF cultures.

Dongmei Zuo preformed indirect immunofluorescence and immunohistochemical staining of human tissues (Figure 2-1, Si Appendix Figure 1). Camille Cassel de Camps mechanically tested engineered fibrotic foci using the Microsquisher (2-3C). Hong Zhao isolated fresh RNA from tissues samples (Figure 2-5), and Yu-Chang Wang performed RNA library generation. Alain Pacis performed RNA sequencing normalization and downstream analysis, as well as Gene Set Enrichment Analysis (Figure 2-5, Figure 2-6). Anne-Marie Fortier and Hellen Kuasne assisted with RNA sequencing analysis through exploring different software's for data analysis as well as data bases for validation. Anne-Marie Fortier immortalized patient derived CAFs using pLVX-SV40LT-

IRES-tdTomato, wrote the immune focused text for the literature review (Chapter 1.3.2), translated the abstract to French, and provided general guidance throughout the project. Monyse Nóbrega with the oversight of Hellen Kuasne preformed gene expression validation by qPCR (Figure 2-6). Annika Pederson midi-prepped AhR and myc-GFP plasmids as well as optimized conditions for transient protein overexpression of AhR (Figure 2-7).

List of Abbreviations

Abbreviation	Definitions
2D	Second dimension
3D	Third dimension
ACTA2	Actin α 2, Smooth muscle
AHR	Aryl hydrocarbon receptor (gene)
AhR	Aryl hydrocarbon receptor (protein)
CAF-pn	Cancer associated fibroblasts derived from primary patients with non-metastatic dissemination
CAF-pm	Cancer associated fibroblasts derived from primary patients with metastatic dissemination
CAFs	Cancer associated fibroblasts
CLF	Clofazimine
CMV	Cytomegalovirus
Col	Collagen
COL1A2	Collagen type I α 2 chain
CXCL12	C-X-C Motif chemokine 12
CYP1B1	Cytochrome p450 1B1
CYP2S1	Cytochrome p450 2S1
DAPI	4’,6-diamidino-2-phenylindole
DDR2	Discoidin domain-containing receptor 2
DEG	Differential gene expression
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
FAP	Fibroblast activated protein
FF	Fibrotic Foci
FFPE	Formalin fixed paraffin embedded
FGF5	Fibroblast growth factor 5
FSP1	Fibroblast specific protein 1
GAS6	Growth arrest specific protein 6
GFP	Green fluorescent protein
H&E	Hemotoxin and eosin
HER2	Human epidermal growth factor receptor
HGF	Hepatocyte growth factor
HR	Hazard ratio
iCAF	Inflammatory CAF
IDO1	Indoleamine 2,3-dioxygenase 1
IF	Immunofluorescence
IL1R1	Interleukin 1 receptor type 1
LAMB2	Laminin subunit β 2

LOX	Lysyl oxidase
LOXL3	Lysyl oxidase like 3
mRNA	Messenger RNA
myCAF	Myofibroblastic CAF
panCK	Pancytokeratin
PDAC	Pancreatic adenocarcinoma
PD-1	platelet derived growth factor receptor
PDGFR	Programmed cell death protein 1
PFA	Paraformaldehyde
PR	Progesterone receptor
qPCR	quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNA-Seq	RNA-sequencing
SDC4	Syndecan 4
SDF1	Stromal cell-derived factor 1
siRNA	Short interfering RNA
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor – β
TGFB1	Transforming growth factor β 1
TGFBR1	Transforming growth factor β receptor 1
TME	Tumour microenvironment
TNBC	Triple-negative breast cancer
TNF-α	Tumour necrosis factor α
αSMA	α – smooth muscle actin

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CHAPTER 1 : LITERATURE REVIEW

1. TRIPLE-NEGATIVE BREAST CANCER

Breast cancer is the most commonly diagnosed cancer among Canadian women, and the second leading cause of death [2].Upon diagnosis, breast cancer is typically stratified based on histopathological subtype, using immunohistochemistry for detection of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2). The majority of diagnosed breast cancers (70%) are ER+ or ER+/PR+ tumours [3-6]. These tumours tend to have the best clinical outcome due to i) the tendency to present as low-grade tumours with low metastatic burden and rates of recurrence, and ii) prevalence of effective targeted therapeutic strategies. HER2+ tumours represent 20% of diagnosed cancers [7], and while they were initially associated with poor prognosis, the development of HER2 targeted therapies have shown significant clinical benefit [8-12]. The remaining 10-15% of breast tumours are negative for all 3 of these markers (ER-/PR-/HER2-) and termed triple-negative breast cancer (TNBC) [13-15]. It has been challenging to treat due to the absence of well-defined molecular targets. Though it is considered a single clinical entity, molecular profiling with RNA sequencing and other “omics” approaches, has shown an unexpectedly high level of heterogeneity [16-20]. Given this, many researchers have instead focused on developing and using therapies aimed at targetting features of the tumour microenvironment (TME). Previous work from our group demonstrated that differential gene expression of breast cancer TME better correlated with clinical outcome than

with tumour subtype [21], suggesting that studying non-cancerous cell populations in the TME can provide further understanding for clinical failings in TNBC.

1.2 CANCER ASSOCIATED FIBROBLAST OVERVIEW

The tumour microenvironment (TME) is a complex landscape, composed of cellular and non-cellular components; it is comprised of tumoral cells, as well as fibroblasts, endothelial cells, various immune cells and non-cellular matrix proteins and ligands, collectively referred to as stroma [22]. Cancer associated fibroblasts (CAFs) are one of the most abundant stromal components of the TME and have been demonstrated to play a prominent role in cancer pathogenesis [21-24].

In normal tissue, fibroblasts remain in a quiescent state, that in response to tissue injury can be activated to repair and regenerate tissue. Activated fibroblasts express α-smooth muscle actin (αSMA) and acquire contractile properties, allowing them to produce and remodel the surrounding extracellular matrix (ECM) [25]. Although this repair effort is usually effective in healthy tissues, where fibroblasts can revert to quiescence, in cancerous lesions where the stress and wounding are continual, CAFs develop [25]. In this state, CAFs display a hyperactive fibroblastic phenotype, and are largely responsible for remodelling the tumour ECM through the contraction, secretion and crosslinking of surrounding collagen, and other fibrillar proteins; this desmoplastic reaction alters both the composition and mechanical properties of the tissue [26] (Figure 1-1).

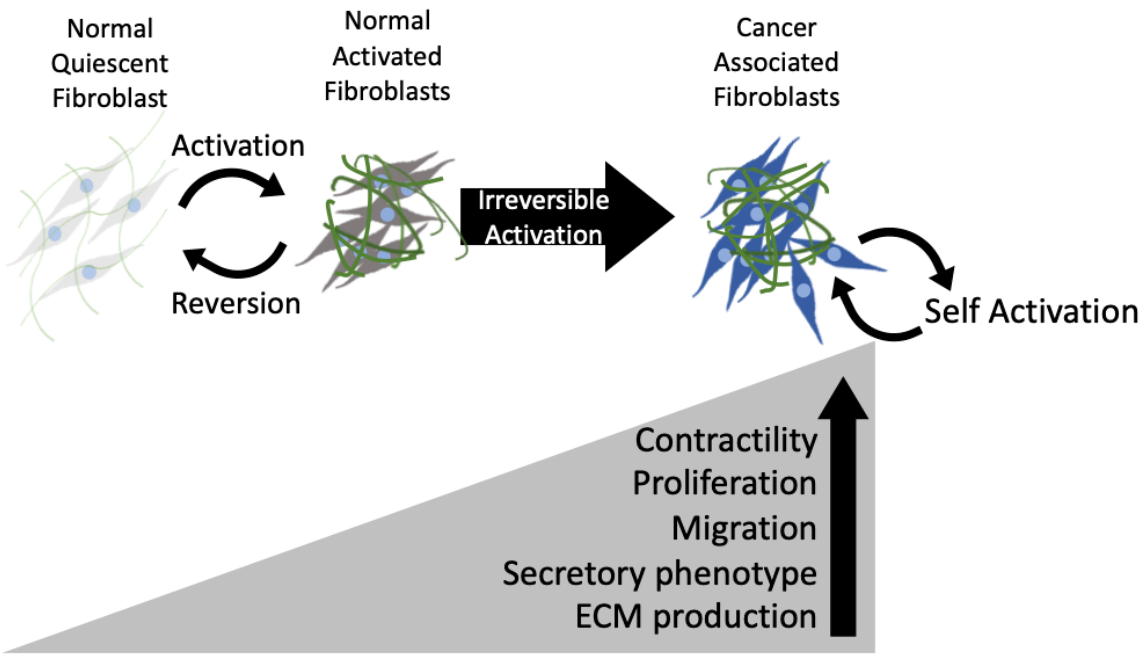


Figure 1-1: CAF activation is step-wise. *Normal quiescent fibroblasts are inert but can be reversibly activated upon external stimuli (stress, chemokines, cytokines, growth factors) to restore tissue homeostasis. Further activation by continuous injury stimuli, cancerous lesions, can lead to an irreversible activation of fibroblasts, termed Cancer Associated Fibroblasts.*

Activation of CAFs depends on tumour induced signals, including transforming growth factor beta (TGF-β), to contract, remodel and secrete extracellular matrix proteins, ultimately altering the TME [25]. Many studies have established a link between patient outcome and CAF number, complexity, or function [27-29]. While the mechanisms of these actions remain unclear, they are likely to require a multidisciplinary understanding of the cancer ecosystem, as CAFs direct remodelling and stiffening of the extracellular matrix (ECM), phenotypes which have been correlated with breast cancer aggression and therefore patient outcome [26]. Fibroblast heterogeneity also contributes to promoting an immunosuppressive microenvironment [30] as well as metastatic progression [31]. Hence, CAFs have become a viable target for anticancer therapies.

Although our knowledge of CAF complexity in the TME is still evolving [32], targeting CAF mediated ECM changes, and associated downstream signalling have become increasingly appealing strategies to modulate CAF-cancer cell communication. However, identifying such targets has not yet translated into clinical benefit. For example, inhibitors of the CAF-dependent hedgehog pathway, IPI-926, failed to recapitulate the overall survival benefits shown in mouse model trials [33-35], and paradoxically decreased patient survival when added to the standard of care [36]. While the reasons for this failure remain unclear, this example highlights the complex roles of CAFs in both stabilizing and supporting the TME.

Since I have started my thesis in September of 2016, there has been a steady growth of papers published focusing on CAFs (Figure 1-2). At the time, 673 papers were published in 2015 with

keywords “cancer associated fibroblast”, in comparison to just 6 years later that number has doubled (1305 papers). Many of these early studies were focused on tumour-CAF interactions [37-42] and were largely limited to immortalized fibroblasts of cancerous and non-cancerous origins, known to acquire phenotypic and genotypic variations characteristic of continual cell culture and immortalization. Moreover those papers focused on CAFs were focused on identifying subtypes of CAFs using combinations of overlapping and non-overlapping biomarkers [25, 43], a static metric that has yet to prove sufficient at predicting patient outcome [30]. These tendencies may be due to the complexity of obtaining and culturing non-immortalized primary human breast CAFs. Though technically challenging, in order to identify CAF functionality driving stiffening and invasion *in vivo*, it is necessary to have assays using primary cells that capture cellular heterogeneity in a physiologically relevant ECM.

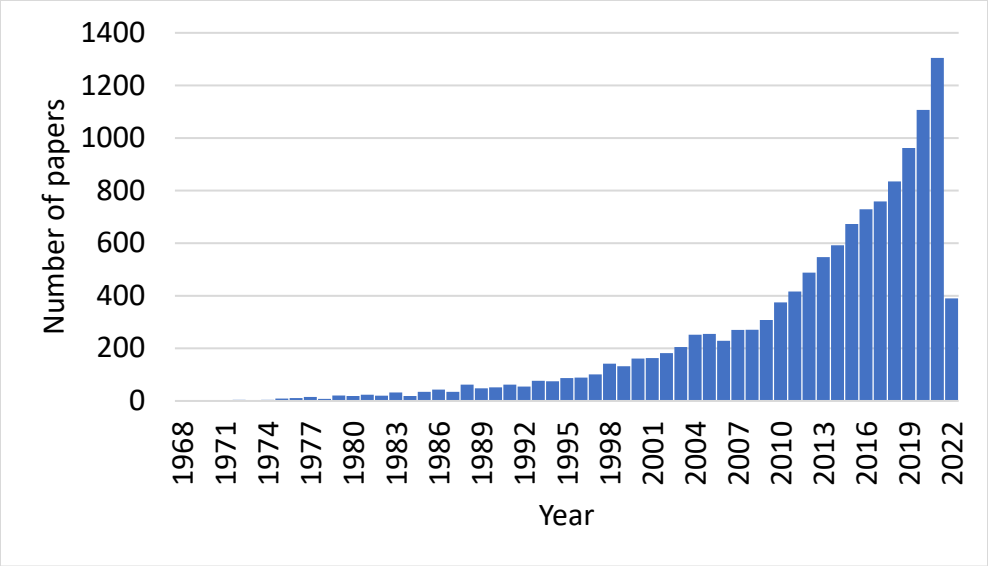


Figure 1-2: Publications in Pubmed with keyword "cancer associated fibroblast" by year.

In the following literature review, I outline the most current understanding of CAF biology, with specific emphasis on the role of CAFs in altering cancer progression. I then discuss the limitations of current models, as well as the complications of studying CAFs in conventional tissue culture systems. I conclude by proposing that specific features arising from the relationship between CAFs and cancer cells should be included in the next generation of *ex vivo* cancer biology studies and suggest technological approaches currently being developed that may be of value in this area.

1.3. CAFs MODULATE CANCER CELL BIOLOGY

Recent reviews have summarized the results of therapeutic strategies focused on modulating CAF behaviour [32, 44-48]. The general lack of success in this area suggests that we do not yet fully understand the role of CAFs in altering cancer cell biology, and particularly response to therapeutic strategies. It is therefore important to briefly review how CAFs are known to modulate cancer cell growth and drug efficacy, as this will affect patient therapeutic response and ultimately disease outcome.

1.3.1. CAFs as positive regulators of cancer cell growth

CAFs have been shown improve organoid and cancer cell growth [49-51] when compared with fibroblasts culture from normal tissue origin [51, 52]. They are a substantial source of growth factors, cytokines and exosomes, where fibroblast expression of TGF-β [41, 53], growth arrest specific protein 6 (GAS6) [54, 55], fibroblast growth factor 5 (FGF5) [56], growth differentiator factor 15 (GDF15) [57] and hepatocyte growth factor (HGF) [58, 59] promotes invasion and metastasis in cancer cells.

1.3.2. Cancer cells modify drug efficacy

Collectively, CAF subpopulations modify therapeutic efficacy in several ways. First, CAFs are highly secretory cells, altering cancer cell phenotypes through paracrine cell-to-cell soluble signalling: modulating cancer cell stemness [60-62], increasing cancer cell epithelial to mesenchymal transition through TGF-β signalling [41], altering chemotherapeutic responsiveness [63-66], as well as immune evasion through production of chemokine C-X-C motif

chemokine 12 (CXCL12) or stromal cell-derived factor 1 (SDF1) [67-69] and TGF-β [70, 71]. Further, a dense fibrotic stroma is also a common feature of immunotherapy resistant tumors, where signatures of TGF-β induced desmoplasia in the stroma is associated with restricted T cell infiltration into the tumor [72]. Interestingly, dual targeting of TGF-β and immune checkpoint inhibitor, PD-1, is currently under clinical trial and showing some promise in improving the success of immune checkpoint therapies [73]. Second, existing therapies can often create fibrotic and tissue-stiffening side effects, which are thought to be mediated by CAFs. These fibrotic reactions are associated with overall worsened survival [26]. For example, the highly publicized B-Raf inhibitor used to treat advanced melanoma, activates stromal fibroblasts [74-76], while radiotherapy in general increases fibrosis [77]. Hence, CAF behaviour may unintentionally be triggered by conventional therapies, in turn modulating the efficacy of said therapy. Taken together, these findings collectively suggest that CAFs are a crucial player in therapeutic response and ultimately in modulating patient outcome. Understanding the effect of therapeutic agents on CAF function and thus the TME, is evidently crucial for the development of new therapeutic strategies.

1.4. CAF FUNCTIONALITY: COMPLICATIONS WITH STUDYING CAF BIOLOGY

Given that CAFs play a complex role in tumour restraint and growth, studies aimed at understanding cancer initiation, progression, therapeutic response, and drug discovery must incorporate CAFs. Here, I overview CAF functionality.

1.4.1. CAF heterogeneity

CAFs are a highly heterogenous "catch-all" description for several sub-populations of activated fibroblasts that function differently depending on their numerous precursors (i.e., tissue-resident fibroblasts, trans-differentiated endothelial or epithelial cells or bone marrow-derived mesenchymal stem cells) [25, 30, 78, 79], and on the local microenvironmental context (i.e. hypoxia or distance to tumour) [49, 80, 81]. Numerous histological markers are established for detecting fibroblasts in the tumour stroma including fibroblast-specific protein 1 (FSP1, or S100A4), vimentin, αSMA, FAP, platelet derived growth factor receptor-α (PDGFRα), PDGFRβ, desmin and discoidin domain-containing receptor 2 (DDR2)[32, 78]; yet none of these markers are specific for fibroblasts or even activated fibroblasts.

Sufficient evidence for the presence and impact of CAF heterogeneity now exists in both *in vivo* and *in vitro* models [30, 78, 79, 82-84] to support considering this complication in cancer studies. For example, CAF subpopulations can undergo metabolic reprogramming to provide a supportive niche for adjacent cancer cells [85]. Moreover, recent single cell studies have demonstrated the broad heterogeneity of CAFs within individual tumours in mice or humans [86-90]. The evolving

nature of CAF subpopulations makes CAFs difficult to study in culture, as conventional culture methods can select and modify the populations, therefore changing the functional behaviour.

It is likely that the subtypes of CAFs are plastic, with capacity to transition between CAF states; activated fibroblasts are known to exhibit multipotency [91-93], and CAFs are dependent on the microenvironment to influence their subtype [94]. Öhlund et al. demonstrated the interconversion of CAF subtypes based on their proximity to tumour cells [49]; where CAFs in direct contact with tumour cells, respond to TGF-β signalling to form a myofibroblastic CAF (myCAF) subtype, and CAFs further from the tumour respond to tumour secreted IL-1, suppressing TGF-β signalling, to drive an inflammatory CAF (iCAF) subtype [86, 95]. Additionally, the heterogeneity of CAF populations is induced and stabilized by CAF signalling [53]. Current studies focusing on changes in CAF populations are limited, even though understanding the interconversion in response to therapies may be key for better patient outcome.

1.4.2. CAFs and tumour invasion

Second, CAFs play a key functional role in tumour invasion by; secreting proteases that break down ECM to enable cancer cell motility [21, 96, 97], clearing tunnels in the ECM [98], YAP-dependent matrix remodelling [99], and physically pulling cancer cells through heterotypic cell junctions [100] (Figure 1-3). To understand drug efficacy on these CAF functional phenotypes, systems must track the movement of individual cells, a process that has been challenging to scale to high-throughput screens, while maintaining a suitable level of robustness. This is challenged by the fact that typical invasion assays follow cumbersome procedures [101, 102] and have

endpoint readouts with low signal to background ratio [103]. Moreover, they require precise, automated, multidimensional microscopy and analysis software, an expensive addition to these studies.

1.4.3. CAFs are mechanically competent cells

Finally, CAFs are mechanically competent cells that both respond to and change their physical environment, by remodelling the tumour ECM through contraction, secretion, crosslinking and aligning of the surrounding collagen and fibrillar proteins (Figure 1-3) [25]. Given the broadly-established impact of 3D tissue mechanics on biological function [104], this can significantly influence the direct response of cancer tumors to candidate therapeutics. Progressive deposition and remodelling of the ECM by CAFs is associated with disease transformation in human breast cancer [26] and *in vitro* analysis shows that changes in the ECM alter breast cancer aggression [105-109]. Moreover, remodelling also aligns, thickens and straightens ECM fibres, where signatures of this are an independent prognostic indicator of poor disease progression [110, 111]. This remodelling, thickening and deposition of the ECM also contributes to an overall increase in tissue stiffening, shown in mouse models and human patient samples to foster tissue transformation and metastasis [26, 112-114]. Although tissue stiffening is recognized as an important factor in cancer drug discovery [115-117] and promoting chemotherapy resistance [118, 119], current drug discovery models lack the dynamic interplay between CAFs and the ECM. In addition to stiffening the environment, aggressive tumours typically have dense and aligned ECM [26], providing highways for cells to invade and altering cancer cell signalling and behaviour. I believe capturing these mechanical phenotypes arising from CAF inclusion is therefore critical

to generating a more thorough understanding of tumour progression, identifying better stromal targeted therapies, and thereby improving patient outcome.

Together this demonstrates that the functionality of CAFs is key to understanding tumour mechanics and progression, implying that assays with a simple live/dead readout are inadequate. Given the microenvironmental impacts outlined in the previous section, I believe that studies aimed at understanding CAF biology must consider i) the heterogeneity of CAF populations, ii) their role in enhancing tumour invasion, and iii) their contribution to dynamic tissue mechanics (Figure 1-3); all of which have been shown to impact cancer cell proliferation, invasion, immune evasion and drug response, and which cannot be easily recreated in standard tissue culture assays.

