Bioengineered 3D collagen-alginate interpenetrating network (IPN) to elucidate phenotypic switching of cancer associated fibroblasts

Cao, Huan

2021

Cao, H. (2021). Bioengineered 3D collagen-alginate interpenetrating network (IPN) to elucidate phenotypic switching of cancer associated fibroblasts. Doctoral thesis, Nanyang Technological University, Singapore. <https://hdl.handle.net/10356/148935>

<https://hdl.handle.net/10356/148935>

<https://doi.org/10.32657/10356/148935>

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0).

Downloaded on 26 Apr 2023 22:17:15 SGT

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**BIOENGINEERED 3D COLLAGEN-ALGINATE
INTERPENETRATING NETWORK (IPN) TO ELUCIDATE
PHENOTYPIC SWITCHING OF CANCER ASSOCIATED
FIBROBLASTS**

CAO HUAN

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2021

**BIOENGINEERED 3D COLLAGEN-ALGINATE
INTERPENETRATING NETWORK (IPN) TO ELUCIDATE
PHENOTYPIC SWITCHING OF CANCER ASSOCIATED
FIBROBLASTS**

CAO HUAN

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2021

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

25 / 03 / 2021

.....
Date

Cao Huan

.....
Cao Huan

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

25/03/2021

.....
Date

.....
Asst. Prof. Dalton Tay Chor Yong

Authorship Attribution Statement

This thesis contains material from one paper published in the following peer-reviewed journals in which I am listed as a co-first author.

Chapter 4 and 5 are published as Huan Cao, Melissa Kao Hui Lee, Haibo Yang, Siu Kwan Sze, Nguan Soon Tan, and Chor Yong Tay. Mechanoregulation of cancer-associated fibroblasts phenotype in three-dimensional interpenetrating hydrogel networks. *Langmuir* 35, 7487-7495 (2019). DOI: 10.1021/acs.langmuir.8b02649.

The contributions of the co-authors are as follows:

- Asst. Prof Tay provided an initial project direction and suggestions on experiment details.
- I have prepared the manuscript draft, and revised the manuscript together with Ms. Lee, Dr. Yang, Assoc. Prof. Tan and Assoc. Prof, Sze.
- I co-designed the study with Asst. Prof. Tay, performed the laboratory works at the School of Materials Science and Engineering, and analyzed the obtained data.

25 / 03 / 2021

.....

Date

Cao Huan

.....

Cao Huan

Abstract

Three-dimensional (3D) biomaterials with physiologically relevant and experimentally tractable biomechanical features are important platforms to advance the understanding on the influence of tissue mechanics in cancer progression. Cancer is the second leading cause of death worldwide, which conservatively estimated to have 13.1 million cases of cancer-related death by the year 2030. It has been shown that cancer-associated fibroblasts (CAF), the most abundant cell type within the tumor stroma, plays a significant role in promoting cancer progression via multiple mechanisms such as paracrine crosstalk, extracellular matrix (ECM) remodeling, etc. However, recent studies have also shown that certain subtypes of CAF may also possess anti-tumorigenic functions. Factors underpinning the existence of the different CAF subtypes with functionally diametrically different (anti and/or pro) tumorigenic properties remain elusive to date. Delineating the spatiotemporal phenotypic switching of CAF phenotype is important as it may uncover novel and specific targeted depletion of certain CAF subsets to effectively impede tumorigenesis. However, current conventional *in vitro* biological toolkits are inadequate to recapitulate the biochemical and biophysical tumor stroma microenvironment that is necessary to systematically investigate CAF heterogeneity.

In this thesis, an interpenetrating polymer network (IPN) hydrogel system composed of rat tail type I collagen and alginate (CoAl-IPN) was used as an *in vitro* tumor stroma surrogate. The distinct crosslinking chemistries of each network offer precise spatiotemporal control over the biochemical and biomechanical properties of the hydrogel. Hydrogels with Young's modulus (E) of ~ 100 Pa and ~ 1000 Pa were utilized as "soft" and "hard" cancerous stroma respectively. The rheological, mechanical, mass transport and microarchitectural properties of the hydrogel were systematically characterized. Using this platform, the effects of 3-dimensional (3D) matrix stiffness and network density on CAF morphology, adhesion, phenotype and paracrine activity were examined. In human breast CAF (b-CAF), it was observed that a soft 3D microenvironment promoted cell adhesion and spreading. In contrast, in a 3D stiff matrix ($E \sim 1000$ Pa) which is characterized by a highly cross-linked and dense network, spreading of b-CAF was severely constrained.

Moreover, nuclear translocation of the YAP/TAZ was also inhibited in b-CAF grown in a stiff matrix, indicative that the intracellular tension is in a “relaxed” state. Importantly, using a 3D MDA-MB-231 tumoroid invasive model, the pro-tumorigenic paracrine activity of b-CAF in the stiff matrix group was significantly muted. Therefore, using the CoAl-IPN hydrogel platform it was shown that the biophysical parameters of the tumor stroma could either promote or restrain the pro-tumorigenic activity of b-CAF.

Interestingly, the matrix stiffness dependent event was recapitulated in human colon-CAF (c-CAF), suggesting that the observed phenomenon was not specific to CAF of a particular tissue origin. Further mechanistic insights were sought via whole transcriptome profiling of the encapsulated CAF using RNA Seq. A total of 1,623 differentially expressed genes (DEGs) (FDR < 0.05) were identified between the c-CAF in the hard and soft matrix group. Further gene enrichment analysis of the DEGs against the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in combination of reverse transcription polymerase chain reaction (RT-PCR) and immunoassays revealed that a soft 3D matrix favors the adoption of a myofibroblastic (m-state) while a stiff 3D matrix favors an inflammatory (i-state) c-CAF phenotype. Specifically, m-state CAF displayed a higher expression level of intracellular α -SMA expression and downregulated IL-6 expression. On the other hand, i-state CAF has a higher secretory of inflammatory phenotype (i.e., increased IL-6) together with downregulated intracellular α -SMA expression. Importantly, phenotypic switching between both cellular states is reversible. Hub gene analysis also unveil reactive oxygen species (ROS)-hypoxia inducible factor 1 alpha (HIF1- α) as a novel mechano-redox transduction axis that can be conditionally switched “on” and “off” between i- and m- state CAF, respectively. The transition from the m- to i-state was accompanied by a significantly attenuated capacity to induce epithelial-mesenchymal transition (EMT) in human DLD-1 colorectal adenocarcinoma by the c-CAF-derived conditioned medium (CM). Consistently, upregulated cytokine/ chemokines genes in the i-state CAF were associated with good overall colorectal cancer patient survival based on the gene expression profiling interactive analysis (GEPIA) platform. Overall, these findings showed that 3D network density and spatial cellular confinements can profoundly determine CAF states, paracrine signaling, and metastatic potential. Deciphering the interplay between ECM mechanics and CAF

states (deleterious vs. beneficial) may reveal critical vulnerabilities of cancer and open-up new avenues of effective combinatorial anti-cancer therapeutic strategies.

Lay Summary

Death from cancer usually occurs when the cancer cells spread to other part of the body, which is also known as cancer metastasis during the late stage of disease progression. Cancer associated fibroblasts (CAF) is one of the major tumor stroma cell types that have been documented to influence tumor spreading by secreting growth factors/cytokines and remodeling the surrounding extracellular matrix (ECM). Hence, studies that target CAF have emerged as one of the promising strategies for cancer therapy. However, recent studies have shown that CAF can exhibit both pro- and anti-tumor effects. Yet, the underlying cause is unknown. In order to study the possible mechanism, a 3D cell culture platform is being proposed to mimic the native tumor stroma ECM to examine the plasticity behavior of the CAF. An interpenetrating polymer network (IPN) hydrogel system that contains at least two separately cross-linked polymer networks is highly suitable in this study due to the autonomous control over the biochemical and biophysical properties of the IPN hydrogel to have exclusive assessment of the cellular behavior. In this study, a CoAl-IPN hydrogel is chosen and fabricated via a two-steps temperature-induced cross-linking of collagen and calcium (Ca^{2+}) cations-mediated cross-linking of alginate. It has been found that 1) CAF would display different morphologies with a spreading and rounding characteristics upon encapsulating in soft and stiff hydrogel respectively; 2) a stiffer CoAl-IPN hydrogel ($E \sim 1000$ Pa) could suppress the pro-tumorigenic capacity of CAF as compared to the soft hydrogel ($E \sim 100$ Pa); and 3) the mechano-redox transduction axis of CAF in CoAl hydrogel could be conditionally switched “on” and “off”. The study understanding implicates that the specific targeting of pro-tumor CAF is critical for the efficacy of cancer therapy. In addition, new therapeutic strategies targeting tumor stroma could be explored, which can contribute to disrupt CAF-cancer cell communication and cancer progression. The CoAl hydrogel platform can be applied for other cancer basic researches and drug test.

Acknowledgements

Over the course of my 4 years postgraduate research journey, I am grateful and thankful to all the persons who have given me the unconditional support, guidance, and concern both at work and in my personal life.

Firstly, I would like to express my sincere gratitude to the generous financial supports and facilities provided by the School of Materials Science and Engineering, Nanyang Technological University Singapore, which includes scholarship, research grants, conference and travel funds, equipment access, and so on. Without these supports, this research work would have been simply impossible.

Special thanks are conveyed to my supervisor, Assistant Professor Tay Chor Yong, for his continuous support, patience, and instructive suggestions throughout my project. His positive working attitude and rigorous habits of critical/logical thinking have deeply influenced me to become a stronger and more positive person, striving for better outcomes.

I would also like to express my thanks to Associate Professor Tan Nguan Soon and Associate Professor Ng Kee Woei by providing valuable research suggestions to me, as well as allowing me to access their facilities and equipment.

Next, I would like to extend my appreciation to my research group leaders, Dr. Nguyen Kim Truc and Dr. Wang Jun Kit, by providing guidance in both my academic and research works, as well as sharing their research experience with me. Also, I am very grateful to Dr. Cao Ye, Dr. Cheng Hong Sheng and Dr. Deng Ya for all the help and encouragement that have been given to me.

Throughout the 4 years of study, I am thankful for all the technical expertise and advice provided by all the technical and administrative staff in MSE to ensure my research works and studies are going smoothly.

I would also like to take this opportunity to thank all my labmates, Dr. Yang Haibo, Mr. Wu Zhuoran, Mr. Lim Hong Kit, Ms. Cigdem Cimenoglu, Ms. Cheam Mein Ji, Dr. Chun Yong Yao, Mrs. Vrushali Baisane, Ms. Ma Yiyuan and Dr. Shi Pujiang for their consistent support and creating a friendly/warm working environment. I am also honored to have the opportunity in mentoring undergraduate students, including Mr. Ng Guang Shao, Mr. Tan Shaojie, Mr. Kelvin Renaldi Haryanto, Mr. Yew Jin Eugene, Ms. Violet Phua Xue Ting, and Ms. Khwar Nyo Thinn. Thank you for all the efforts devoted in exploring the scientific subjects together with me.

Last but not least, I would like to dedicate this thesis to my family and friends for their support and help.

Table of Contents

Abstract.....	i
Lay Summary	v
Acknowledgements	vii
Table of Contents	ix
Table Captions	xvii
Figure Captions.....	xix
Abbreviations	xxv
Chapter 1 Introduction.....	1
1.1 Hypothesis.....	3
1.2 Objectives and Scope	3
1.3 Dissertation Overview.....	4
1.4 Findings and Outcomes/Originality	6
References.....	7
Chapter 2 Literature Review	9
2.1 Overview	10
2.1.1 Tumor microenvironment (TME).....	10
2.1.1.1 Tumor stromal biotic cues	10
2.1.1.1.1 CAF origins	11

2.1.1.1.2	CAF molecular and functional heterogeneity	12
2.1.1.2	Tumor stromal abiotic cues	13
2.1.1.2.1	Stromal matrix biochemical cues	14
2.1.1.2.2	Stromal matrix biomechanical cues	14
2.1.1.3	Remolding of solid stress within tumor tissue.....	16
2.1.2	Mechanotransduction in tumor progression.....	17
2.1.2.1	Cellular mechanotransduction	17
2.1.2.2	Mechanomics approaches to understand cell behavior	20
2.1.2.3	Mechanoregulation within tumor microenvironment.....	21
2.1.3	Biomimetic models for tumor microenvironment study.....	22
2.1.3.1	2D and 3D cell culture.....	22
2.1.3.2	Single component hydrogels	24
2.1.3.3	Engineered hybrid hydrogels.....	27
2.1.3.3.1	Particles incorporated hydrogel.....	27
2.1.3.3.2	Electrospun/printed fibrils embedded hydrogel	28
2.1.3.3.3	Polymeric interpenetrating network hydrogel.....	29
2.1.3.3.4	Polymeric full-IPN hydrogel.....	31
2.1.3.4	Role of hydrogel surface chemistry on cell biology.....	32
2.1.3.5	Stimuli responsive IPN system to gain control in the 4 th dimension	33
2.2	Summary	34
	References.....	35
Chapter 3	Experimental Methodology.....	43
3.1	Preparation of CoAl-IPN hydrogel	44

3.2	Rheological measurements.....	45
3.3	Scanning electron microscope (SEM).....	49
3.4	Mass transport characterization.....	50
3.5	Immunofluorescence staining	51
3.6	Cell viability analysis.....	52
3.7	Intracellular reactive oxygen species (ROS) measurement.....	52
3.8	Enzyme-linked immunosorbent assay (ELISA).....	53
3.9	3D breast MDA-MB-231 tumoroids culture.....	53
3.10	Functional paracrine crosstalk study	54
3.11	RNA extraction from c-CAF embedded in CoAl-IPN hydrogels	56
3.12	Quantitative reverse-transcriptase real-time polymerase chain reaction (RT-PCR)	58
3.13	RNA sequencing (RNA-Seq) and DEG analysis	62
3.14	Gene set enrichment analysis (GSEA).....	63
3.15	Hub gene analysis.....	66
3.16	Gene expression profiling interactive analysis (GEPIA)	66
	References.....	67
 Chapter 4 Fabrication and characterization of mechanically tunable 3D CoAl-IPN hydrogel as an <i>in vitro</i> surrogate of tumor stroma		
4.1	Introduction	72
4.2	Experimental Methods	73
4.2.1	Materials	73
4.2.2	Fabrication of CoAl-IPN hydrogel	73
4.2.3	Rheological properties characterization.....	73
4.2.4	SEM	74

4.2.5	Mass transportation efficiency characterization	74
4.2.6	Statistical analysis	74
4.3	Principle Outcomes	74
4.3.1	Mechanical tunability of 3D CoAl-IPN hydrogel via varying crosslinking density	74
4.3.2	Dynamic mechanical tunability of IPN hydrogel via de- and re- crosslinking process.....	79
4.3.3	Stiffness-dependent microarchitecture of CoAl-IPN hydrogel.....	82
4.3.4	Non-stiffness-dependent mass transport efficiency of CoAl-IPN hydrogel	83
4.4	Conclusion.....	84
	References.....	86

Chapter 5 Effect of CoAl-IPN hydrogel stiffness on breast cancer-associated fibroblasts (b-CAF) phenotype and functional crosstalk with breast tumoroids89

5.1	Introduction	90
5.2	Experimental Methods	91
5.2.1	Materials	91
5.2.2	Fabrication of CoAl-IPN hydrogel	91
5.2.3	2D culture of b-CAF on CoAl-IPN hydrogel	91
5.2.4	3D culture of b-CAF within CoAl-IPN hydrogel and collection of condition medium	92
5.2.5	Live/dead assay of b-CAF within CoAl-IPN hydrogel.....	92
5.2.6	b-CAF morphology analysis.....	92
5.2.7	YAP/TAZ activity assay	93
5.2.8	<i>In vitro</i> breast tumoroids invasiveness assay	93
5.2.9	Statistical analysis.....	93

5.3	Principle Outcomes	93
5.3.1	Non-stiffness-dependent morphology of b-CAF on the CoAl-IPN hydrogel surface	93
5.3.2	Stiffness-dependent morphology of b-CAF within 3D CoAl-IPN hydrogel	95
5.3.3	Effect of hydrogel stiffness on YAP/TAZ activity and its correlation to a phenotypic shift of b-CAF	100
5.3.4	Mechanoregulation of functional crosstalk between b-CAF and breast tumoroids	103
5.3.4.1	Effect of CM derived from hydrogel-encapsulated b-CAF on breast tumoroids invasion.....	105
5.3.4.2	Effect of hydrogel-encapsulated b-CAF on tumoroids invasion via co-culturing	106
5.4	Conclusion.....	107
	References	108

Chapter 6 Effect of CoAl-IPN hydrogel stiffness on colon cancer-associated fibroblasts (c-CAF) phenotype and functional crosstalk with colorectal adenocarcinoma cells

6.1	Introduction	112
6.2	Experimental Methods	112
6.2.1	Materials	112
6.2.2	Fabrication of CoAl-IPN hydrogel	113
6.2.3	2D culture of c-CAF on CoAl-IPN hydrogel.....	113
6.2.4	3D culture of c-CAF within CoAl-IPN hydrogel and collection of condition medium	113
6.2.5	Live/dead assay of c-CAF within CoAl-IPN hydrogel.....	114

6.2.6	c-CAF morphology analysis within CoAl-IPN hydrogel	114
6.2.7	<i>In vitro</i> colorectal adenocarcinoma DLD-1 cells EMT assay.....	114
6.2.8	Statistical analysis	114
6.3	Principle Outcomes	115
6.3.1	Stiffness-dependent morphology of c-CAF on the CoAl-IPN hydrogel surface	115
6.3.2	Stiffness-dependent morphology of c-CAF within 3D CoAl-IPN hydrogel	116
6.3.3	Mechanoregulation of functional crosstalk between c-CAF and colorectal adenocarcinoma cells	117
6.4	Conclusion.....	120
	References	122

Chapter 7 Effect of CoAl-IPN hydrogel stiffness on c-CAF whole transcriptomes and its correlation to colorectal cancer patient overall survival125

7.1	Introduction	126
7.2	Experimental Methods	127
7.2.1	Materials	127
7.2.2	CoAl-IPN hydrogel Fabrication and c-CAF encapsulation.....	127
7.2.3	RNA sequencing and transcriptome analyses.....	128
7.2.4	IL-6 ELISA test.....	129
7.2.5	Intracellular reactive oxygen species (ROS) measurement	129
7.2.6	α -SMA and HIF-1 α immunocytochemical staining	123
7.2.7	Statistical analysis.....	130
7.3	Principle Outcomes	130

7.3.1	NGS analysis unveil matrix stiffness-dependent acquisition of inflammatory and myofibroblastic cell state in c-CAF	130
7.3.2	ROS-HIF-1 α is a mechanotransducible signaling axis in c-CAF	134
7.3.3	Correlation analysis of i-state c-CAF and CRC patient overall survival...	139
7.4	Conclusion.....	141
	References	145
Chapter 8 Conclusions and Recommendations.....		149
8.1	Conclusions	150
8.2	Reconnaissance and Future Work	153
8.2.1	Understand the role of ROS in matrix stiffness-induced breast tumoroids invasiveness	153
8.2.1.1	The relationship between matrix stiffness-induced solid stress and breast tumoroids invasiveness	153
8.2.1.2	The relationship between mechanical stress and ROS generation within tumoroids.....	156
8.2.2	Gelatin-based self-healing hydrogel to engineer tumor microenvironment.....	158
	References	160

Table Captions

Table 3.1	Primer sequence of genes used in qPCR studies.	58
Table 7.1	The details of the cytokines DEGs from enriched ‘cytokine-cytokine receptor interaction pathway’ and the corresponding survival correlation analysis.	143

Figure Captions

Figure 2.1.1.1	Schematic representation of the tumor microenvironment.....	11
Figure 2.1.1.1	Schematic representation of the functional heterogeneity of CAF...13	
Figure 2.1.1.3	Schematic diagram illustrating the physicochemical properties changes of the breast tumor microenvironment during tumor progression.....	16
Figure 2.1.1.4	Solid stress and surrounding matrix stiffness are two distinct biomechanical abnormalities contributing to tumor solid stress build-up.	17
Figure 2.1.2.1	Schematic representation of cellular ECM-integrin-cytoskeleton-nucleus axis mechanotransduction signaling cascades.....	20
Figure 2.1.3.1	Schematic representation of the cellular and microenvironmental differences between 2D and 3D cell culture platforms.	23
Figure 2.1.3.2	Schematic representation of different biomaterials for single hydrogel fabrication and their corresponding crosslinking mechanism.	27
Figure 2.1.3.3	Schematic illustration of single and multi-component hybrid-typed hydrogels.	30
Figure 3.1.1	Schematic of the crosslinking and de-crosslinking chemistry of the devised CoAl-IPN.....	45
Figure 3.2.1	Schematic image of oscillatory characterization mechanism.....	46
Figure 3.2.2	Amplitude and frequency sweep of rheology test of protein-DNA C2IN-C3lim-G205C hydrogel.	47

Figure 3.9.1	Schematic images of tumoroids fabrication process.	54
Figure 3.10.1	The schematic images of the experiment design to investigate the functional paracrine crosstalk between CAF and cancer cells.	55
Figure 3.11.1	The RNA extraction process, including sample homogenization and RNA purification using TRIzol™ Plus RNA Purification Kit.	57
Figure 3.13.1	The RNA sequencing main steps.	62
Figure 3.14.1	The eight collections of the Molecular Signature Database.	64
Figure 3.14.2	Representative enrichment plot image from GSEA.	65
Figure 3.16.1	The correlation analysis between gene (ERBB2) and patient survival with colon cancer.	67
Figure 4.3.1.1	Two-step crosslinking process of CoAl IPN hydrogel.	75
Figure 4.3.1.2	Rheological characterization of hydrogels.	77
Figure 4.3.1.3	Young's modulus of CoAl-IPN hydrogels.	79
Figure 4.3.2.1	Rheological characterization of hydrogels before and after de-/re-crosslinking process.	80
Figure 4.3.2.2	The stiffness of CoAl-IPN hydrogel can be tuned via specifically de-crosslinking of alginate network.	81
Figure 4.3.2.3	Alginate polymer chains could be remained within IPN after 2 days incubation.	82

Figure 4.3.3.1	Representative cross-sectional scanning electron microscope (SEM) images of IPN hydrogels.	83
Figure 4.3.4.1	Crosslinking density of hydrogels would not affect the mass transport efficiency of hydrogel.	84
Figure 4.4	Schematic illustration of CoAl-IPN hydrogel properties for tumor stroma mimicking.	86
Figure 5.3.1.1	The fluorescence images of b-CAF cultured on 2D CoAl-IPN hydrogel surface.	95
Figure 5.3.2.1	The viability assay of b-CAF within CoAl-IPN hydrogel.	96
Figure 5.3.2.2	CoAl-IPN hydrogel stiffness is the key determinants of b-CAF morphology in 3D context.	98
Figure 5.3.2.3	Time-course monitoring of b-CAF behavior within CoAl-IPN hydrogel over 48 h culturing.	99
Figure 5.3.2.4	The enhancement of b-CAF spreading on 2D RGD conjugated alginate hydrogel	99
Figure 5.3.3.1	CoAl-IPN hydrogel stiffness influence the mechanotransduction of the YAP/TAZ signaling axis and CAF marker transcript expression.	102
Figure 5.3.3.2	Actin cytoskeleton of CAF' regulates YAP/TAZ nuclei-cytoplasmic shuttling.	103
Figure 5.3.4.1	The bioactivity of b-CAF CM is highly dependent on the CoAl-IPN hydrogel stiffness.	105

Figure 5.3.4.2	CoAl-IPN hydrogel stiffness modulates functional paracrine dialogue <i>in situ</i> between b-CAF and MDA-MB-231 tumoroids via variant crosslinking density.	106
Figure 5.4	Schematic illustration of CoAl-IPN hydrogel stiffness effect on b-CAF phenotype and functional crosstalk with breast tumoroids....	107
Figure 6.3.1.1	The fluorescence images of c-CAF cultured on 2D CoAl-IPN hydrogel surface.	115
Figure 6.3.2.1	Comparison of c-CAF morphology and proliferation in 3D CoAl-IPN hydrogel.	117
Figure 6.3.3.1	Hydrogel mechanics governs EMT-inducing potency of c-CAF conditioned medium.	119
Figure 6.3.3.2	Representative bright field images of DLD-1 cells after treatment with CM _{CoAl0} and diluted (1.5 times) CM _{CoAl0}	120
Figure 6.4	Schematic illustration of CoAl-IPN hydrogel stiffness effect on c-CAF phenotype and functional crosstalk with colorectal adenocarcinoma DLD-1 cells.	121
Figure 7.3.1.1	Quality and quantity assessment of RNA samples from c-CAF cultured in either CoAl ₀ and CoAl ₁₀₀	131
Figure 7.3.1.2	Comparative transcriptional signatures of c-CAF revealed <i>in situ</i> phenotypic shift govern by hydrogel mechanics.	133
Figure 7.3.1.3	Gene ontology analysis of all upregulated DEGs in i-state c-CAF	134

Figure 7.3.2.1	Gene ontology and gene set enrichment analysis of RNA-Seq data reveals HIF-1 α as a novel upstream mechanotransducer of c-CAF137
Figure 7.3.2.2	Modulation of intracellular ROS levels of c-CAF in hydrogels of varying stiffness and culture conditions138
Figure 7.3.2.3	Modulation of HIF-1 α expression of c-CAF grown in coAl ₁₀₀ hydrogel with or without N-acetylcysteine (NAC) treatment and quantitative assay.138
Figure 7.3.3.1	Significantly cytokine-related DEGs in i-state c-CAF associates with CRC patient overall survival in two independent cohorts.141
Figure 8.2.1.1	Tumoroids response before and after de-crosslinking of alginate network within CoAl-IPN hydrogel.154
Figure 8.2.2.1	The design and biocompatibility of gelatin based self-healing hydrogel.160

Abbreviations

2D	Two Dimension
3D	Three Dimension
ACTA2	ACTin Alpha 2
ANOVA	ANalysis Of VAriance
α -SMA	α -Smooth Muscle Actin
BSA	Bovine Serum Albumin
CaCl ₂	Calcium Chloride
CAF	Cancer-Associated Fibroblasts
CCL	C-C motif Chemokine Ligand
cDNA	complementary DeoxyriboNucleic Acid
CM	Conditioned Medium
CoAl-IPN	Collagen-Alginate InterPenetrating Network
COL1A1	COLlagen type I Alpha 1 chain
CRC	Colorectal Cancer
CSF1	Colony Stimulating Factor 1
C _T	Cycle Threshold
CXCL	chemikine (C-X-C motif) Ligand
dECM	decellularized ECM
DEGs	Differential Expressed Genes
DI water	De-Ionized water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	DeoxyriboNucleic Acid
dNTP	deoxy-riboNucleoside TriPhosphate
dsDNA	double-stranded DeoxyriboNucleic Acid
E-Cad	E-Cadherin
ECM	Extracellular Matrix
EMT	Epithelial-to-Mesenchymal Transition
ES	Enrichment Score
EthD-1	Ethidium homodimer-1

FAK/Src	Focal Adhesion Kinase/Steroid receptor coactivator
FBS	Fetal Bovine Serum
FC	Fold Change
FDR	False Discovery Rate
FITC	Fluorescein IsoThioCyanate
FN1	FibroNectin
FPKM	Fragments Per Kilobase Million
Full-IPN	Full-InterPenetrating Network
GAGs	GlycosAminoGlycans
GelMA	Gelatin MethAcrylate
GEPIA	Gene Expression Profiling Interactive Analysis
GSEA	Gene Set Enrichment Analysis
GTE _x	Genotype-Tissue Expression
HA	Hyaluronic Acid
HIF1	Hypoxia-Inducible Factor 1
HMDS	HexaMethylDiSilizane
HR	Hazard Ratio
iCAF	inflammatory CAF
IL	InterLeukin
INHBA	INHBin Beta A Subunit
IPA	Ingenuity Pathways Analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAMA4	Laminin
LN	LamiNin
LOX	Lysyl OXidase
LPS	LiPopolySaccharide
LVE	Linear Viscoelastic
miRNA	Micro RNA
mM	Millimolar
MMPs	Matrix MetalloProteins

MSigDB	Molecular Signatures DataBase
mRNA	messenger RNA
MT	Melting Temperature
MW	Molecular Weight
myCAF	myofibroblastic CAF
NaCl	Sodium Chloride
N-Cad	N-Cadherin
NES	Normalized Enrichment Score
NFκB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
ODex	Oxidized Dextran
Pa	Pascal
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDGFC	Platelet Derived Growth Factor C
PEG	Poly(Ethylene Glycol)
PFA	ParaFormAldehyde
PHD	Prolyl HyDroxylase
PLGA	Ploy(Lactic-co-Glycolic Acid)
RGD	tripeptide Arginine-Glycine-Aspartate
RhoA	Ras Homolog family member A
RNA	RiboNucleic Acid
RNA-Seq	RNA-Sequencing
ROCK	Rho-associated protein kinase
ROS	Reactive Oxygen Species
RT-PCR	quantitative Reverse-Transcriptase Polymer Chain Reaction
SD	Standard Deviation
SEM	Scanning Electron Microscopy
Semi-IPN	Semi-InterPenetrating Network
SFM	Serum-Free Medium
TAZ	Transcriptional coactivator with PDZ-binding motif
TCGA	The Cancer Genome Atlas Program

TEAD	TEA Domain family member 1
TME	Tumor Microenvironment
VEGFC	Vascular Endothelial Growth Factor C
Vin	Vimentin
YAP	Yes-Associated Protein

Chapter 1

Introduction

Tumor microenvironment (TME) is a complex setting consisting of multiple cell types, extracellular matrix (ECM) proteins and biomolecules. Due to the involvement of stroma tissue (i.e., ECM surrounding tumor cells) in regulating tumor growth, invasion, and metastasis, a myriad of 3D hydrogel models were developed to study the influence of the stroma matrix physicochemical properties on tumor activities. Parameters such as matrix stiffness and biochemical composition have been implicated as important drivers of cancer progression. However, the traditional use of a single-component hydrogel is insufficient to fully emulate and to decouple the mechano-biochemical complexity of the TME necessary to gain the critical structure-activity relationship mechanistic insights. For instance, the increase of the solute concentrations (i.e., collagen) could lead to the overlapping effects of increasing cell adherence ligand density and stiffness. Conversely, hybrid hydrogel system, such as interpenetrating polymer network (IPN) hydrogel, has emerged as an attractive alternative for cancer disease modelling. In this thesis, the hypothesis, objectives, and scope of the study will be presented, followed by a summary of findings.

Tumor microenvironment (TME) is a highly complex and heterogeneous three-dimensional (3D) tissue that is composed of various biotic and abiotic elements. In addition to the cancerous cells, the surrounding tumor stroma is composed of a complex mixture of different cell types, such as cancer-associated fibroblasts (CAF), immune cells and vascular endothelial cells, etc.[1-4] CAF are the most abundant non-cancerous cell type within the TME,[5, 6] which play an active role in remodeling the stroma extracellular matrix (ECM) via deposition of collagen/fibronectin and increasing the local crosslinking density of the ECM proteins, thus resulting in matrix stiffening over time.[7] This matrix stiffening, in turn, mediates the mechanoregulation of CAF and their phenotype expression that impacts the tumor progression in a paracrine crosstalk manner.[8, 9] Simultaneously, matrix stiffening also leads to the accumulation of solid stress experienced by the tumor cells as a result of mechanical confinement and hyperproliferation of tumor cells, which may further affect the tumor cell behavior.[10-12] Subsequently, the growing size of tumoroids will further compress the surrounding stromal matrix, resulting in mechanical properties changes including the network architecture, thus leading to a formation of vicious loop.

While it is generally accepted that CAF are the master architects of the tumor ECM, exactly how CAF reciprocate to these ECM mechanical changes remains elusive to date.

Intensive research has been carried out to study the effect of matrix stiffness on tumor progression. However, most of these studies are limited to a 2D culture model.[13, 14] In the recent years, the use of 3D hydrogels for cancer research study has gained significant attraction.[15, 16] However, most of the hydrogel systems used were fabricated based on a single-component hydrogel system, which is inadequate to emulate the complex TME. In addition, the use of a single-component hydrogel system fails to provide *in situ* control of the matrix stiffness as well as the potential to decouple the influence between biochemical and biophysical factors of the hydrogel. For example, a relatively stiffer collagen hydrogel can be achieved by increasing the concentration of the collagen. However, the increase in cell adherence ligand density could potentially affect cell behavior and signaling. Therefore, a hybrid hydrogel system, such as interpenetrating polymer network (IPN) hydrogel, could potentially be an ideal candidate in overcoming the above-

mentioned drawbacks of mono-hydrogel system. IPN are polymer systems comprising of two or more networks that are interposed non-covalently with each, thereby enabling the autonomous control over the biochemical and biophysical properties of the respective networks.[17, 18]

1.1 Hypothesis

The thesis tests the hypothesis that CAF phenotype, secretory activities and paracrine signaling could be modulated under the influence of ECM mechanics in a 3D setting.

1.2 Objectives and Scope

The overarching aim of this study is to elucidate the (i) direct effect of matrix stiffness on CAF phenotype and secretory activities, as well as the (ii) indirect effect on cancer invasion and metastasis. This is achieved by employing a 3D CoAl-IPN hydrogel base cell culture platform as an *in vitro* tumor stroma surrogate. The stiffness of the CoAl-IPN can be tuned by varying the degrees of crosslinking/de-crosslinking of the alginate component to mimic the native mechanical properties of tumor stroma at different stage (breast stroma E: 200 – 800 Pa [19]; colon stroma E: 0.374 – 68.0 kPa [20]).

The specific objectives are:

1. To fabricate and characterize a mechanically tunable 3D CoAl-IPN hydrogel for CAF culture.
2. To examine the effect of matrix stiffness on CAF phenotype and functional characteristics.
3. To elucidate the mechanomics of CAF within CoAl-IPN hydrogel under the influence of varying matrix stiffness.

1.3 Dissertation Overview

The framework of the proposed research program is based on the mentioned above. The overall flow can be subdivided into four phases:

1.3.1 Phase 1: Fabrication and characterization of mechanically tunable 3D biomimetic CoAl-IPN hydrogel

IPN hydrogel system is known for its capability with independent crosslinking of multiple network that is non-covalently interposed with each other, which allows the autonomous control over the biochemical and biophysical properties of the respective networks. Both collagen and alginate could be crosslinked via different crosslinking mechanisms. Specific to this study, owing to their well-established biocompatibility and facile preparation, collagen (Co) and alginate (Al) has been selected as the respective constituents for the IPN. CoAl-IPN hydrogel was prepared via two-step temperature-induced and cation-mediated (Ca^{2+}) crosslinking process, in which the stiffness could be fine-tuned by varying the crosslinker (Ca^{2+}) concentration (0, 15 and 100 mM) while maintaining the constant components. The mechanical tunability, microarchitecture, and mass transport within hydrogel were first examined, prior to the degradability of the IPN.

1.3.2 Phase 2: Examination of the effect of CoAl-IPN matrix stiffness on breast CAF (b-CAF) phenotype and functional crosstalk with breast tumoroids

The utility of CoAl-IPN hydrogel to investigate the CAF mechanobiology was examined within the context of the breast TME. Stiffness of the CoAl-IPN was varied to mimic the biomechanics of the breast tumor tissue at different stage. Using this platform, the phenotypic changes to the b-CAF, in terms of cell spreading and cytoskeleton development was systematically examined. At the mechanistic level, the mechano-sensitive YAP/TAZ pathway was also evaluated. In addition, functional implications of the matrix stiffness dependent b-CAF phenotypic alterations were drawn through *in vitro* paracrine crosstalk studies involving b-CAF and MDA-MB-231 breast tumoroids.

1.3.3 Phase 3: Investigation of CoAl-IPN hydrogel stiffness on colorectal CAF (c-CAF) phenotype and functional crosstalk with colorectal adenocarcinoma cells

To validate the robustness of the developed platform, the use of CoAl-IPN to study the mechanobiology of c-CAF was demonstrated. Likewise, comparative studies involving the mechanoresponse of c-CAF in CoAl-IPN hydrogel with stiffness mimicking to that of the colorectal tumor stroma at different stage were being conducted. In addition, the stiffness-dependent paracrine crosstalk between c-CAF and colorectal adenocarcinoma cells DLD-1 was examined.