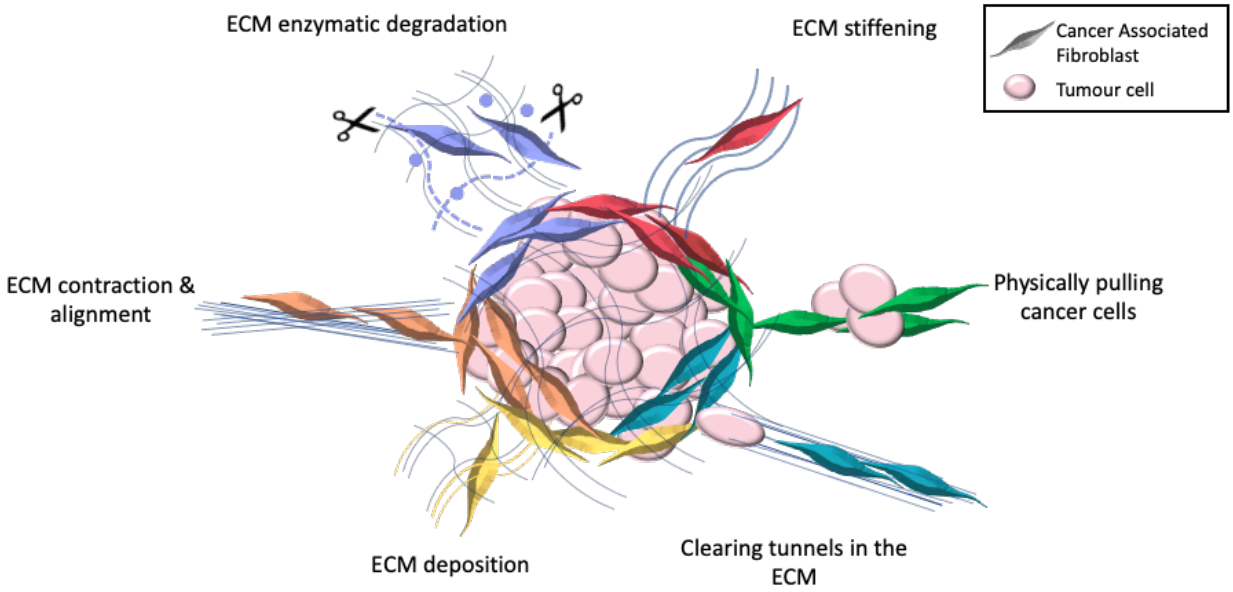


Figure 1-3: The dynamic, functionality of CAFs impacts ECM remodelling and cancer cell invasion. *CAFs (Cancer Associated Fibroblasts) are a highly heterogenous population of cells, with distinct key features that impact cancer progression. CAFs are responsible for dynamically modulating the extracellular matrix (ECM), though contraction and alignment (orange), ECM deposition (yellow), matrix stiffening (red), and enzymatic degradation (purple). CAFs also play a key role in modulating tumour invasion by clearing tunnels in the ECM (blue), or physically pulling cancer cells through cadherins junctions (green).*

1.5. CURRENT MODELS CAPTURING CAF FUNCTIONALITY

The biology of CAFs has been studied using a variety of strategies ranging from conventional 2D culture or histology sections, to mouse models and ex-vivo tissues slices [32]. Current consensus is that 3D models are essential for studying CAFs, as they support the formation of oxygen, nutrient, and growth factor gradients similar to those that occur *in vivo* [120, 121]. They also enable the formation of 3D spatial cellular organization, so that cells simulate bidirectional cell-cell and cell-ECM interactions critical for evaluating stroma-mediated effects on cancer development and progression [122]. Moreover, 2D tissue culture plastic presents mechanical and topographical cues that alter fibroblast behaviour [123], and renders CAFs less secretory than in 3D [124]. Given this established knowledge, I have limited this section to 3D models that incorporate CAF activity for drug discovery.

1.5.1 Mouse Models

Mouse models have been used extensively to build an understanding of CAF function *in vivo*. These models demonstrate that non-specific deletion of CAFs or fibrosis causes rapid tumour progression rather than suppression [24, 125-127]. These findings outlined the foundation of future studies, promoting researchers to focus on altering CAF behaviour rather than ablating CAFs altogether. Although useful, mouse models are far from a perfect system. In co-injection models, where human cancer samples are introduced into the mouse, host-derived fibroblasts will outgrow the co-injected CAFs, leading a study to focus on the interaction of mouse fibroblasts with foreign tissue [32]. With transgenic mouse models, activation of fibroblasts relies on a Cre-

driver, yet no CAF-specific Cre driver exists, so intended CAF inhibition or ablation leads to off-target cellular effects. Moreover, they are extremely low-throughput and not a direct parallel to human disease progression.

1.5.2. Organotypic Culture Systems

Controlled *in vitro* studies of CAF biology are most commonly performed today using low throughput, 3D, collagen or Matrigel ECM scaffolds with tumour organotropic cultures. Mixing epithelial tumour organoids and fibroblast cells in 3D matrices supports CAF-induced improvements in organoid passaging capabilities and enhanced cellular growth through direct cell-cell contact [49, 50, 128-130], highlighting the symbiotic interactions between tumour cells and CAFs. Using an organotypic culture system where cells are seeded on top of a 3D matrix, CAFs have also been shown to enhance ECM remodelling in a manner that supports tumour cell invasion [98, 99]. Moreover, conditioned media from CAF cultures can enhance tumour growth, invasion, and resistance [38, 65, 131], without dynamic cell-cell interactions**.** The use of CAFs instead of normal fibroblasts in these systems is essential; In both premalignant and malignant mammary epithelial cells, CAFs promote epithelial to mesenchymal transition, while normal breast fibroblasts favour the maintenance of epithelial morphology and constrain metastasis [132], therefore altering therapeutic response.

While these organotypic approaches have defined key roles for CAFs in tumour biology, they are limited in throughput. To increase experimental throughput with minimal biological source material, microfluidic systems have been developed for CAF and cancer co-culture studies. These

devices have been used to demonstrate increased cancer cell growth and invasion into physiologically-relevant matrices [133, 134], as well as response to chemotherapy [135].

1.5.3. 3D Bioprinting

3D bioprinting is also gaining traction in studying CAF biology, as it allows the formation of precisely arranged cells within tissue-like structures, while simultaneously controlling the mechanical properties of the bio-printed ECM [136]. These specific capabilities allow the formation of realistic culture environments important for physiologically relevant CAF function. Printing lung cancer epithelial cells and CAFs in a physiologically relevant matrix stiffness [137], demonstrate that robust and manipulable *in vitro* models of human tumours can be bioprinted. Furthermore, Langer *et al.* successfully printed cancer cells, fibroblasts and epithelial cells, demonstrating that distinct microenvironments that differentially effect proliferation, ECM deposition and migration, can be recapitulated [138]. This demonstrates that these models can be used to interrogate complex tumour-stromal interactions in physiologically relevant and manipulable environment. However, the application of these as high-throughput screening methods, is limited by the availability of primary cells and cell detection methods (i.e. imaging techniques to decipher cell types).

These studies collectively demonstrate that the relationship between CAFs and cancer cells is both symbiotic and dynamic. While these studies have led to significant gains in the understanding of tumour biology, future cancer discovery models need to better encompass the

functionality of CAFs and their influence on tumour drug response. In this next section I aim to elucidate the key considerations for future studies.

1.6. MOVING FORWARD: EMERGING TECHNOLOGIES

Several emerging technologies from various fields might allow us to bridge the gaps between cancer drug discovery and patient benefit by incorporating CAFs. While there have been major advances in recent work aimed at targeting CAFs and the TME, implementing these approaches into next-generation high-throughput screening will improve overall drug efficacy. I highlight emerging strategies to improve the drug development process by incorporating CAFs via high-throughput organoid co-cultures (1.6.1), conducting assays in matrices that consider realistic mechano- and biological elements (1.6.2), and integrating techniques designed to measure functional CAF behaviours in living cultures (1.6.3) (Figure 1-4).

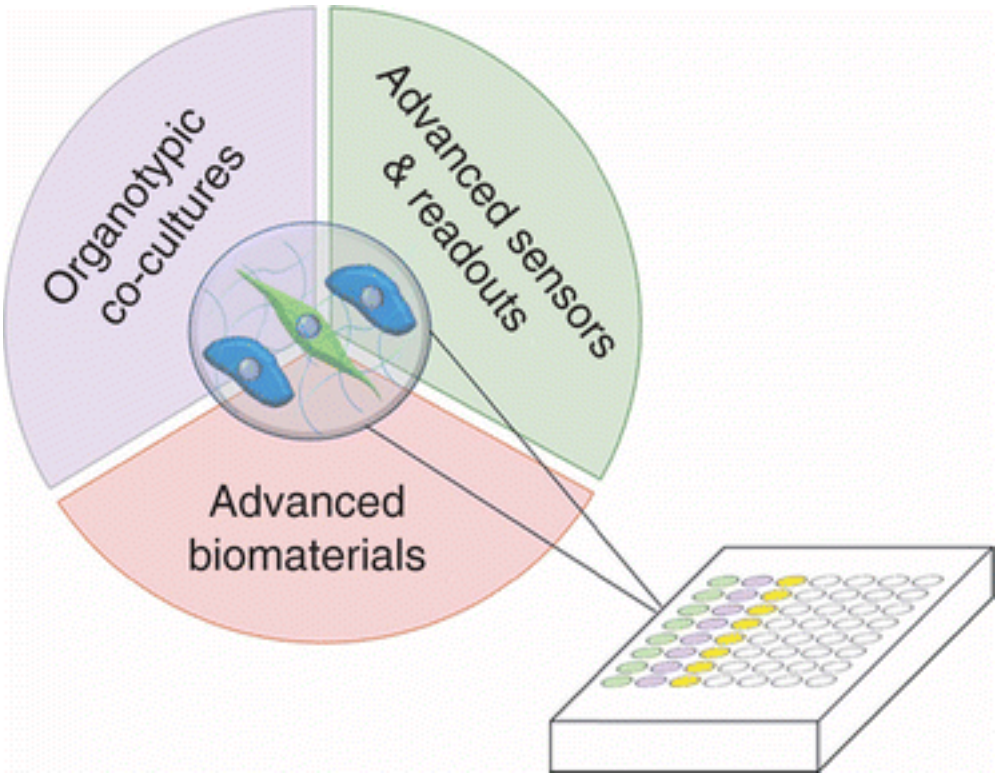


Figure 1-4: Overview of emerging technologies that could be implemented in cancer drug discovery to improve translational impact.

1.6.1. Organoid co-cultures

Patient cancer cell derived organoids have gained increased traction in drug discovery. While they have a 3D, functional ECM for the cancer cells to interact with, conventional cancer drug studies with organoids lack stromal CAFs. Since CAFs play a key role in reshaping the TME, I argue that the addition of CAFs to such organotypic cultures is essential. In fibrosis, by incorporating multiple cell types, a clear resemblance between the *in vitro* cultures and human disease pathophysiology is possible [139, 140]. Similarly, in *in vitro* models of liver fibrosis, 3D multicellular tissues enable preclinical screening of antifibrotic drugs [141, 142], further highlighting the importance of the microenvironment in drug screening. Multiple commercially available systems now exist for high-throughput multi-cellular, physiologically relevant *in vitro* assays. Some examples of these include; i) organ-on-a-chip systems for mechanically realistic lung-blood barriers [143], ii) 3D co-culture chips that support barrier integrity-, transport-, and migration assays [144], iii) tissue culture force sensors to measure human heart health [145] and iv) bioreactors to model human pulmonary fibrosis [146]. The use of these assays would allow for the interrogation of complex biological questions involving cell-cell, and cell-ECM interactions that would encompass the dynamic invasive and mechanical changes induced by CAFs.

1.6.2. Advanced Biomaterials

Physical characteristics of the tissue ECM vary substantially *in vivo*, with changes in fiber length, thickness, density and organization. Given that these changes are induced largely by CAF remodelling, building models that recapitulate the mechanobiological elements of the surrounding TME will reduce the need for CAFs within the system. For example, the use of pre-

aligned matrices [147] or fibroblast preconditioned matrices could be used for invasion assays incorporating the dense and aligned matrix highways seen in aggressive tumours [26]. Advanced biomaterial formulations that consider these factors may therefore capture the mechanical effect of CAFs, without the need to obtain and include live CAFs themselves. While this approach would not capture the dynamic interactions between CAFs and cancer cells, the ability to recreate this important phenotype may improve translational screening efficiency and translational realism while maintaining the assay robustness required for drug screening technologies.

Advanced biomaterials can be tailored *de novo* to present specific characteristics [148], or can be used in blended formulations to modify the properties of existing materials. For example, Matrigel is well-established in many organoid culture protocols, but is challenging to mechanically tune for specific applications. Interpenetrating polymer networks such as gelatin[149-151], hyaluronic acid[152-154], or alginates [117] may be used as a supporting network to modulate substrate stiffness to physiologically relevant levels, while avoiding any modifications to critically important ligand composition or density.

In addition to linear elastic modulations, physiological ECM also exhibits more complex material behaviours such as stress relaxation, or viscoelasticity, parameters proving to be critically important in designing matrices for drug discovery [104]. For example, it has been shown that use of soft substrates with stress relaxation in 3D, promotes cell spreading, fibre remodelling and focal adhesion formation [155-157], emphasising the importance of incorporating physical cues from the ECM in regulating cellular phenotype and therefore drug response. Additionally, human

breast tumour samples exhibit ECM plasticity [158], a permanent deformation of the ECM. Given that, fibroblasts can produce stresses large enough to permanently deform the biomaterials [159, 160], incorporating these forces is critical to recapitulating the effect of CAF functionality, even in their absence.

The contribution of the ECM is more than just mechanical, and due to the diverse range of proteins, proteoglycans, growth factors and other enzymes, it presents a wide range of biological cues to the cells. The use of decellularized ECM (dECM) is well studied in idiopathic pulmonary fibrosis, where it activates myofibroblasts [161] and alters fibroblast gene expression [162]. By implementing this in gut models for intestinal fibrosis it increases the fidelity of disease modelling [163] and the throughput of drug screening [164]. However, the systematic use of dECM is not ideally suited for highly systematic drug screening processes in the context of cancer; biological material available for such assays is limited to the size of the excised tumour and tends to largely vary in composition from patient to patient. While it has proven reliable in other systems, the use of dECM may therefore only be relevant in the context of personalized cancer therapeutic screenings.

1.6.3. Advanced Readouts

If CAFs primarily modulate tumor response via mechanical activity, studies to assess the extent to which CAFs remodel the ECM, exert mechanical forces, and mechanically tune their surroundings will grow in importance. Emerging microscale-engineered technologies that allow quantitative measurements of mechanical changes in tissues, may prove an effective tool in

understanding the changes made to the environment by CAFs, to better understand and ultimately simulate their activity. Several recently developed technologies can provide insight into fibroblast behaviour at this extremely local length-scale. Asmani *et al.* developed a 3D fibrotic microtissue array, in which 3D-cultured fibroblasts remodel the surrounding matrix to deform micro-engineered pillars that anchor the matrix to the substrate. Analyzing the deformation of these pillars provides readouts of forces generated by the CAFs, and therefore enable quantification of fibrosis and drug efficacy testing [87]. This fundamental premise has recently been expanded towards developing dispersible microfabricated sensors that can be applied in a variety of culture contexts to quantify cell-generated mechanical forces [165, 166], mechanical compressive forces [167], residual tissue elasticity [168] and other mechanical properties of tissues [169, 170]. Reducing the size and accessibility of these sensors may hence prove quite valuable in understanding the CAF contributions to the surrounding matrix at the cellular level, to better understand tissue dynamics in response to therapy.

1.7. CONCLUSIONS AND OBJECTIVES

It is evident that tumours can no longer be viewed as static clumps of cancerous cells; the complex and dynamic interactions with the surrounding tumour microenvironment play a key role in altering cancer cell response to therapy and therefore patient outcome. Accumulating work suggests improved strategies could be possible by targeting CAFs, however the disconnect between drug discovery and clinical benefit remains. Therefore, I believe carefully assessing the impact of cancer cell or TME targeted therapies on the mechanical and functional forces within the TME, prior to clinical translation, is critical for narrowing the translational gap. While I propose the use of organotypic co-cultures, advanced biomaterials, and various force sensors as technological advances that will be instrumental in improving the drug discovery pipeline, here I have focused on using advanced biomaterials to study patient derived cancer associated fibroblasts in a physiologically relevant 3D system.

1.7.1 Rationale and objective of thesis

Recent advances in detection and treatment have improved breast cancer survival rates; however, TNBC remains difficult to treat and therefore associated with poor outcome. Although the interactions of the TME and more specifically CAFs are now considered key targets for new interventions in cancer treatment and prevention, there is a clinical disconnect. Though many studies have suggested a putative link between CAF-driven disease progression and microenvironmental mechanics [26, 110, 111, 171-173], the precise role of CAF matrix remodelling in promoting CAF invasion and downstream metastatic dissemination remains poorly understood. Therefore, the overall objective of this thesis is to investigate the functional

role of patient derived CAF populations and response to microenvironmental mechanics in promoting 3D cell invasion in a patient specific manner. The specific aims include:

1. Characterize the role of internal tissue stiffness in promoting 3D cell invasion of patient-derived heterogenous CAF populations.
    a. In Chapter 2.1 of this work, I develop novel micro-engineered approaches capable of working with limited primary CAF populations, to characterize the invasive potential of CAFs derived from human TNBC patients. I systematically develop a multi-material, multi-stage sequence of *in vitro* culture models to separately mimic and manipulate biophysical features of matrix remodeling, stiffening and invasion during CAF-mediated disease progression in a fibrotic foci-like culture model. Strikingly, I find that in contrast with analysis of histological markers, functional CAF invasive patterns and sensitivity to extracellular mechanics correlates with patient metastatic state, where CAFs derived from patients with axillary lymph node metastatic disease display no mechanosensitivity to their surroundings.

2. Identify the mechanogenetic link between fibrotic foci stiffness and CAF invasion.
    a. In Chapter 2.2, pseudo-fibrotic foci cores were expanded to a large-scale assay for RNA sequencing analysis. Using this, I identify unique transcriptional signatures to support findings in chapter 2.1: CAFs from patients with and without lymph node metastatic disease at time of surgery have distinct transcriptional responses to

changes in fibrotic foci stiffness. Here, I determine AHR gene expression and AhR protein levels are significantly increased in CAFs from metastatic patients.

3. Identify the functional role of AhR in facilitating CAF invasion.
    a. In chapter 2.3, I demonstrate through inhibition and activation of AhR, that AhR facilitates CAF invasion. The findings demonstrate that CAFs retain characteristics representative of the associated human tumour in culture. Using external patient datasets, I validate that high AHR expression is associated with poor overall survival, metastatic dissemination, and CAF motility.

Together these findings demonstrate that functional patient-specific CAF phenotypes are retained in culture and suggest that CAF activity can be used to predict patient response to microenvironment targeted therapies. These studies hence support AhR as a novel mechanobiological marker with which to stratify patients, and a target to develop precision treatment strategies.

CHAPTER 2 : RESULTS

2.1. CHARACTERIZE THE ROLE OF INTERNAL TISSUE STIFFNESS IN PROMOTING 3D CELL INVASION OF PATIENT-DERIVED HETEROGENOUS CAF POPULATIONS.

2.1.0 Introduction

Here, I hypothesize that mechanical stimuli arising from CAF extracellular matrix remodeling, plays an important role in stimulating CAF invasion, a phenotype enhancing cancer cell invasion and metastatic progression. By using TNBC patient derived CAFs, I test their contractile and invasive abilities using a simple 3D tissue-engineered model to recreate the collagen- and CAF-dense fibrotic foci seen *in vivo*. I find that CAFs exhibit disease-specific contractility and invasive capabilities, suggesting that local metastasis may be influenced by both CAF type and local microenvironment. To test the role of contraction induced stiffening in promoting invasion, I engineered fibrotic foci cores with stiffnesses of cancer-free breast tissue and early-stage breast tumours. This approach effectively separates fibrotic foci formation from invasion and allows independent control over matrix mechanical cues presented to CAFs. Using this, I demonstrate that CAFs retain mechanosensitivity only at non-invasive stages of disease and become desensitized to mechanical cues as TNBC progresses. This work highlights the role of CAF-induced mechanical cues within the tumour microenvironment and suggests mechanisms responsible for the clinical failure of drugs aimed at targeting CAF activation.

2.1.1 Histological CAF abundance does not correlate with disease state.

CAFs were isolated from four primary grade III, chemotherapy-naïve triple negative breast cancer tumours obtained from surgical resection with informed patient consent. Primary tumours were resected from two patients with axillary lymph node metastasis at time of surgery (labelled CAF-pm1, CAF-pm2) and two with no evidence of nodal dissemination at surgery, and no subsequent recurrence or metastasis within 4 years (labelled CAF-pn1 and CAF-pn2) (Figure 2-1A; detailed clinical information Table 2-1). To characterize the tumour-CAF composition of each tumour, sections of formalin-fixed, paraffin-embedded (FFPE) tissue samples were assessed by H&E to confirm the presence of fibrotic regions within each tumour (Figure 2-1B, *Si Appendix Figure 1*). FFPE sections were immunostained for epithelial cells (via pan-cytokeratin) and activated fibroblasts via smooth muscle actin (αSMA), a common marker of myofibroblasts and desmoplasia (Figure 2-1C) [99].

Although each tumor displayed regions of fibrosis (Figure 2-1B) and expression of both markers (Fig. Figure 2-1C), no consistent significant differences in percentage of stromal area or percentage of active fibroblasts, as defined by panCK and αSMA respectively, were found across whole-tumour sections from all patients (Figure 2-1D). Although activation of CAFs and ensuing desmoplastic responses are associated with clinical outcome, the tumours in our study cannot be differentiated based on relative amount of stroma or presence of activated fibroblasts. However, whether functional heterogeneity of the CAFs might account for variation in disease progression cannot be addressed by static histological differences at defined time points.

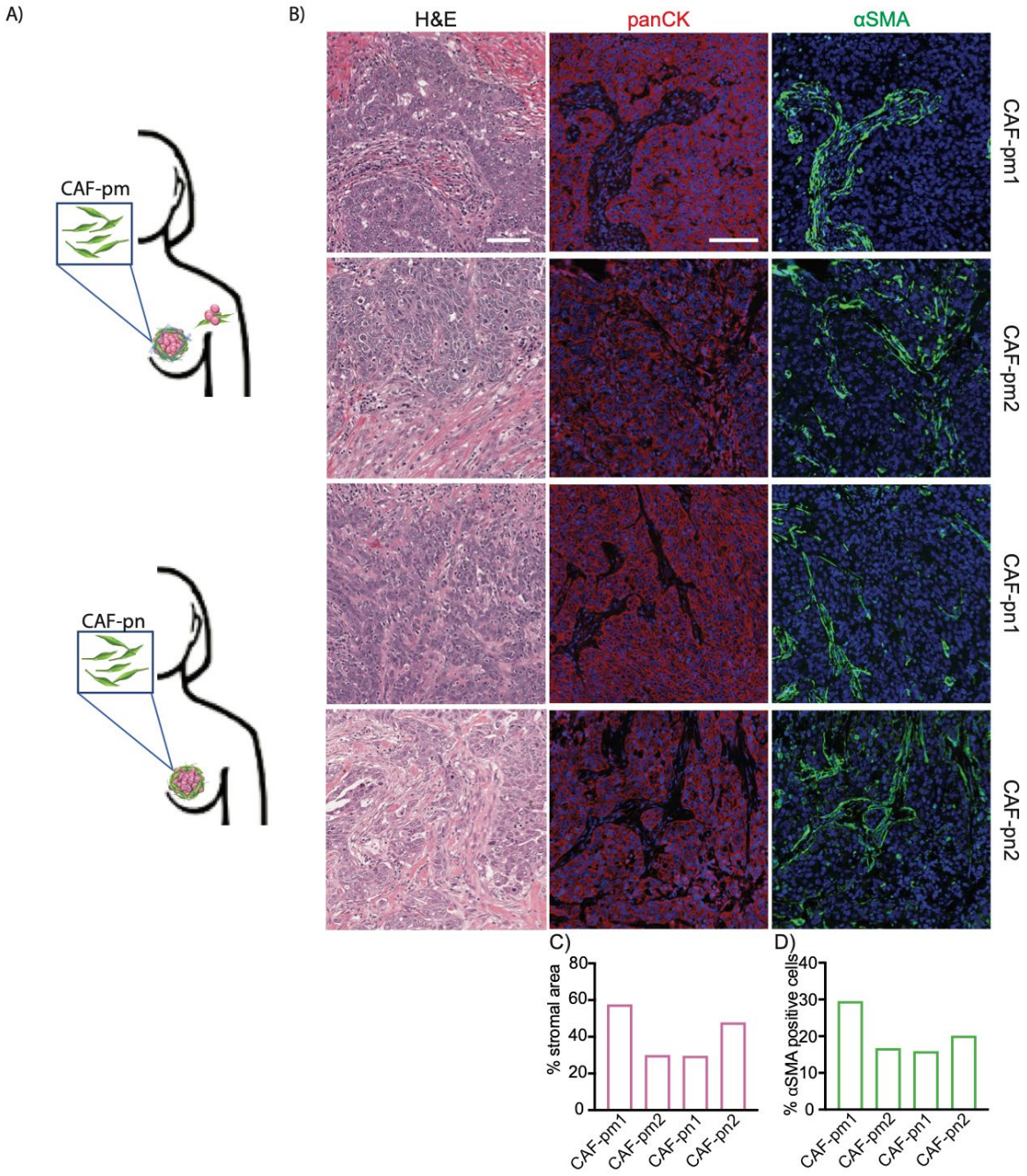
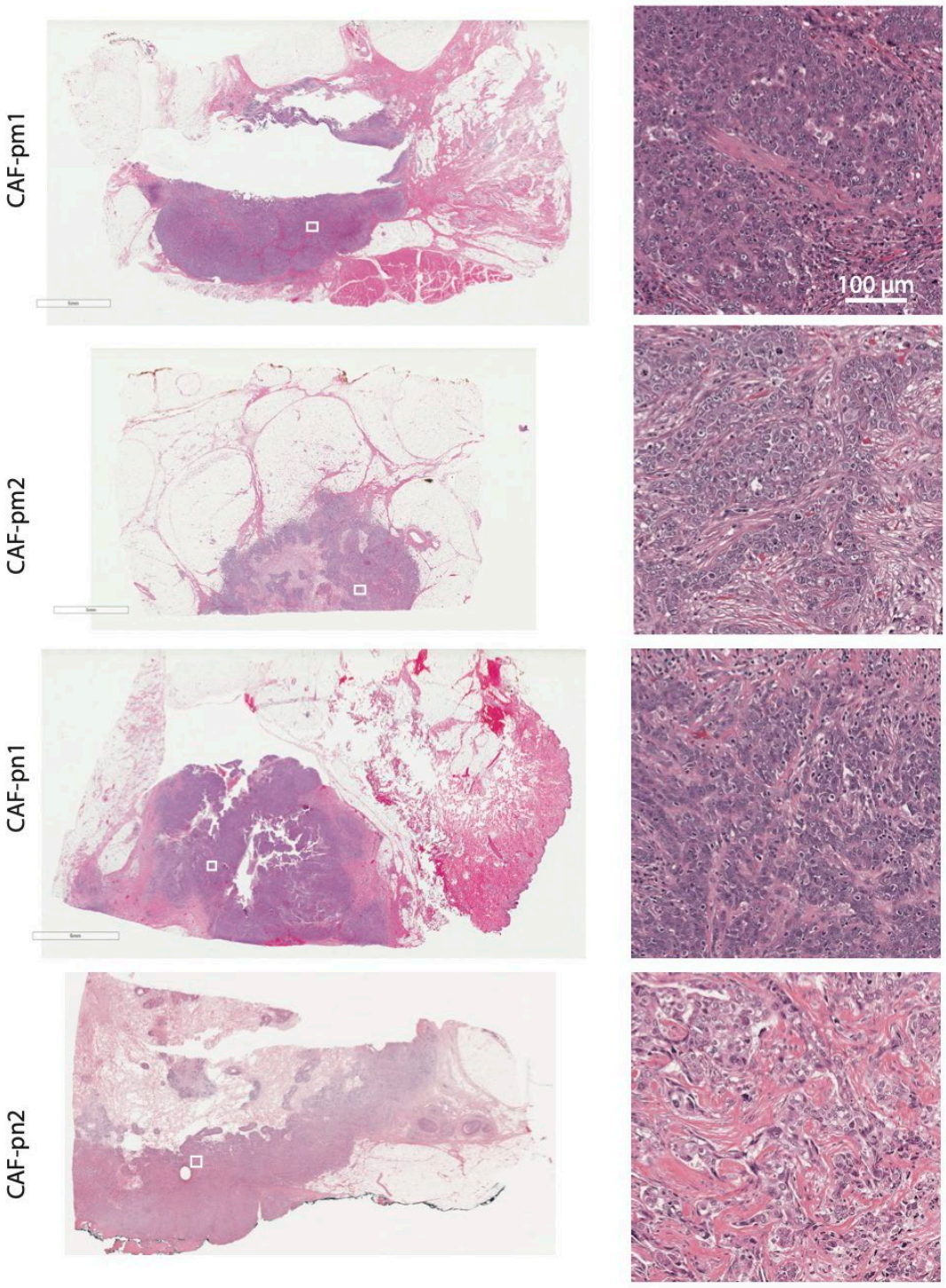


Figure 2-1: Quantifying epithelial content and fibroblast activation does not distinguish patients based on disease progression*. A) Schematic depicting patient status at time of surgery. All CAFs were derived from the primary tumour. B) H&E staining of human patient tumour FFPE sections (Scale bar, 50 µm). C) Multiplexed immuno-fluorescence imaging of human patient tumour FFPE sections, with anti-panCK and anti-αSMA. (Scale bar, 100 µm). D) Quantification of percentage of stromal area by Tissue Classified Add-on algorithm (Halo) and percentage of αSMA positive stromal cells across whole tumour section (n=1).*



Si Appendix Figure 1: Immunohistological staining (H&E) of human patient whole tumour sections.

Table 2-1: Patient clinical information

Patient ID	GCRC-1834	GCRC-1840	GCRC-1882	GCRC-2061
Patient Notation	**CAF-pm1**	**CAF-pm2**	**CAF-pn1**	**CAF-pn2**
Age	72	71	80	38
Tumour size at definitive diagnosis (surgery) (mm)	20	30, 22	40	40
Histological Subtype	IDC-NOS	IDC-NOS	IDC-NOS	IDC-NOS
Nodal Status	Positive	Positive	Negative	Negative
Staging	T1N1	T2N1	T2N0	T2N0
Breast cancer subtype	Basal	Basal	Basal	Basal
Subtype by PAM50	Basal	Basal	Basal	Basal
ER	<1% of cells	Neg	Weak (15% cells)	Low pos (2%)
PR	<1% of cells	<1% of cells	Neg	Neg
Her2_FISH	Neg	Neg	Neg	Neg
Grade	III	III	III	III
Menopausal status	post	post	post	unknown
Neoadjuvant therapy	none	none	yes* (femara)	yes*(tamoxifen)
Adjuvant radiotherapy	none	yes	no	no
Notes			* patient refused chemo but wanted anti-hormone drugs	* for prior IDC on opposite breast

2.1.2. CAFs isolated from primary TNBC with metastatic dissemination are significantly more contractile and invasive.

To assess the functional activity of the CAFs from breast tumours, CAFs were isolated and minimally expanded from the primary tumours of patients with lymph node metastasis (CAF-pm1, CAF-pm2) and without lymph node metastasis (CAF-pn1, CAF-pn2). To determine whether functional contractile and invasive characteristics of the isolated CAFs correlate with disease state, we developed a simple 3D culture model to recreate collagen- and CAF-dense foci within a 3D tissue, an architectural phenotype often observed with adverse prognosis [174-177]. CAF invasion is necessary to facilitate tumour cell invasion [98-100], and therefore a read-out for downstream metastasis. Fibroblast-laden collagen gels were bioprinted using a previously-developed aqueous two-phase printing technology [120, 178], and allowed to contract over two days to form a densified tissue droplet, reminiscent of high-density fibrotic foci that occur in the breast cancer tumour microenvironment [177]. Since activated mouse fibroblasts derived from malignant tissues have previously been shown to have increased contractile capacity over fibroblasts derived from normal or premalignant tissues [99, 179], we first asked whether human CAFs isolated from the primary tumour site can differentially remodel collagen in a disease-specific manner. To assess this, we quantified contraction of the engineered fibrotic foci over 48 hours of culture. While all CAF populations were able to contract the collagen, CAFs from patients with lymph node metastasis were significantly more contractile than CAFs from non-metastatic conditions (Figure *2-2*C).

After contraction, these foci were then embedded into an acellular, background collagen matrix (Figure 2-2A) and imaged via confocal fluorescence microscopy of fixed samples to quantify and characterize CAF invasion into the surrounding collagen matrix. CAFs from patients presenting with lymph node metastasis invaded as strands of cells, while the few invading CAFs from non-metastatic disease migrated individually (Figure 2-2B). CAFs that invaded into the low-density collagen surroundings were spindle-like in morphology (Figure 2-2 B), consistent with in vivo observations [25]. Based on the number of invasive cells (Figure 2-2D, Si Appendix Figure 2) and the distance to which they invade (Figure 2-2E), CAFs isolated from primary tumours with axillary lymph node metastasis are significantly more invasive than those from primary tumours that did not metastasize. Together this correlates with contractile behaviour, such that highly invasive CAFs are more contractile. These differences suggest that downstream metastasis-promoting invasive behaviour may be influenced by both cell type and local microenvironmental conditions.

These pro-metastatic invasive behaviours are reproducible across biological and technical replicates, and are consistent with both the known heterogeneity of CAF populations [78], and the emergence of distinct CAF subtypes at various stages of disease [25]. Taken together, this data provides evidence that invasive CAF populations are distinct and correlate with patient disease status, and that a greater proportion of CAFs in the primary tumour with lymph node metastasizing disease are functionally invasive. Whether the observed changes in invasive behaviour are intrinsic to the contractile phenotype of the CAF population remains unclear.

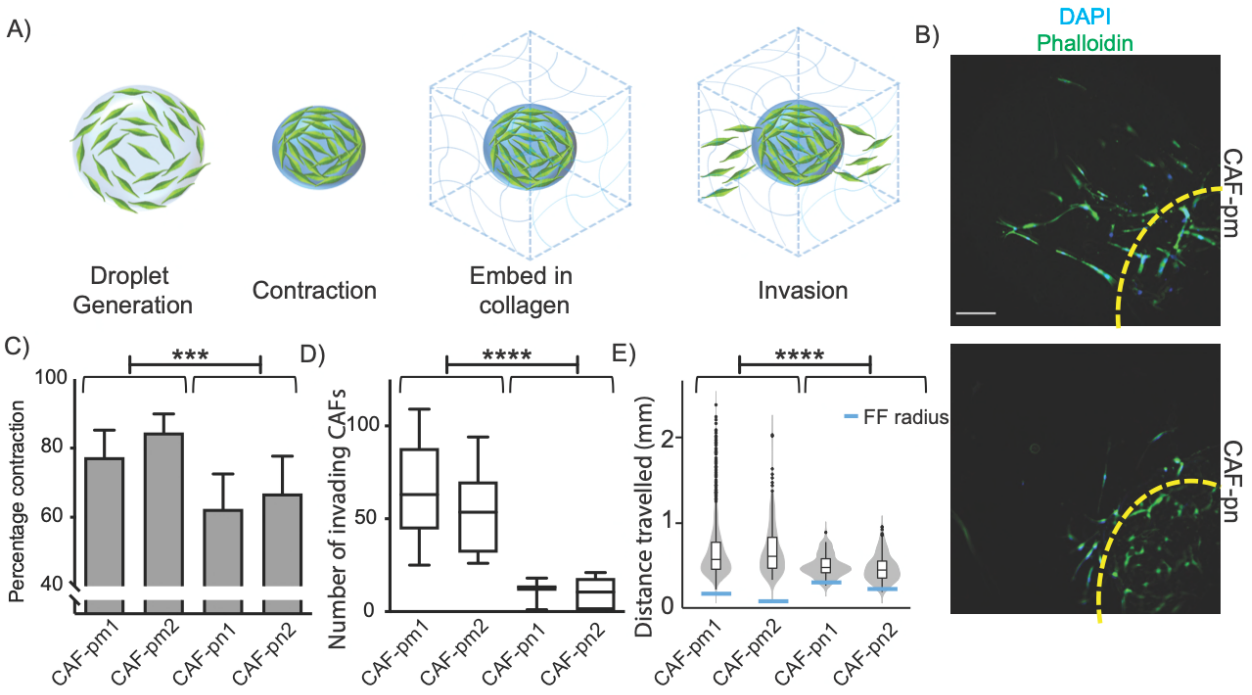
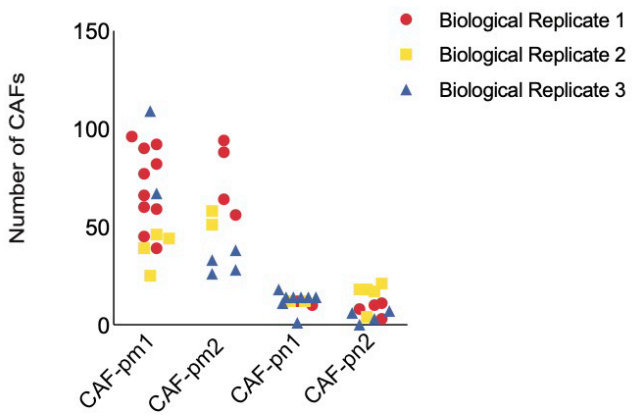


Figure 2-2: CAFS isolated from primary TNBC that display metastatic progression exhibit greater contraction and invasive potential into the surrounding collagen matrix from engineered fibrotic foci. *A) Schematic depicting contraction and subsequent invasion assay; cell-dense collagen droplets are printed, contract and are embedded into Type I collagen gels and assayed for invasion after 24 hours. B) Imaging of filamentous actin (green) and nuclei (blue) in 3D culture section demonstrates CAF invasion is specific to disease state, with increased collective invasion in CAFs associated with metastatic disease (Scale bars, 200µm). C) Collagen droplet contraction after 48 hours, normalized to the original size of the droplet. D) Number of CAFs invading from the contracted collagen gel into the surrounding matrix, (n=3 biological replicates; *p<0.05 by one-way ANOVA with Newman-Keuls post-hoc test for pairwise comparisons), and E) distance travelled by invading CAFs from the centre of the contracted droplet (n=3 biological replicates; *p<0.05 by two-tailed unpaired nonparametric Mann-Whitney Test), after 48 hours in collagen. Box plots indicate median and first to third quartile, and whiskers span the range. Blue lines indicate average contracted droplet radius.*



Si Appendix Figure 2: Quantification of the number of CAFs invading from contracted collagen gel into surrounding matrix, after 48 hours in bulk collagen by biological replicate. Each dot represents one technical replicate.

2.1.3 Engineered pseudo-fibrotic foci with tunable stiffness.

To better understand the specific microenvironmental factors that may drive invasive behaviour, we considered several deconstructed cues arising within the contracted fibrotic foci. The collagen contraction process results in simultaneous microenvironmental changes in matrix stiffness [180], porosity [181], cell-cell contact [182, 183], soluble factor secretion [184-186], and cell alignment [111, 171, 187]; each of which may have competing effects on driving invasion [188-190]. Mechanical matrix stiffness cues in particular have been well-established to drive biological processes in disease [191] and breast cancer progression specifically [26], and I therefore hypothesized that highly contractile CAFs generate stiffer fibrotic foci, which can then prompt enhanced invasion. To assess the specific contribution of mechanical rigidity within fibrotic foci, I designed a stiffness-tunable "core" for the engineered foci. We first estimated that collagen gels stiffen from ~100 Pa initial modulus [192] by approximately two orders of magnitude based on the densification of the material during contraction [193]. These rough calculations were consistent with a range of stiffnesses found in cancer-free adipose tissue (~3 kPa Young's modulus; E_{normal} conditions) through early-stage invasion in breast tumors (>10 kPa Young's modulus; $E_{disease}$ conditions) [194]. To recreate these stiffnesses, we generated stiffness-tunable polyacrylamide hydrogel droplets at similar sizes to our fibrotic foci (Figure 2-3D), using an oil/water two-phase system (Figure 2-3A), and functionalized the surface with Type I collagen (Figure 2-3B) to support cell culture. We verified the stiffness of the polymerized droplet using a cantilever-based mechanical characterization system (MicroSquisher; CellScale Biomaterials Testing Inc.; Figure 2-3C). Adhesion of CAFs to the bead surface was assessed via

immunofluorescence of actin (phalloidin) and nucleus (DAPI), and no significant differences were observed in cell area (Figure 2-3E, F) and viability (Figure 2-3G) between E_{normal} and $E_{disease}$ foci.