1.3.4 Phase 4: Examination of the effect of CoAl-IPN hydrogel matrix stiffness on the transcriptome of c-CAF and its correlation to cancer cell invasiveness

To gain further insights into the global transcriptomics changes accompanying the matrix stiffness dependent phenotypic changes to the c-CAF, next-generation sequencing (NGS) analysis was employed. This approach is in line with the recently introduced concept of mechanomics – codifying the role of mechanical environment on cell function and mapping the global mechanically sensitive proteins and transcripts encoded by its genome.[21, 22] Advanced bioinformatics tools such as Gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Cytospace, and Gene Set Enrichment Analysis (GSEA) were exploited. Non-biased comparative NGS data between the c-CAF in different microenvironment was further validated at the mRNA and protein level. Further correlational analysis of the NGS data was conducted using the obtained *in vitro* data in Phase 3 using the Gene Expression Profiling Interactive Analysis (GEPIA) webserver for patient cohort data.

The dissertation is organized as follows:

Chapter 1 provides the rationale for the research and outlines of the objectives and scope.

Chapter 2 reviews the literature concerning the complexity of the TME, followed by the

evolution of stroma matrix stiffness and the accumulation of tumor solid stress during tumor progression. Subsequently, the phenomenon of mechanoregulation in tumor progression is discussed. The pros and cons of the different hydrogel systems for cancer research are also being covered.

Chapter 3 introduces the detailed experiment methodologies and characterization techniques, together with the principles behind the main experimental techniques used in this dissertation.

Chapter 4 describes the fabrication and characterization of CoAl-IPN hydrogel adopted in this dissertation for CAF culture, including the rheological study, microarchitecture observation, and mass transport efficiency of the hydrogel.

Chapter 5 conducts the bio-characterization of b-CAF cultured within the CoAl-IPN hydrogel prior to assessing the functional crosstalk between b-CAF and breast tumoroids.

Chapter 6 evaluates the behaviors of c-CAF cultured within the CoAl-IPN hydrogel, such as cell spreading, mechanoregulation, and paracrine crosstalk effect on the colorectal adenocarcinoma cells DLD-1.

Chapter 7 begins with the whole transcriptome analyses of c-CAF within the CoAl-IPN hydrogel with different matrix stiffness, followed by assessing the correlation between the obtained matrix stiffness-dependent c-CAF phenotype on the colorectal DLD-1 cancer cells' invasiveness.

Chapter 8 concludes the main findings of this dissertation as well as the proposed future studies.

1.4 Findings and Outcomes/Originality

This research led to several novel outcomes:

1. Established a stiffness-tunable CoAl-IPN hydrogel platform used as an *in vitro* tumor stroma surrogate
2. Examined the effect of matrix stiffness on CAF phenotypes and mechanoregulation signaling via YAP/TAZ transcription factors in a 3D context
3. Identified two states of CAF via whole transcriptome assays, namely m-state (α -SMA^{high}IL6^{low}) and i-state (α -SMA^{low}IL6^{high}). Demonstrated that iCAF encapsulated within stiffer CoAl-IPN hydrogel attained inflammatory (secretory) characteristics, which affected the phenotype expression and muted the tumor cell invasiveness as compared to the corresponding soft hydrogel in a 3D context
4. Implicated reactive oxygen species (ROS)-hypoxia inducible factor 1 alpha (HIF1- α) as a novel mechano-redox transduction axis that can be conditionally switched “on” and “off” in i- and m- state CAF respectively

References

- [1] D. Hanahan, R. A. Weinberg. *Cell*. **2011**, 144, 646-74.
- [2] S. Togo, U. M. Polanska, Y. Horimoto, A. Orimo. *Cancers (Basel)*. **2013**, 5, 149-69.
- [3] R. Kalluri, M. Zeisberg. *Nat. Rev. Cancer*, **2006**, 6, 392-401.
- [4] P. Cirri and P. Chiarugi. *Am. J. cancer Res.* **2011**, 1, 482-497.
- [5] L. Bu, H. Baba, N. Yoshida, K. Miyake, T. Yasuda, T. Uchihara, P. Tan and T. Ishimoto. *Oncogene*. **2019**, 38, 4887-4901.
- [6] M. Augsten. *Front. Oncol.* **2014**, 4, 62.
- [7] R. Malik, P.I. Lelkes and E. Cukierman. *Trends Biotechnol.* **2015**, 33, 230-6.
- [8] F. Calvo, N. Ege, A. Grande-Garcia, S. Hooper, R.P. Jenkins, S.I. Chaudhry, K. Harrington, P. Williamson, E. Moeendarbary, G. Charras and E. Sahai. *Nat. Cell Biol.* **2013**, 15, 637-46.
- [9] S. Ishihara, D.R. Inman, W.J. Li, S.M. Ponik and P.J. Keely. *Cancer Res.* **2017**, 77, 6179-6189.
- [10] J. Cha and P. Kim. *Frontiers in Materials*. **2017**, 4.
- [11] M. Kalli and T. Stylianopoulos. *Front Oncol.* **2018**, 8, 55.
- [12] H. T. Nia, H. Liu, G. Seano, M. Datta, D. Jones, N. Rahbari, J. Incio, V. P. Chauhan,

- K. Jung, J. D. Martin, V. Askoxylakis, T. P. Padera, D. Fukumura, Y. Boucher, F. J. Hornicek, A. J. Grodzinsky, J. W. Baish, L. L. Munn and R. K. Jain. *Nat. Biomed. Eng.* **2016**, 1.
- [13] M. E. Katt, A. L. Placone, A. D. Wong, Z. S. Xu and P. C. Searson. *Front Bioeng. Biotechnol.* **2016**, 4, 12.
- [14] Y. Imamura, T. Mukohara, Y. Shimono, Y. Funakoshi, N. Chayahara, M. Toyoda, N. Kiyota, S. Takao, S. Kono, T. Nakatsura and H. Minami. *Oncol. Rep.* **2015**, 33, 1837-43.
- [15] M. Ebara, Y. Kotsuchibashi, K. Uto, T. Aoyagi, Y.-J. Kim, R. Narain, N. Idota and J. M. Hoffman. *Smart Biomaterials.* **2014**, 9-65.
- [16] K. M. Park, D. Lewis and S. Gerecht. *Annu. Rev. Biomed. Eng.* **2017**, 19, 109-133.
- [17] D. Myung, D. Waters, M. Wiseman, P. E. Duhamel, J. Noolandi, C. N. Ta and C. W. Frank. *Polym. Adv. Technol.* **2008**, 19, 647-657.
- [18] A. Soman, F. Mathew, A. J. Chacko, M. Alias and G.V. Poosan. *The Pharma. Innovation J.* **2014**, 3, 59-66.
- [19] C. M. Kraning-Rush and C. A. Reinhart-King. *Cell Adh. Migr.* **2012**, 6, 274-9.
- [20] S. Kawano, M. Kojima, Y. Higuchi, M. Sugimoto, K. Ikeda, N. Sakuyama, S. Takahashi, R. Hayashi, A. Ochiai and N. Saito. *Cancer Sci.* **2015**, 106, 1232-9.
- [21] L. A. Sawicki, E. M. Ovadia, L. Pradhan, J. E. Cowart, K. E. Ross, C. H. Wu and A. M. Kloxin. *APL Bioeng.* **2019**, 3, 016101.
- [22] J. Wang, D. Lu, D. Mao and M. Long. *Protein Cell.* **2014**, 5, 518-31.

Chapter 2

Literature Review

This chapter introduces the properties of tumor microenvironment (TME) during tumor progression and the current in vitro 3D hydrogel models used in cancer research. Special emphasis will be placed to discuss the origins, molecular and functional heterogeneity of CAF within tumor stroma. Thereafter, the cellular mechanotransduction pathways and the signals derived from TME that influence tumor progression are being discussed at the molecular level from the perspective of mechanomics. Finally, a comprehensive review of the application of hybrid 3D hydrogel models for TME study is presented.

2.1 Overview

2.1.1 Tumor microenvironment (TME)

Cancer is the second leading cause of death globally, which is estimated to have 13.1 million of cancer-related death cases by 2030 (<http://globocan.iarc.fr/factsheets/populations/factsheet.asp?uno=900>). The development of tumors is an elaborate multistep process, which begins with the formation of polyps due to the gene mutations of the epithelial cells (i.e., cancerous cells). Subsequently, these cancerous cells can invade other organs via the process of extravasation by passing through the base-membrane and travel along with the lymphatic and blood vessel system, followed by the intravasation process – escaping of circulating cancer cells from the blood vessels to colonize distal organs.[1] Throughout this process, the tumor cells are engaged in a complex crosstalk with the biotic and abiotic microenvironmental components in a tightly orchestrated fashion. Seminal works by Stephen Paget highlighted the important regulatory of the TME in the “seed & soil” paradigm.[2] The cancer cells (seeds) confined within the specific organ microenvironment (soil) are in constant interactions with the surrounding stroma cues that ultimately influence the tumor progression. As such, gaining a more in-depth understanding of the extrinsic and intrinsic parameters in governing the cancer cell behaviors is important to provide critical insights into the biology of cancer and the development of novel anti-cancer therapeutics.

2.1.1.1 Tumor stromal biotic cues

The TME refers to the environment (stroma) surrounding the tumor (**Figure 2.1.1.1**). This tumor stroma is composed of a broad spectrum of cell types, such as cancer-associated fibroblasts (CAF), immune cells, cancer stem cells, vascular endothelial cells, etc., in which CAF are the majority of the non-cancerous cells within the TME. [3]

Figure 2.1.1.1 Schematic representation of the tumor microenvironment. Adapted with permission [3]. Copyright 2017 The Author(s).

2.1.1.1.1 CAF origins

The origins of CAF remain elusive to date. Resident fibroblasts, bone marrow derived mesenchymal/hematopoietic stem cells, various mutational cell types via epithelial-to-mesenchymal transition (EMT) process, including epithelial cells, endothelial cells, pericytes, adipocyte and even carcinoma cells have all been documented to be possible sources of CAF. [4] As for any single tumor, it is highly possible that the stroma CAF are heterogenous, even though it is hard to pinpoint the exact origin of CAF. Furthermore, there is currently no single biomarker that can definitively confirm the identity of CAF, usually a combination of different markers would be referred, such as α -SMA, Fibroblast associated protein (FAP), fibroblast specific protein-1 (FSP-1) et al. [5]

Native resident fibroblasts found within the tumor stroma which can be “activated” via growth factors and cytokines, is widely believed to be the predominant source of CAF via growth factors and cytokines activation. In addition, apart from the expression of CAF makers at the transcriptional and protein level, it has been reported that CAF can

overexpress IL-6 compared to resident normal fibroblasts, which could favor CAF formation via autocrine signaling loop. [6] Seiichiro Ishihara et al. had reported that the stiffness of ECM can also serve as a potent regulator of trans-differentiation of MSC into CAF via mechano-signal transduction in MSC, which will enhance tumor proliferation by protein prosaposin secretion. [7]

2.1.1.1.2 CAF molecular and functional heterogeneity

Previously, numerous studies have implicated CAF as a critical regulator of tumor progression via secretion of growth factors (VEGF, HGF, SDF-1, PDGF, etc.) and cytokines (ILs and CXCLs) to drive malignancies, [8-9] stroma matrix remodeling and active-active interaction with cancer cells. However, the clinical Phase Ib/II study of CAF targeting using vismodegib, a SHH antagonist of Sonic hedgehog (SHH) derived from CAF, could not enhance the patient survival with metastatic pancreatic cancer. [10] Even worse, genetic depletion of alpha-smooth muscle actin (α -SMA)-positive CAF did not curtail tumor progression, but instead significantly enhanced the aggressiveness of the pancreatic tumor via the upregulation of CD4⁺ regulator T-cells. [11] Therefore, apart from the pro-tumor activities, researches revealed that CAF also show tumor suppression role, [12-13] which may be due to the heterogenous nature of CAF source. **(Figure 2.1.1.2)** For example, CAF-derived slit homolog 2 protein (SLIT2) was also shown to suppress breast cancer cell invasion by inhibiting the phosphoinositide 3-kinase (PI3K) and β -catenin signaling.[14] In addition, Heather M. Brechbuhl revealed that estrogen receptor-positive (ER⁺) breast cancers contain two CAF subtypes defined by CD146 expression. CD146^{neg} CAF suppress ER expression in ER⁺ breast cancer cells, decrease tumor cell sensitivity to estrogen, and increase tumor cell resistance to tamoxifen therapy. [15] Especially, using transcriptomics, it is demonstrated that the gene expression differences between CAF were indeed the molecular basis of its functional heterogeneity. Costa *et al.* revealed the existence of four CAF subsets (i.e., S1-4). Among those, CAF-S1 and CAF-S4 are α -SMA-positive myofibroblastic subsets but have diametrically opposing roles. Fibroblast activation protein (FAP)-positive CAF-S1 is associated with an immunosuppressive TME by promoting the differentiation of T cells into regulator T cells (Tregs), whereas CAF-S4 is

linked to high CD8⁺ cytotoxic T lymphocytes infiltration. [16] A pioneer work conducted by Ankit Kumar Patel revealed that there are two distinct subtypes of CAF within oral tumor stroma, which could influence oral cancer cell stemness via CAF-expressed BMP4. Specifically, the subtype with lower expression of α -SMA would restrict the self-renewal of stem-like cancer cells. [17] Overall, this project is focusing on biophysical factors underpinning the existence of the different CAF subtypes with functionally diametrically different (anti and/ or pro) tumorigenic properties *in vitro* tractable biological toolkits.

Figure 2.1.1.2 Schematic representation of the functional heterogeneity of CAF. Adapted with permission [12]. Copyright 2019 Springer Nature.

2.1.1.2 Tumor stromal abiotic cues

The physicochemical properties of the surrounding stroma ECM are in constant changes during the tumor progression (**Figure 2.1.1.3**), including extracellular matrix (ECM) composition, fibrils architecture, crosslinking density, stiffness, etc. The remodeling of stroma ECM not only has a direct influence on the cell ligand-receptor interactions but also

impose significant impacts on the blood vessel formation and nutrition/waste/oxygen diffusion.[18, 19] The increase in stroma ECM content that is accompanied by the hyperproliferative behavior of tumor cells can further induce the interstitial pressure and solid stress within the tumor, thus indirectly affecting the tumor cell activities.[20]

2.1.1.2.1 *Stromal matrix biochemical cues*

The ECM in the stromal tissue is composed of proteins and glycosaminoglycans (GAGs), such as laminin,[21, 22] fibronectin, [23] hyaluronic acid (HA), [24, 25] chondroitin sulfate (CS), [26, 27] tenascin-C, [28] collagens I, III, and IV, [29] etc. Among the various types of ECM proteins, collagen and fibronectin are the most abundant components in the tumor stroma, with increasing collagen and fibronectin expression being observed during the tumor growth.[19] Depending on the types of collagen, the morphology of the cells can be significantly influenced. For instance, it has been reported that the glioblastoma will adopt a rounded morphology in the presence of collagen IV, while a spindle-shaped morphology in collagen I/III. [30] Similarly, fibronectin has also been shown to influence the cell-ligand interactions as well as the subsequent cell adhesion and growth factors secretion, either due to the different packing density of the ligands or the exposure of cells to the different attachment motifs ($\alpha 5\beta 1$ or $\alpha v\beta 3$).[31,32] Other than collagen and fibronectin, tenascin-C is found to be highly expressed during tumor progression, which can promote the tumor cell survival and proliferation. [33] In addition, the interaction of cancer cells with tenascin-C has also been shown to trigger tumor invasion by weakening the actin stress fibers formation and the cell-matrix adhesion.[34, 35] Other than ECM proteins, GAGs such as HA has also been found to expressed differently during tumor progression. HA interacts with the CD44 receptor, which is considered as a key factor in tumor growth and metastasis.[36,37] As such, high expression of HA is found to be highly associated with poor survivability of patients.[38]

2.1.1.2.2 *Stromal matrix biomechanical cues*

TME is a dynamic landscape whereby the biophysical profile such as stromal ECM

stiffness, pore size, fibers alignment/thickness are constantly being altered. During the tissue remodeling process, collagen and fibronectin proteins will undergo architecture changes, such as fibril arrangement and fiber size, that lead to the different pore sizes of the ECM network. In addition, crosslinking between the fibers is observed during the remodeling that results in the stiffening of the stromal tissue. For example, the progression of breast and colorectal cancers is associated with the increased expressions of collagen I and lysyl oxidase (LOX) that lead to increasing crosslinking of collagen. As such, the stiffness of the surrounding stromal tissues is generally stiffer in the late stages of cancer (III/IV) as compared to the early stages (I/II).[39] It has also been reported that the conformation of fibronectin within a 3D matrix will undergo changes from a compact structure to extended fibrillar form due to the traction forces induced by fibroblasts.[40] The unfolding of these fibronectin fibers exposes the domains for tenascin binding,[41] thus influences the cancer cell behaviors. In addition, the fiber orientation also plays a role in cancer metastasis through an intravasation process.[42] The matrix stiffening of stromal tissues often leads to the activation and increment in contractility of fibroblasts in regulating the ECM orientation and alignment, thus facilitating the migration of cancer cells through the micron-sized channels.[43]

Figure 2.1.1.3 Schematic diagram illustrating the physicochemical properties changes of the breast tumor microenvironment during tumor progression.

2.1.1.3 Remodeling of solid stress within tumor tissue

Apart from the stromal matrix biomechanical stimuli as mentioned above, cells within tumor stroma also experienced other mechanical stress, such as solid stress, shear stress, and interstitial fluid pressure that affect the overall tumor progression.[44] Specifically, solid stress is a critical player in influencing tumor aggression and invasion. In general, solid stress experienced by the cancer cells is generally induced as a result of the hyperproliferative cancer cells confined within the stiffening stromal tissue. In order to understand the relationship between tumor stiffness and solid stress, the analogy of a spring of specific elastic modulus that obeys Hooke's law is being used (**Figure 2.1.1.4**). According to the equation of Hooke's law for linear elastic materials, Young's modulus (E)

is the ratio of stress (σ) in correspondence to the strain (ε). Kalli *et al.* have proposed a theoretical mathematical model to understand the relationship between matrix stiffness and solid stress.[45,46] When a tumor of elastic modulus E_T grows, it exerts an outward force and pushes the surrounding host tissue of elastic modulus E_{HT} . As such, it leads to a deformation ε_T with a stress level of σ_T . Consequently, the host tissue returns an equal and opposite stress σ_{HT} onto the cancer cells, which is also known as ‘externally applied solid stress’. Simultaneously, there is also a growth-induced stress σ_G accumulated in the tumor interior owing to the mechanical interactions among the tumor components. Overall, the total solid stress, which is accumulated intratumorally, is defined by the sum of the externally applied stress and the growth-induced stress. In the case where the tumor is stiffer than surrounding tissue ($E_T > E_{HT}$), the tumor can lead to greater displacement and deformation in the host tissue with much greater σ_T than σ_{HT} . As a result, it pushes against the normal tissue and grow in size. In the theoretical mathematical model proposed by Kalli *et al.*, a tumor stiffness of more than 1.5 times of surrounding tissue is required in order to displace the host tissue and grow.[47]

Figure 2.1.1.4 Solid stress and surrounding matrix stiffness are two distinct biomechanical abnormalities contributing to tumor solid stress build-up. Adapted with permission [45]. Copyright 2018 Kalli and Stylianopoulos.

2.1.2 Mechanotransduction in tumor progression

2.1.2.1 Cellular mechanotransduction

Cellular behaviors and functions in response to mechanical stimuli can be altered via the process of mechanotransduction.[48] Mechanosensitive intracellular signaling events, including post-translational modifications, intracellular shuttling, and protein unfolding, have been uncovered.[49-51] Majority of these mechanosignaling events converge towards the ECM-integrin-cytoskeleton-nucleus axis (**Figure 2.1.2.1**), which consists of ECM–focal adhesion (FA) interaction, the role of cytoskeleton tension as the second messenger for mechanical signals, mechano-actuated shuttling proteins and nuclear mechanotransduction.[52]

Mechanical linkages are formed between the ECM and cell membrane via integrin-interactions, followed by the development of discrete multiprotein complexes known as FA. These adhesion complexes span across the cell membrane, maintaining a direct mechanical continuity between the interior compartment of the cells and the exterior microenvironment. The intracellular segment of the FA complex contains scaffolding, docking, and signaling proteins (vinculin, talin, FAK, paxillin, etc.) that serve as an interface between the transmembrane that is in direct contact with the integrins and the actin cytoskeleton within the cells.[53] The recruitment of these signaling proteins within the intracellular layer is extremely sensitive to ECM composition and mechanics, thus functioning as a mechanosensor of the cells.[54,55]

With the newly formed FA complex structure, the propagation of extracellular and cell-generated forces is ensured via cytoskeleton tension regulation. The cytoskeleton is a dynamic structure composed of filamentous (F-actin, microtubules and intermediated filaments) and crosslinked proteins to provide mechanical support to cells and control cellular activities, such as motility, shape, and tension gene expression.[56, 57] Collectively, the formation of these cytoskeleton structures can profoundly affect cell mechanics, where the cellular cytoskeletal tension is an essential messenger for mechanical signals.[58]

During the propagation of mechanical signals, proteins residing at the cellular membrane or in the cytoplasm will undergo structure modification and nuclear translocation, which in turn can affect the gene expression. Upon mechanical stimulation, tight junction protein

1, c-Abl tyrosine kinase, and β -catenin are the first proteins to be identified in shuttling across the nuclear envelope.[59-61] Another well-established pathway is the YAP/TAZ shuttling from the cytoplasm to the nucleus in response to the mechanical cues. For example, it has been reported that a stiff substrate favors the CAF spreading and the translocation of YAP into nuclear to enhance intracellular tension.[62] In turn, YAP could promote the transcription of the genes involved in cell-matrix interactions, especially the activation of genes encoding for integrins and FA docking proteins,[63] and cytoskeleton integrity like F-actin polymerization.[64]

Additionally, the nucleus also has its mechanosensitive apparatus.[65] Specifically, the nuclear envelope is a vital regulator in connecting the cytoskeleton with the nucleus, in which the force transmission is activated via the nuclear-cytoskeletal coupling. This nuclear envelope can be divided into two main parts, namely inner (lamina) and outer (SUN and nesprin) membrane complexes as linkers for nucleoskeleton and cytoskeleton, respectively.[66] The expression and presence of the complexes proteins are regulated by the extracellular mechanical cues,[67, 68] which can further affect the transcription factor-dependent nuclear translocation such as β -catenin,[69] C-Fos,[70] and NF- κ B[71] that influence the downstream gene expression.

Figure 2.1.2.1 Schematic representation of cellular ECM-integrin-cytoskeleton-nucleus axis mechanotransduction signaling cascades. Adapted with permission [52]. Copyright 2018 Martino, Perestrelo, Vinarský, Pagliari and Forte.

2.1.2.2 Mechanics approaches to understand cell behavior

Mechanics represents the natural progression of knowledge at the intersection of mechanics and biology to codify the role of mechanical environment on biological adaptation.[52,72] Hence, mechanics analysis in mapping the full-set of mechanically sensitive proteins and transcripts encoded by its genome is helpful in deciphering the underlying mechanical regulation cascades. For example, a mechanics study conducted by Darnell M using RNA-Seq shows that the stiffness regulated the expression of mesenchymal stem cells (MSC) immunomodulatory markers in response to cytokine stimulation.[73] In addition, a separate transcriptome profile analysis via single-cell sequencing also reported that by modifying the degree of crosslinking of composite alginate/Matrigel hydrogels, it could fine-tune the neuron-specific gene expression via the

activation of microtubule-associated protein 2 (MAP2) marker, a marker for neuronal maturation and neural network formation and stabilization.[74] Although the biological functions of cells are highly dependent on the biophysical factors of their microenvironment, limited studies have been conducted to explore the detailed mechanisms, and signaling pathways involve between 3D biomechanical cues and biological processes, particularly in the tumor-related field.

2.1.2.3 Mechanoregulation within tumor microenvironment

As mentioned in **Section 2.1.1**, the TME undergoes significant anomalous biomechanical changes during tumor progressions, such as stroma matrix stiffness, topography, interstitial fluid pressure, tumor solid stress, etc., which can profoundly influence the tumorigenic biochemical pathways and the subsequent tumor activities. For example, it has been reported that the tumor cells have the potential in modifying its surrounding microenvironment via the secretion of LOX to promote the crosslinking of collagen and the stiffening of the surrounding matrix. This matrix stiffening is then shown to drive the gastric cancer progression through the activation of focal adhesion kinase/steroid receptor coactivator (FAK/SRC)-signaling pathway and increases the proliferation and invasiveness of the cancer cells both *in vivo* and *in vitro*. [75] Similarly, matrix stiffness-induced solid stress experienced by the cells could potentially influence the cancer cell proliferation, affect the cell apoptosis/necrosis, augment the ECM deposition/organization and enhance the invasive/metastatic potential of tumor cells.[76] One particular study by M. E. Fernandez-Sanchez *et al.* shows that by applying compressive stress around the growing tumors, this compressive stress act as an inducer of tumorigenesis by promoting the translocation of β -catenin from the adherent junctions to the nucleus *in vivo*, thus activating the β -catenin target genes and increased the colon crypt size due to hyperplasia.[77] Based on a mathematical modeling analysis, it predicts that the loss of collective tension (a product of cell-cell adhesion) at the tumor stoma interface as a result of solid stress changes affected the shape of the tumor, and thus promoting the subsequent collective cell migration and metastasis.[78]

2.1.3 Biomimetic models for tumor microenvironment study

Numerous cell culture models have been developed in order to mimic the TME as a means to study the metastasis progression of tumor or drug testing, which can be classified in terms of 2D and 3D cell culture models that comprise of different characteristics.

2.1.3.1 2D and 3D cell culture

Due to its ease of handling, analysis, and cost-effectiveness, conventional plastic-based 2D culture have been the substrate of choice for cellular studies for decades. However, this comes with the cost of low data accuracy, thus limiting the clinical translation of the tested drugs due to the disadvantages of 2D cell culture.[79] On the other hand, 3D culture has emerged as an important cell culture method due to their better mimicking of the cellular microenvironment *in vivo* as compared to the 2D plane as shown in the **Figure 2.1.3.1**. Generally, there are significant cellular and microenvironmental feature differences between 2D and 3D cell culture. Firstly, the biomechanics of both platforms are distinctly different. Hydrogel based 3D scaffold cell culture model can mimic the biomechanical properties of tissue *in vivo* (with stiffness ranging from kPa to mPa), while the conventional tissue culture plates always have a rigid polymeric substrate with a high stiffness reaching GPa. In addition, although there is no limitation in the biomolecular transportation in the 2D cell culture with homogenous distribution of nutrients across the cultured cells, the cellular behaviors such as proliferation and differentiation is often limited to 2D planar with mostly cell-to-substrate interactions. In contrast, the presence of 3D matrix and cell commonly act as physical barriers results in soluble gradients and inhomogeneous distribution of nutrient, which can better mimic the physiology of the *in vivo* microenvironment. Secondly, the cells would behave differently in cellular, molecular and gene level due to the different cell structure. At the cellular level, on one hand, the cell morphology would seem to be flatten with unconstrained spreading on the 2D substrate. However, cells present as spherical, spindle morphology or multicellular aggregates in a 3D context, depending on the adhesion ligands. In addition, as compared to the pre-determined apical-basal polarity in 2D culture, 3D culture provides 360° unrestricted

interactions. At the molecular level, the cytoskeletal structure would be different. There are thick bundles of well-developed ventral stress fiber observed in 2D cell culture, while only few thin stress fibers could be found in cells' cortex within 3D setting. Also, the cell adhesion in 2D is determined by the integrin-based focal adhesions formed at the cell-substrate interface. However, in addition to the focal adhesion forming between cell-matrix, majority of the cells in a 3D context prefer to form cell-cell contacts, like cadherins. Lastly, on the protein or transcript level, 3D culture is more *in vivo* mimicking as compared to the 2D culture.

Cellular and microenvironmental features	2-dimensional (2D) culture	3-dimensional (3D) culture
Cell morphology	Typically flattened and unconstrained spreading on the substrate	Depending on the adhesion ligands used, cells can be spherical, spindle shaped or exist as multicellular aggregates
Cell orientation	Coerced apical-basal polarity that is determined by the relative positioning of focal adhesions in the x-y plane	No pre-determined polarity due to unrestricted formation of focal adhesion contacts in the 3-dimensional space
Cytoskeletal structure	Display thick bundles of well-developed ventral stress fibers	Few thin stress fibers found near the cell cortex
Cell adhesion	Predominantly mediated via integrin-based focal adhesions that are formed at the cell-substrate interface	High proportion of cells forming cell-cell adhesions such as cadherins within a multi-layered cellular construct
Substrate stiffness	Conventional tissue culture plates have high stiffness (GPa range)	Hydrogels used for 3D culture generally softer and have stiffness within the range of kPa-MPa range
Gene and protein expression	Similarities with <i>in vivo</i> counterparts are lacking	Generally more <i>in vivo</i> -like
Biomolecular transport dynamics	Absence of soluble gradients leading to homogenous cellular behaviour (i.e. proliferation, differentiation, etc)	Presence of soluble gradients leading to inhomogeneous microenvironmental conditions and cellular fates

Figure 2.1.3.1 Schematic representation of the cellular and microenvironmental differences between 2D and 3D cell culture platforms. Adapted with permission [80]. Copyright 2016 John Wiley and Sons.

Currently, there are two pre-dominant approaches to culture cells in a 3D context – (i) within porous polymeric pre-fabricated scaffold or (ii) cell encapsulation using *in situ* crosslinked hydrogel/capsules. The pre-prepared scaffold, such as electrospinning

scaffolds, freeze dried porous discs or 3D printed layered scaffold, can provide physical support for cells' activities. Specifically, the electrospinning method endows the possibility to mimic the fibrous microenvironment by controlling fiber diameter and orientation.[81] Generally, upon cell seeding, cells will penetrate into the scaffold via the interconnected pores and aggregate on the surfaces of the pore structure. This approach is generally used for drug testing as the pore size is an critical parameter to determine cells' initial penetration, aggregation, and migration ability within scaffold.[82] As for the encapsulation method, cells are first added to a polymer precursor solution before the network is crosslinked/gelated forming a cell-encapsulated hydrogel system. The extremely high water content (up to 90%) of the hydrogel is highly advantageous as it's not only biomimicking but also provide an optimal microenvironment that would ensure unhindered exchange of nutrients ad waste.[83,84]

2.1.3.2 Single component hydrogels

Over the years, numerous types of hydrogels such as collagen, hyaluronic acid, gelatin, fibrin, alginate, synthetic PEG-derived materials, etc. have been explored for 3D culture (**Figure 2.1.3.2**).[83,85] Among the 29 different types of collagen, collagen I is perhaps the most widely used materials for cell culture applications, which mainly originates from rat tail, bovine tendon, and marine animal skins. Collagen hydrogel can be formed by pH- and salt-induced self-assembly (fibrillogenesis) of the collagen fibers at 37 °C where the fibrillar structure crosslinked and/or entangled to form 3D network structure of hundreds nano-meter thick.[86] Numerous studies have been employing collagen hydrogel as a stroma matrix surrogate to study the ECM stiffness on cancer cell biology with the stiffness being tailored by varying its concentration.

Gelatin, a denatured form of collagen I, is another popular biomaterial for such application. However, gelatin fibers cannot undergo the self-assembly process. Thus chemical crosslinking and modifications have been carried out to promote the formation of stable hydrogel networks, including enzyme-induced crosslinking and photo-crosslinking process.[87] Transglutaminase, a biocompatible enzyme, can be used to induce gelatin

crosslinking. However, the reaction rate is relatively slow.[88] Another approach is by chemically modifying the gelatin, such as methacrylated gelatin (GelMA), for photocrosslinking [89] or by grafting the gelatin with ferulic acid. However, some of these chemical agents may be cytotoxic.

HA is a large ECM glycosaminoglycan composed of D-glucuronic acid and N-acetyl-D-glucosamine. Similar to gelatin, HA cannot form 3D crosslinked network spontaneously. Thus, chemical modifications of HA are required. For example, methacrylated and thiolated HA are the most common approaches used for the chemical modification of HA. In addition, even though HA has been shown to interact with numerous cell surface markers (e.g., CD44 and CD168) to regulate cellular signaling,[90] the interaction between cells and binding sites is relatively weak due to the limited matrix attachment ligands. Furthermore, HA does not have the proteolytic degradable sites (MMP sensitive sites) that are essential for the matrix remodeling process.[91]

Alginate, a polysaccharide derived from seaweed, consists of homopolymeric blocks of (1-4)-linked β -D-mannuronate (M) and C-5 epimer α -L-guluronate (G) residues. The G residues can be crosslinked via divalent cations, such as calcium ions (Ca^{2+}) to form a hydrogel, while the mechanical properties of the hydrogel can be modulated by varying the crosslinking density or changing the ratio of M:G blocks. However, alginate, in its native form, is bioinert in nature that does not contain any cell adhesion ligands to support cell survivability and proliferation.[92] In addition, alginate exhibits poor physiological stability as it can be easily de-crosslinked by chelates such as lactate, citrate or other ions (sodium and magnesium).[93]

Poly(ethylene glycol) PEG-derivatives are widely used as synthetic biocompatible hydrogel materials due to the ease of preparation, relatively low cost, non-biodegradability, ease of chemical modification and controllable mechanical properties.[83] The most common approach to create PEG hydrogel is through photopolymerization of diacrylated PEG (PEGDA) chains. However, pristine PEG-derivatives are non-biofouling, thus unable to support cell adhesion. Therefore, to render the PEG hydrogel bioactive and

biodegradable, chemical modification processes are required by conjugating cell integrin-binding motifs and MMPs onto the backbone of the PEG polymer chains.

Taken together, there are numerous inherent drawbacks that are associated with the abovementioned single component hydrogel systems. The exclusive utility of a single-typed ECM material is also unable to fully recreate the diversity, complexity, and heterogeneity of the native tissue-specific ECM. Therefore, multi-components 3D hydrogel platforms, namely hybrid hydrogels, has attracted much attention for 3D cell culture. Conventional single network hydrogel system has several drawbacks, including i) simple matrix composition that failed to mimic the complex ECM microenvironment; ii) naturally-derived hydrogel exhibit extremely weak mechanical properties; iii) majority of these materials lack cell attachment sites; and iv) the biochemical or biophysical properties are compromised during material modification process. However, hybrid hydrogel system could potentially overcome the abovementioned limitations. In the following section, several representative multi-component hydrogels 3D culture hydrogels are being presented.

Figure 2.1.3.2 Schematic representation of different biomaterials for single hydrogel fabrication and their corresponding crosslinking mechanism. Adapted with permission [84]. Copyright 2017 annual review of biomedical engineering.

2.1.3.3 Engineered hybrid hydrogels

2.1.3.3.1 Particles incorporated hydrogel

There are various types of hybrid hydrogel being developed over the years. One of the common types of hybrid hydrogel is the particles incorporated hybrid hydrogel (**Figure 2.1.3.3b**). [94] On one hand, this composite system has the potential to mimic the TME,

especially mimicking the bone using bioglass nanoparticles.[95] On the other hand, Zhu *et al.* have also developed a novel biomimetic bone microenvironment using chitosan-based hydrogel incorporated with hydroxyapatite to study structure and crystallinity of the hydroxyapatite on the bone metastasis in breast cancer. Findings show that amorphous-HA and nano-HA induced higher cell adhesion and proliferation compared to macro-HA.[96] Additionally, particles embedded within hydrogel promote the nano-roughness topography and the biomechanical properties of the hydrogel. For example, a graphene oxide (GO)-incorporated gelatin methacryloyl (gelMA) hydrogel has also been fabricated with enhanced mechanical properties being observed due to the formation of physical bonds between GO and polymer chains.[97] Another type of particles incorporated-hydrogel has been proposed by Ayyub *et al.*, in which a poly(ethylene glycol) (PEG) network is incorporated with silica nanoparticles to modulate the stiffness of the hydrogel.[98] Another interesting particles incorporated-hydrogel is the development of poly(ethylene glycol) hydrogel with hydrophobic poly(lactic-co-glycolic acid) (PLGA) nanoparticles, which has shown to enhance the permeability of the hydrogel system due to the low crosslinking density at the particle-hydrogel interface.[99]

2.1.3.3.2 *Electrospun/Printed fibrils embedded hydrogel*

In recent years, the concept of electrospun/printed fibrils embedded hydrogel has emerged as a new class of hybrid hydrogel (**Figure 2.1.3.3c**). The main idea of the electrospun/printed fibrils embedded hydrogel is to reinforce the soft hydrogels with highly organized, high-porosity fibrils network, leveraging on the electrospinning or 3D bioprinting technologies. The network of fibers can provide the physical support needed for cells anchoring, while the hydrogel acts as a protective barrier for the encapsulated cells. For example, Visser *et al.* have successfully printed PCL fibrils scaffold, followed by incorporating into either GelMA or alginate hydrogel precursor solution prior to photo- or ionic crosslinking, respectively. The results demonstrated that the stiffness of the obtained composites increased up to 54 fold (~400 kPa) as compared to the pure hydrogel and supported the proliferation and growth of human chondrocytes.[100] In addition, the fibers embedded hydrogel has also been reported for other purposes, such as enhancing

directional neurite outgrowth,[101] ECM-mimicking,[102] and facilitating cell-matrix interactions.[81] For example, a work by Schulte *et al.* demonstrated that with the incorporation of electrospun NCO-sP(EO-stat-PO)/PLGA RGD fibers into the Acr-sP(EO-stat-PO)/HA-SH hydrogel, the system allows the independent control over cell adhesion towards the gel and fibers components to investigate the effect of morphological and biochemical factors of the matrix on cell biology. The results showed that by pre-incubating the cells on the adhesive fibers, it favors the human dermal fibroblasts proliferation and spreading upon embedding within the hydrogel compared to direct encapsulation in the hydrogel alone. In addition, guided cell growth was observed with the incorporation of oriented fibers within the hydrogel, which cannot be achieved via traditional mono-network hydrogel.[103]

2.1.3.3.3 *Polymeric interpenetrating network hydrogel*

The term interpenetrating network (IPN) was coined in 1960, which essentially describe a polymeric system consisting of at least two separately polymer networks. Conceptually, each network interpenetrates and interlaces with another network at the molecular level without any covalent bonds between them. Unless the chemical bonds are broken, the two networks of IPN cannot be separated.[104, 105] Thus, IPN hydrogel allows the fine-tune of biophysical and biochemical properties of the hydrogel independently, in which the comparison between mono and hybrid hydrogel systems are briefly summarized in **Figure 2.1.3.3f.**

Figure 2.1.3.3 Schematic illustration of single and multi-component hybrid-typed hydrogels. Preparation of mono-network hydrogel (a), particles incorporated hydrogel (c), electrospun/printed fibrils embedded hydrogel (d), polymeric semi-IPN (d), and polymeric full-IPN (e) using for tumor stroma matrix biochemical and biophysical properties mimicking. (f) comparison between mono-hydrogel and hybrid hydrogel system.

In general, IPN hydrogel can be classified into two main types, namely semi-IPN and full-IPN. In semi-IPN hydrogel, only one of the polymer network is crosslinked, leaving the other in a linear form, in which the interaction between the two components is governed via physical polymer interwinding, hydrogen bonds, hydrophilic interaction or electrostatic attraction (**Figure 2.1.3.3d**).^[106] From the biochemical standpoint, semi-IPN is an

important strategy to incorporate ECM proteins with poor self-assembly capability by forming an integrated interpenetrating network due to the physical interactions between the additive with the crosslinked polymer chains of the hydrogel. With this method, non-fibrous proteins and GAG-based components can be incorporated into the crosslinked network to recapitulate the complex ECM microenvironment. For example, tenascin-C was incorporated into collagen hydrogels to investigate their effect on glioblastoma invasion. The results revealed that the presence of tenascin-C promoted the glioblastoma cancer cell motility in the 3D environment via the increased matrix metalloproteinase-12 (MMP12), which is not similar to the results obtained from 2D tumor invasion study (MMP-2/9).[28] When incorporating HA into collagen hydrogel, the results showed that HA could trigger spontaneous M2-like polarity of monocyte/macrophage via CD44-mediated activation of STAT3 in THP-1 cells.[107] Additionally, Yali Yang has reported that CS can induce fibril bundling and increase mesh size of hydrogel but inhibit glioma invasion using 3D chondroitin sulfate incorporated collagen hydrogel model.[26]

2.1.3.3.4 *Polymeric full-IPN hydrogel*

The full-IPN hydrogel is formed by the interposition of two or more crosslinked networks. Since the networks are not covalently bonded to each other, physicochemical properties of the respective network can be tailored independently, without affecting the overall integrity of the gel (**Figure 2.1.3.3e**).[104] For instance, a 3D IPN hydrogel consisting of a primary fibrillar collagen network and reinforced with visible light-mediated crosslinkable thiol-ene poly(ethylene glycol) (PEG) network was successfully fabricated with varied stiffness (E) from ~40 Pa to ~4kPa. Hence, bio-mimicking stiffness corresponding to the breast tumor stroma at different stage (0.2 – 0.8 kPa) can be obtained by varying the amount and degree of crosslinking of the modified PEG network. The results showed that an increase in the mechanical stiffness of the full-IPN hydrogel led to the confinement effect onto the breast cancer MDA-MB-231, thus affected the viability, proliferative, and motility of the encapsulated breast cancer cells.[108] Another 3D full-IPN hydrogel system containing GelMA and collagen has been used to study the effect of fiber density and hydrogel stiffness on cancer and endothelial cell behaviors. In this case, the stiffness of hydrogel

(complex modulus; G' of 2, 7 and 12 kPa) can be independently tuned by varying the crosslinking density, while the fiber density can be varied by changing the ratio of GelMA to collagen. Results showed that the changes in fiber density enhanced the cancer cells MDA-MB-231 invasion but not endothelial cells, while the increased stiffness restricted both the cancer cells invasion and sprouting.[109]

A separate transcriptome profile analysis via single-cell sequencing also reported that by modifying the degree of crosslinking of composite alginate/Matrigel hydrogels (G' ranging from ~20 to ~375 Pa), it could fine-tune the neuron-specific gene expression, which is correlated with specific brain regions and development stages, via the activation of MAP2 marker, a marker for neuronal maturation and neural network formation and stabilization.[74] Additionally, a recent study adopted silk-collagen full-IPN hydrogel, in which silk and collagen can be gelled via β -sheet-forming blocks and fibrils polymerization, respectively, to study breast cancer cells invasion behavior. The results showed that invasion of metastatic breast cancer cells exhibited a biphasic dependence on silk fibroin concentration while keeping the collagen I concentration a constant. With increasing hydrogel stiffness (G' ranging from 45 to 345 Pa), the cancer cells showed an increased in cell invasion when adopting a mesenchymal morphology, followed by a decreased in cell invasion with amoeboid morphology as the pore size of silk fibroin decreases resulting in less effective cell-matrix adhesions.[110] Additionally, full-IPN hydrogel can also be used to study the fibrils arrangement effect on cells' fate. For example, in a gelMA/collagen IPN model, the study decoupled the effects of fiber density and hydrogel stiffness on cancer and endothelial cells behaviors. The stiffness of hydrogel can be independently tuned across relevant range of 2-12 kPa, and also various fiber density can be obtained via varying the ratio of gelMA to collagen. The results showed that the presence of fibers can promote cancer cells MDA-MB-231 invasion, but does not necessary to endothelial cells.[109]

2.1.3.4 Role of hydrogel surface chemistry on cell biology

Even though full IPN hydrogel exhibited independently well-tunable mechanical

properties, the surface chemistry of the full IPN hydrogel is also a critical player to influence the cell biology, such as cell adhesion [110, 111], migration [112], differentiation [113], and apoptosis [114]. In general, the survivability and attachment of the cells are mainly governed by the interactions between cell integrins and ligands. Without the proper attachment, the cells could potentially undergo anoikis [114]. In the body, most of the ECM-derived proteins consist of these cell attachment motifs, such as the RGD motifs found in collagen and fibronectin [115] as well as the heparan sulfate binding domains (HSBDs) in fibronectin [112] could be specifically recognized by cellular integrins. As such, many studies have been conjugating RGD to the otherwise bioinert hydrogel polymers, including PEG [116], alginate [117] etc. to improve the biocompatibility of the hydrogel system. On the other hand, cells also play a role in ECM remodeling via enzymatic cleavage of the ECM fiber, which in turn facilitated the cell migratory behavior and ECM remodeling within the hydrogel. Hence, research has also been focusing on incorporating proteases (e.g., MMPs) sensitive oligopeptides onto the polymeric chains to have better mimic of the natural microenvironment for tissue engineering application [118]. In this study, the component collagen within IPN has multifunction, providing cell attachments and MMP sensitive sites. Meanwhile, alginate is a natural polysaccharide in which the saccharides act to provide a similar architectural structure of hyaluronic acid within the tumor stroma. Thus, the combination of collagen and alginate to fabricate full IPN hydrogel can mimic the *in vivo* cellular microenvironment.

2.1.3.5 Stimuli responsive IPN system to gain control in the 4th dimension

Tumor progression is associated with constant evolving changes in the ECM architecture and mechanical properties. Therefore, stimuli responsive polymers that could offer dynamic spatiotemporal control over ECM composition and mechanics have been well-sought in recent years. Broadly speaking, there are two main aspects of TME, which can be mimicked using stimuli responsive IPN system – biochemical and biomechanical. For instance, a handful of stimuli sensitive chemical bonds can be introduced to obtain smart hydrogels, which can control the cell attachment ligands [119] or MMP sensitive peptides

density.[120] In a similar fashion, control over the biomechanical properties of hydrogel can be attained via the introduction of stimuli responsive hydrogel network or nanoparticles to an existing network. For example, Almeida *et al.* have used a combination of semi-flexible/stress-responsive polyisocyanide (PIC) networks embedded within a flexible/thermo-responsive PNIPAM network to develop a smart, dynamic IPN hydrogel to vary the matrix stiffness in response to temperature.[121] Dynamic control over the mechanical properties of full CoAl-IPN hydrogel can also be achieved via selective crosslinking or de-crosslinking of the alginate network.[122] Liu *et al.* have also successfully employed a simple and effective technique of IPN hydrogel fabrication by using the combination of collagen and alginate, and applied the developed system for various cancer research to model the tumor environments.[123] Moreover, a hybrid system with a classic IPN hydrogel with alginate and Matrigel (a replacement of collagen) was developed, in which liposomes encapsulated inside consist of gold nanorods and calcium ions or calcium chelator. Therefore, under near infrared irradiation, the liposomes will become permeabilized due to the loaded gold nanorod-induced heating, leading to calcium ions or calcium chelators release. The hydrogel stiffness is tunable across 150 to 1000 Pa depending on the released amount of calcium ions or chelator.[124]

2.2 Summary

TME is a complex tissue formed by the heterogeneous cells, and the signaling molecules interwoven with the tumor-associated stroma. A series of studies reveal that the biophysical cues and the mechanical stress play important roles in regulating tumor progression. In order to understand the mechanisms on the correlation between matrix stiffness and tumor progression, a hybrid 3D IPN hydrogel was employed as an in vitro tumor surrogate to mimic the TME. By using the full-IPN hydrogel system, it is possible to replicate the complexity of tumor stroma ECM as well as decoupling the biochemical and biophysical characteristics of the hybrid hydrogel, in which most of the single hydrogel systems failed to achieve that particular goal. For instance, the combination of collagen and alginate for IPN fabrication could provide cellular attachment motifs and resemble the similar architectural structure of hyaluronic acid within tumor stroma. Additionally, the stiffness

of CoAl-IPN hydrogel can be tuned systematically and independently by changing the concentrations of the calcium ions as a crosslinking agent to alginate without affecting the collagen properties. Most importantly, calcium-crosslinked alginate network can be de-crosslinked using chelators such as sodium citrate upon crosslinking, thus making it an attractive dynamic hybrid system. In the case of most hydrogel systems, there is no sacrificial component to achieve this active dynamic mechanical tunable-behavior. Hence, CoAl-IPN hydrogel could provide a detailed understanding of the impact of matrix stiffness on the stroma cells, as well as the phenotypic changes in affecting the neighboring cancer cells via paracrine crosstalk manner.

References

- [1] D. Hanahan, R. A. Weinberg. *Cell*. 2011, **144**, 646-74.
- [2] S. Togo, U. M. Polanska, Y. Horimoto and A. Orimo. *Cancers (Basel)*. **2013**, 5, 149-69.
- [3] M. R. Asgharzadeh, J. Barar, M. M. Pourseif, M. Eskandani, M. Jafari Niya, M. R. Mashayekhi and Y. Omid. *Bioimpacts*. **2017**, 7, 115-133.
- [4] V. S. LeBleu, R. Kalluri. *Dis. Model. Mech.* **2018**, 11, dmm029447.
- [5] K. Shiga, M. Hara, T. Nagasaki, T. Sato et al. *Cancers (Basel)*. **2015**, 7, 2443-58.
- [6] M. E. Fiori, S. D. Franco, L. Villanova, P. Bianca, G. Stassi, and R. D. Maria. *Mol. Cancer*. **2019**, 18:70.
- [7] S. Ishihara, D. R. Inman, W. J. Li, S. M. Ponik and P. J. Keely. *Cancer Res.* **2017**, 77, 6179-6189.
- [8] R. Kalluri and M. Zeisberg. *Nat. Rev. Cancer*. **2006**, 6, 392-401.
- [9] P. Cirri and P. Chiarugi. *Am. J. Cancer Res.* **2011**, 1, 482-497.
- [10] D. V. T. Catenacci, M. R. Junttila, T. Karrison, N. Bahary, M. N. Horiba, S. R. Nattam, R. Marsh, J. Wallace, M. Kozloff, L. Rajdev, D. Cohen, J. Wade, B. Sleckman, H-J Lenz, P. Stiff, P. Kumar, P. Xu, L. Henerson, N. Takebe, R. Salgia, X. Wang, W. M. Stadler, F. J. D. Sauvage and H. L. Kindler. *J. Clin. Oncol.* **2015**, 33, 4284-4292.
- [11] B. C. Ozdemir, T. Pentcheva-Hoang, J. L. Carstens, X. F. Zheng, C-C Wu, T. r. Simpson, H. Laklai, H. Sugimoto, c. Kahlert, S. V. Novitskiy, A. D. Jesus-Acosta, P.

- Sharma, P. Heidari, U. Mahmood, L. Chin, H. L. Moses, V. M. Weaver, A. Maitra, J. P. Allison, V. S. LeBleu and R. Kalluri. *Cancer cell*. **2014**, 25, 719-734.
- [12] L. Bu, H. Baba, N. Yoshida, K. Miyake, T. Yasuda, T. Uchihara, P. Tan and T. Ishimoto. *Oncogene*, **2019**, 38, 4887-4901.
- [13] A. K. Patel and S. Singh. *Front. Biosci.* **2020**, 25, 961-978.
- [14] P. Chang, W. W. Hwang-Verslues, Y. Chang, C. Chen, M. Hsiao, Y. Jeng, K. Chang, E. P. Lee, J. Shew and W. Lee. *Cancer Res.* **2012**, 4652-4661.
- [15] H. M. Brechbuhl, J. Finlay-Schultz, T. M. Yamamoto, A. E. Gillen, D. M. Cittelly, A. Tan, S. B. Sams, M. Pillai, A. D. Elias, W. A. Robinson, C. A. Sartorius and P. Kabos. *Clin. Cancer Res.* **2017**, 23, 1710-21
- [16] A. Costa, Y. Kieffer, A. Scholer-Dahirel, F. Pelon, B. Bourachot, M. Cardon, P. Sirven, I. Magagna, L. Fuhrmann, C. Bernard, C. Bonneau, M. Kondratova, I. Kuperstein, A. Zinovyev, A. Givel, M. Parrini, V. Soumelis, A. Vincent-Salomon and Fatima Mechta-Grigoriou. *Cancer Cell*. **2018**, 33, 463-479.
- [17] A. K. Patel, K. Vipparthi, V. Thatikonda, I. Arun, S. Bhattacharjee, R. Sharan, P. Arun and S. Singh. *Oncogenesis*. **2018**, 7:78.
- [18] J. Alexander and E. Cukierman. *Curr. Opin. Cell Biol.* **2016**, 42, 80-93.
- [19] R. Malik, P. I. Lelkes and E. Cukierman. *Trends Biotechnol.* **2015**, 33, 230-6.
- [20] J. Cha and P. Kim. *Frontiers in Materials*. **2017**, 4.
- [21] P. Rousselle and J. Y. Scoazec. *Semin. Cancer Biol.* **2019**.
- [22] X. M. Shen, Y. P. Wu, Y. B. Feng, M. L. Luo, X. L. Du, Y. Zhang, Y. Cai, X. Xu, Y. L. Han, X. Zhang, Q. M. Zhan and M. R. Wang. *Clin. Exp. Metastasis*. **2007**, 24, 541-50.
- [23] J. Xiao, W. Yang, B. Xu, H. Zhu, J. Zou, C. Su, J. Rong, T. Wang and Z. Chen. *BMC Cancer*. **2018**, 18, 976.
- [24] M. Wu, M. Cao, Y. He, Y. Liu, C. Yang, Y. Du, W. Wang and F. Gao. *FASEB J.* **2015**, 29, 1290-8.
- [25] X. Tian, J. Azpurua, C. Hine, A. Vaidya, M. Myakishev-Rempel, J. Abulaeva, Z. Mao, E. Nevo, V. Gorbunova and A. Seluanov. *Nature*. **2013**, 499, 346-9.
- [26] A. Pudelko, G. Wisowski, K. Olczyk and E. M. Kozma. *FEBS J.* **2019**, 286, 1815-1837.
- [27] Y. Liu, S. Wang, D. Sun, Y. Liu, Y. Liu, Y. Wang, C. Liu, H. Wu, Y. Lv, Y. Ren, X.

- Guo, G. Sun and X. Ma. *Scientific Reports*. **2016**, 6.
- [28] S. Sarkar, R. K. Nuttall, S. Liu, D. R. Edwards and V. W. Yong. *Cancer Res*. **2006**, 66, 11771-80.
- [29] P. P. Provenzano, K. W. Eliceiri, J. M. Campbell, D. R. Inman, J. G. White and P. J. Keely. *BMC Med*. **2006**, 4, 38.
- [30] S. S. Rao, J. Dejesus, A. R. Short, J. J. Otero, A. Sarkar and J. O. Winter. *ACS Appl. Mater. Interfaces*. **2013**, 5, 9276-84.
- [31] A. M. Wan, E. M. Chandler, M. Madhavan, D. W. Infanger, C. K. Ober, D. Gourdon, G. G. Malliaras and C. Fischbach. *Biochim. Biophys. Acta*. **2013**, 1830, 4314-20.
- [32] M. J. Bradshaw and M. L. Smith. *Acta Biomater*. **2014**, 10, 1524-31.
- [33] R. H. Green and I. D. Pavord. *The Lancet*. **2001**, 357, 1992.
- [34] W. T. Huang, R. Chiquet-Ehrismann, J. V. Moyano, A. Garcia-Pardo, and G. Orend. *Cancer Res*. **2001**, 61, 8586-8594.
- [35] C. Ruiz, W. Huang, M. E. Hegi, K. Lange, M.-F. Hamou, E. Fluri, E. J. Oakeley, R. Chiquet-Ehrismann and G. Orend. *Cancer Res*. **2004**, 64, 7377-7385.
- [36] S. Misra, V. C. Hascall, R. R. Markwald and S. Ghatak. *Front. Immunol*. **2015**, 6, 201.
- [37] C. Chen, S. Zhao, A. Karnad and J. W. Freeman. *J. Hematol. Oncol*. **2018**, 11, 64.
- [38] L. Y. W. Bourguignon. *Frontiers in Oncology*. **2019**, 9.
- [39] A. M. Baker, D. Bird, G. Lang, T. R. Cox and J. T. Erler. *Oncogene*. **2013**, 32, 1863-8.
- [40] M. L. Smith, D. Gourdon, W. C. Little, K. E. Kubow, R. A. Eguiluz, S. Luna-Morris and V. Vogel. *PLoS Biol*. **2007**, 5, e268.
- [41] K. C. Ingham, S. A. Brew and H. P. Erickson. *J. Biol. Chem*. **2004**, 279, 28132-5.
- [42] W. Han, S. Chen, W. Yuan, Q. Fan, J. Tian, X. Wang, L. Chen, X. Zhang, W. Wei, R. Liu, J. Qu, Y. Jiao, R. H. Austin and L. Liu. *Proc. Natl. Acad. Sci*. **2016**, 113, 11208-11213.
- [43] K. M. Wisdom, K. Adebawale, J. Chang, J. Y. Lee, S. Nam, R. Desai, N. S. Rossen, M. Rafat, R. B. West, L. Hodgson and O. Chaudhuri. *Nat. Commun*. **2018**, 9, 4144.
- [44] J. M. Northcott, I. S. Dean, J. K. Mouw and V. M. Weaver. *Front. Cell Dev. Biol*. **2018**, 6, 17.
- [45] M. Kalli and T. Stylianopoulos. *Front. Oncol*. **2018**, 8, 55.

- [46] A. del Río Hernández, A. Vella, E. M. Eko and A. del Río Hernández. *Emerging Topics in Life Sciences*. **2018**, 2, 739-749.
- [47] C. Voutouri, F. Mpekris, P. Papageorgis, A. D. Odysseos and T. Stylianopoulos. *PLoS One*. **2014**, 9, e104717.
- [48] E. K. Paluch, C. M. Nelson, N. Biais, B. Fabry, J. Moeller, B. L. Pruitt, C. Wollnik, G. Kudryasheva, F. Rehfeldt and W. Federle. *BMC Biol.* **2015**, 13, 47.
- [49] A. D. Rio, R. Perez-Jimenez, R. Liu, P. Roca-Cusachs, J. M. Fernandez, M. P. Sheetz. *Science*. **2009**, 323, 638-641
- [50] J. D. Humphries, P. Wang, C. Streuli, B. Geiger, M. J. Humphries and C. Ballestrem. *J. Cell Biol.* **2007**, 179, 1043-57.
- [51] B. Lohberger, H. Kaltenegger, L. Weigl, A. Mann, W. Kullich, N. Stuendl, A. Leithner and B. Steinecker-Frohnwieser. *Cell Signal*. **2019**, 56, 23-30.
- [52] F. Martino, A. R. Perestrelo, V. Vinarsky, S. Pagliari and G. Forte. *Front. Physiol.* **2018**, 9, 824.
- [53] M. Abercrombie and G. A. Dunn. *Experi. Cell Res.* **1975**, 92, 57-62.
- [54] E. A. Cavalcanti-Adam, T. Volberg, A. Micoulet, H. Kessler, B. Geiger and J. P. Spatz. *Biophys. J.* **2007**, 92, 2964-74.
- [55] H. B. Schiller and R. Fassler. *EMBO Rep.* **2013**, 14, 509-19.
- [56] D. A. Fletcher and R. D. Mullins. *Nature*. **2010**, 463, 485-92.
- [57] K. V. Iyer, S. Pulford, A. Mogilner and G. V. Shivashankar. *Biophys. J.* **2012**, 103, 1416-28.
- [58] T. J. Chen, C. C. Wu, M. J. Tang, J. S. Huang and F. C. Su. *PLoS One*. **2010**, 5, e14392.
- [59] C. J. Gottardi, M. Arpin, A. S. Fanning and D. Louvard. *Proc. Natl. Acad. Sci. USA*. **1996**, 93, 10779-10784.
- [60] J. M. Lewis, R. Baskaran, S. Taagepera, M. A. Schwartz and J. Y. Wang. *Proc. Natl. Acad. Sci. USA*. **1996**, 93, 15174-15179.
- [61] S. Orsulic and M. Peifer. *J. Cell Biology*. **1996**, 134, 1283-1300.
- [62] F. Calvo, N. Ege, A. Grande-Garcia, S. Hooper, R. P. Jenkins, S. I. Chaudhry, K. Harrington, P. Williamson, E. Moeendarbary, G. Charras and E. Sahai. *Nat. Cell Biol.* **2013**, 15, 637-46.
- [63] G. Nardone, J. Oliver-De La Cruz, J. Vrbsky, C. Martini, J. Pribyl, P. Skladal, M. Pesl,

- G. Caluori, S. Pagliari, F. Martino, Z. Maceckova, M. Hajduch, A. Sanz-Garcia, N. M. Pugno, G. B. Stokin and G. Forte. *Nat. Commun.* **2017**, 8, 15321.
- [64] Y. Morikawa, M. Zhang, T. Heallen, J. Leach, G. Tao, Y. Xiao, Y. Bai, W. Li, J. T. Willerson, J. F. Martin. *Sci. Signal.* **2015**, 8, ra41.
- [65] S. Cho, J. Irianto and D. E. Discher. *J. Cell Biol.* **2017**, 216, 305-315.
- [66] S. Neelam, T. J. Chancellor, Y. Li, J. A. Nickerson, K. J. Roux, R. B. Dickinson and T. P. Lele. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, 112, 5720-5.
- [67] J. Swift, I. L. Ivanovska, A. Buxboim, T. Harada, P. C. Dingal, J. Pinter, J. D. Pajerowski, K. R. Spinler, J. W. Shin, M. Tewari, F. Rehfeldt, D. W. Speicher and D. E. Discher. *Science.* **2013**, 341, 1240104.
- [68] A. Buxboim, J. Swift, J. Irianto, K. R. Spinler, P. C. Dingal, A. Athirasala, Y. R. Kao, S. Cho, T. Harada, J. W. Shin and D. E. Discher. *Curr. Biol.* **2014**, 24, 1909-17.
- [69] E. Markiewicz, K. Tilgner, N. Barker, M. van de Wetering, H. Clevers, M. Dorobek, I. Hausmanowa-Petrusewicz, F. C. Ramaekers, J. L. Broers, W. M. Blankestijn, G. Salpingidou, R. G. Wilson, J. A. Ellis and C. J. Hutchison. *EMBO J.* **2006**, 25, 3275-85.
- [70] C. Ivorra, M. Kubicek, J. M. Gonzalez, S. M. Sanz-Gonzalez, A. Alvarez-Barrientos, J. E. O'Connor, B. Burke and V. Andres. *Genes Dev.* **2006**, 20, 307-20.
- [71] J. Lammerding, P. C. Schulze, T. Takahashi, S. Kozlov, T. Sullivan, R. D. Kamm, C. L. Stewart and R. T. Lee. *Clinical Investigation.* **2004**, 113, 370-378.
- [72] V. D. L. Putra, M. J. Song, S. McBride-Gagyi, H. Chang, K. Poole, R. Whan, D. Dean, V. Sansalone and M. L. Knothe Tate. *Front. Cell Dev. Biol.* **2019**, 7, 354.
- [73] M. Darnell, L. Gu and D. Mooney. *Biomaterials.* **2018**, 181, 182-188.
- [74] H. Tekin, S. Simmons, B. Cummings, L. Gao, X. Adiconis, C. C. Hession, A. Ghoshal, D. Dionne, S. R. Choudhury, V. Yesilyurt, N. E. Sanjana, X. Shi, C. Lu, M. Heidenreich, J. Q. Pan, J. Z. Levin and F. Zhang. *Nat. Biomed. Eng.* **2018**, 2, 540-554.
- [75] R. K. Li, W. Y. Zhao, F. Fang, C. Zhuang, X. X. Zhang, X. M. Yang, S. H. Jiang, F. Z. Kong, L. Tu, W. M. Zhang, S. L. Yang, H. Cao and Z. G. Zhang. *J. Cancer Res. Clin. Oncol.* **2015**, 141, 269-81.
- [76] H. Yu, J. K. Mouw and V. M. Weaver. *Trends Cell Biol.* **2011**, 21, 47-56.
- [77] M. E. Fernandez-Sanchez, S. Barbier, J. Whitehead, G. Bealle, A. Michel, H. Latorre-

- Ossa, C. Rey, L. Fouassier, A. Claperon, L. Brulle, E. Girard, N. Servant, T. Rio-Frio, H. Marie, S. Lesieur, C. Housset, J. L. Gennisson, M. Tanter, C. Menager, S. Fre, S. Robine and E. Farge. *Nature*. **2015**, 523, 92-5.
- [78] P. Katira, R. T. Bonnecaze and M. H. Zaman. *Front. Oncol.* **2013**, 3, 145.
- [79] M. Kapałczyńska, T. Kolenda, W. Przybyła, M. Zajączkowska, A. Teresiak, V. Filas, M. Ibbs, R. Bliźniak, Ł. Łuczewski and K. Lamperska. *Archives of medical science : AMS*. **2018**, 14, 910-919.
- [80] C. Y. Tay, M. S. Muthu, S. L. Chia, K. T. Nguyen, S.-S. Feng and D. T. Leong. *Adv. Funct. Mater.* **2016**, 26, 4046-4065.
- [81] S. Lee, H. S. Kim and H. S. Yoo. *RSC Advances*. **2017**, 7, 54246-54253.
- [82] A. Ovsianikov, A. Khademhosseini and V. Mironov. *Trends Biotechnol.* **2018**, 36, 348-357.
- [83] S. R. Caliari and J. A. Burdick. *Nat. Methods*. **2016**, 13, 405-14.
- [84] K. M. Park, D. Lewis and S. Gerecht. *Annu. Rev. Biomed. Eng.* **2017**, 19, 109-133.
- [85] M. W. Tibbitt and K. S. Anseth. *Biotechnol. Bioeng.* **2009**, 103, 655-63.
- [86] J. Zhu and L. J. Kaufman. *Biophysical J.* **2014**, 106, 1822-1831.
- [87] L. Gasperini, J. F. Mano and R. L. Reis. *J. R. Soc. Interface*. **2014**, 11, 20140817.
- [88] M. Hu, E. U. Azeloglu, A. Ron, K.-H. Tran-Ba, R. C. Calizo, I. Tavassoly, S. Bhattacharya, G. Jayaraman, Y. Chen, V. Rabinovich, R. Iyengar, J. C. Hone, J. C. He and L. J. Kaufman. *Sci. Rep.* **2017**, 7.
- [89] J. W. Nichol, S. T. Koshy, H. Bae, C. M. Hwang, S. Yamanlar and A. Khademhosseini. *Biomaterials*. **2010**, 31, 5536-44.
- [90] E. A. Turley, P. W. Noble and L. Y. Bourguignon. *J. Biol. Chem.* **2002**, 277, 4589-92.
- [91] J. M. Cyphert, C. S. Trempus and S. Garantzotis. *Int. J. Cell Biol.* **2015**, 563818.
- [92] K. Y. Lee and D. J. Mooney. *Prog. Polym. Sci.* **2012**, 37, 106-126.
- [93] J. A. Rowley, G. Madlambayan and D. J. Mooney. *Biomaterials*. **1999**, 20, 45-53.
- [94] W. Gao, Y. Zhang, Q. Zhang and L. Zhang. *Ann. Biomed. Eng.* **2016**, 44, 2049-61.
- [95] M. H. Joyce, S. Allen, L. Suggs and A. Brock. *J. Nanotechnol.* **2017**, 1-8.
- [96] L. Bray, C. Secker, B. Murekatete, J. Sievers, M. Binner, P. Welzel and C. Werner. *Cancers*. **2018**, 10.
- [97] A. Pirskanen, A.-M. Kaimio, R. Myllylas and K. I. Kivirikko. *J. Biol. Chem.* **1996**,

- 271, 9398-9402.
- [98] O. B. Ayyub and P. Kofinas. *ACS Nano*. **2015**, 9, 8004-11.
- [99] W. Lee, N. J. Cho, A. Xiong, J. S. Glenn and C. W. Frank. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, 107, 20709-14.
- [100] J. Visser, F. P. Melchels, J. E. Jeon, E. M. van Bussel, L. S. Kimpton, H. M. Byrne, W. J. Dhert, P. D. Dalton, D. W. Hutmacher and J. Malda. *Nat. Commun.* **2015**, 6, 6933.
- [101] R. J. McMurtrey. *J. Neural. Eng.* **2014**, 11, 066009.
- [102] A. Williams, J. F. Nowak, R. Dass, J. Samuel and K. L. Mills. *Front. Physiol.* **2018**, 9, 966.
- [103] V. A. Schulte, K. Hahn, A. Dhanasingh, K. H. Heffels and J. Groll. *Biofabrication*. **2014**, 6, 024106.
- [104] A. Soman, F. Mathew, A. J. Chacko, M. Alias and G. V. Poosan. *Pharma. Innovation J.* **2014**, 3, 59-66.
- [105] A. Lohani, G. Singh, S. S. Bhattacharya and A. Verma. *J. Drug Deliv.* **2014**, 583612.
- [106] D. Myung, D. Waters, M. Wiseman, P. E. Duhamel, J. Noolandi, C. N. Ta and C. W. Frank. *Polym. Adv. Technol.* **2008**, 19, 647-657.
- [107] H. Kim, J. Cha, M. Jang and P. Kim. *Biomater. Sci.* **2019**, 7, 2264-2271.
- [108] D. S. Reynolds, K. M. Bougher, J. H. Letendre, S. F. Fitzgerald, U. O. Gisladdottir, M. W. Grinstaff and M. H. Zaman. *Acta Biomater.* **2018**, 77, 85-95.
- [109] A. J. Berger, K. M. Linsmeier, P. K. Kreeger and K. S. Masters. *Biomaterials*. **2017**, 141, 125-135.
- [110] J.W. Chen, K. Lim, S.B. Bandini, G.M. Harris, J.A. Spechler, C.B. Arnold, R. Fardel, J.E. Schwarzbauer, J. Schwartz, *ACS Appl Mater Interfaces*. **2019**, 11, 15411-15416.
- [111] K. Anselme, L. Ploux, A. Ponche, *J Adhes Sci Technol*. **2010**, 24, 831-852.
- [112] M.D. Mager, V. LaPointe, M.M. Stevens, *Nat Chem*. **2011**, 3, 582-9.
- [113] T.-T. Yu, F.-Z. Cui, Q.-Y. Meng, J. Wang, D.-C. Wu, J. Zhang, X.-X. Kou, R.-L. Yang, Y. Liu, Y.S. Zhang, F. Yang, Y.-H. Zhou, *ACS Biomater. Sci. Eng.* **2017**, 3, 1119-1128.
- [114] William G. Brodbeck, Matthew S. Shive, Erica Colton, Yasuhide Nakayama, Takehisa Matsuda, James. M. Anderson, *Biomed. Mater. Res.* **2001**, 55, 661-668.
- [115] Erkki Ruoslahti, *Annu. Rev. Cell Dev. Biol.* **1996**. 12, 697-715.

- [116] Jason A. Burdick, Kristi S. Anseth, *Biomaterials*. **2002**, 23, 4315–4323.
- [117] N.C. Hunt, D. Hallam, A. Karimi, C.B. Mellough, J. Chen, D.H.W. Steel, M. Lako, *Acta Biomater.* **2017**, 49, 329-343.
- [118] L.D. Amer, S.J. Bryant, *Ann Biomed Eng.* **2016**, 44, 1959-69.
- [119] J. C. Grim, I. A. Marozas and K. S. Anseth. *J. Control Release*. **2015**, 219, 95-106.
- [120] L. D. Amer and S. J. Bryant. *Ann. Biomed. Eng.* **2016**, 44, 1959-69.
- [121] P. de Almeida, M. Jaspers, S. Vaessen, O. Tagit, G. Portale, A. E. Rowan and P. H. J. Kouwer. *Nat. Commun.* **2019**, 10, 609.
- [122] B. M. Gillette, J. A. Jensen, M. Wang, J. T chao and S. K. Sia. *Adv. Mater.* **2010**, 22, 686-91.
- [123] C. Liu, D. Lewin Mejia, B. Chiang, K. E. Luker and G. D. Luker. *Acta Biomater.* **2018**, 75, 213-225.
- [124] R. S. Stowers, S. C. Allen and L. J. Suggs. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, 112, 1953-8.

Chapter 3

Experimental Methodology

This chapter aims to describe the various research methodologies and characterization techniques used in this thesis with the explanation of the underlying principles. This thesis employs an “on demand” mechanically tunable CoAl-IPN hydrogel to investigate the effects of the tumor-stromal microenvironment in terms of matrix stiffness on CAF. The experimental procedures include the preparation and characterization of CoAl-IPN hydrogel, together with the various biological test methods used to examine cell behavior and understand the underlying mechanism are being discussed.