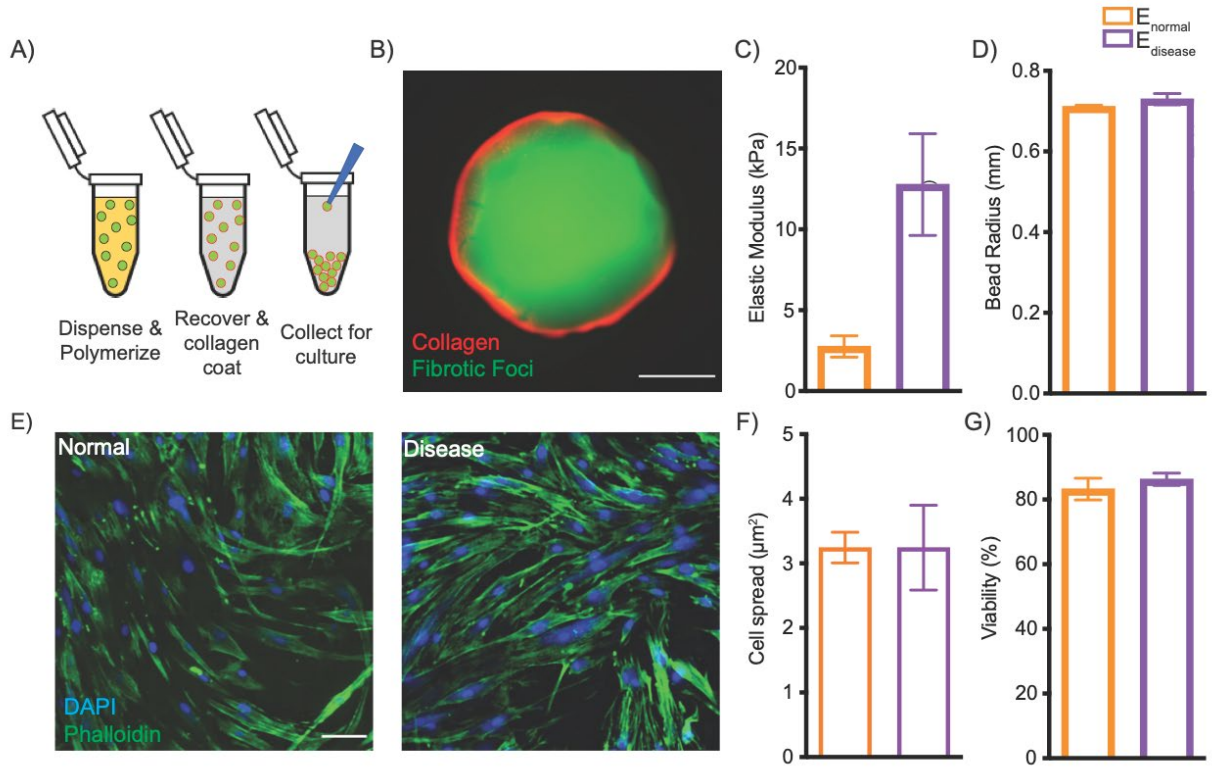


Figure 2-3: Development of a culture model of fibrotic foci cores with tunable internal mechanical stiffness. *A) Schematic depicting polyacrylamide bead formation. Aqueous polyacrylamide components were dispersed in immiscible kerosene phase and allowed to polymerize, coated with collagen and dispensed as an individual bead per well for cell culture. B) Fluorescently labelled (green) polyacrylamide beads were generated and stained for collagen I (red), confirming uniform extracellular matrix coating to support cell attachment and growth. (Scale bar, 500 µm). C) MicroSquisher measurements on engineered cores confirm that stiffnesses represent normal and diseased tissues (3 +/- 0.5 kPa, and 13 +/- 3.1 kPa respectively; n=5). D) Quantification of bead size after swelling confirmed no significant difference in size between E_{normal} and $E_{disease}$ (n=3-4). E) Representative staining of filamentous actin in CAFs (scale bar, 200µm), F) analysis of filamentous actin on cells coating the bead revealed cells spread equivalently on both normal and diseased cores (n=3), and G) cell viability is comparable between both stiffness conditions tested (n=4). Data presented as mean +/- SD.*

2.1.4 Internal stiffness of fibrotic foci regulates CAF invasive potential in CAFs derived from tumours without lymph node metastasis.

CAFs were seeded on E_{normal} and $E_{disease}$ cores, and embedded in collagen to assess invasion into the surrounding matrix (Figure 2-4A). On E_{normal} and $E_{disease}$ foci, CAFs associated with lymph node metastatic disease were significantly more invasive compared to CAFs from non-metastatic disease (Figure 2-4C, *Si Appendix Figure 3*). Interestingly, comparing invasive abilities of each CAF population between normal and disease-stiffened foci demonstrates that CAFs isolated from non-metastatic tumors significantly increased invasive abilities when primed by $E_{disease}$ stiffness cues, but those isolated from tumours that have undergone lymph node metastasis showed no additional stiffness-induced activation (Figure 2-4D, Si Appendix Figure 4). Distance travelled by invading CAF populations was not significantly different across patients or stiffnesses (Si Appendix Figure 4). The stiffness-induced fold change in the fraction of invading cells confirmed that CAFs isolated from non-metastatic tumours were significantly more responsive to increased stiffness cues in the fibrotic foci core than those CAFs from tumours with lymph node metastasis (Figure 2-4D). Taken together, this data suggests that CAFs isolated from tumours that have already undergone lymph node spread are no longer mechanoresponsive or reliant on mechanical stiffness cues for invasion, while CAFs derived from tumours with no metastatic spread remain mechanoresponsive to microenvironmental stiffness cues within the fibrotic foci.

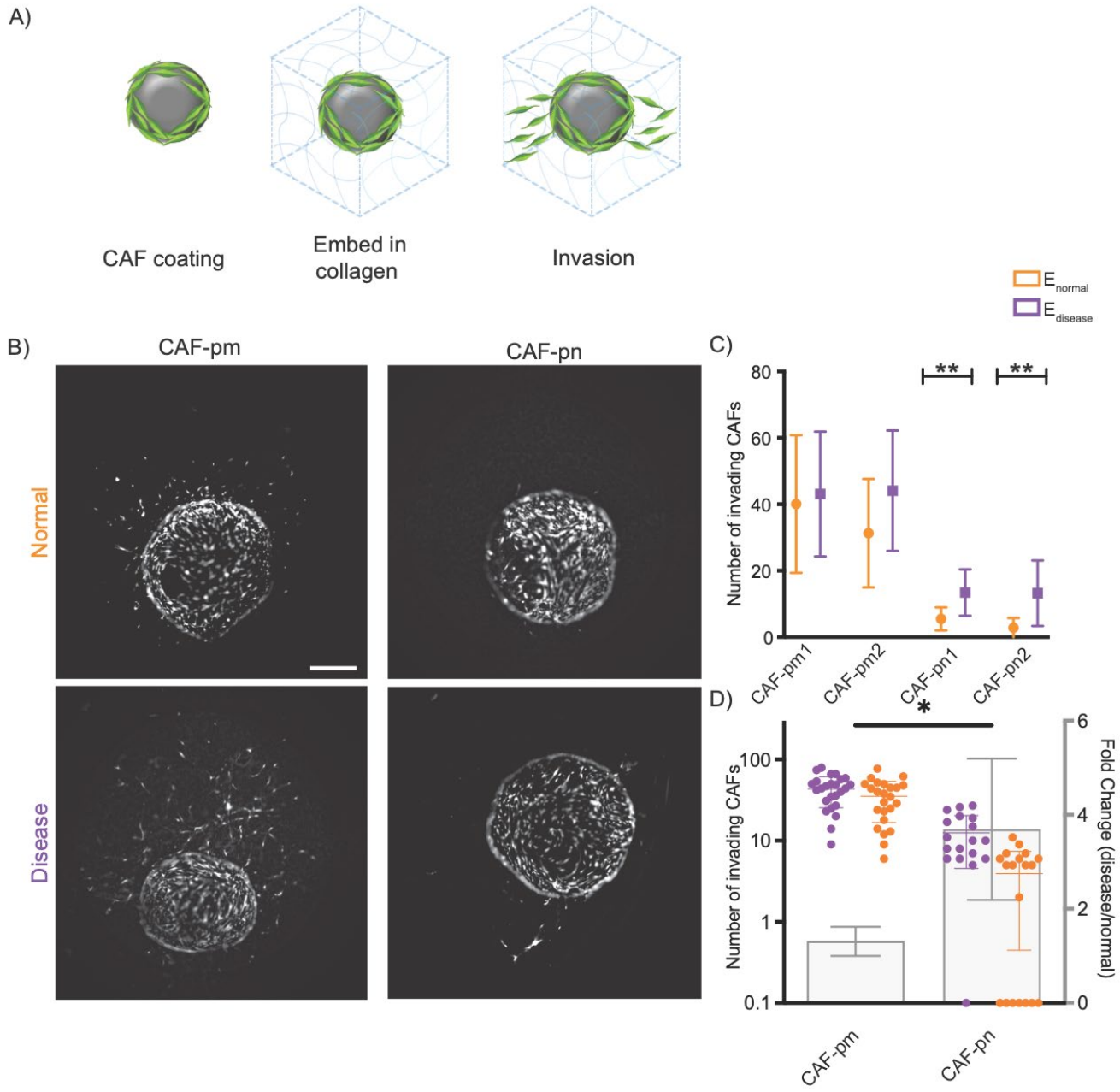
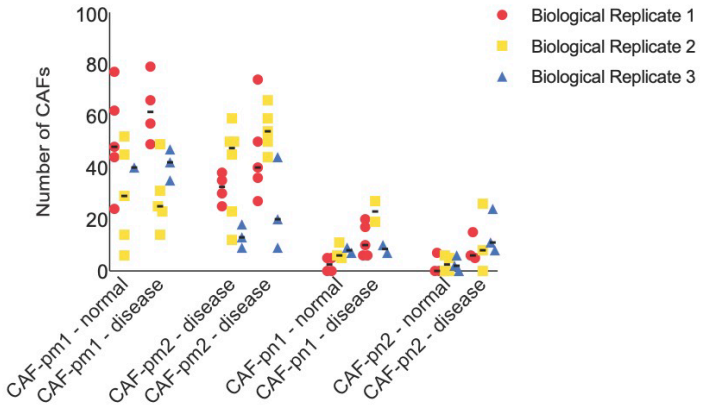
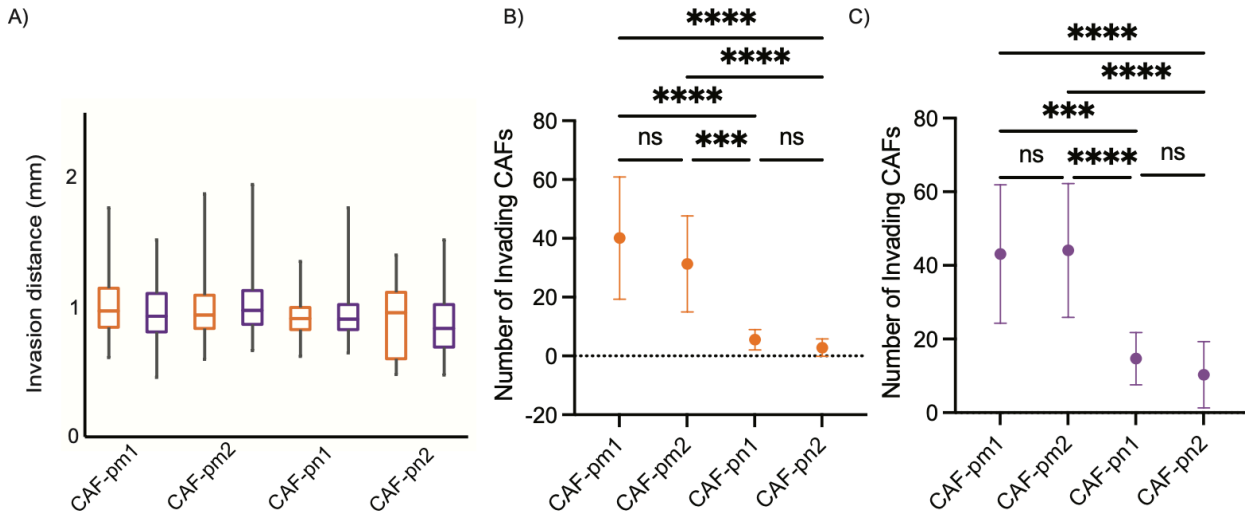


Figure 2-4: CAFs grown on E_{normal} and $E_{disease}$ foci cores embedded in bulk collagen demonstrate loss of mechanoresponsiveness with disease progression. *A) Schematic demonstrating measurements of CAF invasion from engineered fibrotic foci cores with tunable stiffness. B) Representative images of CAFs (DAPI) invading from E_{normal} and $E_{disease}$ foci into bulk collagen matrix. (Scale bar, 500µm). C) Number of CAFs that invade from E_{normal} and $E_{disease}$ foci into the surrounding matrix 48 hours after encapsulation. D) Fold-change in invasion on $E_{disease}$ stiffness polyacrylamide cores relative to E_{normal} stiffness polyacrylamide cores. Each datapoint represents the number of invading CAFs per technical replicate. Grey bars represent the fold change of invasion on diseased to normal polyacrylamide cores. (Data presented as mean +/- SD; biological replicates n=3; * p<0.05 by two-tailed unpaired nonparametric Mann-Whitney Test).*



Si Appendix Figure 3: Quantification of the number of CAFs by biological replicate invading from normal and disease PAA bead into surrounding matrix, after 48 hours in bulk col I.



Si Appendix Figure 4: A) Box-and-whisker plots of distance travelled by invading CAFs from the centre of the bead droplet, after 48 hours in bulk col I. Box plots indicate median and first to third quartile, and whiskers span the range. CAF invasion on B) normal and C) disease stiffened foci.

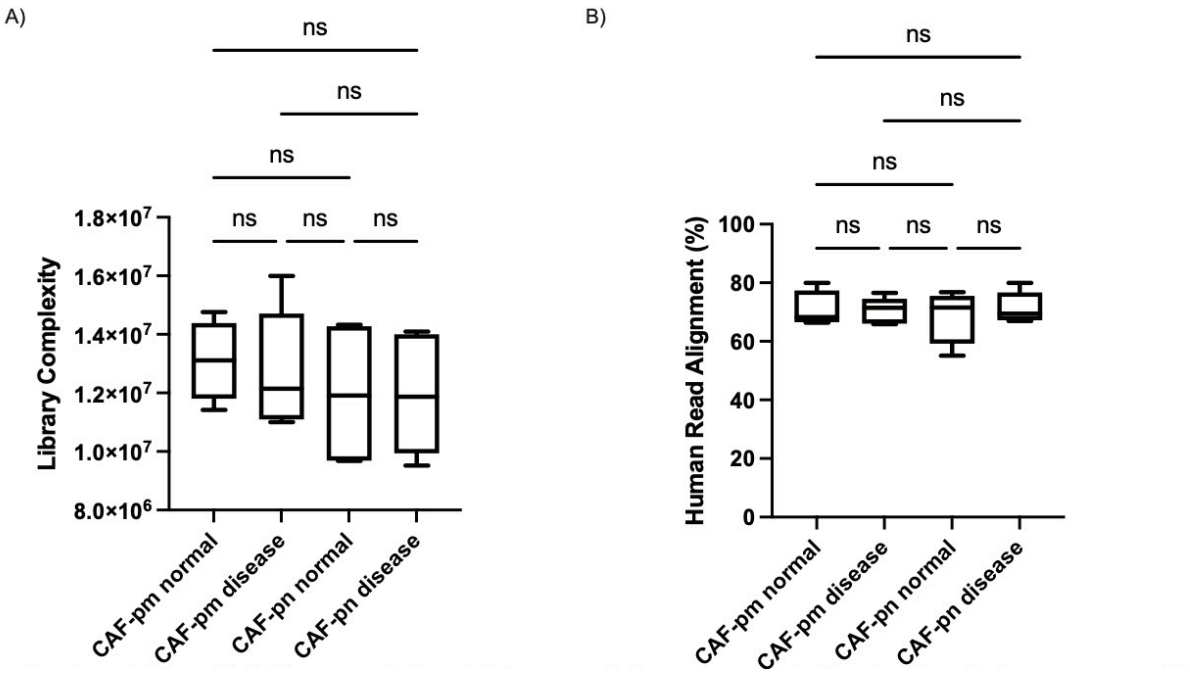
2.2. IDENTIFY MECHANOGENETIC LINK BETWEEN SUBSTRATE STIFFNESS AND CAF INVASION

2.2.0 Introduction

The mechanically tunable 3D invasion assay developed in Chapter 2.1 allows precise control over microenvironmental stimuli and captures the idea of bidirectional stiffness that is present in breast cancer. In this chapter, by scaling up the device size to complete bulk RNA sequencing (RNA-seq), I have identified genes and pathways differentially expressed when CAFs are cultured on E_{normal} and $E_{disease}$ foci cores, as well as between CAF-pn and CAF-pm. We find functional CAF invasive patterns and sensitivity to microenvironmental mechanics correlates with patient metastatic state. Here, we find a correlative link between invasion and the expression of the transcription factor, aryl hydrocarbon receptor (AhR). Previous studies with the transcription factor AhR show a proliferation and migration role in a cell-type dependent manner: Increased AhR levels inhibit proliferation of breast [195], prostate [196], and liver [197] cancer cells and its deficiency promotes epithelial cell migration [198] and metastasis [199]. Interestingly however, in the context of fibroblasts, AhR has been shown to have a regulatory role, where low expression increases cell adhesion and high expression increases fibroblast migration [200, 201]. Here, we find CAFs from patients with non-metastatic disease (CAF-pn) are mechanosensitive, with AhR protein levels that correlate with microenvironmental stiffness, and CAFs from patients with lymph node metastasis (CAF-pm) have high AhR levels independent of stiffness.

2.2.1 CAF-pm and CAF-pn show distinct transcriptional profiles

To determine whether a transcriptional link between mechanosensitivity in early-stage disease (no metastasis) and lack of mechanosensitivity in later-stage (lymph node metastasis) disease, we performed RNA-seq analysis of all 4 CAFs grown on E_{normal} and $E_{disease}$. To obtain sufficient cells for RNA-seq, we scaled up the fibrotic foci cores into flattened devices (Figure 2-5A), coating them with cells and embedding in bulk collagen. After quality control and removal of samples with significantly lower human read alignment a total of 19 samples (CAF-pm E_{normal} = 4, CAF-pm $E_{disease}$ = 5 , CAF-pn E_{normal} = 4 , CAF-pn $E_{disease}$ = 6) were retained for down- stream analyses (Si Appendix Figure 5). Batch effects among the samples were observed and corrected for.



Si Appendix Figure 5: A) Library complexity and B) human read alignment are not significantly different between groups used for RNA-seq analysis.

By comparing the transcriptomes of both CAF subsets on E_{normal} *and* $E_{disease}$, we first identified the differentially expressed genes (DEG) in response to changes in stiffness, in CAFs from patients with lymph node metastasis (CAF-pm) and CAFs from patients with non-metastatic disease (CAF-pn). CAFs from patients with non-metastatic disease had more differentially expressed genes in response to changes in substrate stiffness (1186 for CAF-pm, vs. 3210 for CAF-pn; <0.5 log_2fold, q- value <0.05; Figure 2-5A). This 3-fold reduction in differentially expressed genes in CAFs from patients with lymph node metastasis (CAF-pm) further supports the notion that CAF invasion from later stages of disease are less sensitive to environmental mechanics.

To identify pathways upregulated in each condition, we performed Gene Set Enrichment Analysis (GSEA) of each CAF subset on either substrates of E_{normal} or $E_{disease}$ stiffness (Figure 2-5C). CAFs associated with metastatic disease showed an upregulation in invasion-associated pathways including epithelial-mesenchymal-transition and classical Wnt-beta catenin signaling, genes associated with wound healing and fibrosis[202-206]. In CAFs associated with non-metastatic disease, growth on $E_{disease}$-like substrates of stiffness was enriched for oxidative phosphorylation and mTORC1 signaling suggesting that local microenvironment can drive changes in metabolic state[207] and the upregulation of translation[208], rather than invasion. Given that CAFs associated with non-metastatic disease were invasive on $E_{disease}$, but still not as invasive as CAFs from patients with lymph-node metastasis, it is reasonable that signatures of invasion are not highly enriched for CAF-pn on $E_{disease}$. Interestingly $E_{disease}$ increased TNF-α signaling via NFkB in both CAF subgroups, a signature previously shown to play a key role in driving cancer cell invasion

and in inflammatory CAF formation [40, 53, 60, 209, 210], suggesting microenvironmental stiffness may play a role in influencing TNF-α signaling.

When we compared pro-invasive CAF cultures (CAF-pm E_{normal}, CAF-pm $E_{disease}$, CAF-pn $E_{disease}$) against non-invasive cultures (CAF-pn E_{normal}), fewer genes were uniquely significantly different between these groups (Si Appendix Figure 6). This further supports our previous findings that these cells are functionally distinct in their responses to mechanical cues, and that some consistent differences can be identified even in highly variable patient-specific samples.