3.1 Preparation of CoAl-IPN hydrogel

Principle

The physicochemical properties of tumor-stromal extracellular matrix (ECM) are critical players for tumor progression, especially with the increasing matrix stiffness due to the continuous ECM proteins deposition and crosslinking.[1, 2] As such, a CoAl-IPN hydrogel, whose stiffness can be tuned systematically and independently by changing the concentrations of the calcium ions as a crosslinking agent to alginate but not collagen, becomes a useful substrate in cell mechanomics study.[3-5] Alginate is a natural polysaccharide in which the saccharides act to provide a similar architectural structure of hyaluronic acid within the tumor stroma. The structure of the guluronate (G) block permits a high degree of coordination of the divalent ions, hence, facilitating the binding of divalent calcium ions (Ca^{2+}) solely to the G blocks of the alginate polymer chains. Additionally, the binding of Ca^{2+} ions with the alginate form the widely accepted egg-box junctions between the chains, which subsequently leading to the formation of hydrogel. More importantly, the crosslinking density of the alginate network within hydrogel can be precisely controlled by varying the concentration of Ca^{2+} . [6, 7] On the other hand, collagen could be physically crosslinked via the thermal-induced self-assemble fibril initiation and growth at a neutral condition, which contributes by the amino and carboxyl telopeptides on the collagen chains.[8] The collagen fibrils and fibers formed within the network structure are generally in hundreds of nano-meter thick with abundant cell adherence peptide ligands. Thus, it can mimic the *in vivo* cellular microenvironment.

Due to the different crosslinking and decrosslinking mechanisms of the collagen and alginate network, it is possible to attain precise autonomous control over the biochemical and biomechanical properties of the individual network.[9, 10] In addition, calcium-crosslinked alginate network can be de-crosslinked using chelators such as sodium citrate upon crosslinking, thus making it as an attractive dynamic hybrid system.[11] In fact, the degradation rate between the two hydrogel systems (i.e. alginate and collagen hydrogel) is much different, in which the enzymatic degradation of the collagen hydrogel is much

slower as compared to the chelation process of alginate network due to the small chelating agents that can be easily diffused into the hydrogel for effective interaction between Ca^{2+} and citrate ions. Hence, it would allow rapid fine-tuning of the matrix stiffness by de-crosslinking the alginate without sacrificing the overall structural integrity supported by the collagen hydrogel.

Method

In order to fabricate the IPN hydrogel, the pH of the ice-cold rat tail collagen solution (4 mg /ml, Atlantis Bioscience) was adjusted to 7.4 by the addition of base solution in a volume ratio of 6:1, followed by mixing with 4% (w/v in DI water) of alginate solution (FMC BioPolymer) in a volume ratio of 7:1. The hydrogel precursor solution (40 μL) was then aliquoted into individual wells of 96-well plate and incubated for 20 min at 37 $^{\circ}\text{C}$ and 5% CO_2 environment to allow crosslinking of the collagen component. Thereafter, calcium chloride (CaCl_2) solutions (0, 15, or 100 mM) were added to each well and incubated for 20 min, followed by washing three times with 0.9% sodium chloride (NaCl) solution. The alginate network within hydrogel can be de-crosslinked using sodium citrate solution (55 mM) with 20 min incubation. **Figure 3.1.1** depicts the procedures of the CoAl-IPN hydrogel preparation and de-crosslinking.

Figure 3.1.1 Schematic of the crosslinking and de-crosslinking chemistry of the devised CoAl-IPN. Adapted with permission [12]. Copyright 2019, American Chemical Society.

3.2 Rheological measurements

Principle

Rheology is used to study the deformation and flow behaviors of materials (solid, liquid, and gas) using different models of testing, such as rotation and oscillation.[13] Through the series of testing, it can be used to determine the physical properties and the stability of the materials. In the case of a hydrogel, the oscillation model was being used where the sample is placed in between two plates, and the upper plate oscillates forth and back to apply shear stress onto the sample, as shown in **Figure 3.2.1**. [14]

Figure 3.2.1 Schematic image of oscillatory characterization mechanism. Adapted with permission [14]. Copyright 2013 Wiley Periodicals, Inc.

The applied signal onto the sample is calculated based on **Equation 1**:

$$\gamma(t) = \gamma_A * \sin \omega t \quad \text{Equation 1}$$

The feedback signal is formed based on **Equation 2**:

$$\tau(t) = \tau_A * \sin(\omega t + \delta) \quad \text{Equation 2}$$

Where ω is the applied angle frequency, and δ is the phase difference between the input and feedback sine wave. In general, δ is between 0° to 90° for most viscoelastic materials where an ideal liquid is 90° , while an ideal solid material is 0° . [15]

During the measurement, there are two main variables – strain and frequency, which correspond to amplitude sweep and frequency sweep, respectively. Amplitude is the maximum of the oscillatory motion. During the amplitude sweep test, the amplitude of deformation (shear stress) varies, but the frequency is kept constant. The obtained storage modulus (G') and loss modulus (G''), which reflects the elastic and viscous modulus of the sample, respectively, are then plotted against the deformation (strain), as shown in **Figure 3.2.2a**. When both the G' and G'' have reached a constant value, which is termed by linear-viscoelastic (LVE) range, is the point where the sample has reached a steady state. The obtained data can also be used to measure the yield point, which can be determined as the onset decreasing in G' and G'' . As for frequency sweep mode, the strain/stress is kept constant, but the frequency is varied. The G' and G'' are plotted against the frequency during the test (**Figure 3.2.2b**) to examine the behavior of the hydrogel under stress, such as the possible shear thinning or shear thickening effects.

Figure 3.2.2 Amplitude (a) and frequency (b) sweep of rheology test of protein-DNA C2IN-C3lim-G205C hydrogel. Adapted with permission [16]. Copyright 2017 The Authors.

In general, strain sweep would be conducted first with a fixed frequency (i.e. 1 Hz) to determine the LVE range. Subsequently, frequency sweep will be carried out with pre-determined strain value based on the LVE range to examine the behavior of the hydrogel

under stress. Finally, the complex shear modulus G^* representing the complete viscoelastic behavior of a sample can be depicted as the vector sum of the G' and G'' according to **Equation 3**:

$$G^* = \sqrt{G'^2 + G''^2} \quad \text{Equation 3}$$

The Young's modulus (E) can be calculated according to **Equation 4**:

$$E = 2G^*(1 + \nu) \quad \text{Equation 4}$$

Where ν is the Poisson's ratio of material.[17, 18]

Method

The rheological properties of the fabricated CoAl-IPN hydrogels were characterized using the MCR501 rheometer (Anton Paar) equipped with a parallel plate measuring system. A strain sweep from 0.01% to 100% strain was first conducted at a fixed frequency (i.e., 1 Hz) to determine the LVE range of hydrogels. Then, frequency sweep (0.1 to 5 Hz) was carried out using a constant strain of 0.5% as recommended by the amplitude sweep results. In this study, the G' of the hydrogel, which is also equivalent to the complex shear modulus of the crosslinked hydrogel, was taken based on the linear equilibrium modulus plateau of the frequency sweep. Subsequently, E of hydrogel was calculated as described in **Equation 3** and **Equation 4**. Based on the literature, the Poisson's ratio of alginate is ~0.5, while the Poisson's ratio of collagen is concentration-dependent (0.2% collagen is around 0.495 – 0.485 and 0.4% is around 0.480 – 0.470).[10, 19] Therefore, the Poisson's ratio of CoAl-IPN hydrogel is estimated to be ~ 0.485.

3.3 Scanning electron microscope (SEM)

Principle

SEM is commonly used to image the cross-sectional morphology of the fabricated hydrogels. A high-resolution image was obtained by electron beam interaction with the sample under vacuum conditions.[20] However, hydrogel consists of a high content of water, and inappropriate drying of hydrogel could potentially lead to drying artifacts, such as shrinkage and collapses due to high water surface tension within the small cavities of the hydrogel network. Although the traditional freeze-drying method can be used to remove the water content, the original architecture of the structure might be affected by the ice formation during the freezing process that created a porous bulk structure.[21] Therefore, extra care must be taken into consideration during sample preparation. In the fields of histology, pathology, and cell biology, fixation (glutaraldehyde or paraformaldehyde) is an essential step in preserving the cellular morphology and tissue architecture.[22] As such, prior to SEM imaging, the fabricated hydrogels were dehydrated using an optimized fixation-dehydration method as recommend by the literatures.[22, 23]

Method

The CoAl-IPN hydrogel samples were dehydrated by either traditional freeze-drying method or optimized fixation-dehydration method. In the traditional freeze-drying method, the samples were soaked in a PBS solution overnight, followed by freezing in a -80 °C fridge before drying using a lyophilization machine. Contrarily, the optimized fixation-dehydration method was conducted according to the method of J.T. Lee *et al.*[21] listed as follows:

- 1) Immerse hydrogels with fixation buffer overnight;
- 2) Rinse hydrogels with PBS in the fume hood for three times;
- 3) Ethanol gradient dehydration of hydrogels using 30, 50, 70, 90 and 100% ethanol solutions in consecutive 30 min incubations;

- 4) Critical drying of hydrogels using Hexamethyldisilazane (HDMS): 1:2, 2:1 and 1:0 solution of HDMS:ethanol for 30 min serially;
- 5) Submerge the hydrogel in the absolute HDMS and leave it in a fume hood until the solution evaporated;
- 6) Sputter coat the hydrogels and imaged using SEM.

3.4 Mass transport characterization

Principle

Crosslinking of hydrogel will affect the molecules diffusivity across the crosslinked polymeric network, thus limiting the mass transport of oxygen and nutrition for cell survival, as well as the removal of metabolic waste that will drastically affect the cell survivability and behaviors. As crosslinking is one of the critical variables in the CoAl-IPN hydrogel fabrication, the mass transport efficiency study was conducted using fluorescein isothiocyanate (FITC)-conjugated dextran with different molecular weight as tracer molecules.

Method

FITC-dextran (4, 10, 40 and 70 kDa; Sigma) was mixed with CoAl precursor solution at 0.2 mg/mL, followed by aliquoting the mixture (300 µl) into individual wells of 48-well plate. After gelation, the serum-free medium was added to each well and incubated at 37°C with 5% CO₂. At different time points, the medium containing the released FITC-dextran was collected and replaced with fresh medium. The intensity and the concentration of FITC-dextran were determined using the SpectraMax M2 microplate reader at an ex/em wavelength of 490 and 520 respectively (Molecular Devices, USA), in which the diffusivity can be calculated using the following **Equation 5**.

$$\frac{M_t}{M_\infty} = \frac{4}{L} \sqrt{Dt/\pi} \quad \text{Equation 5}$$

Where M_t is the cumulative amount of released dextran at time t , M_∞ is the total amount of released dextran, L is the thickness of hydrogel, and D is the release diffusion coefficient.[12, 24]

3.5 Immunofluorescence staining

Method

Briefly, the cells embedded in CoAl-IPN hydrogels were washed with PBS, followed by fixation using either 4% paraformaldehyde for YAP/TAZ, α -SMA and F-actin staining (30 min at room temperature) or pre-chilled methanol (Sigma 322415) for HIF1- α staining (30 min). After fixation, the samples were permeabilized with 0.2% Triton X-100 for 20 min at room temperature, followed by incubation with 2% bovine serum albumin (BSA; Sigma A3059) blocking solution for 1 h to minimize unspecific antibody binding. The samples were then washed with PBS three times before the addition of each staining reagent. For immunodetection of the targeted proteins, the samples were incubated overnight at 4°C with primary antibody, all of which were diluted in 2% BSA blocking solution. The primary antibodies used are: YAP/TAZ (cell signaling, D24E4, 1:200), α -SMA (ABCAM, ab124964, 1:500), HIF1A (Cell signaling, D1S7W, 1:800). On the following day, the samples were washed three times with PBS before incubating with Alexa Fluor 488 chicken anti-rabbit (1:200) and Hoechst 33342 (1:1000) for 2 h in the dark at room temperature. For F-actin staining, the samples were stained with Alexa Fluor 568 Phalloidin (Thermo fisher, 1:400) directly for 2 h in the dark at room temperature. The samples were then washed three times with PBS and subsequently mounted using ProLong Gold Antifade Mountant (Thermo Fisher Scientific). Immunofluorescence images were captured using Zeiss Axio Observer Z1 inverted fluorescent microscope. Images were acquired under the same instrument settings and exposure time to ensure a fair comparison of the fluorescence signals between the different sample groups. The fluorescence intensity of the targeted proteins was processed and measured with ImageJ software (<https://imagej.nih.gov/ij/>).

* Notably: all the washing steps were carried out over 15 min intervals with shaking to ensure the removal of the dye. In addition, the hydrogel was cut into tiny pieces (2 mm × 2 mm) to enhance the effectiveness of the staining procedure.

3.6 Cell viability analysis

Method

Cell-embedded CoAl-IPN hydrogels were washed with serum-free medium for three times (15 min interval). Subsequently, the staining solution was prepared by diluting Calcein-AM for live cells staining (Thermo C3099, Ex/Em 494/517 nm) and EthD-1 for dead cells staining (Thermo E1169, Ex/Em 528/617 nm) 1000 times in serum-free medium and incubated for 30 min. At the end of the incubation, the staining solution was removed and replaced with a fresh medium before imaging using Zeiss Observer Z1. The cells treated with 0.3% Triton X-100 for 30 min was used as positive control (dead cells). The obtained pictures were processed using ImageJ software (<https://imagej.nih.gov/ij/>). The number of live (green) and dead (red) cells were counted in which the cells' viability can be calculated based on **Equation 6**:

$$Cell\ Viability = \frac{N_{live}}{N_{live} + N_{dead}} \times 100\% \quad \text{Equation 6}$$

Where N_{live} and N_{dead} represent the number of green and red cells, respectively.

3.7 Intracellular reactive oxygen species (ROS) measurement

Method

ROS are short-lived and highly reactive small molecules containing oxygen, such as hydroxyl radical, singlet oxygen, peroxides, etc., which could regulate both physiological and pathophysiological signaling transduction. Generally, the intracellular ROS homeostasis is regulated by ROS generation and a wide variety of antioxidant defenses.

The intracellular ROS of CAF encapsulated within CoAl-IPN hydrogels was evaluated via staining with CellRox®Green (Thermo #C10444, excitation/emission 545/565nm). Briefly, CAF were incubated with 5 μ M CellRox®Green and 1 μ g/mL Hoechst (Sigma #94403) for 30 min at 37°C, followed by washing with PBS three times. Then, hydrogels were fixed with 4% paraformaldehyde for 20 min. After washing, cells were imaged using the Zeiss Observer Z1. N-acetylcysteine (NAC; Sigma #A9165) was used for ROS scavenging experiments. NAC is an aminothiols and precursor of intracellular cysteine and glutathione.

3.8 Enzyme-linked immunosorbent assay (ELISA)

Method

The cell culture supernatant was probed for IL-6 by using commercially available ELISA kits (BioLegend, San Diego, CA) according to the manufacturer's protocols. Briefly, high binding 96-well plates were coated with anti-human IL-6 overnight on 4 °C fridge and subsequently blocked with provided blocking buffer to reduce non-specific binding and background. IL-6 standards and supernatant were loaded, in which the amount of IL-6 was detected with biotin-conjugated anti-human IL-6. Experiments were quadruplicates for each experimental group.

3.9 3D breast MDA-MB-231 tumoroids culture

Principle

Tumor spheroids are complex tissue consisting of a necrotic core surrounded by a viable layer of tumor cells, with distinct oxygen and nutrient gradients mimicking the tumor *in vivo*. In addition, the tumor cells do secrete ECM proteins in between the cell-cell space, thus truly recapitulate the physiological characteristics of the tumor *in vivo*. [25] Various strategies have been employed to fabricate the tumor spheroids as surrogates for tumor study, including hanging drop, low attachment plat, and magnetic levitation. [26, 27] To

date, the commercially available micromold (Sigma) allows the formation of tumor spheroids of different tumor sizes and shapes based on the model structure.

Figure 3.9.1 Schematic images of the tumoroids fabrication process.

Method

Breast MDA-MB-231 tumoroids were prepared using the MicroTissues 3D Petri Dish® micro-mold Tech Evaluation kit (Sigma) according to the manufacturer protocol. Briefly, agarose mold was fabricated using the micro-mold followed by sterilization. Subsequently, 100 μ l of MDA-MB-231 breast cancer cells (1.5×10^6 cells per mL) were added into the agarose mold and incubated at 37°C and 5% CO₂ with 95% humidity overnight to allow for the formation of the MDA-MB-231 tumoroids as shown in the **Figure 3.9.1**. The tumoroids were then collected via centrifugation.

3.10 Functional paracrine crosstalk study

Method

The paracrine crosstalk between CAF and cancer cells was studied via two approaches, an indirect and direct manner, as shown in **Figure 3.10.1**. For the indirect approach, the collected MDA-MB-231 tumoroids were first embedded into a collagen matrix. Subsequently, CAF were embedded in CoAl-IPN hydrogels with different stiffness and incubated with serum-free high glucose DMEM supplemented with 1% penicillin/

streptomycin solution over 48 h. At the end of the culture, the respective solution was collected and termed as a conditioned medium (CM). The tumoroids cells were then treated with the CM where the cell behaviors were monitored after 24 h of treatment.

As for the direct approach as shown in the **Figure 3.10.1** route 1, a transwell co-culturing system was used where 40 μ l of CAF-encapsulated CoAl-IPN hydrogel was cast into the transwell insert while the MDA-MB-231 tumoroids embedded within collagen matrix was formed at the bottom of the individual wells of 24-well plate. Thereafter, the co-culture (indicated as route 2) was carried out in a serum-free medium. After 24 h of treatment, the tumoroids were imaged, where the dispersed cells from the peripheral of the tumor mass were analyzed. The projected areas of the tumoroids ($n \geq 25$ of uniformly sized tumoroids per experimental group) were measured using ImageJ software (<https://imagej.nih.gov/ij/>). The normalized invasion index is defined as the ratio of the measured tumoroid projected areas between the respective experimental groups and the untreated control group. As for the above two experiments, the CoAl-IPN hydrogels without b-CAF were employed as corresponding control.

Figure 3.10.1 The schematic images of the experiment design to investigate the functional paracrine crosstalk between CAF and cancer cells.

As for the colorectal adenocarcinoma DLD-1 EMT assay, DLD-1 (1×10^4 cells per well) were seeded in 96-well plate in DMEM containing 10% FBS and 1% penicillin/streptomycin for 24 h. Next, the culture medium was removed and replenished with CAF-CM for 24 h. Hydrogels without encapsulated CAF treated with serum-free medium were served as control. The cancer cells colony area and circularity index were computed using ImageJ software (<https://imagej.nih.gov/ij/>) based on the phase-contrast images.

3.11 RNA extraction from c-CAF embedded in CoAl-IPN hydrogels

Principle

There are various RNA extraction strategies available, which is heavily dependent on the starting material for maximizing the RNA quality and yield. However, poor RNA quality and low yield are commonly associated with cells embedded in the hydrogel system due to the interference of polymer material itself. As such, it is important to break down the hydrogel network prior to RNA isolation.[28] There are several approaches that have been commonly used to break down the hydrogel system, including liquid nitrogen-grinding, rotor-stator, microhomogenizer and enzymatic/chemical hydrogel digestion. Among these various methods, microhomogenizer and enzymatic/chemical digestion have been shown to produce high RNA yield. However, the physical and enzymatic actions could potentially cause damage to the isolated RNA, especially for prolonged treatment time. In this thesis, the RNA was extracted via liquid nitrogen–grinding method without involving physical damage or harsh chemicals. In addition, the types of RNA isolation kits also play an important role in the quality of the extracted RNA. Studies have shown that TRIzol™ Plus RNA Purification Kit (ThermoFisher 12183555) produced a better quality of the extracted RNA within a short period (i.e., 2 h) as compared to conventional cetyltrimethylammonium bromide (CTAB) and hot phenol method [29, 30] due to the high lysis capability of TRIzol reagent that can inhibit the RNAase activity efficiently. In addition, the RNA Mini Kit provides the time-saving and straightforward spin column based on RNA purification technology for fast and effective RNA isolation.

Method

The total RNA was isolated from cells embedded in hydrogel using TRIzol (Ambion 15596-018) and PureLink RNA Mini Kit (Thermo #12183018A). Briefly, the hydrogels were washed with PBS three times with 15 min intervals, followed by transferring the hydrogel to a mortar containing excessive liquid nitrogen for rapid freezing of the hydrogel. The frozen hydrogel was then grounded yet ensuring full submersion of the hydrogel within the liquid nitrogen throughout the whole grinding process to avoid interacting with water moisture that can lead to RNA degradation. Thereafter, the sample powder was transferred to a centrifuge tube containing 2 mL Trizol lysis solution and incubated on ice for 1 h. At the end of the incubation, 400 μ L of chloroform was added to the tube and mixed thoroughly via gentle shaking followed by incubating for 3 min. Subsequently, the mixture was centrifuged at 3000 $\times g$ for 5 min to pelletize the hydrogel polymer/fibers where the supernatant was collected for RNA isolation according to the protocol established by the manufacturer (**Figure 3.11.1**).

Figure 3.11.1 The RNA extraction process, including sample homogenization and RNA purification using TRIzol™ Plus RNA Purification Kit from Sigma.

3.12 Quantitative reverse-transcriptase real-time polymerase chain reaction (RT-PCR)

Method

Reverse transcription was done using iScript™ cDNA Synthesis Kit (Biorad #1708891) with the T100™ Thermal Cycler using the following reaction protocol: Priming for 5 min at 25 °C, reverse transcription for 20 min at 46 °C and reverse transcriptase inactivation for 1 min at 95 °C. For qPCR, KAPA SYBR® FAST (Sigma #KK4618) was used, and expression levels of the targeted mRNA transcripts were determined using the CFX Connect™ Real-Time PCR Detection System using the following protocol: Enzyme activation for 3 min at 95 °C (1 cycle), Denaturation for 1-3 sec at 95 °C + annealing/extension/data acquisition (40 cycles). The sequence of the target and housekeeping primers were obtained from primer bank (<https://pga.mgh.harvard.edu/primerbank/>) and synthesized by Sigma. In this thesis, GAPDH was employed as the housekeeping gene. The primers used in this thesis are shown as follows.

Table 3.1. Primer sequence of genes used in qPCR studies

Name	Sequence	Base
a-SMA(ACTA2)	F:5`-GTGTTGCCCCTGAAGAGCAT-3`	20
	R:5`-GCTGGGACATTGAAAGTCTCA-3`	21
E-cad (CDH1)	F:5`-CGAGAGCTACACGTTACGG-3`	20
	R:5`-GGGTGTCGAGGGAAAAATAGG-3`	21
N-cad (CDH2)	F:5`-TGCGGTACAGTGTAAGTGGG-3`	20
	R:5`-GAAACCGGGCTATCTGCTCG-3`	20
Vimentin (VIM)	F:5`-GACGCCATCAACACCGAGTT-3`	20
	R:5`-CTTTGTCGTTGGTTAGCTGGT-3`	21

IL1A	F:5`-TGGTAGTAGCAACCAACGGGA-3`	21
	R:5`-ACTTTGATTGAGGGCGTCATTC-3`	22
IL1B	F:5`-ATGATGGCTTATTACAGTGGCAA-3`	23
	R:5`-GTCGGAGATTCTGTAGCTGGA-3`	20
CXCL2	F:5`-GGCAGAAAGCTTGTCTCAACCC-3`	22
	R:5`-CTCCTTCAGGAACAGCCACCAA-3`	22
IL8	F:5`-ACTGAGAGTGATTGAGAGTGGAC-3`	23
	R:5`-AACCCTCTGCACCCAGTTTTC-3`	21
CXCL3	F:5`-CGCCCAAACCGAAGTCATAG-3`	20
	R:5`-GCTCCCCTTGTTTCAGTATCTTTT-3`	23
VEGFC	F:5`-GAGGAGCAGTTACGGTCTGTG-3`	21
	R:5`-TCCTTTCCTTAGCTGACACTTGT-3`	23
INHBA	F:5`-CAACAGGACCAGGACCAAAGT-3`	21
	R:5`-GAGAGCAACAGTTCACCTCCTC-3`	21
CXCL14	F:5`-CGCTACAGCGACGTGAAGAA-3`	20
	R:5`-GTTCCAGGCGTTGTACCAC-3`	19
INHBB	F:5`-GTGAAGCGGCACATCTTGAG-3`	20
	R:5`-GCGAAGCTGATGATTTTCGAAAC-3`	23
HGF	F:5`-GCTATCGGGGTAAAGACCTACA-3`	22
	R:5`-CGTAGCGTACCTCTGGATTGC-3`	21
LIF	F:5`-CCAACGTGACGGACTTCCC-3`	19
	R:5`-TACACGACTATGCGGTACAGC-3`	21
CCL3	F:5`-AGTTCTCTGCATCACTTGCTG-3`	21
	R:5`-CGGCTTCGCTTGGTTAGGAA-3`	20
IL11	F:5`-CGAGCGGACCTACTGTCCTA-3`	20

	R:5`-GCCCAGTCAAGTGTCAGGTG-3`	20
IL6	F:5`-ACTCACCTCTTCAGAACGAATTG-3`	23
	R:5`-CCATCTTTGGAAGGTTTCAGGTTG-3`	23
BMP2	F:5`-ACCCGCTGTCTTCTAGCGT-3`	19
	R:5`-TTTCAGGCCGAACATGCTGAG-3`	21
CXCL10	F:5`-GTGGCATTCAAGGAGTACCTC-3`	21
	R:5`-TGATGGCCTTCGATTCTGGATT-3`	22
BMP7	F:5`-TCAACCTCGTGGAACATGACA-3`	21
	R:5`-CTTGGAAGATCAAACCGGAAC-3`	23
IL17D	F:5`-GCCCTGGGCCTACAGAATC-3`	19
	R:5`-CGCCCTGTTTGTCGATGCT-3`	19
TNFSF10	F:5`-TGCGTGCTGATCGTGATCTTC-3`	21
	R:5`-GCTCGTTGGTAAAGTACACGTA-3`	22
TNFSF13B	F:5`-CCTCACGGTGGTGTCTTTCTA-3`	21
	R:5`-AACGGCACGCTTATTTCTGCT-3`	21
CSF3	F:5`-GCTGCTTGAGCCAACTCCATA-3`	21
	R:5`-GAACGCGGTACGACACCTC-3`	19
CXCL12	F:5`-ATTCTCAAACTCCAACTGTGC-3`	23
	R:5`-ACTTTAGCTTCGGGTCAATGC-3`	21
IL24	F:5`-CACACAGGCGGTTTCTGCTAT-3`	21
	R:5`-TCCAAGTGTGGAATGCTCTCC-3`	22
INHBE	F:5`-ATCTTCCGATGGGGACCAAG-3`	20
	R:5`-AGAGTTAAGGTATGCCAGCCC-3`	21
CCL8	F:5`-TGGAGAGCTACACAAGAATCACC-3`	23
	R:5`-TGGTCCAGATGCTTCATGGAA-3`	21

CCL5	F:5`-CCAGCAGTCGTCTTTGTCAC-3`	20
	R:5`-CTCTGGGTTGGCACACACTT-3`	20
CSF2	F:5`-TCCTGAACCTGAGTAGAGACAC-3`	22
	R:5`-TGCTGCTTGTAGTGGCTGG-3`	19
PDGFC	F:5`-GACTCAGGCGGAATCCAACC -3`	20
	R:5`-CTTGGGCTGTGAATACTTCCATT-3`	23
CXCL1	F:5`-AAGTGTGAACGTGAAGTCCC-3`	20
	R:5`-GTCAGTGTTCAGCATCTTTTCG-3`	22
IL23A	F:5`-CTCAGGGACAACAGTCAGTTC-3`	21
	R:5`-ACAGGGCTATCAGGGAGCA-3`	19
CCL4	F:5`-CTGTGCTGATCCCAGTGAATC-3`	21
	R:5`-TCAGTTCAGTTCAGGTCATACA-3`	23
CSF1	F:5`-AGACCTCGTGCCAAATTACATT-3`	22
	R:5`-AGGTGTCTCATAGAAAGTTCGGA-3`	23
CCL2	F:5`-CAGCCAGATGCAATCAATGCC -3`	21
	R:5`-TGGAATCCTGAACCCACTTCT-3`	21
TGFB3	F:5`-ACTTGCACCACCTTGGACTTC-3`	21
	R:5`-GGTCATCACCGTTGGCTCA-3`	19
PDGFB	F:5`-CTCGATCCGCTCCTTTGATGA-3`	21
	R:5`-CGTTGGTGCGGTCTATGAG-3`	19
TGFB2	F:5`-CCCCGGAGGTGATTTCCATC-3`	20
	R:5`-GGGCGGCATGTCTATTTTGTAAG-3`	23
CCL20	F:5`-TGCTGTACCAAGAGTTTGCTC-3`	21
	R:5`-CGCACACAGACAACCTTTTCTTT-3`	23
GAPDH	F:5`-GGAGCGAGATCCCTCCAAAAT-3`	21

	R:5`-GGCTGTTGTCATACTTCTCATGG-3`	23
--	---------------------------------	----

3.13 RNA sequencing (RNA-Seq) and DEG analysis

Principle

RNA sequencing (RNA-Seq) is a kind of whole-transcriptome analysis technique based on next-generation sequencing (NGS). The RNA-Seq focuses on the differential expression gene between the sample groups to provide critical information on the particular gene's function as part of the gene ontology (GO) study.[31] In general, the RNA Seq workflow consists of three main steps, namely 1) Total RNA quantification and qualification; 2) Library construction and quality assessment; and 3) Sequencing prior to the subsequent downstream bioinformatics analysis. The overall workflow is illustrated in **Figure 3.13.1**.

Figure 3.13.1 The RNA sequencing main steps, including library preparation via reverse transcription, index addition and PCR enrichment, DNA chains cluster growth via bridge PCR, as

well as the last sequencing of the bases on the DNA chain described by Illumina. Adapted with permission [32].

Method

The RNA samples were barcoded for library construction and sequencing. mRNA was selected using oligo(dT) probes and then fragmented randomly. Subsequently, the first-strand cDNA is synthesized according to the conventional reverse transcription method followed by double-strand DNA with end repair and A/adaptor addition. The constructed library was diluted to 1.5 ng/μL as measured by Qubit2.0 and insert size was determined with Agilent 2100. Q-PCR was subsequently used to accurately quantify the library effective concentration ($> 2\text{nM}$). Next, the enriched cDNA was fed into the Illumina flowcell where the cluster growth of DNA was performed based on the bridge amplification PCR. The processing of fluorescent images into sequences, base calling, and quality value calculations were performed using the Illumina data processing pipeline. Bulk RNA-Seq gene raw data was mapped to reference genome (homo_sapiens_hg19) using the STAR software and gene expression level analysis was performed using EdgeR R package. The gene readout was adjusted by the “Trimmed Mean of M-values” normalization (TMM). Significant differentially expressed genes (DEGs) were selected with a False Discovery Rate (FDR) cutoff of < 0.05 . The GO and KEGG enrichment analysis of the DEGs was conducted using the Goseq R and clusterprofiler R package. Enriched pathways/functions with corrected $p < 0.05$ were considered to statistically significant.

3.14 Gene set enrichment analysis (GSEA)

Principle

GSEA is an analysis tool that uses pre-defined classes of genes that are being over-represented in a large set of genes to identify a significant biological process. All the genes data can be found in a Molecular Signatures Database (MSigDB), which has been categorized into eight major collections for easy browsing and viewing as shown in **Figure 3.14.1**. [33] GSEA is a reliable tool with the database which is actively continuously

updated and improved by the incorporation of experimental profile data.[34] Thus, GSEA covers a wide range of information.

Figure 3.14.1 The eight collections of the Molecular Signature Database. Adapted with permission [33]. Copyright 2015 Elsevier Inc.

Based on the MSigDB collections and the respective subsets' information, a smaller gene list of more easily interpretable pathways is obtained. Thereafter, the analysis was carried out by comparing the gene level of those pre-defined gene sets between the sample groups using rank-based statistics by analyzing the fold change values calculated from FPKM values, and the statistical significance is determined by false discovery rate (FDR). In general, there are three main steps for this analytical process: 1) the genes in a certain gene set are ranked based on the fold change values calculated from FPKM values. Subsequently, the ranked gene list of two samples is being evaluated by assigning an enrichment score (ES) using a Kolmogorov-Smirnov-like statistic tool; 2) a normalized enrichment score (NES) will then be calculated based on the respective gene set size; and 3) Thereafter, the NES statistical significance will be determined by calculating the p value based on the phenotypic-based permutation test as well as FDR statistical tool. Overall, all three NES value, p value, and FDR value are crucial to determine whether the test sample has a similar (in)activation pathway or phenotype to that of the certain gene set. For example, a standard result with statistical significance are generally expressed as $NES > 1$, $p < 0.05$ and $FDR < 0.25$, and the figure of a certain gene set is presented in **Figure 3.14.2**. [35, 36]

Figure 3.14.2 Representative enrichment plot image from GSEA. The Gene set is “Glycosaminoglycan biosynthesis chondroitin sulfate”. The green line is the ES value of all the genes in the gene set. If $ES > 0$, indicating that the functional gene is enriched at the front of the sequence. Larger ES value means that these genes in the list are enriched and not scattered in the pathway. The dark bar-code shows the position of a gene in a gene set in the background gene (gene list), and each vertical line represents the gene in that pathway. The followed heatmap reflects the gene expression level. The butterfly graph shows a positive and negative correlation between the gene order and the sequencing metric score. Adapted with permission [37]. Copyright 2019 Bian et al.

Method

GSEA analysis was performed using GSEA software 4.0.1 enriched with C2.all.v7.1.symbols.gmt curated gene set under default parameters where the genes associated with the certain biological process can be annotated automatically. Subsequently, the gene set was pre-ranked based on the fold change values and stored as a .rnk file. Subsequently, the pathway enrichment of the ranked gene list was evaluated

based on NES (weighted statistic of a gene set size ranging from 15 to 500 and a normalization mode of meandiv), p value, and FDR calculation.

3.15 Hub gene analysis

Method

The DEGs from enriched gene sets were imported into online STRING (<http://string-db.org/>) database and used as the seed genes for developing a network of DEGs-encoded proteins and PPIs (Protein-protein interaction). The parameters include a minimum required interaction score > 0.4 (medium confidence), and only queried proteins were displayed. Subsequently, the hub genes, defined as key genes with significant biological regulatory functions, were analyzed and identified using the CytoHubba application in Cytoscape software, where five calculation methods were employed, including Degree, EPC, Eccentricity, MCC, and MNC.

3.16 Gene expression profiling interactive analysis (GEPIA)

Principle

GEPIA is a web-based tool for cancer and normal gene expression profiling. It has a user-friendly interface to provide customizable functions based on established databases, such as cancer genome atlas (TCGA) and genotype-tissue expression GTEx.[38] The information provided by GEPIA contains differential expression analysis, survival analysis and profiling plotting, etc. where the correlation analysis between a particular gene and patient survival can be obtained (**Figure 3.16.1**).

Figure 3.16.1 The correlation analysis between gene (UBE2C) and patient survival with colon cancer. The red and solid blue lines represent the survival plot of patients with higher/ lower expression of ERBB2, respectively. High Hazard ratio (HR) is correlated to poorer patient prognosis using TCGA data. N is the sample number. Generally, $p < 0.05$ indicates the result is statistically significant. In this case, the higher expression will elongate patient survival. Adapted with permission [39]. Copyright 2019 by the authors.

Method

Based on the information obtained from GSEA, RNA-Seq and PCR, genes (i.e., cytokines) that were statistically differentially expressed ($\text{Log2FoldChange} \geq 1$ and $\text{FDR} \leq 0.05$) were further processed using GEPIA to explore the correlation between gene expression level and patient survival rate. When the Logrank p value of the survival analysis is less than 0.05, the corresponding gene was considered to have a significant contribution to patient survival.

References

- [1] R. Malik, P. I. Lelkes and E. Cukierman. *Trends Biotechnol.* **2015**, 33, 230-6.

- [2] D. Hanahan and R. A. Weinberg. *Cell*. **2011**, 144, 646-74.
- [3] B. M. Gillette, J. A. Jensen, M. Wang, J. Tchao and S. K. Sia. *Adv. Mater.* **2010**, 22, 686-91.
- [4] C. Liu, D. Lewin Mejia, B. Chiang, K. E. Luker and G. D. Luker. *Acta Biomater.* **2018**, 75, 213-225.
- [5] C. B. D. Cunha, D. D. Klumpers, W. A. Li, S. T. Koshy, J. C. Weaver, O. Chaudhuri, P. L. Granja and D. J. Mooney. *Biomaterials*. **2014**, 35, 8927-36.
- [6] N. J. Hoglebe, J. W. Reinhardt and K. J. Gooch. *J. Biomed. Mater. Res. A*. **2017**, 105, 640-661.
- [7] S. R. Caliali and J. A. Burdick. *Nat. Methods*. **2016**, 13, 405-14.
- [8] C. H. Chuang, R. Z. Lin, J. M. Melero-Martin and Y. C. Chen. *Artif. Cells Nanomed. Biotechnol.* **2018**, 46, S434-S447.
- [9] A. Soma, F. Mathew, A. J. Chacko, M. Alias and G. V. Poosan. *The Pharma. Innova. J.* **2014**, 3, 59-66.
- [10] D. Myung, D. Waters, M. Wiseman, P. E. Duhamel, J. Noolandi, C. N. Ta and C. W. Frank. *Polym. Adv. Technol.* **2008**, 19, 647-657.
- [11] K. Y. Lee and D. J. Mooney. *Prog. Polym. Sci.* **2012**, 37, 106-126.
- [12] H. Cao, M. K. H. Lee, H. Yang, S. K. Sze, N. S. Tan and C. Y. Tay. *Langmuir*. **2019**, 35, 7487-7495.
- [13] T. G. Mezger. *The Rheology handbook*. **2014**.
- [14] J. M. Zuidema, C. J. Rivet, R. J. Gilbert and F. A. Morrison. *J. Biomed. Mater. Res. B Appl. Biomater.* **2014**, 102, 1063-73.
- [15] A. Franck. TA instruments, New castle, DE, USA AN004.
- [16] J. Gačanin, A. Kovtun, S. Fischer, V. Schwager, J. Quambusch, S. L. Kuan, W. Liu, F. Boldt, C. Li, Z. Yang, D. Liu, Y. Wu, T. Weil, H. Barth and A. Ignatius. *Adv. Health. Mater.* **2017**, 6.
- [17] J. R. Tse and A. J. Engler. *PLoS One*. **2011**, 6, e15978.
- [18] J. Hay and Y. J. Meng. *Nanomechanics*, INC.
- [19] A. P. Castro, P. Laity, M. Shariatzadeh, C. Wittkowske, C. Holland and D. Lacroix. *J. Mater. Sci. Mater. Med.* **2016**, 27, 79.
- [20] G. D. Danilatos and V. N. E. Robinson. *Scanning*. **1979**, 2, 72-82.

- [21] J. T. Lee and K. L. Chow. *Scanning*. **2012**, 34, 12-25.
- [22] S. Thiberge, A. Nechushtan, D. Sprinzak, O. Gileadi, V. Behar, O. Zik, Y. Chowers, S. Michaeli, J. Schlessinger and E. Moses. *PNAS*. **2004**, 101, 3346-3351.
- [23] J. Lee. Centre for advanced microscopy. Austrian National university.
- [24] J. Crank. The mathematics of diffusion (book). **1975**.
- [25] M. Zanoni, F. Piccinini, C. Arienti, A. Zamagni, S. Santi, R. Polico, A. Bevilacqua and A. Tesei. *Sci. Rep.* **2016**, 6, 19103.
- [26] S. A. Langhans. *Front. Pharmacol.* **2018**, 9, 6.
- [27] A. Ovsianikov, A. Khademhosseini and V. Mironov. *Trends Biotechnol.* **2018**, 36, 348-357.
- [28] N. Koster, A. Schmiermund, S. Grubelnig, J. Leber, F. Ehlicke, P. Czermak and D. Salzig. *Tissue Eng. Part C Methods*. **2016**, 22, 552-60.
- [29] L. Wang and J. P. Stegmann. *Biomaterials*. **2010**, 31, 1612-8.
- [30] M. Heidary and M. P. Kakhki. *J. Sci. I. R. Iran*. **2014**, 25, 13-17.
- [31] Z. Wang, M. Gerstein and M. Snyder. *Nat. Rev. Genet.* **2009**, 10, 57-63.
- [32] Illumina. The next generation of gene Expression profiling with RNA-Seq (e-book).
- [33] A. Liberzon, C. Birger, H. Thorvaldsdottir, M. Ghandi, J. P. Mesirov and P. Tamayo. *Cell Syst.* **2015**, 1, 417-425.
- [34] A. L. Tarca, G. Bhatti and R. Romero. *PLoS One*. **2013**, 8, e79217.
- [35] C. Weidner, M. Steinfath, E. Wistorf, M. Oelgeschlager, M. R. Schneider and G. Schonfelder. *J. Vis. Exp.* **2017**, 126.
- [36] A. Subramanian, P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, A. Paulovich, S. L. Pomeroy, T. R. Golub, E. S. Lander and J. P. Mesirov. *PNAS*. **2005**, 102, 15545-15550.
- [37] Q. Bian, J. Chen, W. Qiu, C. Peng, M. Song, X. Sun, Y. Liu, F. Ding, J. Chen and L. Zhang. *Oncol. Lett.* **2019**, 18, 5043-5054.
- [38] Z. Tang, C. Li, B. Kang, G. Gao, C. Li and Z. Zhang. *Nucleic Acids. Res.* **2017**, 45, W98-W102.
- [39] H. Dastsooz, M. Cereda, D. Donna and S. Oliviero. *Int. J. Mol. Sci.* **2019**, 20.

Chapter 4*

Fabrication and characterization of mechanically tunable 3D CoAl-IPN hydrogel as an *in vitro* surrogate of tumor stroma

This chapter aims to fabricate a mechanically tunable hydrogel as an in vitro surrogate of tumor stroma, namely 3D collagen-alginate interpenetrating network (IPN) hydrogel. Using this platform, different stiffness of hydrogel, covering the stiffness range of breast and colon tumor stroma matrix, could be obtained independent of biochemical component changes. Specially, the capacities of de-crosslinking and re-crosslinking of alginate network within IPN endow the hydrogel with dynamic properties, allowing for in situ continuously study of cell behavior under varying biomechanical conditions. Findings show that the increase of crosslinking density would change the microstructure of hydrogels from sparse to dense network structure without significantly affecting the diffusion coefficient.

*This section published substantially as Huan Cao, Melissa Kao Hui Lee, Haibo Yang, Siu Kwan Sze, Ngan Soon Tan and Chor Yong Tay. Langmuir 2019, 35, 23, 7487-7495. DOI : 10.1021/acs.langmuir.8b02649. Reproduced with permission. Copy right (2019) American Chemical Society.

4.1 Introduction

TME is a complex assemble, composes of different cell types and tumor associated stroma matrix.[1] During tumor progression, the physicochemical properties of the surrounding stromal ECM, including stiffness, fibril alignment, network pore size, etc., are in constant changes due to the deposition and crosslinking of proteins by the CAF and the compression force induced by the tumor growth. This in turn will impose different functionally effects on the surrounding cells' activities, thus acting in a dynamic reciprocal way.[2] Similarly, the increased in stroma ECM would also affect the overall substance diffusivity (nutrition/waste/oxygen) [3, 4] as well as inducing interstitial pressure in tumor, both of which would further affect the tumor cells' activities.[5]

Although studies have been conducted using different 3D model systems, such as collagen [6] and polysaccharide polymers [7, 8] to mimic tumor stroma, most of these systems do not allow the exclusive study on the effect of biophysical factors on cell behaviors. For instance, different concentrations of collagen were commonly used to develop hydrogels with varying stiffness, resulting in difficulties to distinguish the biophysical and biochemical factors due to the increasing in cell adhesion sites.[9, 10] On the other hand, polysaccharide polymers is bioinert in nature,[11, 12] which could not resemble native TME-like properties. Hence, in this study, a 3D IPN composed of collagen and alginate crosslinked polymers is fabricated via a two-step temperature-induced and cation-mediated (Ca^{2+}) crosslinking process. The multicomponent crosslinked polymers were formed without any covalent bonds formed between them. Thus, one of the major advantages of CoAl-IPN hydrogel is that the biochemical and biomechanical properties of the hydrogel can be controlled autonomously.

4.2 Experimental Methods

4.2.1 Materials

Sodium alginate (Na-Al; PRONOVA UP MVG) was obtained from FMC BioPolymer, Norway. Rat tail type I collagen solution (FibriCol, ~4 mg/mL) was purchased from Atlantis Bioscience (Singapore). Phosphate-buffered saline (PBS) (10× without Ca^{2+} and Mg^{2+} , Cat.70013032) was obtained from Gibco. Calcium chloride (CaCl_2), sodium chloride (NaCl), sodium citrate, hexamethyldisilazane (HDMS), collagenase (C9891) were purchased from Sigma (Sigma-Aldrich). All reagents were used without further purification.

4.2.2 Fabrication of CoAl-IPN hydrogel

The fabrication of the IPN hydrogel was achieved via the thermal-induced gelation of the collagen component in holding the hydrogel scaffold in shaped, followed by ionotropic crosslinking of the alginate component in tailoring the mechanical properties of the IPN hydrogel. Briefly, the pH of the ice-cold rat tail collagen solution (4 mg /ml dissolved in 20 mM of acetic acid, Atlantis Bioscience) was adjusted to 7.4 by the addition of base solution provided by manufacturer in a volume ratio of 6:1, followed by mixing with 4% (w/v in DI water) of alginate solution (FMC BioPolymer) in a volume ratio of 7:1. Next, 300 μL of the hydrogel precursor solution was added to the individual well of the 48-well plate and incubated for 20 min at 37 °C to allow the thermal-induced gelation of the collagen component via fibrillogenesis process according to protocol described (**Figure 4.3.1.1**).

4.2.3 Rheological properties characterization

Rheological characterization of the fabricated hydrogels was done as explained in **Section 3.2**. Specifically, hydrogels (CoAl_{15} and CoAl_{100}) were de-crosslinked with sodium citrate (50 mM, 20 min) followed by rheological assessment to evaluate the dynamic property of

hydrogel. Also, both the collagen and CoAl₀ hydrogels were treated with Ca²⁺ (100 mM, 20 min) to assess the remnants of alginate network within the CoAl₀ hydrogel after 2 days of incubation.

4.2.4 SEM

The microarchitecture imaging of hydrogels (CoAl₀ and CoAl₁₀₀) was conducted using SEM as shown in **Section 3.3**.

4.2.5 Mass transportation characterization

FITC-dextran with different molecular weight was used as a tracer to study the diffusion coefficient of both CoAl₀ and CoAl₁₀₀ hydrogels as illustrated in **Section 3.4**.

4.2.6 Statistical analysis

All experiments were repeated at least three times with duplicates. Statistical significance was determined by one-way analysis of variance (ANOVA) using SPSS among three or more than three groups. Comparison between two groups was analyzed by two-tailed Student's t test. The differences are considered as statistically significant with $p < 0.05$.

4.3 Principle Outcomes

4.3.1 Mechanical tunability of 3D CoAl-IPN hydrogel via varying crosslinking density

In this study, collagen- and alginate-based IPN hydrogels were employed as the bona fide *in vitro* surrogate of the tumor stroma. Collagen I, being one of the most abundant acellular components in the tumor stroma was included to provide structural support and integrin binding ligands such as RGDs and GxOGER to promote cell adhesion.[13] Conversely,

the alginate network was interlaced into the 3D pre-formed collagen matrix as the second network to fine-tune the hydrogel mechanics via inotropic gelation.[11, 14]

As seen from **Figure 4.3.1.1a**, the hydrogel precursor appeared to be highly transparent prior to the collagen gelation. On the other hand, the turbidity of CoAl hydrogel precursor increased from a highly transparent state to semi-translucent state due to the self-assembly of collagen fibers in forming collagen network during the fibrillogenesis process as shown in **Figure 4.3.1.1b** (also known as CoAl₀) [15].

Figure 4.3.1.1. Two-step crosslinking process of CoAl IPN hydrogel preparation. a) Fully transparent hydrogel precursor solution. b) Semi-translucent hydrogel indicating the partially gelated IPN hydrogel due to the collagen fiber's self-assembly process. c) Final fully crosslinked IPN hydrogel upon washing and exposed to DMEM medium.

This partial gelation of the collagen component was confirmed by measuring the rheological properties of the IPN hydrogel using the MCR501 rheometer (Anton Paar) equipped with a parallel plate measuring system. Briefly, 8 mm disk-shape sample was punched out from the 48-well plate hydrogel using a leather puncher. Subsequently, 10 mm spindle was applied with a measurement gap of around 0.5-1 mm. A strain sweep from 0.01% to 100% strain was first conducted at a fixed frequency (i.e., 1 Hz) to determine the LVE range of hydrogels. As shown in the **Figure 4.3.1.2a**, the storage modulus (G') of all the hydrogels exhibited a constant value across the strain ranging from 0% to ~30%, suggesting that this range is the linear viscoelastic region (LVE) of the hydrogels. Thereafter, a frequency sweep (0.1 - 1.58 Hz) was performed with a fixed strain (0.5%) as recommended by the amplitude sweep results. As shown in **Figure 4.3.1.2b**, the storage modulus (G') of the hydrogels maintained a constant value, suggesting that the hydrogels

were predominantly elastic within these experimental conditions. More importantly, the storage modulus of the CoAl₀ hydrogel is higher than the loss modulus of the IPN hydrogel, which is an indication of the ‘solid state’ of the hydrogel system [16, 17].

Thereafter, calcium chloride (CaCl₂) solution was added to the partially gelated IPN hydrogel (CoAl₀) and incubated for 20 min to allow the ionotropic crosslinking of the alginate component to produce fully crosslinked IPN hydrogel (CoAl₁₀₀). An incubation period of 20 mins was chosen for alginate network crosslinking as it is more than sufficient for the diffusion of small molecule such as calcium ions throughout the hydrogel network when immersed within calcium ions crosslinking bath [18-21]. At the end of the crosslinking, the newly fabricated hydrogel was washed for 3 times using 0.9% NaCl with 5 mins interval prior to the addition of fresh cell culture medium (**Figure 4.3.1.1c**). In this case, NaCl was chosen as a washing solution instead of PBS in removing the excessive/unreacted Ca ions solely aimed to prevent the formation of sediments between the calcium ions and the PBS salt contents. Similarly, cells can hardly survive for prolonged period of time without adequate nutrient and the same sediment issue occurs when excessive Ca ions are in contact with the cell culture medium. As such, NaCl is an ideal washing solution in removing the excessive Ca ions. The successful ionotropic crosslinking of the IPN hydrogel was evaluated by measuring the rheological properties of the final hydrogel system. As seen from **Figure 4.3.1.2b**, the modulus of the fully crosslinked hydrogel CoAl₁₀₀ has higher storage modulus as compared to partially gelated IPN hydrogel CoAl₀.

Figure 4.3.1.2 Rheological characterization of hydrogels. Representative amplitude (a) and frequency (b) sweep of CoAl-IPN hydrogels treated with different Ca^{2+} ions concentrations (0, 15 and 100 mM). All experiments were triplicated. Data represent the mean $\pm$ standard deviation.

Moreover, the Young's modulus (E) was further calculated. Poisson's ratio is defined as the negative ratio of the lateral strain to the axial strain for a uniaxial stress state[22]. It has been reported that the Poisson ratio of collagen and alginate gel will decrease with increasing concentrations [23, 24]. For example, the Poisson ratio for 0.2% collagen gel is around 0.495–0.485, while a 0.3% collagen gel has a Poisson ratio of 0.480–0.490 and a Poisson ratio of 0.480–0.470 for 0.4% collagen gel [23]. In this study, the collagen used was 3 mg/mL (0.3%), as such a ν_{col} of 0.485 was chosen as the Poisson ratio for the collagen component. Additionally, a Poisson ratio value of 0.5 has been reported for alginate gel ranging from 0.5% to 2.5% [25–27] as the alginate gel have been shown to be nearly incompressible and behaved like Mooney material (rubber like characteristics) as reported by A.V. SALSAC *et al.* and M. Ahearne *et al.* [28–29]. In our study, the alginate used was 5 mg/ml (0.5%), as such in order to simplify the calculation, a Poisson ratio of ν_{Al} 0.5 was selected for the alginate component.

As for the Poisson ratio calculation for the composite scaffold, Johan Helsing *et al.* have studied on a fiber-reinforced system using an interface integral equation method, in which he has demonstrated the effective calculation of the Poisson's ratio particularly for two-

phase fiber reinforced composites over the whole parameter space (fraction ratio f_i , Poisson ratio ν_i and Young's modulus E_i) ($i = 1; 2$) [30]. The results showed that the effective Poisson's ratio for a system with $\nu_1 = \nu_2 = 0.5$ and stiffness ratios $(E_2/E_1) = 2000$ and $(E_2/E_1) = 0.0005$ is approximately equivalent to rigid inclusions and voids, respectively. In our case, the f_{Col} ratio between alginate and collagen is 0.125. Moreover, the storage modulus of pure collagen and alginate gel are around 30 Pa and 260 Pa, respectively, and the ratio of E_{Al}/E_{Col} is around 8.45. As such, the ν_{eff} of composite at $f_2 = 0.125$ and $E_2/E_1 = 20$ slightly decrease, reaching to around 0.49. Hence, it is expected that the ν_{eff} of CoAl hydrogel system will follow the similar change, whose value will be around or slightly lower than the ν_{eff} value of each polymer network.

Specifically, we calculated the ν_{eff} value based on the following equation.

$$\nu_{eff} = \nu_{col} * \frac{V_{col}}{V_{total}} + \nu_{Al} * \frac{V_{Al}}{V_{total}}$$

Where $\frac{V_{col}}{V_{total}}$ and $\frac{V_{Al}}{V_{total}}$ are the volume fraction ratio of collagen and alginate within scaffold. ν_{col} and ν_{Al} are the Poisson ratio for collagen and alginate respectively. ν_{eff} is the calculated Poisson ratio for scaffold.

$$\nu_{eff_{CoAl}} = 0.485 * \frac{7}{8} + 0.5 * \frac{1}{8} = 0.487$$

The computed Young's modulus (E) of CoAl₀, CoAl₁₅ and CoAl₁₀₀ were found to be around 150, 450 and 1200 Pa respectively (**Figure 4.3.1.3**), which falls within the range of the reported ECM stiffness for several tumor stroma at different stage (breast stroma E : 200 – 800 Pa [31]; colon stroma E : 0.374 – 68.0 kPa [32]). The colon tumor stroma stiffness is highly dependent on the tumor-stages, the presence of lymph node/ distant, as well as the gender and age of the patient that lead to a wide range properties. For example, the stiffness of colon tumor stroma varied from 1.91-3.96 kPa for T1 stage, 1.08-12.2 kPa for T2 stage, 2.6-43.2 kPa for T3 stage, and 5.58-68 kPa for T4 stage. Additionally, the stiffness varied during the metastasis process, in which the stiffness would be ranging from 2.1-68 kPa for lymph node metastasized tumor stroma, while 1.08-47.8 kPa for non-metastasized one [32]. The fabricated IPN hydrogel has a stiffness of approximately 1.2 kPa could represent the colon stroma stiffness to some degree, yet from the materials

standpoint, it is sufficient to determine the threshold network density to trigger the phenotypic as an alternative 3D hydrogel-based tumor stroma mimic as discussed below.

Hence, CoAl-IPN hydrogel is a promising platform to serve as a tumor stroma proxy *in vitro*. From cell aspect, on one hand, the CoAl-IPN hydrogel can be used to study the tumor stroma cells (e.g., CAF, macrophages, etc.) mechanoresponse and the related mechanosignaling pathways. On the other hand, the tumor solid stress effect induced by the surrounding matrix on tumoroids activities (e.g., growth and invasiveness) can be explored using the developed CoAl-IPN hydrogel. It is known that the growing tumor would compress the surrounding stroma matrix, resulting in the matrix stiffness increase. In turn, the stiffing matrix could exert compression force on tumoroids during tumor growth, inducing solid stress formation within tumoroids.[33]

Figure 4.3.1.3 Young's modulus of CoAl-IPN hydrogels. All experiments were triplicated. Data represent the mean $\pm$ standard deviation.

4.3.2 Dynamic mechanical tunability of IPN hydrogel via de- and re-crosslinking process

It has been reported that the citrate ion is a great chelator to the calcium ions by forming calcium citrate complexes.[34] Hence, sodium citrate has been widely used to dissolve calcium-crosslinked alginate hydrogels. **Figure 4.3.2.1** shows the characterization of the various CoAl-IPN hydrogels before and after the sodium citrate treatment. As shown in the **Figure 4.3.2.1a**, when the crosslinked CoAl₁₀₀ hydrogel treated with 50 mM sodium citrate for 20 mins, the hydrogel became slightly compliant yet maintaining a cylindrical shape. The rheology measurement shows that both de-crosslinked hydrogels (CoAl₁₅ and CoAl₁₀₀) exhibited storage modulus of approximately 70 and 80 respectively (**Figure 4.3.2.1b and 4.3.2.2**), which is close to CoAl₀ hydrogel (~50 Pa), hence, confirming the successful decrosslinking of the alginate networks and demonstrating the mechanical tunability of the CoAl-IPN hydrogel.

Figure 4.3.2.1 Rheological characterization of hydrogels before and after de-/re-crosslinking. Representative digital images (a) and frequency (b) sweep of CoAl-IPN hydrogels before and after de-/re-crosslinking process. All experiments were triplicated. Data represent the mean $\pm$ standard deviation.

Figure 4.3.2.2 The stiffness of CoAl-IPN hydrogel can be tuned via specifically de-crosslinking process of alginate network. The rheological characterization of hydrogels after decrosslinking of alginate network within IPN using sodium citrate (50 mM).

CoAl₀ hydrogel compose of crosslinked collagen network interpenetrated with uncrosslinked alginate polymer chains. During the cell-material interaction studies, there is a possibility that the alginate polymer would be diffusing out from the hydrogel, causing a rapid deterioration of the hydrogel biomechanical properties. In order to examine the diffusion rate of the alginate polymer, both the collagen (Col) and CoAl₀ were treated with Ca²⁺ (100 mM) after 2 days incubation in PBS. At the end of the re-crosslinking procedure, the hydrogel appeared in a more compact nature with a slight shrinkage in the size (**Figure 4.3.2.1b**) as well as a final storage modulus (**Figure 4.3.2.3**) close to that of the fully crosslinked IPN hydrogel due to the interlaced of the alginate into the 3D pre-formed collagen matrix as the second network [35]. As shown in the **Figure 4.3.2.3**, the G' of CoAl₀ hydrogel treated with calcium chloride solution would increase from 50 to 300 Pa, while the Col hydrogel would maintain a similar G' of 40 Pa after same treatment. This suggest that the alginate network is stably integrated with the crosslinked collagen fibrils to form the IPN network.

Figure 4.3.2.3 Alginate polymer chains could be remained within IPN after 2 days incubation. The rheological characterization of hydrogels (Col and CoAl₀) after re-crosslinked with Ca²⁺ solution (100 mM). * $p < 0.05$.

4.3.3 Stiffness-dependent microarchitecture of CoAl-IPN hydrogel

The cross-sectional SEM images of the CoAl₀ and CoAl₁₀₀ hydrogels is shown in **Figure 4.3.3.1**. In both types of hydrogels, a 3D interconnected fibril network composed of collagen fibers with diameters ranging from 50 to 300 nm was clearly evident. In the CoAl₁₀₀, zonal Ca²⁺-induced crosslinking of the alginate network resulted in a highly crosslinked matrix that is characterized by the formation of interspersed nanoporous mesh within the collagen network.[36,37] The resulting complex micro-architecture resembled the basement membrane (~10 nm) in the TME.[38]

Figure 4.3.3.1 Representative cross-sectional scanning electron microscope (SEM) images of CoAl₀ (-Ca²⁺) and CoAl₁₀₀ (+Ca²⁺ 100 mM) IPN hydrogel.

4.3.4 Non-stiffness-dependent mass transport efficiency of CoAl-IPN hydrogel

In 3D cell culture, effective mass transport and oxygen diffusion are highly crucial to ensure the survivability of encapsulated cells and the subsequent cell activities.[39] It has been reported that most of the cell-derived proteins are less than 80 kDa in size.[40] As such, FITC-conjugated dextran with different molecular weight (4, 10, 40 or 70 kDa) was used to test the permeability of both IPN hydrogels over 48 h of study. The results (**Figure 4.3.4.1**) showed that both the hydrogels exhibit high diffusion capacity with decreasing diffusion rate being observed with increasing molecular weight. However, no significant differences were detected between the two sample groups, indicating that the crosslinking process did not adversely affect the mass transport and the subsequent cell biological activities. Overall, the results show that the CoAl-IPN hydrogel can be fine-tuned by introducing crosslinking to the alginate component to produce hydrogels with different biomechanical properties.

Figure 4.3.4.1 Crosslinking density of hydrogels would not affect the mass transport efficiency of CoAl-IPN hydrogel. CoAl-IPN Diffusion coefficient of fluorescein-labeled dextran (FITC-dextran; Mw: 4/10/40/70 kDa) released from CoAl-IPN hydrogels. All experiments were triplicated. Data represents mean $\pm$ standard deviation. The n.s. denotes no statistical difference between compared experimental groups. Statistical significance is determined at $p < 0.05$.

4.4 Conclusion

In this study, thorough characterization has been carried out to verify the feasibility of the CoAl-IPN hydrogel as a potent *in vitro* surrogate of tumor stroma. The mechanical properties of this IPN platform shows crosslinker (Ca^{2+}) concentration dependent. Specifically, 1) its stiffness (E: 100- 1000 Pa) could be fine-tuned to cover the range of tumor stroma (breast stroma E: 200 – 800 Pa; colon stroma E: 0.374 – 68.0 kPa) independent of hydrogel component changes (collagen and alginate mass ratio within hydrogel); 2) The varying hydrogel architecture according to crosslinking density is similar to the tumor stroma matrix changes during tumor progression (from sparse to dense). A micro-fibrillar architecture was observed in soft hydrogel in comparison to nanopores structure in stiff hydrogel due to the crosslinked alginate network; 3) the mass transport efficiency would not be affected by the hydrogel crosslinking density significantly, thus ensuring good permeability of hydrogel in maintaining good cell survivability. Additionally, the alginate network within CoAl-IPN hydrogel could be de-crosslinked using sodium citrate to better mimic the dynamic microenvironment surrounding cells *in*

vivo, which also allows *in situ* dynamic regulating matrix mechanical properties over time to have a better understanding of the cell mechanoresponse in real time. As compared to other single-gel system, CoAl-IPN hydrogel would not comprise hydrogel integrity during alginate network decrosslinking process due to of the independent crosslinking mechanisms of collagen (thermal-induced gelation at neutral condition) and alginate (coordination of the divalent ions). Moreover, CoAl-IPN hydrogel is not only suitable for tumor stroma cells but also for tumoroids studies, as the varied hydrogel stiffness could mimic the tumor stroma to exert different compression force on growing tumoroids, resulting in the build-up of solid stress within tumor. Overall, the CoAl-IPN hydrogel is a suitable platform to mimic the tumor stromal biophysical properties, including component, architecture, as well as varied and dynamic stiffness, for tumor mechanobiology studies.

Figure 4.4 Schematic illustration of CoAl-IPN hydrogel properties for tumor stroma mimicking.

References

- [1] D. F. Quail, J. A. Joyce. *Nat. Med.* **2013**, 19, 1423-37.
- [2] D. C. Hinshaw, L. A. Shevde. *Cancer Res.* **2019**, 79, 4557-4566.
- [3] J. Alexander, E. Cukierman. *Curr. Opin. Cell Biol.* **2016**, 42, 80-93.
- [4] R. Malik, P. I. Lelkes and E. Cukierman. *Trends Biotechnol.* **2015**, 33, 230-6.
- [5] J. Cha, P. Kim. *Frontiers in Materials.* **2017**, 4.
- [6] W. Han, S. Chen, W. Yuan, Q. Fan, J. Tian, X. Wang, L. Chen, X. Zhang, W. Wei, R. Liu, J. Qu, Y. Jiao, R. H. Austin and L. Liu. *Proc. Natl. Acad. Sci. USA.* **2016**, 113, 11208-11213.
- [7] D. S. Reynolds, K. M. Bougher, J. H. Letendre, S. F. Fitzgerald, U. O. Gisladdottir, M. W. Grinstaff and M. H. Zaman. *Acta Biomater.* **2018**, 77, 85-95.
- [8] A. V. Taubenberger, S. Girardo, N. Träber, E. Fischer-Friedrich, M. Kräter, K. Wagner, T. Kurth, I. Richter, B. Haller, M. Binner, D. Hahn, U. Freudenberg, C. Werner, J. Guck. *Adv. Biosyst.* **2019**, 3, e1900128.
- [9] V. Gkretsi, A. Stylianou, M. Louca and T. Stylianopoulos. *Oncotarget.* **2017**, 8, 27364-27379.
- [10] V. Gkretsi, A. Stylianou and T. Stylianopoulos. *Exp. Cell Res.* **2017**, 352, 281-292.
- [11] J. A. Rowley, G. Madlambayan and D. J. Mooney. *Biomaterials.* **1999**, 20, 45-53.
- [12] S. R. Caliari, J. A. Burdick. *Nat. Methods.* **2016**, 13, 405-14.
- [13] N. Davidenko, C. F. Schuster, D. V. Bax, R. W. Farndale, S. Hamaia, S. M. Best and R. E. Cameron. *J. Mater. Sci. Mater. Med.* **2016**, 27, 148.
- [14] K. M. Park, D. Lewis and S. Gerecht. *Annu. Rev. Biomed. Eng.* **2017**, 19, 109-133.
- [15] J. Zhu, Laura J. Kaufman, *Biophys. J.* **2014**, 6, 1822-1831.
- [16] L. Weng, X. Chen, W. Chen, *Biomacromolecules* **2007**, 8, 1109-15.
- [17] P. F. Duan, N. Kandemir, J. J. Wang, J. J. Chen, *MRS advances* **2017**. DOI: 10.1557/adv.2017.8.
- [18] C. Liu, D. Lewin Mejia, B. Chiang, K.E. Luker, G.D. Luker, *Acta Biomater.* **2018**, 75, 213-225.
- [19] R.A. Perez, M. Kim, T.H. Kim, J.H. Kim, J.H. Lee, J.H. Park, J.C. Knowles, H.W. Kim, *Tissue Eng Part A.* **2014**, 20, 103-14.

- [20] G.Z. Jin, H.W. Kim, J. Tissue Eng. **2018**, 9, 2041731418802438.
- [21] S.R. Moxon, N.J. Corbett, K. Fisher, G. Potjewyd, M. Domingos, N.M. Hooper, Mater. Sci. Eng. C Mater. Biol. Appl. **2019**, 104, 109904.
- [22] N.W. Tschoegl and W.G. Knguss and I. Emri. Mech Time Depend Mater. **2002**, 6, 3-51.
- [23] A.P. Castro, P. Laity, M. Shariatzadeh, C. Wittkowske, C. Holland, D. Lacroix, J. Mater. Sci. Mater. Med. **2016**, 27, 79.
- [24] R. Camba, J. Biotechnol. Biomater. **2013**, 03.
- [25] G. Kaklamani, D. Cheneler, L.M. Grover, M.J. Adams, J. Bowen, J. Mech. Behav. Biomed. Mater. **2014**, 36, 135-42.
- [26] M. Cavo, M. Fato, L. Peñuela, F. Beltrame, R. Raiteri, S. Scaglione, Sci. Rep. **2016**, 6(1).
- [27] L. G. Zhang, P. Y. He, J. Y. Zhang, Z. Y. Ji, Z. L. Zhang, H. L. Xu and D. D. Fan, Adv. Mater. Process. **2016**, 4, 52-58.
- [28] A.V. Salsac, L. Zhang and J.M. Gherbezza, 19^eme Congres Franc,ais de M `ecanique ´ Marseille. **2009**, aout 24-28.
- [29] M. Ahearne, Y. Yang, A.J. El Haj, K.Y. Then, K.K. Liu, J. R. Soc. Interface. **2005**, 2, 455-63.
- [30] H. Christiansson and J. Helsing. J. Appl. Phys. **1996**, 79, 7582.
- [31] C. M. Kraning-Rush, C. A. Reinhart-King. *Cell Adh. Migr.* **2012**, 6, 274-9.
- [32] S. Kawano, M. Kojima, Y. Higuchi, M. Sugimoto, K. Ikeda, N. Sakuyama, S. Takahashi, R. Hayashi, A. Ochiai and N. Saito. *Cancer Sci.* **2015**, 106, 1232-9.
- [33] M. Kalli, T. Stylianopoulos. Front. Oncol. 2018, 8, 55.
- [34] Z. Wu, X. Su, Y. Xu, B. Kong, W. Sun and S. Mi. *Sci. Rep.* **2016**, 6, 24474.
- [35] S.-L. Huang, Y.-S. Lin, The Size Stability of Alginate Beads by Different Ionic Crosslinkers, Advances in Materials Science and Engineering 2017 (2017) 1-7.
- [36] N. J. Hogrebe, J. W. Reinhardt and K. J. Gooch. *J. Biomed. Mater. Res. A.* **2017**, 105, 640-661.
- [37] H. Liu, M. Wu, Y. Jia, L. Niu, G. Huang and F. Xu. *NPG Asia Materials.* **2020**, 12.
- [38] A. Glentis, V. Gurchenkov and D. Matic Vignjevic. *Cell Adh. Migr.* **2014**, 8, 236-45.
- [39] L. Gasperini, J. F. Mano and R. L. Reis. *J. R. Soc. Interface.* **2014**, 11, 20140817.

- [40] K. F. Chung. *Asthma and COPD: Basic Mechanisms and Clinical Management*, chapter 27, cytokines.

Chapter 5*

Effect of CoAl-IPN hydrogel stiffness on breast cancer-associated fibroblasts (b-CAF) phenotype and functional crosstalk with breast tumoroids

This chapter aims to examine the influence of matrix stiffness changes on b-CAF behavior and the functional paracrine crosstalk with the neighboring tumor cells in a 3D microenvironment. Thus, CoAl-IPN hydrogel system was employed as an in vitro surrogate to mimic the stiffness of breast tumor stroma at different stage, whose mechanical properties were able to precisely fine-tune with Young's modulus ~ 100 and 1000 Pa respectively. Using this platform, the mechanoresponse of b-CAF to the effect of matrix stiffness were investigated. Specifically, matrix-stiffness dependent characteristics of b-CAF in terms of morphology, phenotype, and paracrine activity were examined. Findings show that hydrogel with higher stiffness ($E \sim 1000$ Pa) imposed a “physical confinement” effect on b-CAF that limits its spreading and cells were coerced to adopt a phenotype to dampen their crosstalk in driving the in vitro metastasis and invasion of the highly aggressive MDA-MB-231 human breast cancer tumouroids.

* This section published substantially as Huan Cao, Melissa Kao Hui Lee, Haibo Yang, Siu Kwan Sze, Ngan Soon Tan and Chor Yong Tay. *Langmuir* 2019, 35, 23, 7487-7495. DOI : 10.1021/acs.langmuir.8b02649. Reproduced with permission. Copy right (2019) American Chemical Society.

5.1 Introduction

Breast cancer is the most frequent cancer among women worldwide, with only 27% of the 5 years survival rate being documented when the cancer cells has spread to a distant part of the body (metastasis). (<https://www.cancer.net/cancer-types/breast-cancer/statistics#:~:text=The%20average%205%2Dyear%20survival,with%20breast%20cancer%20is%2099%25.>) Breast tumor microenvironment (TME) is a complex tissue, which plays an important role in tumor progression by providing biophysical support and biochemical signals in promoting tumor growth and metastasis.[1-3] Thus, many research efforts have been devoted to study the bidirectional functional interactions between the cancer cells and their surrounding stroma. Breast cancer-associated fibroblasts (b-CAF) are the primary stroma cell type within the TME and a critical regulator of tumor progression via the secretion of growth factors/cytokines, as well as the remodeling of the stromal ECM. Increased deposition of collagen by the b-CAF in the tumor-stromal space can led to the stiffening of stroma tissue.[4,5] It is reported that the elastic modulus of breast tumor stroma will increase from 200 Pa at early stage to 800 Pa at late stage.[6] Although studies have shown that the biomechanical changes of tumor tissue have direct and indirect influence on the b-CAF phenotype, majority of these studies have been conducted on the traditional 2D substrate that fails to accurately replicate the 3D architecture and the complexity of stromal tumor.[7] In addition, the precise biophysical interactions between the TME and b-CAF phenotype, as well as the further downstream influence of the paracrine signaling with the surrounding tumor cells, have yet to be thoroughly examined.

In this study, a 3D IPN composed of collagen and alginate crosslinked polymers is fabricated and used as an *in vitro* surrogate of tumor stroma. Specific to this study, two CoAl-IPN hydrogel systems were chosen to be employed as the 3D cell culture model testing platforms, namely CoAl₀ and CoAl₁₀₀ as described in the **Chapter 4**. Breast tumor MDA-MB-231 are invasive cancer cell type, and its tumoroids were adopted to investigate the cancer cells invasiveness within 3D matrix under the influence of CAF-derived secretions.