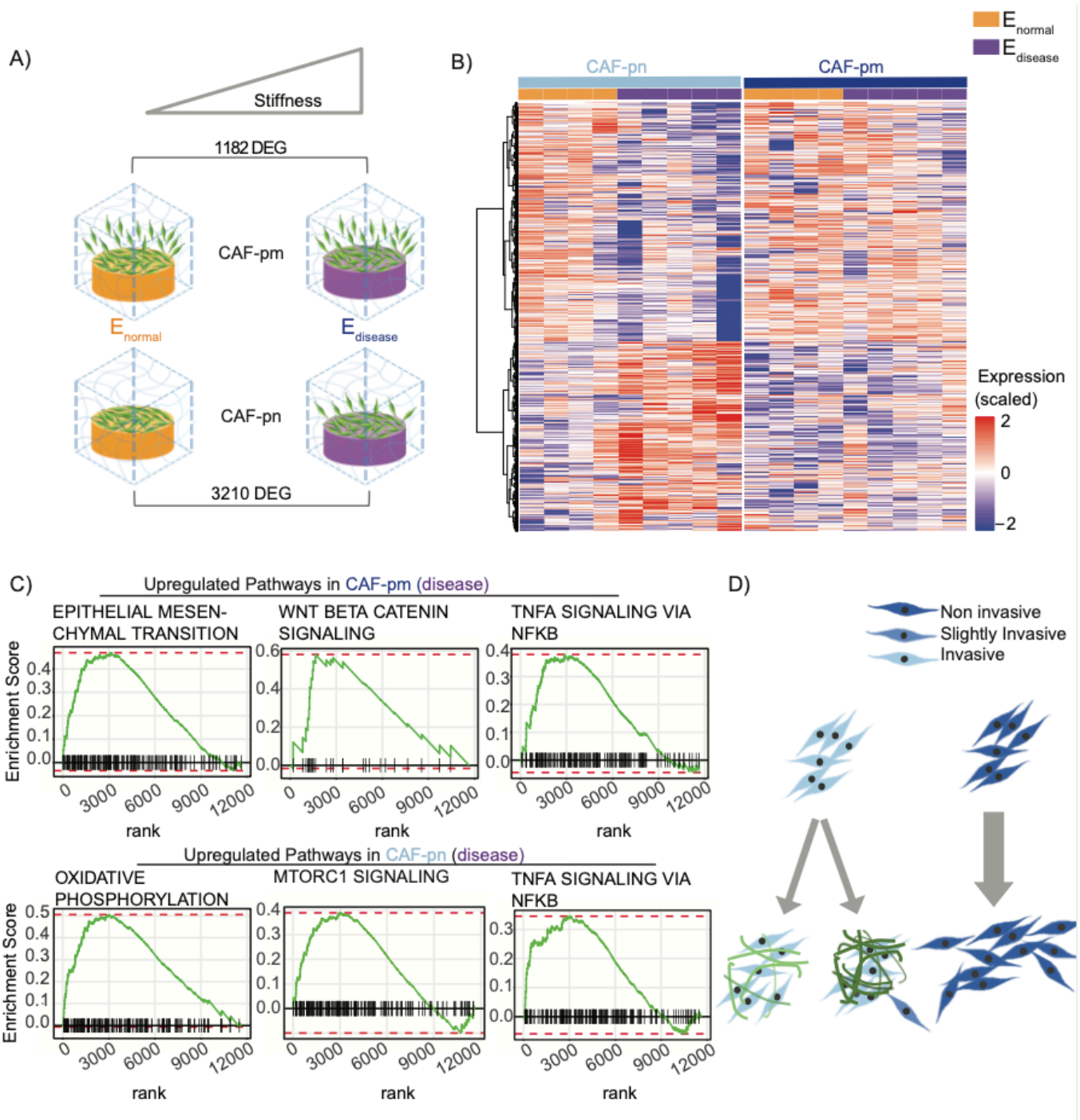
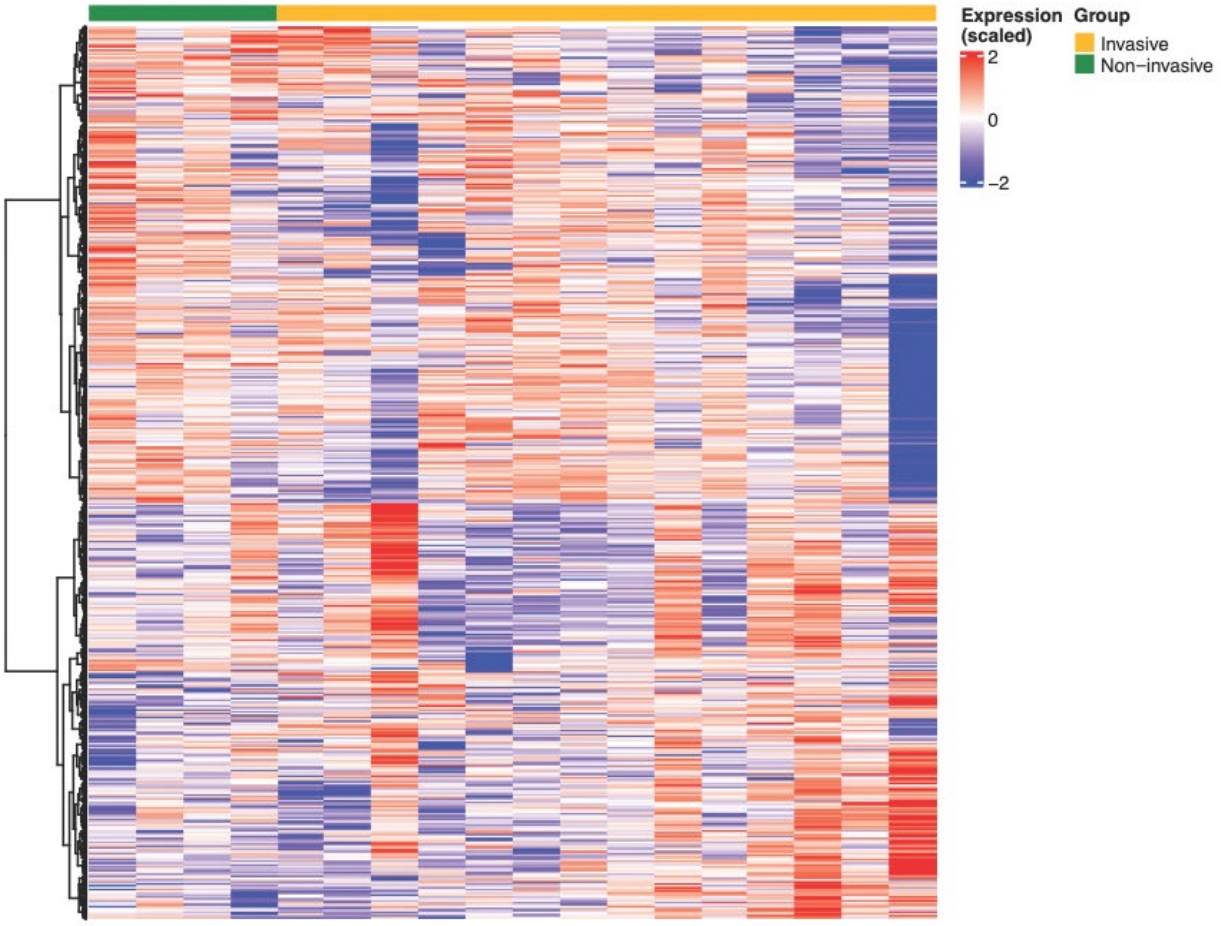


Figure 2-5: CAF-pm and CAF-pn have distinct transcriptional responses to substrate stiffness*. A) Schematic displaying differentially expressed genes across the various conditions tested (Q<0.10). B) RNA sequencing analysis of CAFs on E_{normal} and $E_{disease}$. The heat map shows differentially expressed genes between the E_{normal} and $E_{disease}$ for CAF-pm and CAF-pn(Q < 0.1, log_2FCI>0.5). C) GSEA of up-regulated pathways in CAF-pm (top) and CAF-pn (bottom) on $E_{disease}$ compared with self on E_{normal}. D) Working model demonstrating the response to substrate cues to support CAF invasion.*



Si Appendix Figure 6: RNA sequencing analysis comparing differentially expressed genes of invasive CAFs (CAF-pm normal, CAF-pm disease, CAF-pn disease) compared with non-invasive CAFs (CAF-pn normal)(Q < 0.1, log_2FCI>0.5). Heatmap supports distinct transcriptional profile between CAF-pm and CAF-pn.

2.2.2 AhR protein expression is significantly elevated in non-mechanosensitive CAF-pm

Given the correlation between disease state and stiffness-independent invasion, we speculated that comparing differentially expressed genes in CAF-pm and CAF-pn independent of substrate stiffness, would allow for identification of differential gene expression inherent to each CAF grouping. (Figure 2-6A). In particular, genes associated with the Aryl Hydrocarbon Receptor (AhR) Pathway (AHR, CYP1B1, CYP2S1, IL1R1, SDC4, REL and IDO1) were uniquely elevated in CAF-pm (Figure 2-6B). Interestingly, the activation of AhR is being explored for treating fibrotic eye disease, as its expression leads to an increase in MMP protein levels, a phenotype associated with cell invasion, and a decrease in TGF-β signaling, a hallmark of fibrosis [211]. Supporting this, in CAF-pn (low AhR expression) we noted an increase in pro-fibrotic genes (ACTA2, JUNB, COL1A2, TGFBR1, LAMB2, TGFB1, LOX, LOXL3). Moreover, the expression of downstream cytochrome p450 enzyme (CYP1B1) is associated with activated human hepatic stellate cells [212], suggesting a role in CAF activation. I therefore hypothesized that elevated AhR levels were responsible for a pronounced invasive phenotype in CAFs derived from patients presenting with metastatic disease (CAF-pm).

To begin validation of the gene expression data, we immortalized the primary CAFs using pLVX-SV40-LT-IRES-tdTomato to expand their long-term culturing abilities and used immortalized CAFs for all subsequent experiments. Using quantitative polymerase chain reaction (qPCR), I validated a significant increase in mRNA expression of AHR and CYP1B1 in CAF-pm (Figure 2-6C), and a significant increase in AhR protein levels by western blot (Figure 2-6D). To characterize the cellular localization of AhR in response to microenvironmental mechanics, CAFs were cultured

for 72 hours on E_{normal} and $E_{disease}$, fixed and immunostained for AhR (Figure 2-6E, Si Appendix Figure 7). Consistent with western blot data, total AhR protein levels were significantly elevated for CAF-pm (Figure 2-6E). Consistent with previous observations [199, 200, 211], the nuclear accumulation of AhR was significantly higher in CAF-pm. Taken together, this data confirms that AhR protein expression is significantly upregulated in the primary tumour CAFs from patients with metastatic disease (CAF-pm).

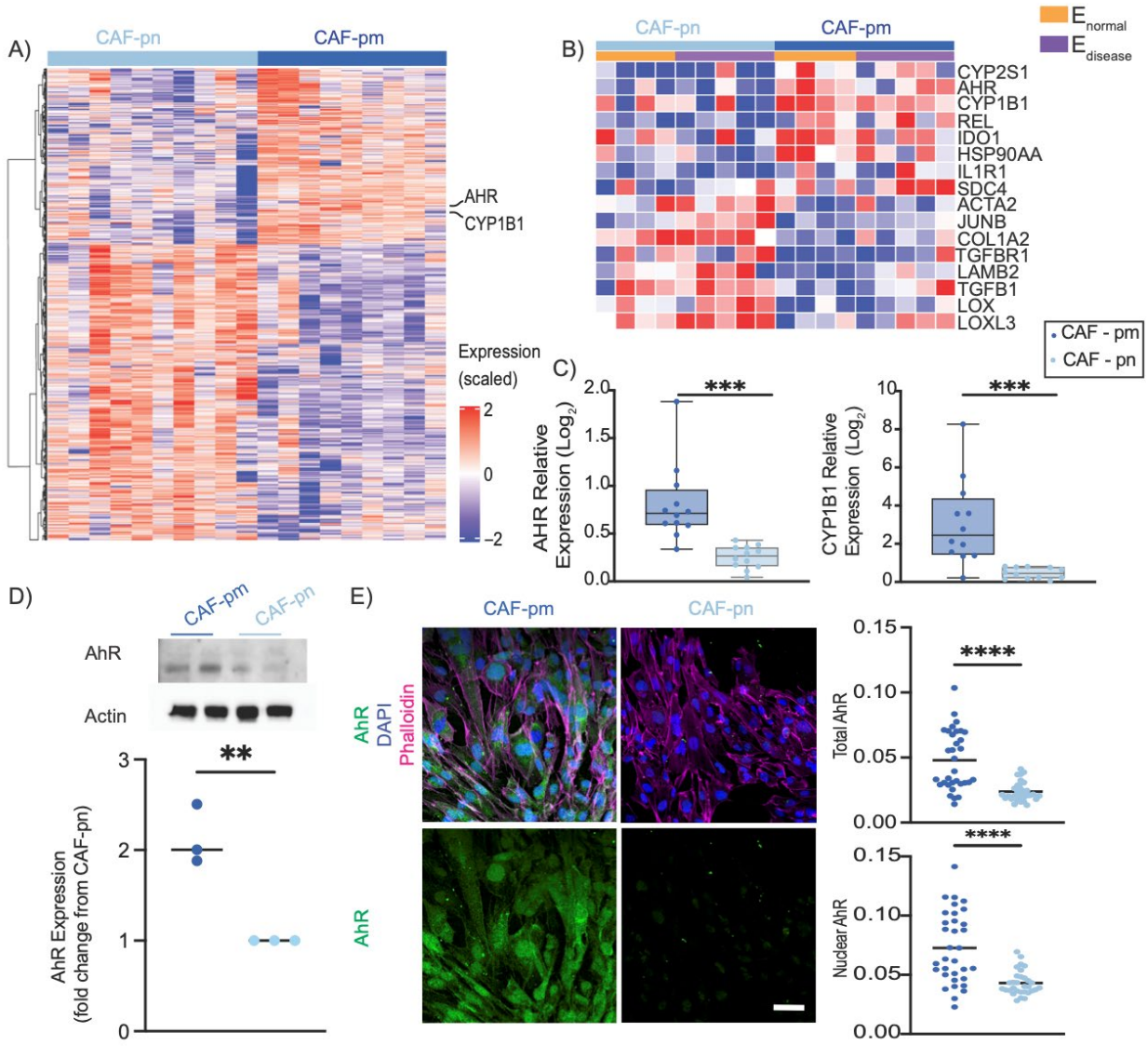
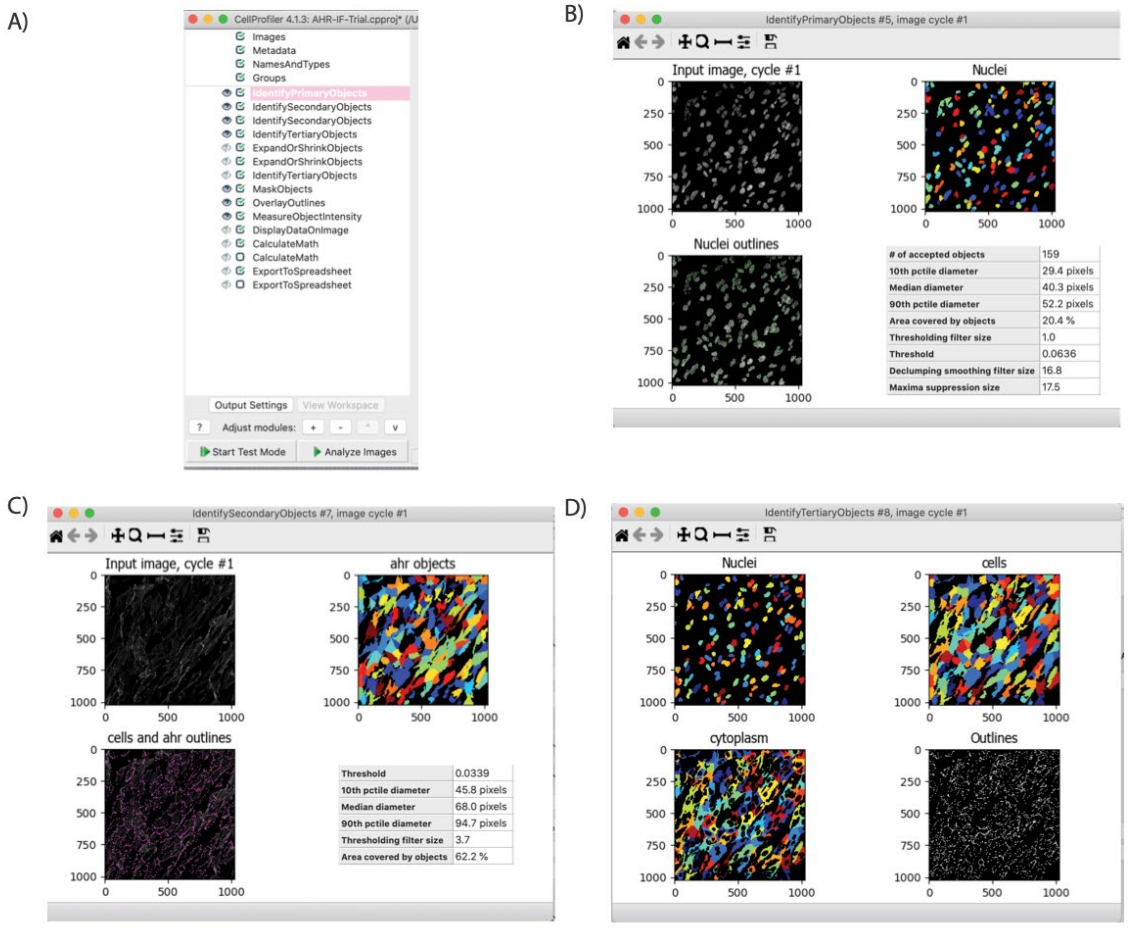


Figure 2-6: AhR protein expression is significantly upregulated in CAFs derived from patients with lymph node metastatic disease. *A) RNA sequencing analysis of differentially expressed genes of CAF-pm compared with CAF-pn on E_{normal} and $E_{disease}$ ($Q < 0.1$, $log_2FCI>0.5$). B) Heatmap showing normalized mRNA expression of genes encoding AhR related proteins and fibrosis associated proteins. C) qPCR validation of AHR and CYP1B1 in CAF-pm and CAF-pn (n=3 biological replicates; * $p < 0.05$ by unpaired t-test). D) Western blot analysis of AhR in CAF-pm and CAF-pn. Analysis was normalized against β-Actin (loading control) and represented as a fold change from CAF-pn (n=3, $p <0.05$ by unpaired t-test). E) Representative immunofluorescence staining of AhR (green), DAPI (blue) and phalloidin (magenta) (Scale bar, 50 µm). Quantification of total AhR protein levels and localization demonstrates CAFs from patients with metastatic disease have increased AhR protein levels and nuclear localization. Box plots indicate median and first to third quartile, and whiskers span the range.*



Si Appendix Figure 7: CellProfiler pipeline for quantifying nuclear and cytoplasmic immunofluorescent staining of AhR per cell. A) Overview of pipeline. Algorithm for B) identifying nuclei based off DAPI, C) for classifying total cell based off phalloidin, and identifying cytoplasmic area by subtracting nuclei from total cell.

2.3. IDENTIFY THE FUNCTIONAL ROLE OF AHR IN FACILITATING CAF INVASION.

2.3.0 Introduction

Given the established link between mechanosensitivity and elevated AHR/AhR levels, we next aimed to assess the function role of AhR in facilitating CAF invasion. Here, using siRNA knockdown and chemical inhibition with clofazimine, we demonstrate AhR is needed to facilitate CAF invasion. Through AhR over expression experiments, we demonstrate high AhR protein levels are sufficient to facilitate CAF-pn invasion. Lastly, we demonstrate that late-stage disease stiffness is sufficient to increase CAF-pn invasion, and link this with a concomitant increase in AhR levels. Due to the novelty of the culture method for RNA-seq and the limited number of patient samples, we then validate AHR in external datasets, establishing a role for AHR expression in disease progression and patient outcome.

2.3.1 AhR function is necessary and sufficient for CAF invasion

To assess the functional activity of AhR in promoting CAF invasion, we used chemical inhibition and short interfering RNA (siRNA) knockdown of AhR. Since we aimed to compare knockdown of AhR against CAF invasion, both CAF-pms and -pns were seeded onto substrates of stiffness $E_{disease}$ on which both cells exhibited invasion. Once seeded, cells were treated with clofazimine (CLF), a potent inhibitor of AhR nuclear translocation [213], or two siRNAs targeting AhR (Si Appendix Figure 8 and Si Appendix Figure 9, respectively). Importantly, we aimed to prevent CAF invasion without effecting cell viability, as the depletion of CAFs has had deleterious effects *in vivo* [24, 125]. We first confirmed that tested concentrations of clofazimine did not significantly affect cell viability (Si Appendix Figure 8), and neither clofazimine nor si-AhR significantly impact CAF

proliferation (Si Appendix Figure 8 and Si Appendix Figure 9, respectively). Clofazimine and si-AhR (Figure 2-7B, Figure 2-7C, Si Appendix Figure 10) both significantly decreased CAF invasion, consistent with the previously established role for AhR in fibroblast migration [199, 200]. Hence, AhR function is necessary for CAF invasion.

2.3.2 AhR overexpression promotes CAF-pn invasion

We next investigated whether AhR activation or overexpression alone was sufficient to drive invasion in CAFs derived from non-metastatic disease. Using L-kynurenine, a metabolite of tryptophan known to induce AhR nuclear translocation [214], CAF-pn were seeded onto E_{normal} and treated with L-Kynurenine (same flow as Si Appendix Figure 8). Interestingly, although CAF-pn were trending towards an increase in CAF invasion, there was no significant increase in invasiveness (Figure 2-7D). We reasoned that the nuclear localization of very low levels of AhR in CAF-pn cells was not significant to drive invasion.

To address whether AhR overexpression drives invasion, we transiently overexpressed an AhR plasmid under CMV promoter with a GFP tag. After plasmid transfection (Figure 2-7E) and on quantification, activation of AhR by overexpression significantly increased CAF-pn invasion (Figure 2-7E, F). Together, this data demonstrates that high levels of AhR fosters CAF invasion.