5.2 Experimental Methods

5.2.1 Materials

Most of the materials employed in this chapter are as being described in **Section 3.2.1**. Additionally, RGD-coupled alginate (NOVATACH MVG GEGDSP) was purchased from Novamatrix. CAF culture medium Vitroplus III (PC00B1-500) was purchased from Neuromics (USA). Dulbecco's modified Eagle's medium (DMEM), paraformaldehyde (PFA), Hoechst (#94403) and KAPA SYBR® FAST (#KK4618) were purchased from Sigma (Sigma-Aldrich). All reagents were used without further purification. Calcein-AM (C3099), ethidium homodimer-1 (EthD-1; E1169), PureLink RNA Mini Kit (#12183018A), Alexa Fluor 488 chicken anti-rabbit, Hoechst 33342, ProLong Gold Antifade Mountant, and Alexa Fluor 568 Phalloidin were obtained from Thermo Fisher Scientific. iScript™ cDNA Synthesis Kit (#1708891) was obtained from Biorad. TRIzol (#15596-018) was obtained from Ambion.

Human primary b-CAF were purchased from Neuromics (<https://www.neuromics.com>).

5.2.2 Fabrication of CoAl-IPN hydrogel

CoAl-IPN hydrogels with different stiffness were fabricated by varying the CaCl_2 concentrations (0 or 100 mM), as described in **Section 3.1**. In this particular study, pure rat tail collagen and RGD-conjugated alginate hydrogels were also prepared for cell culture and used as control groups.

5.2.3 2D culture of b-CAF on CoAl-IPN hydrogel

Human primary b-CAF were cultured and expanded in MSC-GRO™ (VitroPlus III, low serum, complete) medium supplemented with 5% FBS. In this particular study, 2D cell culture was also carried out by seeding b-CAF directly on the surface of CoAl-IPN hydrogels to assess the b-CAF behaviors and to demonstrate the role of hydrogel stiffness

in affecting the cellular morphology. Briefly, blank CoAl₀ and CoAl₁₀₀ hydrogels without encapsulated cells were prepared in 48-well plate as described in **Section 5.2.2**. Thereafter, 5×10^3 of b-CAF were seeded on the surface of the hydrogel. After 48 h of culture, cells were fixed and stained with FITC-conjugated phalloidin. The cell morphology was captured with Zeiss Axio Observer Z1 inverted fluorescent microscope and the cell spreading area was calculated using the Analyze-measure function in ImageJ software (<https://imagej.nih.gov/ij/>).

5.2.4 3D culture of b-CAF within CoAl-IPN hydrogel and collection of condition medium

For the fabrication of b-CAF-encapsulated CoAl-IPN hydrogel, b-CAF were trypsinized and mixed with the precursor solution at a density of 5×10^5 cells/mL prior to gelation of collagen and crosslinking of alginate. Subsequently, b-CAF-encapsulated hydrogels were cultured using a serum free DMEM medium for 48 h. At the end of incubation, the solution, termed as condition medium (CM), was collected and filtered to remove impurities. The CM collected from hydrogel without cells was used as control.

5.2.5 Live/dead assay of b-CAF within CoAl-IPN hydrogel

b-CAF viability was tested using Calcein-AM and EthD-1 as illustrated in **Section 3.6**.

5.2.6 b-CAF morphology analysis

In order to investigate the effect of matrix stiffness on b-CAF behaviors, samples were fixed at different time points (i.e. 2 h, 4h, 24h and 48 h). The morphology of the b-CAF was visualized by staining with FITC-conjugated phalloidin and Hoechst 33342 as described in **Section 3.5**. Fluorescent images were captured and analyzed as shown in the **Section 5.2.3**.

5.2.7 YAP/TAZ activity analysis

YAP/TAZ staining was conducted following steps as described in **Section 3.5**. The signals intensity ratio between nuclei and cytoplasm was calculated using ImageJ software (<https://imagej.nih.gov/ij/>). Firstly, the background of captured images was subtracted using the subtract background function. Thereafter, the signal threshold was adjusted to fixed value (Minimum value 0 and Maximum value 90). All of the images were treated in the same way. The markers (α -SMA and FAP) expression at transcript level was evaluated using RT-PCR as described in **Section 3.12**.

5.2.8 *In vitro* breast tumoroids invasiveness assay

Breast cancerous MDA-MB-231 cells were maintained in high glucose DMEM, supplemented with 10% FBS and 1% penicillin/ streptomycin solution. Subsequently, the MDA-MB-231 tumoroids were formed following the steps, as described in **Section 3.9**. Thereafter, the tumoroids invasion study was carried out, as explained in **Section 3.10**.

5.2.9 Statistical analysis

All experiments were repeated at least three times with duplicates. Statistical significance was determined by one-way analysis of variance (ANOVA) using SPSS among three or more than three groups. Comparison between two groups was analyzed by two-tailed Student's t test. The differences are considered as statistically significant with $p < 0.05$.

5.3 Principle Outcomes

5.3.1 Non-stiffness-dependent morphology of b-CAF on the CoAl-IPN hydrogel surface

Changes to the mechanical properties of the matrix have been shown to affect the cell behaviors via the mechanotransduction process.[8, 9] For example, human mesenchymal

stem cells (MSC) have been shown to be mechanoresponded to the methacrylated hyaluronic acid (MeHA) matrix stiffness (from ~3 to 30 kPa), including the increased cell spreading area and traction force.[10] In contrast, a more rounded morphology of cells (poor cell spreading) is observed on fibronectin-conjugated polyacrylamide (PAA) hydrogel soft substrates (1.5 kPa) due to the lack of cell-mediated traction on the substrate.[11, 12] As a comparison, the effect of the matrix stiffness on the cell spreading was conducted first using the traditional 2D cell culture model. **Figure 5.3.1.1** shows that a poor cell spreading was being observed when b-CAF were cultured on both CoAl₀ and CoAl₁₀₀ hydrogel surfaces. In addition, quantitative analysis shows that no significant differences in the extent of cell spreading between the two sample groups despite the fully crosslinked IPN hydrogel is ~ 8 times stiffer as compared to CoAl₀ (**Figure 4.3.1.2**). In an earlier study conducted by Yeung *et al.*, it was demonstrated that fibroblasts adopted a larger spreading area when cultured on a stiff 2D substrate of > 3 kPa.[13] In this case, the stiffness of both IPN hydrogels is less than 1 kPa, thus the spreading capacity of b-CAF cultured on both the CoAl₀ and CoAl₁₀₀ hydrogel surfaces is limited. It is well-established that there have significant differences between 2D and 3D cell culture (**Chapter 2, Section 2.1.3.1**), and the latter one would be better to replicate the cell surrounding microenvironment *in vivo*. Hence, the next study by encapsulating the b-CAF within CoAl-IPN hydrogel with varied stiffness would be conducted and their subsequent behaviors would be assessed.

Figure 5.3.1.1 The fluorescence images of b-CAF cultured on 2D CoAl-IPN hydrogel surface, CoAl₀ (a) and CoAl₁₀₀ (b) hydrogels, respectively. (c) quantitative measured of b-CAF spreading area. Scale bar = 100 μm. n = 20; * $p < 0.05$.

5.3.2 Stiffness-dependent morphology of b-CAF within 3D CoAl-IPN hydrogel

To ensure that the CoAl-IPN hydrogels are cytocompatible for 3D cell encapsulation, the viability of the encapsulated b-CAF was firstly assessed by live/dead staining after 48 h of culture. As seen from **Figure 5.3.2.1**, most of the encapsulated b-CAF are highly viable even after 48 h of culture, which is an indication that the hydrogel materials, fabrication process, crosslinking conditions and the resulted microenvironments are suitable to be used for the subsequent studies.

Figure 5.3.2.1 The viability assay of b-CAF within CoAl-IPN hydrogel. Live/dead staining of b-CAF encapsulated within CoAl₀ (a) and CoAl₁₀₀ (b) hydrogels. (c) Quantification of cell viability. Scale bar = 100 μ m, * $p < 0.05$.

To examine the effect of matrix stiffness on b-CAF, the cells were encapsulated in either the CoAl₀ or CoAl₁₀₀ where the cell morphology were being compared. As shown in the **Figure 5.3.2.2**, b-CAF adopted a spindle-shaped with a larger spreading area ($1043 \pm 221 \mu\text{m}^2$) upon culturing in soft CoAl₀ hydrogel ($E \sim 100$ Pa), specially b-CAF spreading equilibrium was attained as early as 4 h post-culture (**Figure 5.3.2.3**). In contrast, a more rounded form ($331 \pm 22 \mu\text{m}^2$) could be seen for b-CAF encapsulated within a stiffer CoAl₁₀₀ hydrogel. However, the exact relationship between hydrogel network structure and the restriction of cell spreading is yet to be clear. In addition, the use of excess Ca^{2+} to crosslink the alginate network could potentially lead to the disruption of the intracellular electrolyte balance, resulting in the damage of the cell membrane that lead to the cell rounding effect. Hence, in order to verify the exact mechanism of cell spreading differences, b-CAF were encapsulated within Col hydrogel, which was subsequently treated with Ca^{2+} (100 mM). As can be seen in the **Figure 4.3.2.2**, the Young's modulus of Col hydrogel was found to be around 100 Pa (E) after Ca^{2+} treatment. Yet, the cells grown in the Col + Ca^{2+} group retained their spindle shaped morphology ($1200 \pm 399 \mu\text{m}^2$; **Figure 5.3.2.2c**). This suggest that the amount of CaCl_2 used to crosslink the alginate network is not significantly

high to exert a cytotoxic effect nor impairing the cell spreading within the 3D microenvironment.

Similarly, the spreading of b-CAF is also highly dependent on the interactions of cell membrane receptor ligands with the surrounding matrix. Studies have shown that the engagement of integrin sub-units with the ECM protein will influence the spreading and migration of human epithelial cells.[14, 15] However, alginate does not contain any integrin-binding sites.[16] Therefore, the round morphology of b-CAF observed within the CoAl₁₀₀ hydrogel system ($E \sim 1000$ Pa) could be possibly due to the calcium-crosslinked of alginate polymer chains, resulting in the shielding the b-CAF from making contact with the attachment-permissive collagen network. To examine this possibility, we repeated the experiment using molecular weight-matched arginine-glycine-aspartic (RGD)-conjugated alginate (Novamatrix MVG) to foster cell-matrix interaction via $\alpha 5 \beta$ integrins-mediated adhesion as shown in the **Figure 5.3.2.4**. [17] However, despite the presence of RGD-enriched network, b-CAF within CoAl₁₀₀-RGD hydrogel retained its rounded morphology ($335 \pm 36 \mu\text{m}^2$; **Figure 5.3.2.2d**). Interestingly, enhancement ($736 \pm 200 \mu\text{m}^2$) in cell spreading was observed for b-CAF encapsulated in the CoAl₁₀₀ hydrogel upon de-crosslinking of the IPN hydrogel system using 50 mM sodium citrate solution (**Figure 5.3.2.2e**). The quantitative calculation of cells circularity and spreading exhibited a similar trend, as shown in **Figure 5.3.2.2 f and g**. Taken together, the findings suggest that ECM mechanics is the predominant factor governing the morphology of b-CAF in 3D context.

Figure 5.3.2.2 CoAl-IPN hydrogel stiffness is the key determinants of b-CAF morphology in 3D context. Representative fluorescent images of b-CAF cultured in (a)CoAl₀, (b)CoAl₁₀₀, (c)Col + Ca²⁺ (100 mM), (d) CoAl₁₀₀-RGD, and (e) CoAl₁₀₀ + sodium citrate counter stained for filamentous actin (red) and the cell nucleus (blue). Corresponding quantitative measurement of the b-CAF (f) spreading area and (g) circularity index. Scale bar = 50 μm. n = 20; * *p* < 0.05.

Figure 5.3.2.3 Time-course monitoring of b-CAF behavior within CoAl-IPN hydrogel over 48 h culturing. The fluorescence images of b-CAF cultured within CoAl₀ and CoAl₁₀₀ hydrogels for a) 2h, b) 4h, c) 24h and d) 48h, respectively. Corresponding quantitative measurement of b-CAF circularity (e) and spreading area (f). Scar bar = 50 μm.

Figure 5.3.2.4 The enhancement of b-CAF spreading on 2D RGD conjugated alginate hydrogel. Representative phase contrast images of b-CAF cultured on a) alginate and b) alginate-RGD hydrogel surface. Scale bar = 200 μm.

Collectively, the difference in the cellular spreading between the 2D and 3D culture systems clearly highlights the importance of culture dimensionality. In 2D cell culture, a monolayer of cells is being formed in which the spreading is highly limited by the basolateral x-y plane. In contrast, 3D encapsulated cells are able to interact with the surrounding matrix as well as neighboring cells in a 360-degree manner, all of which will pose restrictions to the cell spreading.[18] In this specific study, the nano-microarchitecture of the crosslinked alginate in CoAl₁₀₀ imposes physical confinement on the encapsulated b-CAF, resulting in restrained spreading of the encapsulated b-CAF. In contrast, the sparse fibril network of CoAl₀ could be beneficial for b-CAF spreading inside.

5.3.3 Effect of hydrogel stiffness on YAP/TAZ activity and its correlation to a phenotypic shift of b-CAF

Recent studies have implicated the transcriptional regulator Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) as an important mechano-sensitive pathway. It is reported that the mechanical changes of stroma during cancer progression would trigger the activation and nuclear translocation of the YAP and TAZ proteins via RhoA-mediated signaling and actomyosin-induced contractility of CAF.[19] Studies have shown that when the cells were cultured on 2D stiff (Elastic modulus, 20 kPa) substrates using a NorHA hydrogel, modification of hyaluronic acid (HA) with norbornene groups, YAP/TAZ will be activated and translocated into the nucleus to bind with the transcriptional enhanced associate domain (TEAD) transcription factors to promote cell growth, proliferation, migration and survival as compared to the soft one (Elastic modulus, 1 KPa).[20, 21] However, findings from **Section 5.3.1 and 5.3.2** highlight the specificity of b-CAF having different responses/interactions (i.e., cell spreading) with matrix stiffness between the 2D surface culture and 3D encapsulation systems. As such, a similar phenomenon could potentially be observed where cells encapsulated in soft hydrogel ($E \sim 100$ Pa) exhibiting higher activation of YAP/TAZ as compared to the stiff hydrogel ($E \sim 1000$ Pa). Indeed, **Figure 5.3.3.1a and b** showing intense YAP/TAZ signaling

accumulated within the nuclei of b-CAF encapsulated in soft CoAl₀ hydrogel but not stiff CoAl₁₀₀ hydrogel.

CAF cultured on stiff substrates (well plate) have been shown to promote the recruitment of alpha-smooth actin (α -SMA), a type of cytoskeleton marker, forming stress fibers that can augment the cell tension leading to YAP/TAZ nucleus translocation, which in turn further stabilize the actomyosin protein simultaneously.[22, 23] In addition, the CAF-mediated ECM remodeling leads to the formation of fibrous collagen networks, which further contributes to the stiffening of the stroma matrix and the activation of YAP/TAZ translocation. Thus, this stiffness-dependent fibroblast activation can initiate a vicious feed-forward cycle of matrix stiffening, fibroblast activation and further cancer progression. In this study, limited b-CAF spreading in the CoAl₁₀₀ hydrogel can prevent the nucleus shuttling of the YAP/TAZ complex by relieving the intracellular tension of the cells. Conceptually, this can be explained by the “physical confinement” effect, which disrupt the cytoskeleton integrity, leading to decreased cell tension to facilitate YAP/TAZ nucleus translocation. Conversely, b-CAF encapsulated within soft CoAl₀ hydrogel ($E \sim 100$ Pa) showing intense YAP/TAZ nuclear translocation expressed higher levels of α -SMA and FAP mRNA transcripts (**Figure 5.3.3.1c and d**). FAP is a type II integral membrane serine protease, which selectively expressed in reactive CAF.[24] Collectively, apart from the morphological differences, the results also indicate that CAF regulated the YAP/TAZ signaling pathway differently in response to the surrounding mechanical cues under a 3D setting. Thus, it could potentially lead to the phenotypic shift of CAF.

Figure 5.3.3.1 CoAl-IPN hydrogel stiffness influence the mechanotransduction of the YAP/TAZ signaling axis and CAF marker transcript expression. (a) Representative immunofluorescence images of b-CAF countered stained for YAP/TAZ protein (green) and the cell nucleus (blue) cultured within CoAl₀ and CoAl₁₀₀ hydrogel. Scale bar = 50 μ m. Magnified images of the corresponding region of interest (ROI) reveal the spatial distribution of both the YAP/TAZ protein and cell nucleus signals. (b) the normalized level of nuclear and cytosolic YAP/TAZ expression. Gene expression levels of (c) α -SMA and (d) FAP transcript of the b-CAF cultured in CoAl₀ and CoAl₁₀₀ hydrogel. $n = 20$; * $p < 0.05$.

To further examine the relationship between the actin cytoskeleton of CAF and YAP/TAZ nuclei-cytoplasmic shuttling, both cytochalasin D (CytoD, 10 μ M) and Y27632 (20 μ M) were used to treated CAF for 2 h. CytoD is an inhibitor of cellular actin polymerization, resulting in cell cycle arrest in G1-S transition. Y27632 has been generally shown to have effects on cells behaviors, including the cell morphology, cytoskeleton arrangement, and the cell migratory behavior via the inhibition of Rho-associated coil containing protein

kinase [25]. For instance, Zhang *et al.* have demonstrated that osteoblastic cells treated with Y27632 over a duration of 9 h have stellate morphology with a limited volume of perinuclear cytoplasm enriched an exhibiting long and thin actin, which resembling the disrupted cytoskeleton [26]. As shown in the **Figure 5.3.3.2**, as compared to the Ctrl group, the F-actin is disrupted in both CytoD and Y27632 groups obviously. Meanwhile, both CytoD and Y27632 treatment of CAF could efficiently regulate YAP/TAZ nuclear translocation, indicating that the actin cytoskeleton is an important regulator for YAP/TAZ signaling axis.

Figure 5.3.3.2 Actin cytoskeleton of CAF' regulates YAP/TAZ nuclei-cytoplasmic shuttling. Representative immunofluorescence images of b-CAFs countered stained for nucleus protein (**blue**), YAP/TAZ (**green**) and F-actin (**red**) cultured with either CytoD or Y27632. Scale bar = 50 μ m. Magnified images of the corresponding region of interest (ROI) clearly reveal the actin stress fiber arrangement.

5.3.4 Mechanoregulation of functional crosstalk between b-CAF and breast tumoroids

5.3.4.1 Effect of CM derived from hydrogel-encapsulated b-CAF on breast tumoroids invasion

The biological significance of the matrix stiffness-dependent switch in CAF phenotype (spindle vs. round) was examined. Specifically, the paracrine activity of the CAF cultured in either the stiff ($E \sim 1000$ Pa) or mechanically compliant ($E \sim 100$ Pa) CoAl-IPN hydrogel was examined. After 48 h of culture in the respective hydrogels under serum free condition, b-CAF conditioned medium (CM) was collected. The potency of the b-CAF derived CM to drive malignancy was assessed using the 3D MDA-MB-231 tumoroids laden collagen model. In this study, an indirect paracrine crosstalk effect of b-CAF on tumor cells was first being explored. Briefly, the CM was harvested from b-CAF encapsulated within soft CoAl₀ ($E \sim 100$ Pa) and stiff CoAl₁₀₀ ($E \sim 1000$ Pa) hydrogels after 48 h of incubation, which are denoted as CM_{CoAl0} and CM_{CoAl100}, respectively. CM collected from CoAl₀ and CoAl₁₀₀ hydrogels without CAF encapsulation were used as control. Thereafter, the various CM was added to the MDA-MB-231 tumoroids embedded within a 3D collagen hydrogel, as illustrated in **Figure 5.3.4.1a**. The result (**Figure 5.3.4.1b**) shows that a relatively compact, spherical shape with minor peripheral cellular protrusions is being observed for the tumoroids exposed to serum-depleted medium (control groups). Contrarily, **Figure 5.3.4.1c and d** shows that the tumoroids exhibited a typical “starburst” invasion pattern when exposed to the CAF-derived CM, indicating that the growth factors and cytokines secreted by the CAF successfully promoted the tumors’ invasion. In order to quantitatively measure the bioactivity of b-CAF-derived CM, the invasion index was introduced via quantifying the degree of peripheral cancer cell spreading from the tumoroids. **Figure 5.3.4.1e** reveals that tumoroids treated with CM_{CoAl0} has a significantly larger mean normalized invasion index of ~6.0-fold compared to the CM_{CoAl100} with approximately ~3.5-fold.

Figure 5.3.4.1 The bioactivity of b-CAF CM is highly dependent on the CoAl-IPN hydrogel stiffness. (a) Schematic illustrating the experimental design. Representative phase contrast images of MDA-MB-231 tumoroids exposed to (b) the control medium, (c) CM_{CoAl0} , and (d) $CM_{CoAl100}$. Scale bar = 200 μm . The yellow dashed line indicates the boundary of spreading tumor cells. (e) Box and whisker plots of the normalized invasion index of MDA-MB-231 tumoroids treated with variants of b-CAF CM. $n \geq 25$. * $p < 0.05$.

5.3.4.2 Effect of hydrogel-encapsulated b-CAF on tumoroids invasion via co-culturing

A co-culture system was also being used to explore the direct paracrine crosstalk effect of b-CAF on tumor cells using a transwell insert, which can better recapitulate the spatiotemporal cell-cell and cell-matrix interactions within tumor stroma (**Figure 5.3.4.2a**). After 24 h of co-culture, the tumoroids were fixed and imaged in which the invasion index was measured. Similarly, the results (**Figure 5.3.4.2b-e**) show that the MDA-MB-231 cells exhibited a greater extent of invasion and showing more aggressive behavior when co-

cultured with b-CAF encapsulated within CoAl₀ hydrogel compared to b-CAF encapsulated within CoAl₁₀₀ hydrogel. Collectively, all of the results indicate that the paracrine activity of b-CAF can be mechanically regulated, and the stromal matrix mechanic is an important modulator for the functional crosstalk between b-CAF and cancer cells.

Figure 5.3.4.2 CoAl-IPN hydrogel stiffness modulates functional paracrine dialogue *in situ* between b-CAF and MDA-MB-231 tumoroids via variant crosslinking density. (a) Schematic illustrating the co-culture experimental setup. Representative phase-contrast images of MDA-MB-231 tumoroids co-cultured with (b) control and CAF embedded in (c) CoAl₀ and (d) CoAl₁₀₀ hydrogels. Scale bar = 200 μ m. The yellow dashed line indicates the boundary of spreading tumor cells. (e) Box and whisker plots of the normalized invasion index of MDA-MB-231 tumoroids treated with variants of b-CAF-encapsulated CoAl-IPN hydrogels. $n \geq 25$. * $p < 0.05$.

5.4 Conclusion

Mechanically tunable CoAl-IPN hydrogel is a potent platform for the study of mechanotransduction signaling events. The b-CAF is greatly confined in a round morphology embedded within stiff CoAl₁₀₀ hydrogel with denser fibril microarchitecture and smaller pore size, while a greater extent of cell spreading was observed in b-CAF encapsulated within soft (sparse microarchitecture) CoAl₀ hydrogel ($E \sim 100$ Pa) with spindle shape morphology. On the molecular level, b-CAF will reciprocate to the stiff CoAl₁₀₀ hydrogel ($E \sim 1000$ Pa) by restricting the nuclear translocation of mechanosensitive YAP/TAZ protein with decreased molecular markers expression, such as α -SMA and FAP. In addition, b-CAF encapsulated within stiff CoAl₁₀₀ hydrogel significantly mute the invasion of MDA-MB-231 tumoroids compared to that of the soft CoAl₀ hydrogel system, which is possibly affected by the restricted functional effects of YAP/TAZ expression and nuclear translocation in b-CAF. Collectively, all of the results reveal that the phenotype and functional characteristics of b-CAF are highly mechanically dependent, which provides important guidance for tumor mechanobiology study.

Figure 5.4 Schematic illustration of CoAl-IPN hydrogel stiffness effect on b-CAF phenotype and functional crosstalk with breast tumoroids.

References

- [1] D. Hanahan, R. A. Weinberg. *Cell*. **2011**, 144, 646-74.
- [2] D. F. Quail, J. A. Joyce. *Nat. Med.* **2013**, 19, 1423-37.
- [3] F. Mbeunkui, D. J. Johann. *Cancer Chemother. Pharmacol.* **2009**, 63, 571-82.
- [4] R. Malik, P. I. Lelkes and E. Cukierman. *Trends Biotechnol.* **2015**, 33, 230-6.
- [5] Z. Liao, Z. W. Tan, P. Zhu and N. S. Tan. *Cell Immunol.* **2018**.
- [6] C. M. Kraning-Rush, C. A. Reinhart-King. *Cell Adh. Migr.* 2012, 6, 274-9.
- [7] K. Stock, M. F. Estrada, S. Vidic, K. Gjerde, A. Rudisch, V. E. Santo, M. Barbier, S. Blom, S. C. Arundkar, I. Selvam, A. Osswald, Y. Stein, S. Gruenewald, C. Brito, W. V. Weerden, V. Rotter, E. Boghaert, M. Oren, W. Sommergruber, Y. Chong, R. D. Hoogt and R. Graeser. *Sci. Rep.* **2016**, 6, 28951.
- [8] C. Y. Tay, S. A. Irvine, F. Y. Boey, L. P. Tan and S. Venkatraman. *Small*. **2011**, 7, 1361-78.
- [9] C. Y. Tay, C. G. Koh, N. S. Tan, D. T. Leong and L. P. Tan. *Nanomedicine (Lond)*. **2013**, 8, 623-38.
- [10] M. Guvendiren, J. A. Burdick. *Nat. Commun.* **2012**, 3, 792.
- [11] C. Y. Tay, Y.-L. Wu, P. Cai, N. S. Tan, S. S. Venkatraman, X. Chen and L.P. Tan. *NPG Asia Materials*. **2015**, 7, e199-e199.
- [12] H. Yang, K. T. Nguyen, D. T. Leong, N. S. Tan and C. Y. Tay. *ACS Appl. Mater. Interfaces*. **2016**, 8, 26591-26599.
- [13] T. Yeung, P. C. Georges, L. A. Flanagan, B. Marg, M. Ortiz, M. Funaki, N. Zahir, W. Ming, V. Weaver and P. A. Janmey. *Cell Motil Cytoskeleton*. **2005**, 60, 24-34.
- [14] W. W. Chen, M. S. Tjin, A. W. C. Chua, S. T. Lee, C. Y. Tay and E. Fong. *ACS Appl. Mater. Interfaces*. **2017**, 9, 36483-36492.
- [15] E. Fong, D. A. Tirrell. *Adv. Mater.* **2010**, 22, 5271-5275.
- [16] J. A. Rowley, G. Madlambayan, D. J. Mooney. *Biomaterials*. **1990**, 20, 45-53.
- [17] B. Erdogan, M. Ao, L. M. White, A. L. Means, B. M. Brewer, L. Yang, M. K. Washington, C. Shi, O. E. Franco, A. M. Weaver, S. W. Hayward, D. Li and D. J. Webb. *J. Cell Biol.* **2017**, 216, 3799-3816.
- [18] M. W. Tibbitt, K. S. Anseth. *Biotechnol. Bioeng.* **2009**, 103, 655-63.

- [19] R. Kaukonen, A. Mai, M. Georgiadou, M. Saari, N. De Franceschi, T. Betz, H. Sihto, S. Ventela, L. Elo, E. Jokitalo, J. Westermarck, P. L. Kellokumpu-Lehtinen, H. Joensuu, R. Grenman and J. Ivaska. *Nat. Commun.* **2016**, 7, 12237.
- [20] E. Rozengurt, J. Sinnett-Smith and G. Eibl. *Signal Transduct. Target Ther.* **2018**, 3, 11.
- [21] S. R. Caliali, S. L. Vega, M. Kwon, E. M. Soulas and J. A. Burdick. *Biomaterials.* **2016**, 103, 314-323.
- [22] K. Shiga, M. Hara, T. Nagasaki, T. Sato, H. Takahashi and H. Takeyama. *Cancers (Basel).* **2015**, 7, 2443-58.
- [23] F. Calvo, N. Ege, A. Grande-Garcia, S. Hooper, R. P. Jenkins, S. I. Chaudhry, K. Harrington, P. Williamson, E. Moeendarbary, G. Charras and E. Sahai. *Nat. Cell Biol.* **2013**, 15, 637-46.
- [24] P. Carin-chesa, L. J. Oldt and W. J. Rettigt. *Proc. Natl. Acad. Sci. USA.* **1990**, 87, 7235-7239.
- [25] M. Amano, M. Nakayama, K. Kaibuchi. *Cytoskeleton (Hoboken)* **2010**, 67, 545-54.
- [26] X. Zhang, C. Li, H. Gao, H. Nabeka, T. Shimokawa, H. Wakisaka, S. Matsuda, N. Kobayashi, *Cell Mol. Biol. Lett.* **2011**, 16, 279-95.

Chapter 6*

Effect of CoAl-IPN hydrogel stiffness on colon cancer-associated fibroblasts (c-CAF) phenotype and functional crosstalk with colorectal adenocarcinoma cells

This chapter aims to apply the CoAl-IPN hydrogel to examine the impact of 3D matrix stiffness on colon cancer-associated fibroblasts (c-CAF) activities and the functional characteristics. Specifically, matrix-stiffness dependent characteristics of c-CAF morphology, phenotype, and paracrine activity were examined. Findings show that hydrogel with higher stiffness ($E \sim 1000 \text{ Pa}$) would also impose a “physical confinement” effect on c-CAF by limiting its spreading, in which the cells were coerced to adopt a phenotype to dampen their crosstalk in driving the in vitro epithelial-to-mesenchymal transition (EMT) potential of colorectal adenocarcinoma cells DLD-1.

6.1 Introduction

In the previous study (**Chapter 5**), it was known that the surrounding matrix would affect b-CAF phenotype and the functional crosstalk between CAF and breast cancer tumoroids. Unfortunately, the experience with the primary b-CAF was that the cells exhibited slow growth rate, with an estimated doubling time of 48 h. Moreover, the cells displayed gross morphological features that reminisce senesced cells after 5 number of passages. Therefore, the long-term usage of the b-CAF has become untenable for this study. In view of these technical limitations, the focus of the study switched to colon cancer-associated fibroblasts (c-CAF).

Colorectal cancer (CRC) is the third leading cause of the cancer related deaths worldwide.[1, 2] Over 90% of under-five year mortality rate of patients with stage IV CRC is linked to metastasis.[3, 4] The CRC cells invasion-metastasis cascade is a multi-steps and highly orchestrated process, where tumor cells from the primary site are disseminated to the distal organs.[5] The initial escape of the cancer cells from the primary tumor is widely believed to be associated with the EMT program, whereby the cancer cells losses their epithelial traits (e.g., cell-cell attachments) and is accompanied by the acquisition of mesenchymal characteristics (e.g., increased motility) to enable the cells to escape, to migrate, and to invade to distal organs.[6, 7] Studies have shown that the paracrine crosstalk between CRC tumor and the tumor stromal-residing CAF has emerged as a key mechanism to trigger EMT.[8, 9] In addition, colorectal DLD-1 is a non-invasive epithelial cancer cells, and thus could be employed as a EMT model to investigate the crosstalk between CAF and DLD-1 cells.[10]

6.2 Experimental Methods

6.2.1 Materials

Most of the materials employed in this chapter are as shown in **Section 4.2.1 and Section 5.2.1**.

Human primary colorectal cancer c-CAF (source from human colorectal adenocarcinoma tumor, clinical stage III-B, tumor type pT3) was purchased from Neuromics (<https://www.neuromics.com>). Human colorectal adenocarcinoma DLD-1 was obtained from American Type Culture Collection (ATCC).

6.2.2 Fabrication of CoAl-IPN hydrogel

CoAl-IPN hydrogels with different stiffness were fabricated by varying the CaCl_2 concentrations (0 or 100 mM), as described in **Section 3.1**.

6.2.3 2D culture of c-CAF on CoAl-IPN hydrogel

Human primary c-CAF were cultured and expanded in MSC-GROTM (VitroPlus III, low serum, complete) medium supplemented with 5% FBS. In this particular study, 2D cell culture was also carried out by seeding c-CAF directly on the surface of CoAl-IPN hydrogels to evaluate the role of hydrogel stiffness in affecting the c-CAF morphology and behaviors. The experimental details followed the steps in **Section 5.2.3**.

6.2.4 3D culture of c-CAF within CoAl-IPN hydrogel and collection of condition medium

For the fabrication of c-CAF-encapsulated CoAl-IPN hydrogel, c-CAF were trypsinized and mixed with the precursor solution at a density of 5×10^5 cells per mL prior to gelation of collagen, followed by the crosslinking of alginate. Subsequently, c-CAF-encapsulated hydrogels were cultured using a serum free DMEM medium for 48 h. At the end of incubation, the solution, termed as condition medium (CM), was collected and filtered to remove impurities. The CM collected from hydrogel without cells was used as control.

6.2.5 Live/dead assay of c-CAF within CoAl-IPN hydrogel

c-CAF viability was tested using Calcein-AM and EthD-1 as shown in **Section 3.6**.

6.2.6 c-CAF morphology analysis within CoAl-IPN hydrogel

The morphology of the c-CAF was visualized by staining with FITC-conjugated phalloidin and Hoechst 33342 as described in **Section 3.5**. The spreading area of cells was calculated with ImageJ software (<https://imagej.nih.gov/ij/>). The analyses details followed the steps in **Section 5.2.6**.

6.2.7 *In vitro* colorectal adenocarcinoma DLD-1 cells EMT assay

DLD-1 (1×10^4 cells per well) were seeded in 96-well plate in DMEM containing 10% FBS and 1% penicillin/streptomycin for 24 h. Next, the culture medium was removed and replenished with CAF-CM for 24 h. Hydrogels without encapsulated CAF treated with serum-free medium were served as a control. The cancer cells colony area and circularity index were computed using ImageJ (<https://imagej.nih.gov/ij/>) based on the phase-contrast images. CAF-CM treated DLD-1 samples were also analyzed using RT-PCR as described in **Section 12** for EMT markers such as CDH1, CDH2 and VIM. Experiments were triplicated.

6.2.8 Statistical analysis

All experiments were repeated at least three times with duplicates. Statistical significance was determined by one-way analysis of variance (ANOVA) using SPSS among three or more than three groups. Comparison between two groups was analyzed by two-tailed Student's t test. The differences are considered as statistically significant with $p < 0.05$.

6.3 Principle Outcomes

6.3.1 Stiffness-dependent morphology of c-CAF on the CoAl-IPN hydrogel surface

As shown in the **Figure 6.3.1.1**, c-CAF exhibited a spreading morphology upon culturing on both of the CoAl₀ ($916 \pm 278 \mu\text{m}^2$) and CoAl₁₀₀ ($3634 \pm 1530 \mu\text{m}^2$) hydrogel surfaces, which is significantly different from b-CAF, indicating that different CAF types would demonstrate different cellular behaviors. This phenomenon also indicated that c-CAF may have higher inner contraction force as compared to b-CAF due to the abundant F-actin.[11, 12] Between the two sample groups, c-CAF have a greater extend of spreading on the CoAl₁₀₀ hydrogel surface with more obvious F-actin bundles as compared to that on the CoAl₀ hydrogel, which showed a more spindle shape. The possible reason is that CoAl₀ is sparsely crosslinked and the architecture is loose, hence, c-CAF could drag and re-arrange the fibrils network and spread along the fibrillar structures.[13] On the other hand, the CoAl₁₀₀ hydrogel was too rigid and c-CAF could not remodel the fibrillar structure of hydrogel.

Figure 6.3.1.1 The fluorescence images of c-CAF cultured on 2D CoAl-IPN hydrogel surface, CoAl₀ (a) and CoAl₁₀₀ (b) hydrogels, respectively. c) Quantitative measurement of c-CAF spreading on the surface of hydrogel.