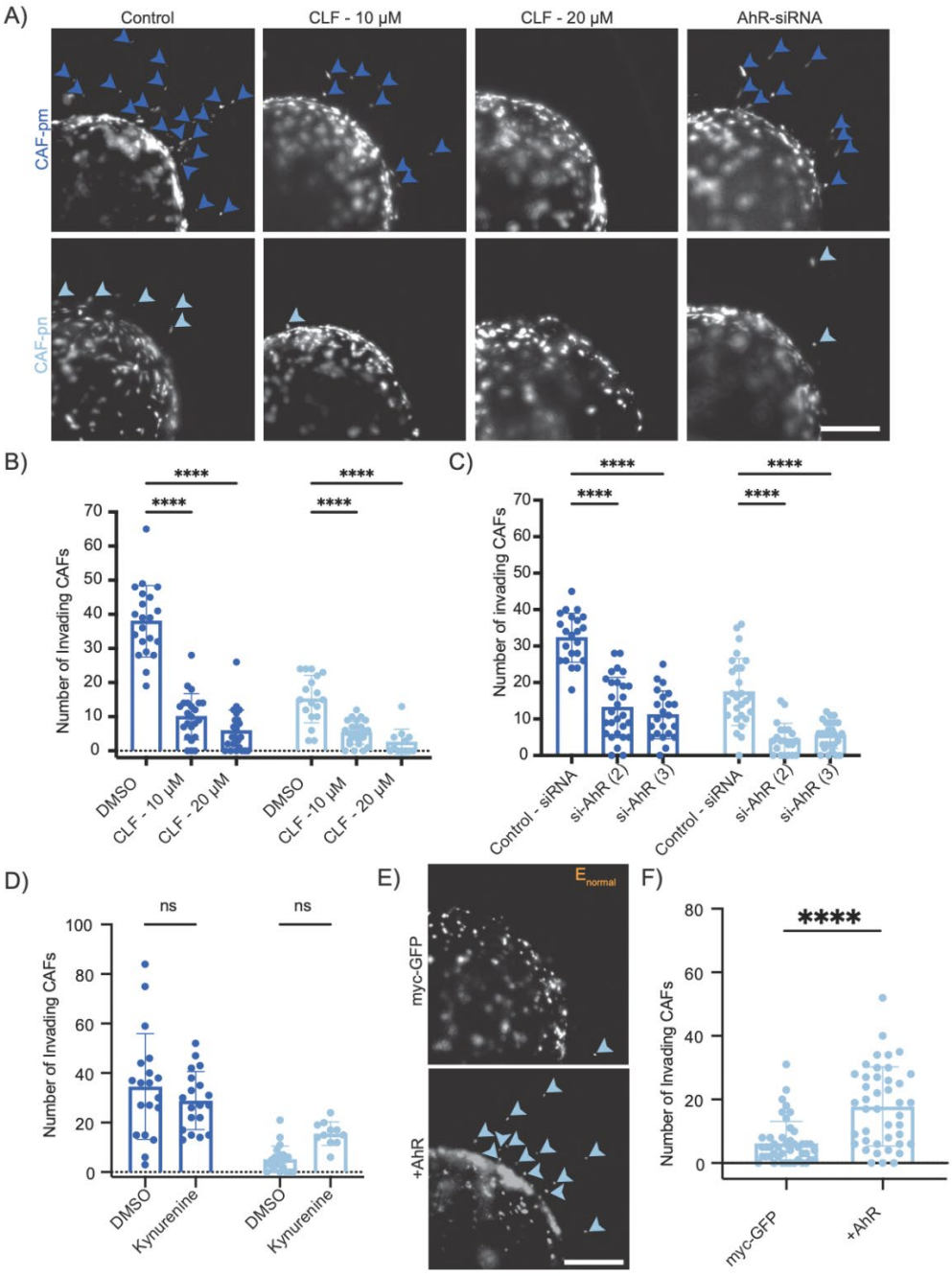
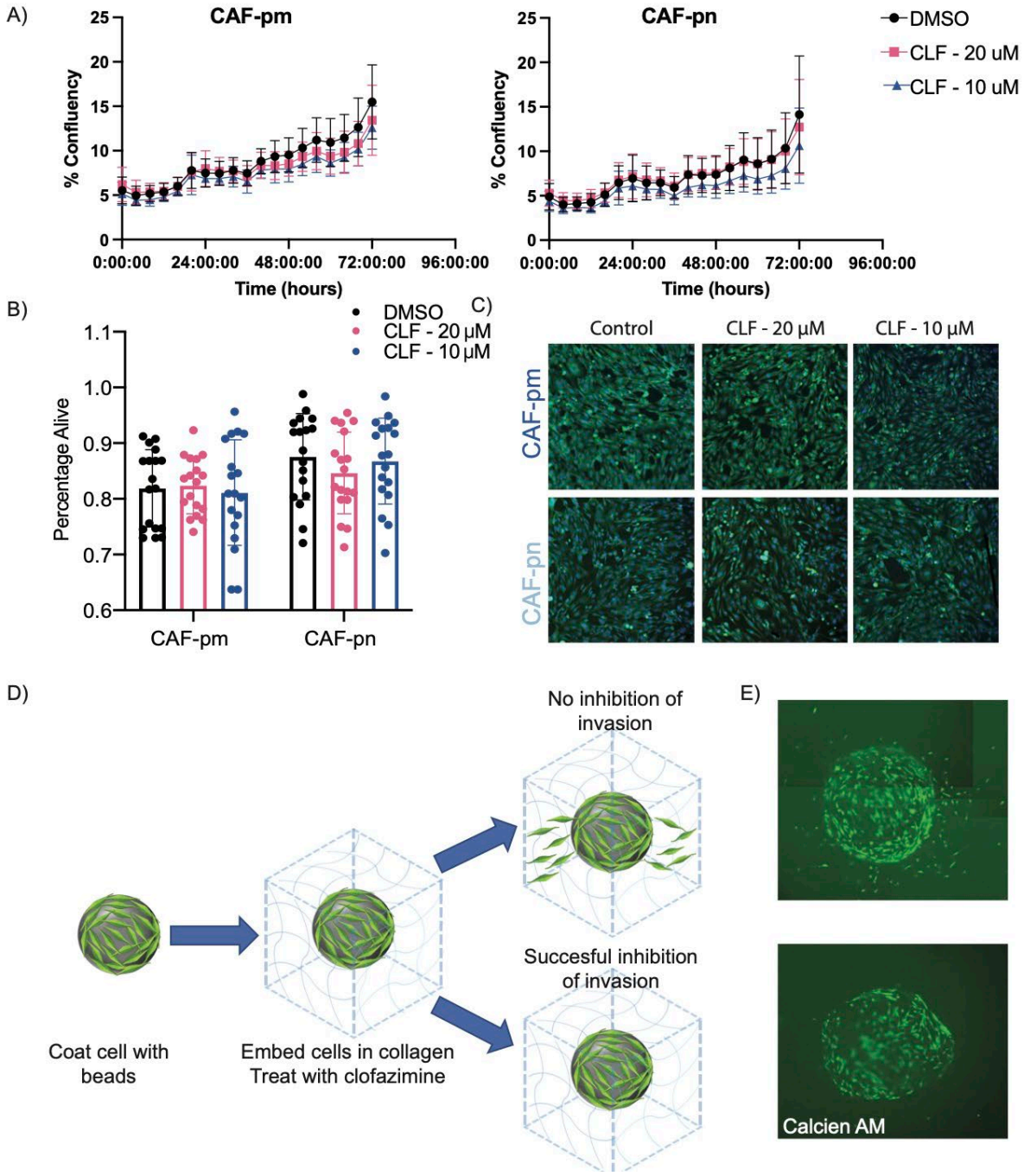
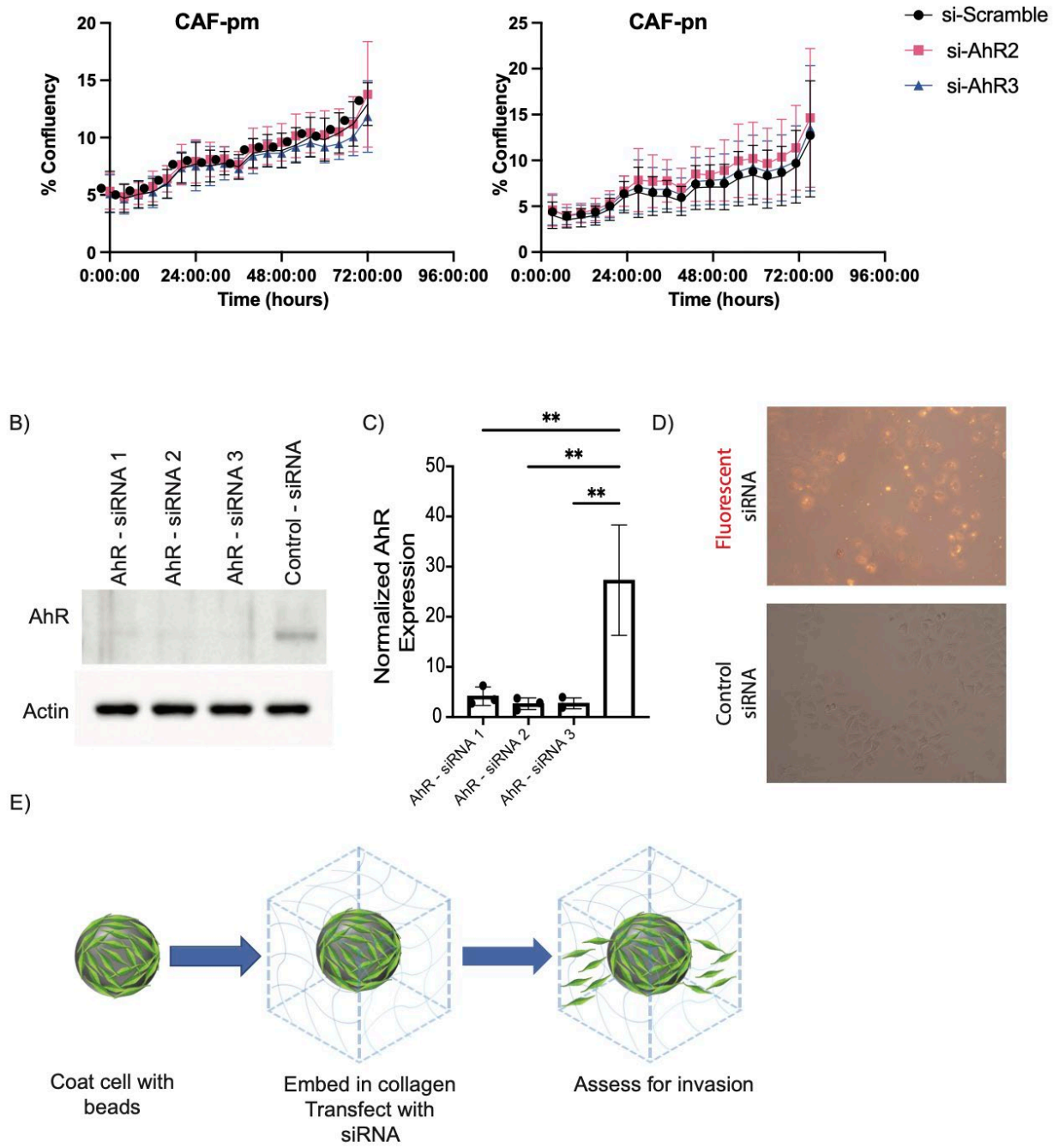


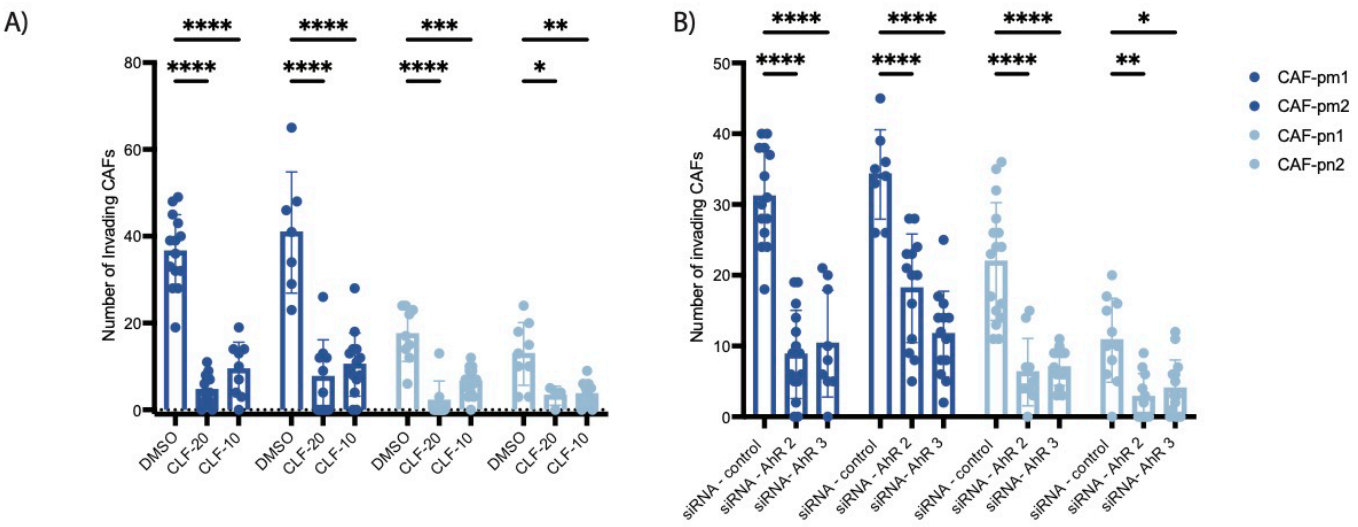
Figure 2-7: AhR function is required for stromal fibroblast invasion. *A) Representative images of CAF invasion after treatment with clofazimine or siRNA knockdown of AhR (Scale bar, 300 µm). Arrows indicate invasive cells. B) Clofazimine significantly decreases CAF invasion at 10 µM and 20 µM of Clofazimine in a dose dependent manner. C) CAFs transfected with si-AhR are significantly less invasive. D) L-Kynurenine does not significantly increase CAF-pm or CAF-pn invasion on E_{normal}. E) Representative image of CAF-pn invasion transfected with control myc-GFP or AhR. F) Overexpression of AhR prompts CAF-pn invasion on E_{normal}. (Biological n=3) *p <0.05 by one-way ANOVA, Tukey post-hoc).*



*Si Appendix Figure 8: AhR inhibition does not significantly affect proliferation or viability of CAFs. A) Proliferation of CAFs is not significantly different between control and clofazimine treated cells (biological replicate n=3, p<0.05 by one way ANOVA). B) Viability is not significantly different between vehicle and clofazimine treated cells (biological replicates, n=3, * p < 0.05 by one-way ANOVA). C) Representative images of live/dead as determined by Calcien AM/DAPI staining. D) Schematic representing the flow of clofazimine invasion assays. E) Representative images of invasion assay with and without invasion inhibition as imaged using Calcien AM.*



Si Appendix Figure 9: Confirming si-RNA knockdown of AhR. A) siRNA knockdown of AhR does not significantly change proliferation of CAFs. B) Western blot analysis reveals significant knock-down of AhR in CAF-pm2. C) CAF-pm2 were transfected with 3 siRNA (biological replicate, n=3). B) No significant different in normalized AhR expression in siRNA 1,2, or 3. D) HeLa cells were transfected with fluorescent and non-fluorescent control siRNA in 3D collagen gel. Fluorescently labelled cells confirm siRNA transfection in 3D. E) Schematic representing flow of siRNA transfection in invasion assay.



Si Appendix Figure 10: The effect of AhR A) inhibition by Clofazimine and B) knockdown by siRNA is independent of CAF lines.

2.3.3 AhR protein levels increase with disease stiffness in CAF-pn

Thus far our work has shown CAFs derived from the primary tumour of patients with lymph node metastasis are more contractile and invasive, regardless of microenvironmental cues (Chapter 2.1). In our models, the stiffnesses of the fibrotic foci cores are representative of cancer-free adipose tissue through early-stage invasion in breast tumors [194], however it is becoming increasingly apparent that late-stage breast cancer reaches much higher stiffnesses [168, 215, 216]. Given that increasing stiffness significantly increases invasion of CAFs derived from non-metastatic disease, we asked whether generating fibrotic cores at $E_{late\text{-}stage}$ (>25 kPa) would increase CAF-pn invasion, and if this correlated with AhR levels.

Consistent with our earlier findings on non-immortalized CAFs, CAFs associated with metastatic disease were only slightly more invasive on $E_{late\text{-}stage}$. CAFs associated with non-metastatic disease showed a significant increase in invasiveness correlating with increasing foci core stiffness (Figure 2-8E). To validate AhR protein levels, sections were immunofluorescently stained for AhR (Fig. 6A), and quantified (Figure 2-8B). Interestingly on late-stage disease stiffness, CAF-pm and CAF-pn had no significant difference in AhR total protein levels. When comparing $E_{late\text{-}stage}$ to E_{normal}, CAF-pm had no significant change in AhR protein levels, while CAF-pn had a strong and significant increase in AhR protein expression, suggesting a role for AhR in mechanono-response and memory. This was further validated by western blot (Figure 2-8C, D, *Si Appendix Figure 11*) and unsurprisingly, AhR chemical inhibition with clofazimine is still sufficient to decrease CAF invasion on late-stage disease-stiffened cores (*Si Appendix Figure 11*).

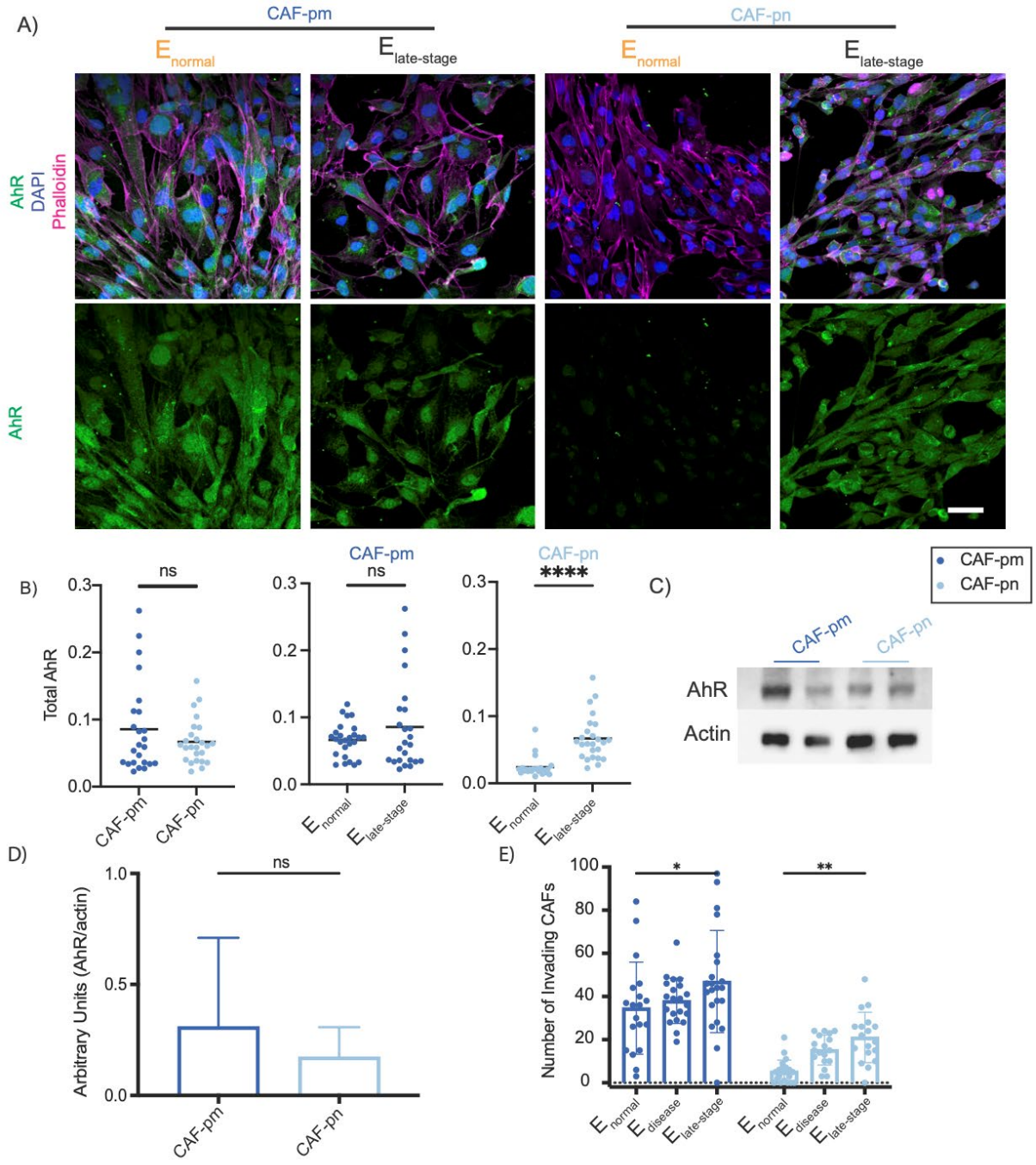
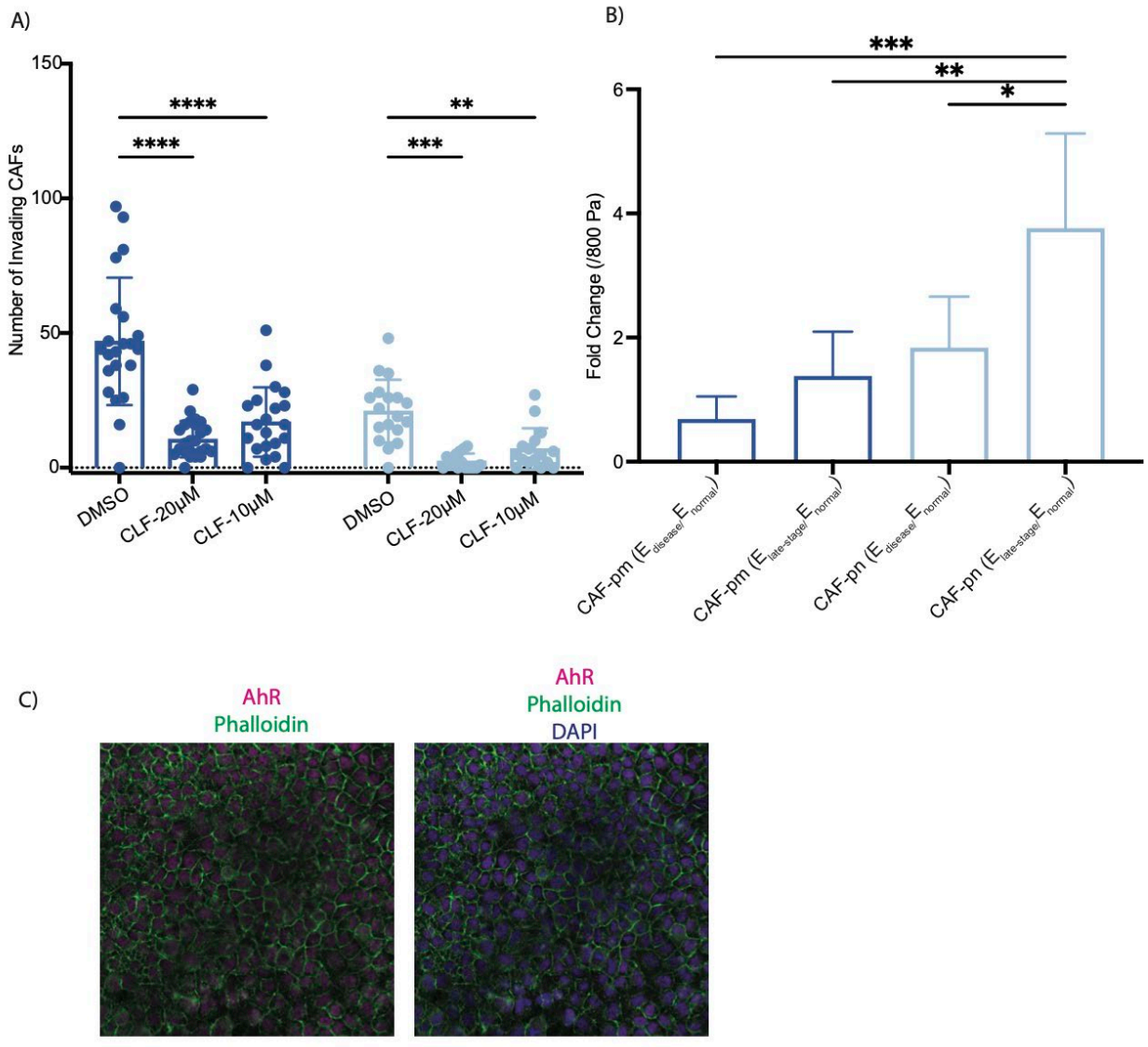


Figure 2-8: $E_{late\text{-}stage}$ significantly increases AhR expression and invasion. *A) Immunofluorescent staining of cellular localization of AhR (green), DAPI (blue) and actin cytoskeleton (magenta) in CAFs on E_{normal} and $E_{late\text{-}stage}$ (Scale bar, 50 µm). B) Quantification of total AhR protein levels demonstrates on $E_{late\text{-}stage}$ stiffness AhR is not significantly different between CAF-pm and CAF-pn. CAF-pm has no significant change in AhR protein levels between E_{normal} and $E_{late\text{-}stage}$, while CAF-pn has a significant increase in AhR. C) Western blot and D) quantification of AhR protein level on $E_{late\text{-}stage}$. (* p< 0.05 by unpaired t-test). E) Increased expression of AhR is closely associated with increased invasive phenotype (Biological replicate n=3, *p<0.05 by one-way ANOVA).*



Si Appendix Figure 11: AhR expression is significantly increased for CAF-pn grown on late-stage disease stiffness substrate. A) Clofazimine significantly inhibits invasion at late-stage disease stiffness. B) Quantification of AhR protein expression by western blot, represented as fold-change in expression relative to the normal stiffness (n=3, p <0.05 by one-way ANOVA). C) Immunofluorescent staining of AhR (magenta), actin (green) and DAPI of HeLa cells (positive control).

2.3.4 AHR is associated with poor outcome & disease progression

Finally, to address the putative link between elevated AHR expression in CAFs and clinical prognosis, we used various datasets. We clustered tumour samples from highly fibrotic tumours in larger patient datasets. Given that AhR in epithelial cells is associated with lack of proliferation [195-197] and decreased migration [198, 199], we reasoned that using highly fibrotic tumours would enable the detection of CAF associated signatures. In cervical and pancreatic tumours, high AHR expression is associated with poor overall survival (Figure 2-9A, B). Furthermore using gene expression analysis of CAFs from the primary tumour of 26 triple negative breast cancer patients [30], demonstrated a significantly elevated expression of AHR in CAF subset associated with motility and invasion [31] (Figure 2-9C). Using gene expression analysis of normal fibroblasts, CAFs isolated from the primary tumour and CAFs isolated from the metastatic site [217], AHR RNA levels progressively increase from normal to primary to metastatic CAFs (Figure 2-9D). Taken together, this evidence suggests that high AHR expressing CAFs not only increase with disease progression, but also play a key role in leading to downstream metastatic spread.

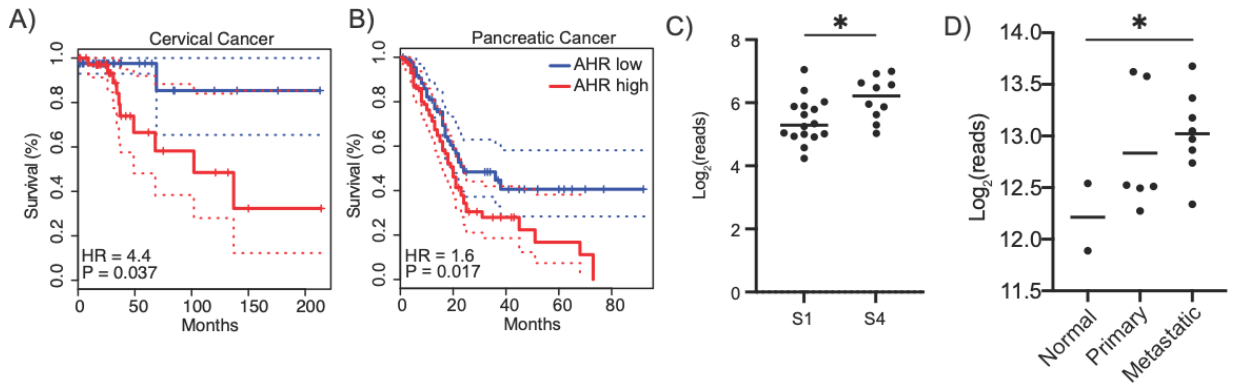


Figure 2-9: High levels of AhR expression in cancer associated fibroblasts are associated with disease progression and motility in triple negative breast cancer patients. *A, B) Kaplan-Meier analyses of highly fibrotic cancers, A) cervical cancer (n=82) and B) pancreatic cancer (n=178), associated AHR expression with overall survival (HR = hazard ratio, P value is denoted, and doted lines represent 95% confidence interval). C) AHR is significantly elevated in triple negative breast cancer tumour subtype S4 (motile CAF subpopulation) in comparison to S1 (immunosuppressive CAF subpopulation). D) AHR expression increases with disease state in the primary and metastatic tumours ($p<0.05$, by unpaired t-test).*

CHAPTER 3 : DISCUSSION

3.1 SUMMARY OF FINDINGS

CAF activation has been shown to correlate with aggressiveness of TNBC patient disease and to functionally promote downstream cancer cell metastasis [26, 30]. Here by developing a sequentially deconstructed experimental culture model of fibroblastic activity that recapitulates formation of foci-like microdomains in the tumor microenvironment, we demonstrate that CAFs isolated from primary TNBC have distinct contractility, invasion and mechanosensing abilities that depend on disease state; where CAFs isolated from primary TNBC with lymph node dissemination are no longer dependent on mechanical cues to prompt invasive phenotypes. By comparing the transcriptomes of both CAF subsets by RNA-seq analysis, uniquely in 3D mechanically controlled assays, we confirm CAFs from primary tumours of patients with lymph-node metastasis have distinct transcriptional responses to substrate stiffness. In particular, we find a role for Aryl Hydrocarbon Receptor (AhR) in facilitating CAF invasion and validate its clinical significance. This indicates that in the primary tumour, CAFs with increased AhR protein levels are more invasive, and these CAFs retain these behaviors independent of mechanobiological activation cues.

We first highlight in Chapter 2.1, using a microscale contraction and invasion assay, CAFs from primary tumours, isolated from patients presenting with lymph node metastasis have enhanced contractility and invasive abilities. Consistent with our findings, others have shown similar increases in both collagen contraction and invasion of murine fibroblasts, as a function of

premalignant through malignant transitions [99, 179], and our work confirms this functional phenotype for fibroblasts isolated from human patients.

Using hydrogel-based tissue engineering strategies, we recapitulate fibrotic foci with cores of tunable stiffness. This approach effectively separates the process of fibrotic foci formation from invasion, while allowing independent control over mechanical cues presented to the CAFs. Using this strategy, we demonstrate that CAFs become increasingly unresponsive to microenvironmental mechanical cues as disease progresses: while CAFs from patients without metastatic disease can be mechanically induced to invade, CAFs derived from patients with axillary lymph node metastasis are intrinsically invasive. Collectively, this functional *in vitro* phenotype is consistent with previous studies demonstrating that various degrees of CAF activation occur *in vivo*, where early lesions are infiltrated or encircled by tumour suppressive fibroblasts [24, 125] and that subsequent events drive transition towards pro-tumorigenic fibroblasts [25]. Our findings capture phenotypes inherent to CAFs regardless of cancer cell cues, as others have shown co-culture changes CAF activation, secretion and invasive behaviors [49, 218]. The data presented here therefore adds an understanding that CAFs that become mechano-insensitive retain this phenotype in culture independent of cancer cell cues, possibly reflecting an irreversible transition during *in vivo* disease progression.

In Chapter 2.2, by transcriptomic analysis, we confirm CAF-pm are unresponsive to changes in mechanical cues and this correlates with elevated AhR gene expression and proteins levels. In Chapter 2.3, we highlight the role of AhR in facilitating CAF-pm invasion, consistent with previous

studies showing AhR activation can facilitate fibroblast migration and invasion [199, 200]. Interestingly however, AhR activation is associated with a non-fibrotic and non-responsive TGF-β phenotype [211, 219], where AhR knockout mouse fibroblasts elicit higher levels of TGF-β secretion [198]. However, given that we established AhR protein levels were mechanoresponsive in CAFs derived from patients without metastatic disease (CAF-pn), where high stiffness increases AhR levels, we hypothesize that AhR low cells represent early stages of disease progression. Based on this data and previous literature, we therefore propose that high AhR expression in CAFs is secondary to generating a stiffened microenvironment, whereby TGF-β responsive CAFs can contract, remodel and secrete ECM over time to increase matrix stiffness [174, 175, 177]. Notably in CAF-pn, genes associated with fibrosis are uniquely upregulated (Figure 2-6B, Figure 3-1A), suggesting they are generating a pro-fibrotic tumour microenvironment. As disease progresses and microenvironmental stiffness increases, AhR expression increases, inhibiting profibrotic mechanisms where it becomes pro-invasive and mechano-insensitive (Figure 3-1). This is consistent with previous studies showing CAFs have potential to transition from one state to another [32, 53]. In particular, Wang *et al.* identified CAF interconversion by analyzing PDAC by single-cell RNAseq. While AhR was not significantly differentially expressed in their publicly available dataset, CAFs associated with dense fibrotic PDAC (C3) could convert into, clusters (C0 and C4) with elevated CYP1B1, a read out of AhR activation, where cluster 4 was associated with tumours with worsened prognosis [220].

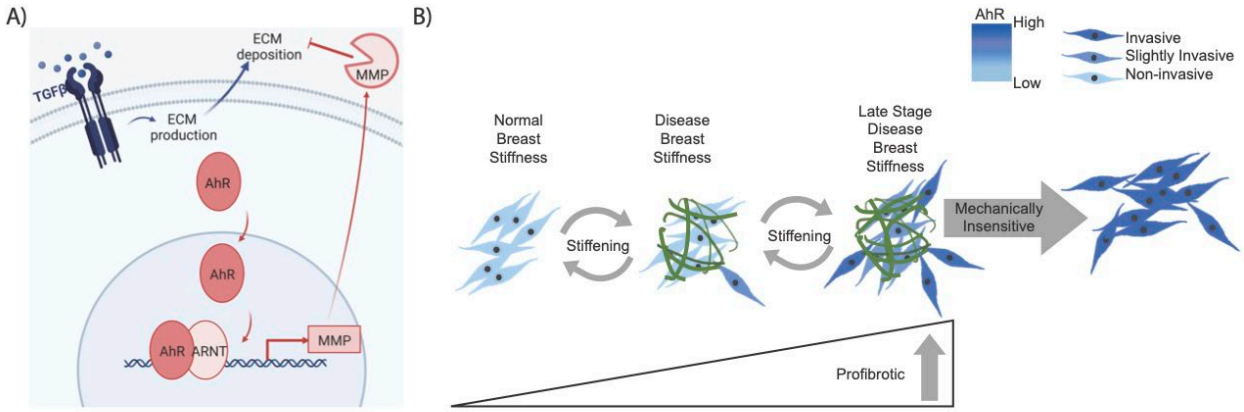


Figure 3-1: Contraction inducing stiffening generates and maintains AhR high, mechanically independent CAF invasion. *A) Schematic outlining the role of AhR in blocking TGFβ induced collagen deposition and myofibroblast formation. B) Model outlining the role of contraction-induced stiffening in the generation and maintenance of AhR high, mechanically independent CAF invasion.*

Moreover, in support of the model, it is possible that CAFs are displaying memory, created or imprinted by the patient tumour state. Mechanical memory is a 2D tissue culture concept that cells retain behavioral features of previous mechanical environments, whereby pre-exposure to stiff mechanical ques, delays cell response to environments with different properties. Previous work highlights that extended exposure to stiffness can permanently transition lung fibroblasts to myofibroblast state [123], or bias mesenchymal stem cell differentiation [123, 221]. Other studies suggest that several mechanisms that contribute to matrix stiffening also promote positive feedback loops that generate and keep CAFs in an activated state [99, 222]. Therefore, it seems likely that extended exposure to a possibly self-generated, highly stiffened matrix may also foster CAFs to adapt into an irreversible, non-mechanosensitive, high AhR expressing cellular state. Previous studies suggest that several mechanisms that contribute to matrix stiffening also promote positive feedback loops that generate and keep CAFs in an activated state [99, 222], and our current work suggests these feedback loops may also be involved in CAF activation and AhR expression *in vitro*.

The multi-stage deconstructed culture model presented in Chapter 2.1, overcomes several key challenges in studying CAFs in a patient-specific and physiologically relevant manner. First, CAFs were obtained without selection against pre-specified biomarkers. Other studies in this area commonly isolate primary CAFs using specific combinations of molecular markers, which therefore only capture a fraction of the complexity inherent in CAF populations [49, 78]. Moreover, these studies are largely limited to immortalized fibroblasts of cancerous and non-cancerous origins, known to acquire functional, genetic and epigenetic changes during virus-

based immortalization and prolonged culture *in vitro* [223-226]. For example, Costa *et al.* recently demonstrated that two CAF subtypes can accumulate differentially in TNBC, but that these subtypes do not correlate with patient survival [30]. Here by using non-immortalized, non-selected CAFs, in relatively limited numbers needed for our initial microscale 3D assays (Figure 2-2A, Figure 2-4A) and RNA-seq experiments (Figure 2-5A), we were able to correlate patient disease state with CAF functional characteristics. Given the limited cell material available and needed by these assays, these 3D models may ultimately allow high-throughput screening of functional activity in primary CAFs, to identify drug targets for patient-specific therapeutic development.

3.2 LIMITATIONS AND FUTURE DIRECTIONS

Some limitations must be considered in interpreting the results of this work. First, the present *in vitro* models lack the dynamics of stiffening and fibrotic foci formation that likely occur *in vivo*, where the stiffening process itself may have a different impact than the end stiffness alone [227]. Second, validating this proposed model of CAF activation *in vivo* will be challenging, due to the lack of suitable animal models and the difficulties associated with controlling changes in live fibrotic foci stiffness. Further, due to the complexity of obtaining and culturing non-immortalized CAFs, the cohort is limited to 4 patient samples that were immortalized for the purposes of validating the RNA-seq.

While these findings provide evidence for a role of AHR in fibroblast driven disease progression and are therefore novel, the mechanism for overexpression of AHR in CAFs remains to be elucidated. In this study we did not include or consider the effect of cancerous epithelial cells in dictating the expression of AHR or the potential of epigenetic changes in CAFs. It seems plausible that cancerous epithelial cells can produce ligands or soluble factors responsible for activating AHR. Future experiments should involve culturing CAFs with tumoral epithelial cells and associated conditioned media from patients with CAFs expressing high levels of AhR (CAF-pm1, CAF-pm2).

Moreover, the mechanism for constitutive, mechano-insensitive activation of AHR and concomitant increase in invasiveness is not clear. Further studies are warranted to explore i) if priming CAFs with 3D *in vitro* stiffness can lead to mechano-insensitive invasion and ii) what the

epigenetic changes are regulating AHR activation in fibroblasts. Previous studies have demonstrated AhR influences chromatin remodeling in cancerous cells through interaction with SWI/SNF chromatin remodeling complex [228], steroid receptor co-activator-1 complex (SRC-1) [229] and by displacing histone deacetylase complexes [230], but how this translates to CAF activation remains unknown.

This study also lacks 3D *in vivo* relevance, due to difficulties in correlating AHR expression levels and patient tumour stiffness. Recent data from the Moraes lab, suggests FFPE tissues lose all residual elasticity on fixation and therefore lack good methods to prob for the stiffness found *in vivo.* Developing more approaches to dynamically assess matrix stiffening during disease progression [168] *in vivo* may help to understand the interplay between CAF remodeling, AhR expression and downstream invasion. For now, one such way to investigate the role of stiffness in AhR elevation might be to profile CAFs in PDX models by immunohistochemistry or immunofluorescence against AhR, comparing levels in models with high and low degrees of fibrosis. In this model, for larger scale tissue stiffness measurements the use of shear wave elastography [216, 231] to measure *in vivo* tumour stiffness during tumour progression could allow for quantitating changes in elastic moduli over time to correlate with AHR expression. Although outside the scope of this work, in order to validate the correlation of stiffness and AHR *in vivo,* one could create a much more in-depth project by employing the use of thermally responsive hydrogel probes as previously established by our group [168] in a knock-in mouse model expressing fluorescence or bioluminescent tags with AHR expression.

The validation of AHR gene expression in this study is limited by the availability of fibroblast focused cohorts with longer-term patient follow-up. At this time, databases are largely focused on bulk or epithelial sorted tumour cell sequencing and lack the fibroblast component. Given that previous studies with AhR show upregulation of AhR inhibits proliferation of breast [195], prostate [196], and liver [197] cancer cells and its deficiency promotes epithelial cell migration [198] and metastasis [199], we chose to avoid validating AhR in these settings. Moreover, cohorts that encompass the tumour microenvironment [21] include immune cell populations which confound the data because of the various roles of AhR in immune cells [232, 233].

In future, data arising from single cell sequencing studies with long-term follow-up will be useful for validating and correlating fibroblast specific expression of AHR. Moreover, the use of single cell sequencing in CAFs might allow for identification of small subsets of CAFs expressing higher levels of AHR. It remains unknown whether it is small amounts of AHR facilitating CAF-pn invasion as seen on $E_{disease}$, or another mechanism at hand.

3.3 CONSIDERATIONS FOR CLINICAL SIGNIFICANCE OF AHR

The lack of mechanoresponsiveness in AhR expressing CAFs, provides reasoning as to why drugs aimed at reducing microenvironmental stiffening have previously failed in clinical trials. However, given the varied roles AhR plays in different cell populations (outlined in 3.2) [195-199, 232, 233], simply targeting AhR may prove harmful and difficult.

Most research on AhR has extensively focused on its response to man-made chemical pollutants, like polycyclic aromatic hydrogens, with a strong emphasis on the human carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin (colloquially termed dioxin, or TCDD), a high affinity ligand. Interestingly AhR is evolutionarily conserved across vertebrates and invertebrates, yet AhR in invertebrates cannot bind dioxin or other polyaromatic hydrocarbons, suggesting it is important for other development processes and maintaining physiological functions. For example, i) in *D. melanogaster* the AhR protein homologue, spineless, is required for eye, leg and wing development [234], and ii) in *C. elegans* AHR-1 is essential for neuronal differentiation and migration [235, 236]. Given this, the current view in the field is that xenobiotic dependent AhR function represents an adaptative mechanism in vertebrates, that overlaps with physiologically conserved roles. Therefore, a deeper understanding the role of AhR in homeostasis and development may be necessary before therapeutically targeting it.