6.3.2 Stiffness-dependent morphology of c-CAF within 3D CoAl-IPN hydrogel

To examine the influence of matrix crosslinking density on the 3D cell behavior and spreading, c-CAF were encapsulated in the CoAl-IPN hydrogels. Representative fluorescence images of CAF counterstained with live/dead staining are shown in **Figure 6.3.2.1 a and b**. After 48 h of culture, the embedded CAF displayed high viability in the 3D- constructs. c-CAF encapsulated within in the mechanically compliant CoAl₀ hydrogel were bipolar, spindle-shaped with thin micro-filament F-actin staining, while the c-CAF embedded within the stiffer CoAl₁₀₀ hydrogel ($E \sim 1000$ Pa) were distinctively rounded with diffuse actin structure (**Figure 6.3.2.1 c and d**). As compared to the spindle-shaped c-CAF, the spherical c-CAF in the CoAl₁₀₀ group are lacked of long-range ordering of the F-actin network, indicating that F-actin polymerization was disrupted.[14] Additionally, a large discrepancy in terms of cell spreading area was observed between the two groups. c-CAF encapsulated within the CoAl₀ and CoAl₁₀₀ have a mean spreading area of $676 \mu\text{m}^2$ and $162 \mu\text{m}^2$ respectively (**Figure 6.3.2.1e**).

The rounded morphology of c-CAF embedded within the CoAl₁₀₀ group could be attributed to the spatial confinement of the densely crosslinked microenvironment, thereby preventing the c-CAF from spreading.[15, 16] Due to the extremely small pore-size of the ionically crosslinked alginate, the cells were completely entrapped and physically constrained within the 3D nano-porous mesh-like architecture matrix.[13] The lack of adhesion as a reason for the c-CAF rounding has been ruled out, since similar observation was also documented in human breast CAF in stiff CoAl-IPN hydrogel ($E \sim 1000$ Pa) that was composed of RGD-modified alginate. Furthermore, there was a higher (~ 1.5 -fold) number of c-CAF within the CoAl₀ as compared to CoAl₁₀₀ group after 48 h of culture in serum-free condition (**Figure 6.3.2.1f**). This finding was consistent with earlier reports that restricting cell spreading reduced actin-polymerization and arrested cells in G1 phase of mitosis.[17, 18]

Figure 6.3.2.1 Comparison of c-CAF morphology and proliferation in 3D IPN hydrogel. Representative fluorescence images of CAF cultured in CoAl-IPN hydrogels counterstained with (a) Live/ Dead dyes (scale bar=200 μm) and (b) Nucleus/ F-actin (scale bar=20 μm). Quantification of (c) cell spreading area and (d) cell proliferation of CAF. All experiments were triplicated. Data represents mean $\pm$ standard deviation. Asterisk * denotes statistical difference between compared experimental groups. n.s.: not significant. Statistical significance is determined at $p < 0.05$.

6.3.3 Mechanoregulation of functional crosstalk between c-CAF and colorectal adenocarcinoma cells

To determine whether c-CAF morphology difference could influence the paracrine crosstalk and EMT potential, DLD-1 human colorectal adenocarcinoma cells were treated with conditioned medium (CM) harvested from c-CAF cultured in either CoAl₀ (CM_{CoAl0}) or CoAl₁₀₀ (CM_{CoAl100}) as shown in (Figure 6.3.3.1a). DLD-1 cells treated with serum-free medium serves as a negative control. After 24 h exposure to the CM_{CoAl0}, scattering of the DLD-1 cells was apparent, as opposed to the compact DLD-1 colonies observed in the CM_{CoAl100} and control groups (Figure 6.3.3.1b). In stark contrast, reduced size and circularity of the DLD-1 cells colonies were observed for the CM_{CoAl0} treated samples (Figure 6.3.3.1c and d).

Next, the expression levels of EMT mRNA transcripts of the CM-treated DLD-1 cells were examined. CDH1 encodes the E-cadherin protein, which is a crucial cell-cell adhesion protein to hold the epithelial cells together. Conversely, CDH2 and VIM encode the N-cadherin and vimentin, respectively, and are positive regulators of the EMT process.[6, 19, 20] As compared to the control group, a 24 h treatment of DLD-1 cells with CM_{CoAl0} induced a significant downregulation of CDH1 (1.69-fold), while CDH2 (2.11- fold) and VIM (1.97-fold) were strongly overexpressed. By contrast, CM_{CoAl100} treated DLD-1 cells displayed an approximately 2-fold reduction in CDH2 and VIM expression levels relative to the untreated control, suggesting that the mesenchymal phenotype is repressed. Implicit in this finding is that the pro-EMT paracrine interaction between CAF and cancer cells may be mitigated by changing the TME mechanics.

Figure 6.3.3.1 Hydrogel mechanics governs EMT-inducing potency of c-CAF conditioned medium. (a) Schematic illustrating the experimental design. (b) Representative fluorescent images of F-actin (red) and nucleus (blue) stained colorectal adenocarcinoma DLD-1 cells exposed to CM_{Ctrl} (i.e. serum-free DMEM), CM_{CoAI0} and $CM_{CoAI100}$ respectively. Scale bar = 50 μm . Corresponding box and whisker plots of the DLD-1 cells (c) colony area and (d) circularity index after 24 h of c-CAF-CM treatment ($n \geq 25$). Expression level of EMT mRNA transcripts namely (e) CDH1, (f) CDH2 and (g) VIM for indicated experimental groups. All experiments were triplicated. Data represents mean $\pm$ standard deviation. n.s. denotes no statistical difference between compared experimental groups. Statistical significance is determined at $p < 0.05$.

Figure 6.3.3.2 Representative bright filed images of DLD-1 cells after treatment with CM_{CoAl0} and diluted (1.5 times) CM_{CoAl0}.

It is possible that the reduced cells number in the CoAl₁₀₀ group may result in less secreted factors being produced, and therefore may interfere with the EMT-inducing potential of CM_{CoAl100}. To further investigate this likelihood, the *in vitro* EMT assay was repeated using CM_{CoAl0} that was diluted by 1.5x, which corresponded to the fold-change difference in cell number between the CoAl₁₀₀ and CoAl₀ groups (**Figure 6.3.3.2**). Interestingly, even in its diluted form, CM_{CoAl0} was able to trigger the scattering of the DLD-1 epithelial clusters. This suggests that the differences in CM concentration is unlikely to account for the varying EMT-inducing potential of both types of CM. Importantly, the observations further strengthen the critical role of matrix confinement-dependent effects on CAF phenotype and secretory potency.

6.4 Conclusion

In this study, c-CAF was used to replace b-CAF to study the CAF mechanoresponse within mechanically tunable 3D matrix. The studies, including cell morphology observation on 2D or within 3D hydrogel, paracrine signaling, and EMT potential, were conducted. The results showed the different morphological responses between c-CAF and b-CAF cultured on the surface of CoAl-IPN hydrogel, with a better spreading capacity being observed in c-CAF on both soft CoAl₀ (E ~100 Pa) and stiff CoAl₁₀₀ (E ~1000 Pa) hydrogels, with a

cell area of 916 and 3644 μm^2 respectively, as compared to b-CAF (798 and 898 μm^2 respectively). Specifically, the c-CAF showed sharp spindle morphology on the soft hydrogel due to its abundant F-actin bundles, while b-CAF is round morphology. However, a similar trend was observed in c-CAF encapsulated within the 3D hydrogel, stiff CoAl₁₀₀ hydrogel ($E \sim 1000$ Pa) could impose physical entrapment on c-CAF and coerced them into spherical morphology, while the sparse CoAl₀ hydrogel ($E \sim 100$ Pa) would favor c-CAF spreading. Most importantly, the c-CAF encapsulated within stiff CoAl₁₀₀ hydrogel significantly mute the EMT potential of DLD-1 colon cancer cells compared to that of the soft CoAl₀ hydrogel system, which is similar to the b-CAF. Collectively, c-CAF is also a mechanoresponsive cell type in 3D context, which is a suitable cell model to explore the underlying mechanisms using transcriptome analyses.

Figure 6.4 Schematic illustration of CoAl-IPN hydrogel stiffness effect on c-CAF phenotype and paracrine crosstalk with colorectal adenocarcinoma cells.

References

- [1] N. Keum, E. Giovannucci. *Nat. Rev. Gastroenterol. Hepatol.* 2019, 16, 713-732.
- [2] R. L. Siegel, K. D. Miller, A. Goding Sauer, S. A. Fedewa, L. F. Butterly, J. C. Anderson, A. Cercek, R. A. Smith, and A. Jemal. *CA Cancer J. Clin.* 2020, 70, 145-164.
- [3] R. L. Siegel, K. D. Miller and A. Jemal. *CA Cancer J. Clin.* 2019, 69, 7-34.
- [4] R. L. Siegel, S. A. Fedewa, W. F. Anderson, K. D. Miller, J. Ma, P. S. Rosenberg and A. Jemal. *J. Natl. Cancer Inst.* 2017, 109, 2017.
- [5] T. N. Seyfried and L. C. Huysentruyt. *Crit. Rev. Oncog.* 2013, 18, 43–73.
- [6] V. Mittal. *Annu. Rev. Pathol.* 2018, 13, 395-412.
- [7] S. Heerboth, G. Housman, M. Leary, M. Longacre, S. Byler, K. Lapinska, A. Willbanks and S. Sarkar. *Clin. Transl. Med.* 2015, 4.
- [8] L. J. Hawinkels, M. Paauwe, H. W. Verspaget, E. Wiercinska, J. M. van der Zon, K. van der Ploeg, P. J. Koelink, J. H. Lindeman, W. Mesker, P. ten Dijke and C. F. Sier. *Oncogene.* 2014, 33, 97-107.
- [9] A. De Boeck, A. Hendrix, D. Maynard, M. Van Bockstal, A. Daniels, P. Pauwels, C. Gespach, M. Bracke and O. De Wever. *Proteomics.* 2013, 13, 379-88.
- [10] M. Rokavec, D. Horst and H. Hermeking. *Cancer Res.* 2017, 77, 1854-1867.
- [11] J. Stricker, T. Falzone and M. L. Gardel. *J. Biomech.* 2010, 43, 9-14.
- [12] V. Maruthamuthu, Y. Aratyn-Schaus and M. L. Gardel. *Curr. Opin. Cell Biol.* 2010, 22, 583-8.
- [13] N. J. Hogrebe, J. W. Reinhardt and K. J. Gooch. *J. Biomed. Mater. Res. A.* 2017, 105, 640-661.
- [14] C. Y. Tay, Y.-L. Wu, P. Cai, N. S. Tan, S. S. Venkatraman, X. Chen and L. P. Tan. *NPG Asia Materials.* 2015, 7, e199-e199.
- [15] N. Huebsch, P. R. Arany, A. S. Mao, D. Shvartsman, O. A. Ali, S. A. Bencherif, J. Rivera-Feliciano and D. J. Mooney. *Nat. Mater.* 2010, 9, 518-26.
- [16] D. S. Reynolds, K. M. Bougher, J. H. Letendre, S. F. Fitzgerald, U. O. Gisladdottir, M. W. Grinstaff and M. H. Zaman. *Acta Biomater.* 2018, 77, 85-95.
- [17] O. D. Lohez, C. Reynaud, F. Borel, P. R. Andreassen and R. L. Margolis. *J. Cell Biol.* 2003, 161, 67-77.

- [18] J. Melendez, K. Stengel, X. Zhou, B. K. Chauhan, M. Debidda, P. Andreassen, R. A. Lang and Y. Zheng. *J. Biol. Chem.* 2011, 286, 15132-7.
- [19] J. Baulida. *Mol. Oncol.* 2017, 11, 847-859.
- [20] Z. Ling, X. Yang, X. Chen, J. Xia, B. Cheng and X. Tao. *J. Oral Pathol. Med.* 2019, 48, 477-482.

Chapter 7*

Effect of CoAl-IPN hydrogel stiffness on c-CAF whole transcriptomes and its correlation to colorectal cancer patient overall survival

Using the 3D CoAl-IPN culture platform in combination with RNA sequencing analysis, this study unveils the matrix stiffness-dependent phenotypic switching of human colorectal cancer-associated fibroblasts (c-CAF) between an inflammatory state (i-state, α -SMA^{low} IL-6^{high}) and myofibroblastic state (m-state, α -SMA^{high} IL-6^{low}). Further bioinformatic analysis reveals that ROS-HIF-1 α mechanosensitive axis is closely associated to the i-state CAF phenotype, accounting for its secretory phenotype. Interestingly, the clinical relevance using GEPIA demonstrated that differentially expressed mRNA transcripts in i-state CAF such as VEGFC, IL8, IL1A, IL1B, and CXCL2/3 were significantly correlated with colorectal cancer patient survival. These results indicate that physical cues could determine CAF-states, paracrine signaling, and metastatic potential, which may offer new biological insights to disrupt the CAF-cancer cell communication and tumor progression.

7.1 Introduction

Studies have shown that the activation of the epithelial-to-mesenchymal transition (EMT) process is highly dependent on the paracrine crosstalk between colorectal cancer (CRC) tumor and the tumor stromal-residing colon cancer-associated fibroblasts (c-CAF). However, c-CAF are notoriously heterogeneous with diverse tumor-promoting or tumor-suppressing functions. For instance, genetic depletion of alpha-smooth muscle actin (α -SMA)-positive CAF did not curtail tumor progression, but instead significantly enhanced the aggressiveness of the pancreatic tumor via the upregulation of CD4⁺ regulator T-cells.[1] Additionally, CAF-derived slit homolog 2 protein (SLIT2) was also shown to suppress breast cancer cell invasion by inhibiting the phosphoinositide 3-kinase (PI3K) and β -catenin signaling.[2] The conundrum that CAF can either promote or restraint tumor progression is largely due to its pervasive phenotypic diversity and plasticity displayed by the different sub-populations of CAF. CAF are a heterogeneous population of stromal cells. In the context of triple-negative breast cancer (TNBC), Costa *et al.* revealed the existence of four CAF subsets (i.e., S1-4).[3] Among those, CAF-S1 and CAF-S4 are α -SMA-positive myofibroblastic subsets but have diametrically opposing roles. Fibroblast activation protein (FAP)-positive CAF-S1 is associated with an immunosuppressive TME by promoting the differentiation of T cells into regulator T cells (Tregs), whereas CAF-S4 is linked to high CD8⁺ cytotoxic T lymphocytes infiltration. Pioneer works by Tuveson *et al.* have also demonstrated the occurrence of 2 major types of CAF in pancreatic ductal adenocarcinoma (PDAC), namely myofibroblastic CAF (myCAF) and inflammatory CAF (iCAF). myCAF are localized to the peri-tumor region while iCAF are in the dense fibrous stroma, distance from the tumor. Phenotypically, myCAF have a transcriptional profile that resembles the activated myofibroblasts (i.e., high expression of α -SMA), while iCAF represent a sub-population of CAF that lacks α -SMA, while expressing high levels of cytokines and chemokines (e.g., IL-6) transcript.[4]

Different CAF subsets have distinctive roles in the regulation of tumorigenesis and metastasis. The existence of different CAF subsets suggests a ‘continuum’ of CAF phenotypes. However, it is unclear whether these different CAF subtypes remain a stable,

self-subsistent population or a transitory state that can be dynamically reversed between the different phenotypes. Moreover, factors underpinning the existence of the different CAF subtypes remains elusive to date. In general, cells response to their biomechanics of the microenvironment by changing their shape, cytoskeletal architecture, and intrinsic mechanical properties through cytoskeletal adaptation, up- and down-regulation of structural proteins that result in different mechanomics.[5] Hence, a comprehensive study was carried out using RNA Seq, a powerful technology that allows unbiased bioinformatic analysis on these mechanomics changes to provide a better understanding on the effect of matrix stiffness on CAF, and the subsequent paracrine crosstalk with the cancer cells.[6]

7.2 Experimental Methods

7.2.1 Materials

The materials used in this study were described in the **Section 4.2.1 and 5.2.1**. On the other hand, α -SMA (124964) primary antibody was purchased from ABCAM. HIF-1 α (36169) primary antibody was obtained from Cell Signaling Technology. IL-6 ELISA kits were purchased from BioLegend (San Diego, CA).

7.2.2 CoAl-IPN hydrogel fabrication and c-CAF encapsulation

CoAl-IPN hydrogels with different stiffness were fabricated by varying the CaCl₂ concentration (0 and 100 mM) as described in **Section 3.1**. c-CAF were trypsinized, followed by mixing with the CoAl precursor solution (cells density of 5×10^5 cells per mL) prior to the hydrogel fabrication. The cell-encapsulated hydrogels were then cultured using serum free medium over 48 h.

7.2.3 RNA sequencing and transcriptome analyses

After 48 h of culture, RNA was extracted from c-CAF as described in **Section 3.11** before sending to Novogene Co., Ltd for RNA sequencing profiling via Illumina HiSeq™. The expression of some targeted genes was further evaluated by PCR as shown to **Section 3.12**.

The whole transcriptome analysis was conducted as follows. Bulk RNA-Seq gene raw data were mapped to reference genome (homo_sapiens_hg19) using the STAR software and gene expression level analysis was performed using EdgeR R package. The gene readout was adjusted by the Trimmed Mean of M-values (TMM) normalization. Significant differentially expressed genes (DEGs) were selected with a False Discovery Rate (FDR) cutoff of < 0.005 . The gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis of the DEGs was conducted using the Goseq R and clusterprofiler R package. Enriched pathways/functions with corrected $p < 0.05$ were considered to statistically significant. Functional analysis of the full gene list was further analyzed by performing the Gene Set Enrichment Analysis (GSEA) using the GSEA software 4.0.1 to determine enriched biological processes and pathways based on the C2.all.v7.1.symbols.gmt curated gene set. Significant gene sets with a FDR $< 25\%$ and a nominal $p < 0.05$ were identified. The DEGs from enriched gene sets were imported into online STRING (<http://string-db.org/>) database and used as the seed genes for developing a network of DEGs-encoded proteins and protein-protein interaction (PPIs). The parameters include a minimum required interaction score > 0.4 (medium confidence), and only queried proteins were displayed. Subsequently, the hub genes, defined as key genes with significant biological regulatory functions, were analyzed and identified using the CytoHubba application in Cytoscape software, where five calculation methods were employed, including Degree, EPC, Eccentricity, MCC, and MNC. Based on the GSEA analysis, the cytokine-cytokine receptor interaction gene set was significantly enriched (FDR q-value 0.054). According to the RNA-Seq and PCR gene expression results, the cytokines genes with significant expression change were filtered out for survival analysis using the Gene Expression Profiling Interactive Analysis (GEPIA; <http://gepia.cancer-pku.cn/index.html>), with parameters including median group cutoff (a suitable 50%

expression threshold for splitting the high-expression and low-expression cohorts). The colon adenocarcinoma (COAD) database is based on the cancer genome atlas (TCGA) program and the genotype-tissue expression (GTEx) projects. The final prognostic survival plots were presented as hazard ratio (HR) at 95% confidence interval (CI), patient number n , and logrank p value. The significant correlation between cytokines and patients overall was defined as Logrank $p < 0.05$.

7.2.4 IL-6 ELISA test

The c-CAF cell culture supernatant was probed for IL-6 by using commercially available ELISA kits according to manufacturer's directions. Briefly, high binding 96-well plates were coated with anti-human IL-6 overnight in 4 °C fridge and subsequently blocked with the provided blocking buffer to reduce non-specific binding and background. IL-6 standards and supernatant were loaded and detected with biotin-conjugated anti-human IL-6. Experiments were quadruplicates for each experimental group.

7.2.5 Intracellular reactive oxygen species (ROS) measurement

The intracellular ROS of c-CAF encapsulated within CoAl-IPN hydrogels was evaluated via staining with CellRox®Green. Briefly, c-CAF were incubated with 5 μ M CellRox®Green and 1 μ g/mL Hoechst for 30 min at 37 °C, followed by washing three times with PBS. Then, the hydrogels were fixed with 4% PFA for 20 min. After washing, the cells were imaged using the Zeiss Observer Z1. N-acetylcysteine NAC was used for ROS scavenging experiments.

7.2.6 α -SMA and HIF-1 α immunocytochemical staining

The α -SMA expression and HIF-1 α bioactivity of the encapsulated c-CAF within CoAl-IPN hydrogels was analyzed via immunofluorescence staining as shown in the **Section 3.5**.

7.2.7 Statistical analysis

All experiments were repeated at least three times with duplicates. Statistical significance was determined by one-way analysis of variance (ANOVA) using SPSS among three or more than three groups. Comparison between two groups was analyzed by two-tailed Student's *t* test. The differences are considered as statistically significant with $p < 0.05$.

7.3 Principle Outcomes

7.3.1 NGS analysis unveil matrix stiffness-dependent acquisition of inflammatory and myofibroblastic cell state in c-CAF

Changes to the cell morphology alter critical cell functions and phenotypes via mechanotransduction.[7-10] To understand the biological significance of c-CAF shape changes in the CoAl-IPN hydrogel systems, a non-biased genome-wide transcriptome profiling was conducted. C-CAF were cultured in either hydrogel for 48 h prior to the bulk RNA-seq. The quality of the extracted RNA was evaluated as shown in **Figure 7.3.1.1**. Generally, the quality of the extracted RNA was analyzed via bioanalyzer Agilent 2100, which can provide information on the pre region, marker region, 5s region, fast region, 18s region, inter region, 28s region, precursor region and post region of each sample. Based on the specific system algorithm, the RNA Integrity Number (RIN) ranging from 10 to 1 will be obtained, in which 10 is a representation of a highly intact RNA while 1 as a representation of the completely degraded RNA. The RIN value for the extracted RNA from both CoAl₀ and CoAl₁₀₀ hydrogels were found to be 9 and 10 respectively, which is an indication that RNA isolation process used in this study managed to preserve the quality of the extracted RNA.

Figure 7.3.1.1 Quality and quantity assessment of RNA samples from c-CAF cultured in either CoAl₀ (a) and CoAl₁₀₀ (b) hydrogel.

By comparing the gene expressed by the c-CAF in both the CoAl₀ and CoAl₁₀₀ hydrogels, 2 gene sets were obtained. Significant differences in the transcriptome of CAF as a function of cell shape were observed. Of the 1623 DEGs with a FDR value of < 0.005, 608 genes were upregulated in the spindled shaped CAF (CoAl₀ group), while 1015 genes were upregulated in the rounded CAF (CoAl₁₀₀ group) as shown in **Figure 7.3.1.2a**. To gain further biological insights into the identified DEGs, gene enrichment analysis was performed against the KEGG pathway. R program package clusterprofiler was used to analyze and to annotate the upregulation genes in c-CAF cultured in CoAl₀ and CoAl₁₀₀. The top 10 KEEG enriched pathways for each experimental group are shown in **Figure 7.3.1.2b and c**. For c-CAF grown in the CoAl₀ group, the enriched upregulated DEGs were involved in signaling pathways such as cell cycle, actin formation, focal adhesion, and ECM-receptor interaction (**Figure 7.3.1.2b**). These findings agree with the associated well-spread morphology and increased cellular proliferation of c-CAF cultured in CoAl₀ (**Figure 6.3.2.1**), indicating robust cell-matrix interaction. In contrast, a large proportion of upregulated DEGs in the c-CAF within the CoAl₁₀₀ group were implicated in cytokine-cytokine receptor, calcium signaling, cell adhesion molecules and TGF-beta signaling pathways, suggestive of a secretory phenotype (**Figure 7.3.1.2c**).

Since earlier studies have shown that CAF can be subdivided into iCAF and myCAF,[4, 11] DEGs relating to inflammation and myofibroblasts were further scrutinized in the samples of this study. Distinct genes relating to inflammatory markers (i.e., IL-1A, B, 11, 6 and CXCL 1-3, 8 and 10) were found to be exclusively upregulated in the spherical c-CAF encapsulated within the CoAl₁₀₀ hydrogel. In contrast, elongated c-CAF grown in the CoAl₀ hydrogel displayed elevated myofibroblastic transcripts (i.e. PALLD, MYL6, CFL1, COL1A1, CNN1, COL1A2, LOXL1, TAGLN, MYLK, ACTG2) as shown in **Figure 7.3.1.2d**. These findings revealed for the first time that CAF could switch between an inflammatory (i-) of myofibroblastic (m-) state, that is contingent on the matrix-mechanics dependent confinement effect in a 3D context. The rounded and elongated cells were termed as CAF in their i-state and m-state, respectively. The RNA-seq data was further assessed by ELISA and RT-PCR. The i-state cells secreted significantly (~2-fold) higher of IL-6 (~ 563 pg/mL) as compared to the m-state counterpart (**Figure 7.3.1.2e**). Furthermore, the mRNA (**Figure 7.3.1.2f**) and protein (**Figure 7.3.1.2g**) levels α -SMA in the m-state CAF were significantly higher than the i-state CAF. Therefore, by using CoAl-IPN platforms, myCAF (α -SMA^{high} IL-6^{low}) and iCAF (α -SMA^{low} IL-6^{high}) phenotypes could be recapitulated.[4, 11] It is also important to note that the mechanoregulated i- and m-states of c-CAF were derived from the same parent cell. In contrast, iCAF and myCAF identified by Daniel *et al.*, reported two distinctive CAF sub-types defined in part by their anatomical locations in the pancreatic ductal adenocarcinoma (PDAC) stroma. However, the findings also raise the possibility that ECM mechanics can drive the switch between different CAF sub-types.

Figure 7.3.1.2 Comparative transcriptional signatures of c-CAF revealed *in situ* phenotypic shift govern by hydrogel mechanics. (a) Volcano plot of transcriptional landscape profiled by RNA-Seq comparing c-CAF in CoAl₁₀₀ vs CoAl₀. The x-axis shows the log transformed fold change while y-axis denotes log adjusted p value. KEGG pathway enrichment analysis for relative upregulated genes in c-CAF cultured in (b) CoAl₀ and (c) CoAl₁₀₀. (d) Fold change expression of targeted DEG related to inflammation and myofibroblasts in c-CAF (CoAl₁₀₀) compared to CAF (CoAl₀). (e) Quantification of secreted IL-6 by c-CAF. (f) Expression level of ACTA2 mRNA transcripts in c-CAF. (g) Immunofluorescent images of CAF counter-stained for F-actin (red), α-SMA (green) and nucleus (blue). Scale bar = 20 μm. All experiments were triplicated. Data represents mean ± standard deviation. n.s. denotes no statistical difference between compared experimental groups. Statistical significance is determined at $p < 0.05$.

The notion is further supported by the GO analysis for upregulated DEGs in i-state CAF. As shown in **Figure 7.3.1.3**, the upregulated DEGs were related to molecular functions (MF) such as cytokine activity (GO:0005125) and growth factor activity (GO: 0048018). Several

biological processes, such as response to lipopolysaccharide (GO: 0071222), to a molecule of bacterial origin (GO: 0002237), to the bacterium (GO: 0009617) and to acid chemical (GO: 0001101) were highly scored in i-state CAF. These findings indicate that i-state CAF are associated to a secretory and inflammatory phenotype, consistent with the increased secretion of IL-6 as observed earlier.

Figure 7.3.1.3 Gene ontology analysis of upregulated DEGs in i-state c-CAF. Bar charts showing top 10 GO terms for molecular function, cellular component and biological processes ranked by fold enrichment.

7.3.2 ROS-HIF-1 α is a mechanotransducible signaling axis in c-CAF

The above results highlighted the pivotal role of ECM mechanics in the regulation of CAF states, with the assumption that the m- to i- state transition of CAF is dependent on a mechanoregulated process caused by the change in cell shape. To examine the possible mechanosensitive targets, GSEA of the full gene expression list was performed, which similarly showed that the inflammatory pathway (M9673) was enriched in the i-state CAF (**Figure 7.3.2.1a**). Another enriched pathway of i-state CAF was hypoxia 11 (M2345) (**Figure 7.3.2.1b**). Cellular adaptations to hypoxia are regulated by the hypoxia-inducible factors (HIFs). In normoxia, HIF-1 α is hydroxylated by HIF prolyl hydroxylase 1 to form

the von-Hippel Lindau (pVHL)-E3 ligase complex lead to its polyubiquitination and subsequent proteasomal degradation.[12] Under hypoxia, O₂-dependent hydrolyzation of HIF-1 α is reduced and therefore is not degraded. Instead, HIF-1 α is translocated into the nucleus, where it binds with HIF-1 β to transcriptionally upregulates hypoxia-responsive genes. In tumor stromal cells, hypoxia reduced the expression of α SMA, periostin, and contractile force, culminating in the deactivation of CAF and impairment of CAF-mediated cancer invasion.[13] Additionally, knockout of HIF-1 α in stromal fibroblasts accelerated tumorigenesis in a murine model of mammary cancer.[14]

The protein-protein interaction (PPI) network of the upregulated genes ($p_{adj} < 0.05$) of i-state CAF from the inflammatory and hypoxia gene sets were analyzed using the STRING database (**Figure 7.3.2.1c**). The constructed PPI network consisted of 32 nodes and 33 interactions, of which 24 of the 32 DEGs were captured. Based on the cytoHubba ranking, HIF1A (score = 6) has emerged as one of the top 5 hub gene candidates (**Figure 7.3.2.1d**). This revelation is in line with earlier work, where HIF-1 α was established as a novel mechano-redox sensitive protein in human adipose-derived mesenchymal stem cells (AMSCs).[15] Rounding of AMSCs on soft polyacrylamide substrate ($E = 0.15\text{kPa}$) was shown stabilize ROS-dependent induction of HIF-1 α expression under normoxic condition, prompting the cells to secrete enhanced amount of angiogenic factors.

Similarly, the GO analysis of DEGs also revealed that the ROS metabolism was upregulated, such as ROS metabolic process (GO: 0072593), ROS biosynthetic process (GO: 1903409), positive regulation of ROS metabolic process (GO: 2000379), and response to ROS (GO: 0000302) (**Figure 7.3.2.1 e**). The ROS staining further confirmed that the ROS intensity was higher within iCAF compared to mCAF (**Figure 7.3.2.2**). Importantly, treating the cells with NAC, a potent antioxidant, suppressed both the level of intracellular ROS and HIF1- α in the i-state CAF. This suggests that the mechanoregulated ROS level lies upstream of the induction HIF-1 α (**Figure 7.3.2.3**). These findings collectively suggest that HIF-1 α may be also involved in the mechano-switching of CAF between the i and m states. To examine this end, c-CAF cultured in either the 3D CoAl₀ or CoAl₁₀₀ hydrogels were counter-stained for HIF-1 α . As indicated in **Figure 7.3.2.1f**, while

HIF-1 α staining was absent in the spindle-shaped m-state CAF (i.e. CoAl-IPN group), nuclear HIF-1 α staining was evident in the physically confined round-up i-state CAF. As compared to the CoAl₀ group, cells in the CoAl₁₀₀ expressed 6-fold higher nuclear HIF-1 α (**Figure 7.3.2.1g**).

Another question was raised whether the observed HIF-1 α stabilization in the CoAl₁₀₀ gel represents a transitory or permanent biological end state. Interestingly, when the alginate network was selectively de-crosslinked in the CoAl₁₀₀ group with sodium citrate (50 mM), the cells resume the fibroblastic spindle-shaped morphology, and the expression of HIF-1 α was abolished (**Figure 7.3.2.1g and h**), which could be explained by the decreased ROS intensity simultaneously (**Figure 7.3.2.3**). These findings suggest that c-CAF can adapt to the changes in the mechanical deformation of the cell by undergoing a reversible switching between the m- and i-states. The underlying mechanism involves HIF-1 α as a novel mechanosensitive protein (**Figure 7.3.2.1h**).

Figure 7.3.2.1 Gene ontology and gene set enrichment analysis of RNA-Seq data reveals HIF-1 α as a novel upstream mechanotransducer of c-CAF. GSEA enrichment plots of (a) inflammatory response LPS and (b) hypoxia 11. (c) PPI network analysis and (d) computed CytoHubba scores of candidate genes from the inflammatory and hypoxia gene sets. (e) GO analysis of upregulated GO terms for biological processes related to ROS metabolism in i-state c-CAF ranked by fold enrichment. (f) Immunostaining and phase contrast images of c-CAF counterstained for HIF-1 α (green) and nucleus (blue) in c-CAF cultured under different conditions. Scale bar = 50 μ m. (g) Quantification of nucleus bound HIF-1 α expression of c-CAF. (h) Schematic depicting the microenvironmental regulation of HIF-1 α expression in c-CAF. All experiments were triplicated. Data represents mean $\pm$ standard deviation. n.s. denotes no statistical difference between compared experimental groups. Statistical significance is determined at $p < 0.05$.

Figure 7.3.2.2 Modulation of intracellular ROS levels of c-CAF in hydrogels of varying stiffness and culture conditions. ROS generation analysis of CAF within CoAl₀ hydrogel, CoAl₁₀₀ hydrogel treated without or with 2.5 mM N-acetylcysteine (NAC) and CoAl₁₀₀ hydrogel decrosslinked with 50 mM sodium citrate solution (a), as well as corresponding quantitative assay (b). Scale bar = 50μm. Asterisk * denotes statistical difference at $p < 0.05$.

Figure 7.3.2.3 Modulation of HIF-1α expression of c-CAF grown in CoAl₁₀₀ hydrogel with or without NAC (2.5 mM) treatment. HIF-1α expression cultured within CoAl₁₀₀ hydrogel with or without 2.5 mM N-acetylcysteine (NAC) treatment (a) and quantitative assay (b). Scale bar = 20μm. Asterisk * denotes statistical difference at $p < 0.05$.

7.3.3 Correlational analysis of i-state c-CAF and CRC patient overall survival

So far, the findings revealed that in a stiff and physically confined microenvironment, c-CAF can assume an i-state that is characterized by a low α -SMA and high IL-6 expression, and nuclear-HIF1- α expression. Moreover, EMT-inducing potential of the CM derived from i-state c-CAF was muted as compared to the m-state c-CAF CM according to the results from **Chapter 6**. Interestingly, the observed anti-EMT properties of the i-state CAF appears to contradict the pro-tumor roles of iCAF, as was reported in several other types of cancer such as pancreatic,[16] breast,[17] and lung[18] cancers. However, using single-cell transcriptomics analysis of head and neck cancer, it was observed that there are two distinct types of CXCL12 and IL-6 enriched “inflammatory” fibroblasts, which suggest that additional heterogeneity within the iCAF subsets with different functionality may exist.[19]

To identify the possible specific i-state CAF-derived paracrine signals that are responsible suppressing the induction of EMT in DLD-1 cells, 31 DEGs (FDR < 0.05) were curated in the KEGG cytokine-cytokine receptor interaction gene set in the i-state CAF and confirmed their expression levels using RT-PCR (**Figure 7.3.3.1a**). To further validate the clinical relevance of the findings, overall patient survival analysis from the DEGs was performed using GEPIA and Kaplan-Meier plotters.[20] GEPIA is a web server that can deliver a non-biased analysis of the targeted genes based on the publicly available cancer genome atlas (TCGA) and Genotype-Tissue Expression (GTEx) databases. Among the examined candidate genes, 6 genes were found to be statistically different in prognosticating CRC patients' (sample size = 270) survival (logrank $p < 0.05$). The genes were VEGFC (HR(high) 1.7, Logrank $p = 0.039$), IL8 (HR(high) 0.61, Logrank $p = 0.05$), IL1B (HR(high) 0.61, Logrank $p = 0.043$), IL1A (HR(high) 0.58, Logrank $p = 0.031$), CXCL2 (HR(high) 0.61, Logrank $p = 0.042$) and CXCL3 (HR(high) 0.62, Logrank $p = 0.047$). It was interesting to note that the classical pro-tumor inflammatory proteins such as IL-6 (Logrank $p = 0.45$), CXCL12 (Logrank $p = 0.68$), and LIF (Logrank $p = 0.61$) that were associated with iCAF did not have a significant effect on CRC patient survival (**Table 7.2**). Except

for VEGFC, the expression levels of the remaining genes were significantly upregulated in the i-state CAF relative to the m-state CAF.

VEGFC is a growth factor belonging to the platelet-derived growth factor family, which could bind to Flt4/VEGF receptor 3 (VEGFR3) on cancer cells to promote tumor progression.[21] CXCL8(IL-8) is a multifunctional chemokine interacting with C-X-C chemokine receptor 1/2 and modulates proliferation and invasion of cancer cells, immunosuppression via N2 tumor-associated neutrophils (TAN) recruitment.[22] Both IL-1 α and IL-1 β are proinflammatory cytokines, which could either promote or suppress tumor progression.[23] For example, IL-1 β encourages cancer metastasis because of the pro-invasive inflammation microenvironment, while also inhibit cancer progression via improving adaptive T cell-mediated immunity (CD4+ and CD8+ T cells expansion and maturation).[24] CXCL2/3 belongs to the CXC chemokine family and its role in tumor progression is controversial. For instance, the overexpression of CXCL2 inhibited hepatocellular carcinoma (HCC) cells proliferation via the ERK1/2 signaling pathway and induced apoptosis to attenuate tumor growth.[25] However, studies also showed that CXCL2 could promote tumor progression.[26] Also, the CXCL3 could induce ERK1/2 and ETS1 phosphorylation and promote CD133 expression, which is associated with vascular invasion.[27] 6 genes (i.e. CXCL2/3/8, IL1A/B, and VEGFC) were significantly correlated with the overall survival of the CRC patients. Specifically, the over-expression of CXCL2/3/8, IL1A/B, and under-expression of VEGFC can improve patient survival (HR <1 and Logrank p < 0.05) as shown in **Figure 7.3.3.1b-g**.