Moreover, using transgenic mouse models, a number of studies support a role for AhR in key physiological and homeostatic roles in the body; AhR null mice (AhR-/-) show developmental defects in hepatic [237, 238], hematopoietic [239], detoxification [240], and immune [238]

systems. In the liver, AhR has been shown to regulate metabolism, where it's inhibition in mouse models leads to significant weight gain [241, 242]. Given the correlation between obesity and cancer [243-247], obesity as a side effect of inhibiting AhR should be explored carefully. Moreover, AhR-/- mice develop extensive fibrosis in the heart and kidneys [248] as well as heart defects [239, 249]. While fibrosis has been demonstrated to have a caging effect in cancer[24, 125], the full body ramifications of this make it difficult to inhibit AhR. Perhaps with the growth in nano-based drug delivery technologies and therefore more localized treatments, specifically inhibiting AhR in CAFs may prove a viable target.

Moreover while environmental toxicants bind AhR with high affinity, more recent work has demonstrated naturally derived endogenous ligands play a role in cell cycle regulation [250], cell differentiation [251] and immune response [238, 251-253]. These natural ligands can come from host metabolic breakdown, microbiota and dietary intake, where the majority of dietary AhR ligands are plant derived [214, 254-256]. Interestingly recent research highlights links between AhR levels and gut microbiota; AHR expression is attenuated in germ free mice, suggesting AhR acts as a mediator in communication between host and gut microbiota [257]. With a growing interest in microbiome mediated cancer cell response and therapy resistance, connecting microbiome-cancer axis with AhR protein levels may help to understand it's mechanisms of activation and signaling for the purpose of therapeutically targeting.

3.4 CONCLUSIONS

Unfortunately, nearly 25 years after the initial studies showing the physiological relevance of 3D culture systems [122, 258, 259], the vast majority of cancer biology research is still in 2D. This is likely due to the ease of culture, growth, and biochemical testing in 2D systems as well as the relative success of these technologies in identifying useful molecules. However, it would appear that the low-hanging fruit of easily identifiable therapeutic molecules have already been identified, as evidenced by the dwindling number of novel therapeutic discoveries, despite increases in economic resources allocated to this problem [260]. Furthermore, the poor clinical translatability for many seemingly promising drugs suggests that 2D systems are no longer sufficient in this area, and that more complex 3D culture systems will be required to identify next-generation therapeutics. Moving forward, I believe advanced technologies like those presented in this thesis will help bridge this gap, in improving the physiological relevance of discoveries, while also improving the ease of setting up, operating, and data analysis. Moreover, I anticipate the inclusion of immune cell populations will become more and more important, as numerous recent studies have demonstrated a key role for immune cells in tumour progression [26, 68, 72, 73, 261]. Given that there are small subsets of patients who respond to immune checkpoint inhibitors [262], perhaps other microenvironmental factors dictate patient responsiveness to checkpoint blockade. Recent findings are demonstrating that CAFs may be critically important in these microenvironmental feature sets that drive immune evasion [30, 67, 70, 71, 263-268], and so these advanced discovery systems may better pair patient populations with successful therapeutics. The consideration of immune cell infiltration in future cancer biology studies will be significant.

The presented data highlights the importance of contraction-induced mechanical remodeling by CAFs in triple negative breast cancers. Here I have demonstrated that subsets of CAFs in primary TNBC tumours, transition to a state where they are expressing pro-invasive transcriptional factor AhR and therefore invade regardless of microenvironmental stiffness cues. The high expression of AhR in tumours with lymph node dissemination may explain why drugs aimed at reducing CAF contractility and microenvironmental stiffening fail to improve cancer outcomes [36, 269], as CAFs are inherently invasive. Patient levels of AhR at time of surgery may therefore be a useful screening strategy to identify patients likely to present with metastasis and those who might respond to therapies aimed at mechanically inactivating CAFs. Furthermore, the data highlights the importance of the physical properties of fibrotic cores observed in poor outcome TNBC *in vivo* and underscores the necessity to study the tumour microenvironment in models of physiologically relevant stiffness. These findings broadly suggest that integrative multidisciplinary knowledge of CAF mechanical state is important in developing and testing novel therapeutics or biomarkers. Ultimately because of this process, patient stratification based on AHR expression represents a strategy to identify patients at risk for lymph node dissemination.

CHAPTER 4 : METHODS

Unless otherwise stated, all cell culture materials and supplies were purchased from Fisher Scientific (Ottawa, ON) and chemicals from Sigma Aldrich (Oakville, ON).

4.1 Clinical samples and human CAFs

Tumour specimens and tissue samples used in this study were obtained with informed consent from all subjects and in accordance with the McGill University Health Center research ethics board (SUR-99-780). For the purposes of this study, samples were selected according to clinically documented lack of expression or amplification of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor2 (HER2); a histological subtype assignment of IDC (not otherwise specific [NOS]). Briefly, CAFs were harvested from dissociated tumour suspensions by centrifugation at 500xg for 5 minutes at 4 °C and plated on collagen coated plates. This was done to minimize selection of CAFs by markers, as it is widely described that no one marker is exclusive to, or encompasses all, activated fibroblasts [25]. To best maintain the heterogeneity of the CAF population, they were cultured on collagen coated tissue culture plastic, to a maximum of 10 passages.

4.2 Cell culture

CAFs were isolated from patients as described above and grown on collagen coated tissue culture plates. They were cultured in fully supplemented Dulbecco's Modified Eagle Medium (DMEM, with 10% Fetal Bovine Serum (FBS), 50 µg/mL gentamicin, 2.5 µg/mL amphotericin B) at 20% O_2,

5% CO_2. Experiments were carried out at 3% O_2, and 5% CO_2. Post RNA-sequencing analysis CAFs were immortalized with pLVX-SV40-LT-IRES-tdTomato, generously gifted from Dr. David Tuveson's lab.

Functionalized polyacrylamide beads were dispensed 1 per well in ultra-low attachment, 96 well plates. CAFs were collected at 150,000 c/mL in complete media, dispensed into each bead containing well, resuspended to ensure sufficient coating (4 x 15min) and incubated overnight at 37°C. CAF coated polyacrylamide beads were overlaid with collagen as previously described. Tissues were incubated for 48 hours, fixed, stained, and imaged as previously described. Cell invasion and distance was quantified as previously described. Due to the heterogeneity of CAFs, outliers were removed using the 1.5xIQR rule.

4.3 Immunofluorescence on human tissue sections

Immunofluorescence staining of nuclei and actin cytoskeleton was performed by fixing sections in 4% PFA. FFPE tissue was deparaffinized and underwent heat-mediated antigen retrieval in citrate buffer pH6.0 or EDTA buffer pH9.0. Slides were blocked with Power Block (Biogenex, San Ramon, CA, United States) for 5 min at room temperature and incubated with the primary antibody for 30 min at room temperature followed by washing with TBST (3 x 3 min). Slides were incubated with secondary antibody-HRP for 30 min at room temperature, washing with TBST (3 x 3 min) and stained with Opal fluorophore working solution for 10 min. This was followed by heat-mediated antibody stripping to remove the primary and secondary antibodies to repeat additional rounds for labeling with other primary antibodies. The primary antibodies are αSMA

(Cat# M0851, DAKO (Glostrup, Denmark)), Vimentin (Cat# 760-2512, Ventana (Oro Valley, AR, United State)), Pan-Keratin (Cat# 760-2595, Ventana (Oro Valley, AR, United States)). The antibody specificity and dilution were tested before multiplex assay. Tissue images were captured using AxioScan Z1 scanner. Quantification was done using Tissue Classifier Add-on, and Area Quantification FL from HALO v 3.1.1076.

4.4 Collagen Contraction and Invasion Assay

Free floating collagen microdroplets were fabricated using an aqueous two-phase droplet printing technique by an automated liquid handler. The 2 aqueous phases, poly(ethylene glycol) (PEG) and dextran were prepared as follows. 35 kDa PEG (Sigma-Aldrich, 94646) at a concentration of 6% w/v in supplemented DMEM was sterile filtered through a 0.22 µm pore size sterile filter cup. 500 kDa dextran (www.dextran.ca) at a concentration of 20% w/v in sterile RO water was sterilized under UV light for 45 min. Solution were stored at 4 °C when not in use.

Free floating collagen droplets were generated as previously described [120]. Briefly, CAFs were passaged at 90% confluency, centrifuged at 800RPM for 5 min and resuspended at a final concentration of 2.7 x 10^6 cells/mL in collagen and supplemented DMEM containing 15% v/v dextran solution. 100 µL of PEG solution was robotically dispensed into each well of a round bottom 96-well plate by an automated liquid handler (Pipetmax, Gilson (Middleton, Wisconsin)). 2 µL of cell-laden dextran-collagen solution was robotically dispensed into each well. The plate was incubated at 37 °C for 60 min to allow collagen gelation. After gelation, PEG-rich DMEM was removed and replaced with 100 µL of supplemented DMEM and incubated at 37 °C and 5% CO2 over 2 days to assess contraction.

Contracted collagen microdroplets were overlaid with collagen gels to produce 3D constructs. Media was aspirated from each device and collagen was polymerized using established protocols [43]. Briefly, Type I bovine collagen (Advanced Biomatrix, 3 mg/mL) was diluted to 1.5 mg/mL in PBS and 10× DMEM solution to obtain a final 1× DMEM concentration and kept on ice. The acidic solution was neutralized with 1M NaOH by titration based on the color of the phenolphthalein indicator in DMEM. Cold collagen gel solution was dispensed over each contracted droplet and allowed to incubate at 37 °C for 45 min to gel. After gelation, 100 μl of complete media was added to each well.

4.5 Polyacrylamide core preparation

Stiffness-tunable polyacrylamide hydrogel formulations were prepared using the following acrylamide (Biorad, 1610140) to bisacrylamide (Bio-rad, 1610142) ratios with the stated nominal shear modulus values determined by shear rheology of bulk samples: 1450 Pa (3.0 wt%/0.10 wt%); 5770 Pa (7.5 wt%/0.24 wt%). To polymerize 1 mL of pre-polymer mixture, 100 μL of 1% w/v ammonium persulfate (APS; Bio-rad, 1610700) in phosphate buffered saline (PBS) and 1.5 μL of tetramethylethylenediamine (TEMED; Sigma-Aldrich, T7024) were added to initiate and catalyze the polymerization reaction.

Droplets of polyacrylamide pre-polymer with TEMED and ammonium persulfate initiator were manually dispensed, in volumes of 0.5uL and 1 uL for 400Pa and 7500Pa gels respectively, into kerosene with 6% w/v polyglycerol polyricinoleate (PGPR 4150). The hydrogel cores were allowed

to polymerize in a kerosene bath. The surfactant-rich kerosene was replaced with PBS after multiple centrifugations using Labnet Mini Centrifuge C-1200, before being left overnight at room temperature in PBS to allow for swelling. Due to the large swelling ratios of soft polymer formations, we optimized the aqueous polymer dispersion volume to produce cores of identical radius and similar in size to the starting collagen droplet from the invasion assay.

Recovered cores were resuspended in 0.05mg/mL Sulfo-SANPAH (G-Biosciences, BC38) in PBS and irradiated under UV light for 4 min to activate the cross-linker. Microspheres were rinsed with PBS, resuspended in 0.05 mg/mL collagen I (VWR, CACB354231) in PBS, and stored overnight at 4 °C. Collagen I coated microspheres were resuspended in PBS and stored at 4 °C until use.

4.6 Stiffness readings with microsquisher

Parallel plate compression testing was performed on polyacrylamide cores with a MicroSquisher (CellScale Biomaterials Testing Inc. (Kitchener, ON, Canada) to determine their elastic moduli. Samples were loaded onto the instrument and then core diameter was measured under its magnification. Samples were compressed at a rate of 10 µm/s by at least 20% [nominal] strain, and data was collected at a rate of 5 Hz.

Elastic moduli were calculated in R computing environment as follows. Core diameter was used to calculate cross-sectional area as an estimate of the area in contact with the compressing plate, and nominal stress was calculated by dividing the force data output from the MicroSquisher by this calculated contact area. Nominal strain was calculated from displacement data by dividing

the change in sample height at each collected time-point by its initial height. Plots of stress as a function of strain were generated, and linear regression was used to calculate the slope of the curve for data from 0-10% strain, yielding an estimate of the elastic modulus.

4.7 Fluorescent labelling of polyacrylamide cores

Collagen I functionalization was verified with anti-collagen I mouse primary antibody (ab6308, Abcam (Cambridge, United Kingdom)) and goat anti-mouse secondary antibody tagged with Alexafluor 594 (ab150116, Abcam (Cambridge, United Kingdom). CAFs in microdroplet or on polyacrylamide core assays were stained with DAPI and Alexafluor546 Phalloidin (A22283, Invitrogen (Carslbad, CA, United States). For viability assays, CAFs were cultured on polyacrylamide core of normal and diseased stiffness and the surrounding media was replaced with 4 μM Calcein-AM and 2 μM Ethidium Homodimer (EtHD) in PBS for 30 minutes. The media was washed and droplets were fluorescently imaged to assess viability.

4.8 RNA sequencing

CAF-pm1, CAF-pm2, CAF-pn1 and CAF-pn2 cultured on matrices of various stiffnesses as indicated were collected, and the total RNA was extracted using RNAeasy kit (Qiagen) according to the manufacturer's instructions. High RNA quality was verified using the Bioanalyzer RNA 6000 Nano assay (Agilent). cDNA was PCR amplified for 18 cycles using poly T primer. All cDNA was produced the same day to avoid batch effects. The final sequence ready library was generated using Illumina DNA Prep and IDT for Illumina DNA/RNA UD Indexes. The sequencing-ready library was cleaned up with SPRIselect and quantified by qPCR (KAPA Biosystems Library Quantification Kit

for Illumina platforms). 1 nM sequencing libraries were loaded on an Illumina HiSeq Instruments and ran using the following parameter: 151 bp Read1, 8 bp I7 Index, 8 bp I5 Index and 151 bp Read2.

Adaptor sequences and low-quality score bases (Phred score < 30) were first trimmed using Trimmomatic (Bolger et al., 2014). The resulting reads were aligned to the GRCh38 human reference genome assembly, using STAR (Dobin et al., 2012). Read counts were obtained using HTSeq (Anders et al 2015) with parameters -m intersection-nonempty -stranded=no. For all downstream analyses, we excluded lowly-expressed genes with an average read count lower than 10 across all samples. Raw counts were normalized using edgeR's TMM algorithm (Robinson et al., 2010) and were then transformed to $\log_2$-counts per million (logCPM) using the voom function implemented in the limma R package (Ritchie et al., 2015). To assess differences in gene expression levels, we fitted a linear model with the covariates Patient_ID and Mycoplasma_Contamination_Rate using limma's lmfit function (method = "robust"). Nominal p-values were adjusted for multiple testing using the Storey's Q-values method (R package qvalue; https://bioconductor.org/packages/qvalue). Gene set enrichment analysis (pre-ranked mode) was performed using the R package fgsea (https://bioconductor.org/packages/fgsea/).

4.9 Gene expression by quantitative PCR with reverse transcription

RNA was isolated using the RNAeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesised using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. qPCR reactions were performed using SYBR Green

I Master on a LightCycler480 (Roche). Primers sequences used are *CYP1B1* forward 5' – CCAAGAGACTCGAGTGGGAG – 3' and reverse 5' - TGCCCATGCTGCGGG – 3', and *AHR* forward 5'-TGGTTGTGATGCCAAAGGAAG – 3' and reverse 5' – ATAACCTGAGCCTCTCGTGC – 3'. GAPDH was selected as reference. Relative quantification of the expression levels was calculated according to Pfaffl method[270]. For RNA-Seq gene expression validation, the data presented represent the merge of three biological replicates for CAF-pm1, CAF-pm2, CAF-pn1 and CAF-pn2.

4.10 Western blotting

CAF lines were lysed using RIPA Lysis Buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate supplemented with freshly added (1 mM phenylmethylfulphonyl fluoride (PMSF), 1 mM Na3VO4, 1 mM NaF, 10 µg/ml aprotinin). Whole cell lysates were resolved by SDS/PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% Bovine Serum Albumin and probed with primary antibodies (AhR SA210, Enzo Life Sciences and beta-actin AC15, Sigma Aldrich) overnight at 4 °C. After TBS-Tween washes, membranes were incubated with HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (Cell Signalling) for 1h at room temperature for signal detection using ChemiDoc Imaging System (Biorad) and quantified using Image Studio.

4.11 Immunofluorescence (IF) on polyacrylamide gels

Cell samples were fixed in 4% paraformaldehyde for 10 min, washed with PBS three times and permeabilized with 0.2% Triton-X100 solution for 20 min. Non-specific protein adsorption was blocked with 2% bovine serum albumin in IF buffer (phosphate buffer saline with 0.2% Triton X-

100 and 0.05% Tween 20) for 30 minutes at room temperature. After washing with IF buffer, AhR primary antibody (AhR SA210, 1:100, Enzo Life Sciences) at room temperature for 2 hours and washed in IF buffer 3 times. Samples were incubated with secondary antibody (1:500, Alexa fluorophore 488 Donkey-anti-Rabbit) and phalloidin-546 (1:100, Thermofisher) for 1 hour at room temperature. Finally, samples were incubated with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) diluted in PBS for 10 min at room temperature and washed three times with PBS. All samples were from 3 independent biological replicates, and each replicate was processed in parallel.

4.12 Chemical inhibition and activation of AhR

AhR inhibition experiments were performed adding 10 µM or 20 µM of Clofazimine to CAFs on beads within 3D collagen gels for 48 hours. AhR activation experiments were performed adding 100 µM of L-kynurenine to CAFs on beads within 3D collagen gels for 48 hours.

4.13 Transfections

Transient siRNA-mediated knockdown of AHR was performed using TriFECTa RNAiKit (Integrated DNA Technologies). For siRNA experiments, cells were transfected with 10 nM siRNA lipofectamine RNAiMax (Thermo Fisher) according to manufacturer's instructions. Human aryl hydrocarbon receptor gene ORF cDNA clone, C-GFPSpark Tag was purchased through SinoBiological. Transient transfections for protein expression were performed using Invoitrogen Lipofectamine and Lipofectamine Plus according to the manufacturer's instructions, and 3D invasion assays were conducted within 3 hours post-transfection.

4.14 Short term growth and cell viability assays

Single cell suspensinos were seeded into 96-well plates and treated with DMSO, Clofazimine, si-control or siRNA. Cells were incubated for 72 hours imaged by Incucyte S3 and analyzed using Incucyte analysis software.

4.15 Imaging and analysis

Live collagen microgels were imaged at selected timepoints using the EVOS FL and a 4× objective. Measurement of collagen droplet area was performed by analysis of threshold images and measuring droplet size in ImageJ (NIH). Data was generated using the change in droplet size (final size divided by initial) and analyzed by one-way ANOVA. Data plotted is displayed to be representative as a percentage of contraction.

For all fixed culture experiments, fluorescent images were collected using Olympus IX73 spinning disc confocal microscope or LSM800 laser scanning confocal microscope (Carl Zeiss) using 4X and 10X or 20X objectives, respectively.

Using ImageJ invasion distances and count were quantified by nuclear count. Each cell was given a relative position within the image and used to calculate the distance to the centre of the droplet. Cell spread area was quantified using ImageJ to calculate the area of phalloidin expression relative to the number of nuclei. Percentage of viable cells on polyacrylamide beads, was calculated as the number of cells labeled with Calcein AM, divided by the total number of cells identified with Calcein AM and EtHD. Percentage of viable cells on flat assays, was calculated

as the number of cells labeled with Calcein AM, divided by the total number of cells identified with DAPI.

Immunofluorescent images of AhR were analyzed using Cell Profiler v4.0 [271]. In brief, the nuclei and cell were identified (DAPI and phalloidin respectively) and masked to identify the cytoplasm only (Cell-Nuclei). AhR fluorescent intensity was quantified on a per cell basis, and reported expression in total cell, nuclei and cytoplasm. The same pipeline settings were used on all images of each immunostaining, where replicates were stained on the same day to control for fluorescent variation. 4 images were taken and analyzed per individual biological replicate.

4.16 Statistical Analysis

Statistical analyses were performed using Prism software (GraphPad Software Inc.). Mean values and standard deviation (SD) are shown on graphs that were generated from three repeats of biological experiments. P values were obtained from t tests with unpaired samples, with significance set at $p < 0.05$. For paired samples, one-way ANOVA test was run, followed by a post-hoc analysis using Newman-Keuls multiple comparison test, with significance set at $p<0.05$. Graphs show either the actual p value or symbols describing it (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

For Kaplan–Meier analyses of AHR in cervical cancer patients and pancreatic cancer patients, the TCGA database was mined on GEPIA2 server using recommended parameters[272].

For AHR expression analysis, the breast cancer data sets from Costa, et al.[30] (EGAS00001002508) and Gui, et al. [217] (ENA number PRJEB34465) were used.

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