Figure 7.3.3.1 Significantly cytokine-related DEGs in i-state CAF associates with CRC patient overall survival in two independent cohorts. (a) Fold change (log2 scale) of DEGs in i-state CAF as assessed by RT-PCR. Kaplan-Meier survival analysis of (b) IL1A, (c) IL1B, (d) CXCL2, (e) CXCL3, (f) CXCL8 and (g) VEGFC in patient groups with low expression (blue) and high expression (red).

7.4 Conclusion

Recent advancements in 3D culture platforms and bioinformatics have equipped researchers with the necessary tools to interrogate complex biology at an unprecedented level and precision. In this study, a mechanically tunable 3D CoAI-IPN hydrogel was employed as an *in vitro* surrogate of CRC stroma and RNA-seq analyses to examine the phenotypic diversity of c-CAF. The results revealed that ECM mechanics exert profound alterations to the morphology, transcriptome, and EMT-inducing capacity of c-CAF. Using

various bioinformatic approaches, the study unveiled that a soft and sparsely crosslinked microenvironment ($E \sim 100$ Pa) favors the m-state phenotype, while stiff and highly crosslinked networks ($E \sim 1000$ Pa) significantly limited cells spreading and coerced the cells to transit into an i-state or secretory phenotype.

This study highlights HIF-1 α as a novel mechanosensitive protein that is associated with the phenotypic switch in CAF. While the rounded CAF displayed expression of nuclear HIF-1 α , the activation is transient and can be “switched-off” when the cells are relieved from the physical entrapment in the 3D hydrogel. CAF constantly survey the TME and respond to the mechanical changes by switching between the m- and i-state, and their associated mechanotransduction signaling. Therefore, far from being a static and fixed state, CAF is a dynamic and consequently, a heterogeneous stromal cell population at different tumor progression. Importantly, the study showed that i-state CAF secrete factors that restrain CRC EMT and spreading. The clinical relevance and significance of the findings were further validated by the correlation between DEGs and patient’s survival using the GEPIA database.

In conclusion, the findings clearly showed that CAF are intrinsically responsive to mechanical and architectural changes in the stromal compartment, which will ultimately influence the aggressive potential of tumor.

Table 7.1 The details of the cytokines DEGs from enriched ' cytokine-cytokine receptor interaction pathway ' and the corresponding survival correlation analysis. The statistic values of PCR results are all less than 0.05.

Gene name	Receptor	Receptor in tumor	Receptor in normal tissue	Log2(FC) (RNA seq)	FDR	Log2(FC) (PCR)	HR	log rank p
IL1A	IL1R1	6.51	21.66	4.8979	9.32E-17	2.587973	0.58	0.031
IL1B	IL1R2	5.13	11.17	3.2236	1.43E-10	1.552878	0.61	0.043
CXCL2	IL8RB	0.29	0.88	1.9463	0.0002938	2.504678	0.61	0.042
IL8	IL8RA	0.99	2.39	2.551	4.04E-07	2.840902	0.61	0.05
CXCL3	IL8RB/A	0.99	2.39	1.7643	0.0011433	3.615707	0.62	0.047
VEGFC	KDR	3.29	3.76	-1.32	0.031448	-2.953355	1.7	0.039
INHBB	ACVR2	4.02	6.11	3.6415	0.028395	0.116211	1.5	0.11
HGF	MET	28.84	3.61	1.4164	0.016685	1.128443	1.1	0.7
LIF	LIFR	0.27	6.68	1.3089	0.033474	1.986994	1.1	0.61
CCL3	CCR5	1.33	0.64	6.6046	1.35E-21		0.97	0.91
IL11	IL1RA	6.51	21.66	3.8866	1.23E-14		1.5	0.12
IL6	IL6R	2.12	13.1	3.2903	4.74E-11	3.09123	1.2	0.45
BMP2	BMPR2	6.63	8.09	1.8903	0.0003722	1.129224	0.83	0.45
CXCL10	CXCR3	3.94	0.53	3.2407	0.024999	0.567961	0.86	0.56
BMP7	ACVR2/	4.02/	6.11/	6.5335	0.029326	1.400694	1	0.89
	BMPRII	0.1	0.52					
IL17D	NA			-1.5978	0.0098843	-0.54822	1	1

TNFSF10	NA			3.2407	0.0007287	2.368887	1	0.89
TNFSF13B	NA			3.8922	2.34E-14	1.878113	1.4	0.13
CSF3	CSF3R	2.72	4.78	2.8313	1.60E-08	1.817909	1.3	0.22
CXCL12	CXCR7/4	19.48/0	11.19/0	-1.4281	0.018434	-2.82682	1.1	0.68
IL24	IL20RB	0.46	0.65	3.7265	1.80E-13	0.712858	1.1	0.67
INHBE	ACVR1B			4.421	0.0019801	0.654677	1.3	0.3
CCL8	CCR3	0.11	0.05	-4.1122	0.0034749	-0.69883	1.2	0.55
CCL5	CCR1/5	2.52/1.33	1.71/0.64	3.5388	0.0061738	1.562362	0.77	0.29
CSF2	CSF2RA/B	2.86/2.25	2.16/2.97	-2.0274	0.0002142	-2.27603	1.1	0.71
PDGFC	PDGFRA	5.65	26.14	-1.2752	0.044039	-0.44781	1.2	0.57
CXCL1	IL8RB/A	0.99	2.39	1.8067	0.0007833	0.658792	0.69	0.13
IL23A	IL23R	2.1	0.3	3.347	0.014277	1.206651	1	0.97
CCL4	CCR5	1.33	0.64	6.7379	0.019327	1.355489	0.71	0.16
CSF1	CSF1R	6.76	12.25	-1.6461	0.0031147	-2.09034	1	0.88
CCL2	CCR2	0.5	1.25	1.4311	0.020003	2.706547	1.3	0.24
TGFB3	TGFBR1	9.27	11.63	3.6383	4.97E-10	1.733598	1.5	0.11
PDGFB	PDGFRB	19	29.55	4.807	4.83E-09		1	0.95
TGFB2	TGFBR2	31.67	61.2	3.1795	5.13E-06	1.984658	1.5	0.086
CCL20	CCR6	1.22	0.1	5.5571	5.25E-25	5.467068	0.84	0.48

References

- [1] B. C. Ozdemir, T. Pentcheva-Hoang, J. L. Carstens, X. Zheng, C. C. Wu, T. R. Simpson, H. Laklai, H. Sugimoto, C. Kahlert, S. V. Novitskiy, A. D. Jesus-Acosta, P. Sharma, P. Heidari, U. Mahmood, L. Chin, H. L. Moses, V. M. Weaver, A. Maitra, J. P. Allison, V. S. LeBleu and R. Kalluri. *Cancer Cell*. **2014**, 25, 719-34.
- [2] P. H. Chang, W. W. Hwang-Verslues, Y. C. Chang, C. C. Chen, M. Hsiao, Y. M. Jeng, K. J. Chang, E. Y. Lee, J. Y. Shew and W. H. Lee. *Cancer Res*. **2012**, 72, 4652-61.
- [3] C. Neuzillet, A. Tijeras-Raballand, C. Ragulan, J. Cros, Y. Patil, M. Martinet, M. Erkan, J. Kleeff, J. Wilson, M. Apte, M. Tosolini, A. S. Wilson, F. R. Delvecchio, C. Bousquet, V. Paradis, P. Hammel, A. Sadanandam and H. M. Kocher. *J. Pathol*. **2019**, 248, 51-65.
- [4] D. Ohlund, A. Handly-Santana, G. Biffi, E. Elyada, A. S. Almeida, M. Ponz-Sarvisse, V. Corbo, T. E. Oni, S. A. Hearn, E. J. Lee, Chio, II, C. I. Hwang, H. Tiriach, L. A. Baker, D. D. Engle, C. Feig, A. Kultti, M. Egeblad, D. T. Fearon, J. M. Crawford, H. Clevers, Y. Park and D. A. Tuveson. *J. Exp. Med*. **2017**, 214, 579-596.
- [5] L. A. Sawicki, E. M. Ovadia, L. Pradhan, J. E. Cowart, K. E. Ross, C. H. Wu and A. M. Kloxin. *APL Bioeng*. **2019**, 3, 016101.
- [6] Z. Wang, M. Gerstein and M. Snyder. *Nat. Rev. Genet*. **2009**, 10, 57-63.
- [7] J. Xie, M. Bao, S. M. C. Bruekers and W. T. S. Huck. *ACS Appl. Mater. Interfaces*. **2017**, 9, 19630-19637.
- [8] H. B. Yang, K. T. Nguyen, D. T. Leong, N. S. Tan and C. Y. Tay. *ACS Appl. Mater. Interfaces*. **2016**, 8, 26591-26599.
- [9] H. B. Yang, N. M. J. Cheam, H. Cao, M. K. H. Lee, S. K. Sze, N. S. Tan and C. Y. Tay. *Adv. Health. Mater*. **2019**, 8, 1900929.
- [10] C. Y. Tay, C. G. Koh, N. S. Tan, D. T. Leong and L.P. Tan. *Nanomedicine (Lond)*. **2013**, 8, 623-38.
- [11] G. Biffi, T. E. Oni, B. Spielman, Y. Hao, E. Elyada, Y. Park, J. Preall and D. A. Tuveson. *Cancer Discov*. **2019**, 9, 282-301.
- [12] E. Berra, D. Roux, D. E. Richard and J. Pouyssegur. *EMBO reports*. **2001**, 2, 615-20.
- [13] C. D. Madsen, J. T. Pedersen, F. A. Venning, L. B. Singh, E. Moeendarbary, G. Charras, T. R. Cox, E. Sahai and J. T. Erler. *EMBO Rep*. **2015**, 16, 1394-408.

- [14] J. W. Kim, C. Evans, A. Weidemann, N. Takeda, Y. S. Lee, C. Stockmann, C. Branco-Price, F. Brandberg, G. Leone, M. C. Ostrowski and R. S. Johnson. *Cancer Res.* **2012**, 72, 3187-95.
- [15] K. O. Osuala, M. Sameni, S. Shah, N. Aggarwal, M. L. Simonait, O. E. Franco, Y. Hong, S. W. Hayward, F. Behbod, R. R. Mattingly and B. F. Sloane. *BMC Cancer.* **2015**, 15, 584.
- [16] K. Kesh, V. T. Garrido, A. Doesch, B. Durden, V. K. Gupta, N. S. Sharma, M. Lyle, N. Nagathihalli, N. Merchant, A. Saluja and S. Banerjee. doi: <https://doi.org/10.1101/2020.04.23.041509>
- [17] Y. Yu, C. H. Xiao, L. D. Tan, Q. S. Wang, X. Q. Li and Y. M. Feng. *Br. J. Cancer.* **2014**, 110, 724-32.
- [18] L. M. Wang, L. M. Cao, H. M. Wang, B. N. Liu, Q. C. Zhang, Z. W. Meng, X. Wu, Q. H. Zhou and K. Xu. *Oncotarget.* **2017**, 8, 76116-76128.
- [19] S. V. Puram, I. Tirosh, A. S. Parikh, A. P. Patel, K. Yizhak, S. Gillespie, C. Rodman, C. L. Luo, E. A. Mroz, K. S. Emerick, D. G. Deschler, M. A. Varvares, R. Mylvaganam, O. Rozenblatt-Rosen, J. W. Rocco, W. C. Faquin, D. T. Lin, A. Regev and B. E. Bernstein. *Cell.* **2017**, 171, 1611-1624 e24.
- [20] Z. Tang, C. Li, B. Kang, G. Gao, C. Li and Z. Zhang. *Nucleic. Acids. Res.* **2017**, 45, W98-W102.
- [21] N. Khromova, P. Kopnin, V. Rybko and B. P. Kopnin. *Oncogene.* **2012**, 31, 1389-97.
- [22] Q. Liu, A. Li, Y. Tian, J. D. Wu, Y. Liu, T. Li, Y. Chen, X. Han and K. Wu. *Factor Rev.* **2016**, 31, 61-71.
- [23] E. Voronov and R. N. Apte. *Cancer Microenviron.* **2015**, 8, 187-200.
- [24] I. Kaplanov, Y. Carmi, R. Kornetsky, A. Shemesh, G. V. Shurin, M. R. Shurin, C. A. Dinarello, E. Voronov and R. N. Apte. *Proc. Natl. Acad. Sci. USA.* **2019**, 116, 1361-1369.
- [25] J. Ding, K. Xu, J. Zhang, B. Lin, Y. Wang, S. Yin, H. Xie, L. Zhou and S. Zheng. *BMB Reports.* **2018**, 51, 630-635.
- [26] Y. Cheng, X. L. Ma, Y. Q. Wei and X. W. Wei. *Biochim. Biophys. Acta Rev. Cancer.* **2019**, 1871, 289-312.
- [27] L. Zhang, L. Zhang, H. Li, C. Ge, F. Zhao, H. Tian, T. Chen, G. Jiang, H. Xie, Y. Cui,

M. Yao and J. Li. *Sci. Rep.* **2016**, 6, 27426.

Chapter 8

Conclusions and Recommendations

This chapter summarizes all the research findings presented in this thesis. Overall, the thesis focuses on using the CoAl-IPN platform to examine the phenotypic plasticity of CAF. The detailed mechanisms by which matrix mechanics regulate CAF state and their paracrine activity crosstalk manners with the aid of RNA seq techniques are being highlighted. This chapter also describes the potential future work based on the current findings. In addition, other possible studies of the direct impact of matrix mechanics on the tumor invasiveness such as matrix stiffness-induced solid stress will also be proposed. According to the current understanding of the complex tumor microenvironment (TME), reconnaissance work regarding the possible exploitation of gelatin-based self-healing hydrogel for cell co-culture platform as a tumor surrogate is being presented.

8.1 Conclusions

The central aim of this thesis is to test the hypothesis that CAF phenotype, secretory activities and paracrine signaling could be modulated under the influence of ECM mechanics in a 3D setting. This is aided by exploitation of the mechanically tunable CoAl-IPN hydrogel platform as an *in vitro* tumor stroma surrogate.

By varying the crosslinker (Ca^{2+}) concentration (0, 15 and 100 mM), a stiffness gradient of CoAl-IPN hydrogel with Young's modulus (E) ranging from 100 to 1000 Pa was obtained, which covers the stiffness range of breast and colon tumor stroma. Despite the different matrix stiffness, all of the fabricated IPN hydrogels exhibit similar biochemical component (consistent collagen and alginate concentration), which allows precise study of the effect of matrix stiffness on the cells. In addition, the alginate network, even without crosslinking with Ca^{2+} ions, are well-entangled within the gelated collagen fibrils, indicating the high stability of CoAl-IPN hydrogel platform. Moreover, the Ca-crosslinked alginate network could be easily de-crosslinked with sodium citrate chelating agent, as such it endows the possibility of *in situ* tunability of the hydrogel mechanical properties to monitor the cellular behavior over time. Findings show that the microarchitecture would become denser with increasing alginate network crosslinking density, yet without adversely affecting the mass transport efficiency of the different IPN hydrogels that could ensure excellent cell survivability within the hydrogel. Collectively, the data implicate that the CoAl-IPN hydrogel is a versatile tumor stroma mimicking platform, which can be applied for the tumor parametric studies, such as ECM component, stiffness, architecture and matrix-induced solid stress.

Using the CoAl-IPN platform, the matrix stiffness effects on breast cancer associated fibroblast (b-CAF) phenotype (Cat. No. CAF06, Neuromics) and functional characteristics were explored. b-CAF encapsulated within 3D soft CoAl₀ hydrogel ($E \sim 100$ Pa) exhibited a spindle shape, while rounded morphology within stiff CoAl₁₀₀ hydrogel ($E \sim 1000$ Pa). Several *in vitro* experiments were designed to pinpoint the possible cause of the cell rounding activity by using different types of hydrogel system, including i) collagen soaked

in Ca^{2+} solution, ii) substituting the alginate component with RGD-conjugated alginate, and iii) decrosslinking of CoAl-IPN with sodium citrate. Findings confirmed that the rounding activity of b-CAF was not governed by the biochemical factors (Ca^{2+} in damaging the cell membrane or the crosslinking of alginate wrapping around the collagen fibers that hindered the interactions of b-CAF with collagen). Instead, the physical entrapment induced by the increased stiffness of CoAl-IPN hydrogel serves as a critical mechano-determinant of the b-CAF morphology, which was confirmed by the spreading of b-CAF upon decrosslinking of the stiff CoAl₁₀₀ hydrogel. At the mechanistic level, this phenomenon is accompanied, by a matrix-stiffness dependent regulation of the nuclear translocation of YAP/TAZ mechano-signaling protein and upregulation of b-CAF hallmark transcripts. Interestingly, a “soft” microenvironment was observed to support CAF pro-invasive paracrine signaling in 3D MDA-MB-231 tumoroids, while a stiff matrix would suppress the pro-invasive capacity of b-CAF. Collectively, the study is the first-time adopting CoAl-IPN hydrogel platform to reveal the mechano-response behaviors of b-CAF. The findings implicate that 1) the phenotype of b-CAF exhibit high plasticity characteristics; 2) b-CAF could reciprocate to the surrounding mechanical cues via mechano-regulation pathway in 3D context; and 3) the mechano-response of b-CAF would influence its functions on tumor invasiveness. However, the underlying mechanism is yet to be explored and required further investigation.

Considering the extremely slow growth rate of b-CAF, colon cancer associated fibroblast (c-CAF) was as the *in vitro* CAF for subsequent studies. Similarly, the stiff CoAl₁₀₀ hydrogel ($E \sim 1000$ Pa) could impose physical confinement on the c-CAF that limits the spreading of c-CAF, while the compliant and soft CoAl₀ hydrogel ($E \sim 100$ Pa) favor the cellular spreading in a 3D setting. Additionally, the cells coerced to adopt the round morphology would dampen their crosstalk to drive the epithelial-to-mesenchymal transition (EMT) potential of colon cancerous DLD-1 cells *in vitro*. These findings implicate that 1) c-CAF exhibit a similar mechano-responsive behaviors in 3D context to that of b-CAF; and 2) the stiffness-dependent behaviors of CAF within 3D CoAl-IPN matrix could be a universal phenomenon, providing important mechano-insights into the complex biological roles of CAF.

Finally, in order to provide a mechanistic understanding on how the CoAl-IPN hydrogel stiffness regulate CAF phenotype and functional characteristics, whole transcriptome profiling via RNA-sequencing and correlative analysis were conducted. Findings show that soft matrix CoAl₀ (E ~ 100 Pa) could result in myofibroblastic characteristic (m-state, $\alpha\text{SMA}^{\text{high}}$ IL-6^{low}) in the encapsulated c-CAF, whereas a stiff hydrogel CoAl₁₀₀ (E ~ 1000 Pa) promotes an inflammatory phenotype (i-state, $\alpha\text{SMA}^{\text{low}}$ IL-6^{high}) in the encapsulated c-CAF. Interestingly, the reactive oxygen species (ROS)-hypoxia inducible factor 1 alpha (HIF1- α) was revealed as a novel mechano-redox transduction axis that can be conditionally switched “on” and “off” in i- and m- state CAF respectively. The non-biased GEPIA analysis suggests that the growth factors/cytokines, such as downregulation of VEGFC and upregulation of INHBA, IL8, IL1A/B, CXCL2/3 are more likely to be exerting anti-tumorigenic properties in restraining cancer cells’ EMT process. Collectively, the findings suggest that 1) the ECM mechanics play a role in regulating the phenotypic switch between different CAF sub-types; 2) the matrix stiffness could be a promising therapeutic target to disrupt the paracrine crosstalk between CAF and cancer cells; and 3) The mechano-redox transduction axis provides novel biomarkers for CAF function diagnosis and study.

In summary, the thesis addressed the hypothesis, and successfully demonstrated the feasibility of using the CoAl-IPN hydrogel platform as tumor stroma matrix proxy for tumor microenvironment (TME) study. In the process, both b-CAF and c-CAF were adopted for their mechano-response studies in 3D context, and RNA-seq was applied for transcriptome stratification and correlation analysis. Taken together, the findings in this thesis shed important insights on the role of CoAl-IPN matrix stiffness on the CAF phenotype and pro-tumor potential, as well as provide a mechanistic understanding of the CAF heterogeneity at transcript level. Besides CAF mechanobiology, the CoAl-IPN hydrogel platform is technically amenable to investigate the effects of ECM mechanics on tumor progression involving other cell types.

8.2 Reconnaissance and Future Work

The obtained data suggest that IPN is a versatile platform to study crosstalk between CAF and tumor cells. However, its application can be extended in the broader context of tumor mechanobiology, such as examining matrix-stiffness-induced breast tumoroids invasiveness and crosstalk studies of other stroma cell types and cancer cells.

8.2.1 Understand the role of ROS in matrix stiffness-induced breast tumoroids invasiveness

8.2.1.1 *The relationship between matrix stiffness-induced solid stress and breast tumoroids invasiveness*

The relationship between tumor stiffness and solid stress can generally be described using the analogy of a spring of specific elastic modulus that obeys Hooke's law (**Chapter 2, section 2.1.1.3**). According to the equation of Hooke's law for linear elastic materials, the Young's modulus (E) is the ratio of stress (σ) to the corresponding strain (ϵ). Kalli *et al.* has proposed a theoretical mathematical model to understand the relationship between matrix stiffness and matrix stiffness.[1, 2] When a tumor of elastic modulus E_T grows, it exerts an outward force and pushes the surrounding host tissue of elastic modulus E_{HT} . As such, it leads to a deformation ϵ_T and a subsequent stress σ_T . Consequently, the host tissue returns an equal and opposite stress σ_{HT} , which is also known as 'externally applied solid stress'. Concurrently, there is a growth-induced stress σ_G accumulated in the tumor interior owing to the mechanical interactions among the tumor components. Overall, the total solid stress, which is accumulated intratumorally is defined by the sum of the externally applied stress and the growth-induced stress. Along with the solid stress, the tumor interior experiences radial and circumferential compression, while the surrounding of tumor periphery experiences radial compression and circumferential tension.[3, 4]

In the case where the tumor is stiffer than surrounding tissue ($E_T > E_{HT}$), the tumor can

lead to greater displacement and deformation in the host tissue with much greater σ_T than σ_{HT} to push against the normal tissue and grow in size. In the theoretical mathematical model proposed by Kalli *et al.*, a tumor stiffness of more than 1.5 times of surrounding tissue is required in order to displace the host tissue and grow.[5] As observed in **Figure 8.2.1.1**, the growth of tumoroids was observed in both CoAl₁₅ (E ~ 450) and CoAl₁₀₀ (E ~ 1200) hydrogels. As such, the stiffness of the encapsulated tumoroids was estimated to be in the range of 450 – 1350 kPa (i.e. 1.5× greater than the matrix stiffness), which is close to the range of 1 – 2 kPa as reported by others using pure alginate capsules to measure the encapsulated MDA-MB-231.[6, 7] Hence, further validating the proposed model illustrated by Kalli *et al.* regarding the effect of matrix stiffness in tumoroids behaviors.

Figure 8.2.1.1 Tumoroids response before and after de-crosslinking alginate network within CoAl-IPN hydrogel (Day 3). (a) Brightfield images of representative tumoroids in CoAl₀, CoAl₁₅ and CoAl₁₀₀ IPN hydrogels. Scale bar = 100µm. (b) Tumoroid sizes between CoAl₁₅ and CoAl₁₀₀ tumoroids at day 3. (c) Calculated increased percentage of tumor area in CoAl₁₅ and CoAl₁₀₀ hydrogels after 3 days culture. * $p < 0.05$.

Between the two hydrogel systems, different tumor growth and invasion behaviors were observed. The encapsulated tumoroids experience different degrees of resistance force generated from the surrounding hydrogel matrix, thus resulting in the difference in tumoroid sizes. Similarly, this confinement effect that is highly dependent on the matrix stiffness could potentially lead to the accumulation of solid stress (i.e. matrix stiffness-induced solid stress) and the subsequent tumor invasion. A study by Gabriel Helminger *et al.* adopted an agarose hydrogel system with varying stiffness to estimate the core compression stress and peripheral tensile stress experienced by the encapsulated tumoroids. In that particular study, 0.5% (~ 2 kPa) and 1% (~ 4 kPa) agarose hydrogels were used to culture tumoroids over 5 days. The initial size of the tumoroids was ~ 20 μm , while the final size of the tumoroids encapsulated within soft and stiff agarose was found to be ~ 90 and ~ 30 μm , respectively. The reduced tumoroid size observed in the stiff hydrogel is due to the confinement effect and matrix stiffness-induced solid stress experienced by the agarose hydrogel.[8] Mathematical modeling analysis has estimated that the core compression stress and peripheral tensile stress experienced by the tumoroids encapsulated in soft 0.5% agarose were found to be 6.0 mmHg (800 Pa) and 7.0 mmHg (930 Pa), respectively. On the other hand, the corresponding stiff 1% agarose led to the core compression stress and peripheral tensile stress of 9.0 mmHg (1200 Pa) and 10.5 mmHg (1400 Pa), thus confirming that the matrix stiffness has a direct relationship to the tumor solid stress.[9] This built-up of solid stress could potentially lead to the greater tumoroid activity, such as tumoroid invasion upon removal of the confinement effect during stress relaxation test.

In this case, tumoroids with initial size of 107.14 μm were encapsulated within CoAl-IPN hydrogel system and cultured for 3 days, in which the final diameter of the encapsulated tumoroids was found to be 133.47 and 112.85 μm for soft CoAl₁₅ and CoAl₁₀₀ hydrogels, respectively. Assuming that i) human colon adenocarcinoma cells (LS174T) and human breast adenocarcinoma cells (MDA-MB-231) have similar proliferation rate, ii) the hydrogel stiffness follows a simple linear relationship with the tumor solid stress, and iii) in accordance to the theoretical mathematical model proposed by Tiina Roose *et al.*, the interior compression stress and peripheral tensile stress experienced by the tumoroids

encapsulated within CoAl₁₅ hydrogel can be estimated to be in the range of 130-200 Pa and 150-250 Pa, respectively. As such, the core compression stress and peripheral tensile stress experienced by the tumoroids encapsulated within CoAl₁₀₀ can be estimated to be in the range of 400-600 Pa and 450-700 Pa, respectively.

Based on an *in vitro* 2D study,[10] compressive stress of around 200-600 Pa will lead to the upregulation of invasive phenotype in MDA-MB-231 breast cancer cells via Pizo1 activation. In addition, a greater increase in the invasive phenotype was observed with increasing substrate stiffness. Similar findings were being observed in demonstrating the impact of compressive stress on tumor cells invasion, both in 2D [11] and 3D context.[12] Overall, the findings confirm that the relatively higher solid stress built-up in the tumoroids encapsulated within CoAl₁₀₀ hydrogel has led to the greater tumoroids invasiveness compared to the compliant CoAl₁₅ hydrogel. In the future, the build-up solid stress needs to be further thoroughly measured.

8.2.1.2 *The relationship between mechanical stress and ROS generation within tumoroids*

8.2.1.2.1 *The relationship between ROS and tumor invasiveness*

ROS are highly reactive oxygen-containing molecules generated as a by-product of cellular metabolism,[13] in which the accumulation of ROS molecules will trigger oxidative stress in the cells. As such, the ROS levels are constantly regulated by the healthy cells by managing the redox homeostasis and antioxidant signaling. However, under unfavorable conditions, the cells may loss the capacity to maintain intracellular homeostasis, thus leading to oxidative-stress-induced damage and even death.[14] Studies have shown that the cancer progression is highly associated with increased levels of ROS generated by mitochondria as a result of mutations and polymorphisms of the mitochondria in affecting the proper mitochondrial function.[15-17] Even though the molecular mechanisms of ROS-driven tumor progression are not well understood, but it is important to consider that sublethal concentrations of ROS are second messengers that upregulated the expression of

pathways involved in tumor growth and metastasis.[18] On the other hand, increased expression of mitochondrial targeted catalase (mCAT), which is an antioxidant enzyme has successfully reduced the tumor invasiveness and metastasis by the activation of p38MAPK marker and quenching of the intracellular ROS.[19, 20] Collectively, these studies clearly showed that redox signaling plays a significant role as a cellular messaging system in regulating tumor invasion and metastasis.

8.2.1.2.2 The effect of mechanical stress on ROS generation

The effect of mechanical stress has been evaluated in various types of tissues. For instance, it has been reported that ROS production from mitochondrial complex I could be enhanced in response to mechanical insult in articular cartilage. Additionally, by introducing actin polymerase inhibitor, such as cytochalasin B to the articular cartilage cells, an increased in ROS production was observed as a result of cytoskeletal distortion under excessive compressive strain.[21] The interplay between the cellular redox metabolism and physical forces is also well-studied in endothelial cells as the physiologic shear stress (regular pulsatile or laminar flow) is crucial in maintaining the normal vascular functions.[22] For example, the elevated or irregular turbulent/oscillatory shear stress under abnormal conditions has shown to promote upregulation of Rac1 activity in the endothelial cells, which fueled the activation of NF- κ B and ROS production.[23] In the case of ischemia, irregular shear stress will lead to the PI3K/AKT activation and ROS production. [24] Similarly, the impact of physical stimulation on the activation of intracellular ROS was examined using cancerous cells. For example, lung cancer cells show an increased production of ROS in response to shear stress.[25, 26] In addition, continuous oscillatory shear causes a sustained activation of pro-oxidant processes resulting in redox-sensitive gene expression in human endothelial cells.[27] Collectively, several studies have demonstrated mechanistic links between mechanical stress and intracellular redox state.

8.2.1.2.3 *The potential effect of ROS level on tumoroids invasiveness*

The findings have shown that the increased in matrix stiffness gradually lead to the accumulation of solid stress experienced by the tumor, thus affected the tumor invasion and its metastasis process. On the other hand, mechanical stress has also been shown to regulate the activation of ROS in other studies. Thus, the potential ROS-mediated signaling mechanism of the matrix stiffness-induced solid stress on tumoroids invasiveness is being proposed to have better understanding on the mechanistic influence on tumor progression.

In the future, more studies, such as effect of ROS inhibition on tumor invasiveness and ROS intensity study within the well-developed CoAl-IPN hydrogels should be conducted to explore the ROS-mediated signaling pathway associated with the matrix stiffness-induced solid stress on tumor progression.

8.2.2 Gelatin-based self-healing hydrogel to engineer tumor microenvironment

Using a transwell system, it has been shown that matrix stiffness could regulate CAF phenotype, which in turn could affect the functional paracrine signalling with tumor cells. (**Chapter 5 - 7**). However, in the actual tumor settings, mechanical continuity exist between the tumor mass and stroma. Although it is possible to directly mix two different cell types within a single matrix, such strategy is insufficient to emulate the precise spatial distribution of the tumor and stroma compartments.[28] Alternatively, microfluidic co-culture systems could be employed. However, such an approach would require the utilization of advanced micro-fabrications techniques and extensive optimization [29,30]. Therefore, a gelatin-based self-healing hydrogel is being developed to capture these spatiotemporally complex interactions between the CAF and cancer cells *in vitro*. It is envisioned that the successful development of such 3D co-culture systems will be useful for drug screening applications as it is a better representative of the 3D tumor tissue mimicking platform.

Briefly, the development of the self-healing hydrogel involves the synthesis of oxidized dextran, prior to mixing with pristine gelatin in forming an oxidized dextran/gelatin precursor. Subsequently, gelation of the oxidized dextran/gelatin precursor will take place via dynamic Schiff base reaction between aldehyde groups from oxidized dextran and amine groups from gelatin, in which the gelation process will be completed within 10 min to form the self-healing hydrogel. In order to produce a biomimicking TME, CAF or tumoroids can be pre-incorporated into the oxidized dextran/gelatin precursor prior to gelatin separately. Upon gelation, the CAF-Odex/Gel and tumoroids-Odex/Gel will be placed together to initiate the self-healing process. Thereafter, the interactions between the CAF and tumoroids (i.e., migratory behavior) will be monitored. Preliminary data (**Figure 8.2.2.1**) shows the self-healing capacity of the Odex/Gel hydrogel. In addition, the cells can be evenly distributed throughout the hydrogel owing to the high viscosity of the Odex/Gel precursor. Moreover, the fabricated self-healing hydrogel is highly cytocompatible and stable, which supported the growth and viability of the cells over 14 days of culture. Thus, making it a suitable platform for the proposed study.

Figure 8.2.2.1 The design and biocompatibility of gelatin based self-healing hydrogel. Self-healing capacity of fabricated hydrogel (a) and the underlying mechanism (dynamic Schiff base) (b). c) The biocompatibility of hydrogels for both colon DLD-1 cancer cells and colon associated fibroblasts.

References

- [1] M. Kalli and T. Stylianopoulos. *Front. Oncol.* **2018**, 8, 55.
- [2] A. D. R. Hernández, A. Vella, E. M. Eko and A. D. R. Hernández. *Emerg. Top. Life Sci.* **2018**, 2, 739-749.
- [3] T. Stylianopoulos. *J. Biomech. Eng.* **2017**, 139.

- [4] H. T. Nia, H. Liu, G. Seano, M. Datta, D. Jones, N. Rahbari, J. Incio, V. P. Chauhan, K. Jung, J. D. Martin, V. Askoxylakis, T. P. Padera, D. Fukumura, Y. Boucher, F. J. Hornicek, A. J. Grodzinsky, J. W. Baish, L. L. Munn and R. K. Jain. *Nat. Biomed. Eng.* **2016**,1.
- [5] C. Voutouri, F. Mpekris, P. Papageorgis, A. D. Odysseos and T. Stylianopoulos. *PLoS One.* **2014**, 9, e104717.
- [6] K. Alessandri, B. R. Sarangi, V. V. Gurchenkov, B. Sinha, T. R. Kiessling, L. Fetler, F. Rico, S. Scheuring, C. Lamaze, A. Simon, S. Geraldo, D. Vignjevic, H. Domejean, L. Rolland, A. Funfak, J. Bibette, N. Bremond and P. Nassoy. *Proc. Natl. Acad. Sci. USA.* **2013**, 110, 14843-8.
- [7] P. V. Liedekerke, J. Neitsch, T. Johann, K. Alessandri, P. Nassoy and D. Drasdo. *PLoS Comput. Biol.* **2019**, 15, e1006273.
- [8] G. Helmlinger, P. A. Netti, H. C. Lichtenbeld, R. J. Melder and R. K. Jain. *Nat. Biotech.* **1997**, 15, 778-783.
- [9] T. Roose, P. A. Netti, L. L. Munn, Y. Boucher and R. K. Jain. *Microvasc. Res.* **2003**, 6, 204-212.
- [10] M. Luo, K. K. Y. Ho, Z. T. Deng and A. P. Liu. doi: <https://doi.org/10.1101/513218>
- [11] J. M. Tse, G. Cheng, J. A. Tyrrell, S. A. Wilcox-Adelman, Y. Boucher, R. K. Jain and L. L. Munn. *Proc. Natl. Acad. Sci. USA.* **2012**, 109, 911-6.
- [12] T. Morikura and S. Miyata. *Micromachines (Basel).* **2019**, 10.
- [13] J. S. Armstrong and M. Whiteman. *Mitochondria, 2nd Edition.* **2007**, 355-377.
- [14] T. Finkel and N. J. Holbrook. *Nature.* **2000**, 408, 239-247.
- [15] L. Enns and W. Ladiges. *J. Bioenerg. Biomembr.* **2012**, 44, 635-8.
- [16] P. L. D. S. Junior, D. A. D. Camara, A. S. Porcacchia, P. M. M. Fonseca, S. D. Jorge, R. P. Araldi and A. K. Ferreira. *Oxid. Med. Cell Longev.* **2017**, 2467940.
- [17] R. K. Bai, S. M. Leal, D. Covarrubias, A. Liu and L. J. C. Wong. *Cancer Res.* **2007**, 67, 4687-4694.
- [18] M. Nishikawa. *Cancer Lett.* **2008**, 266, 53-9.
- [19] J. Goh, L. Enns, S. Fatemie, H. Hopkins, J. Morton, C. PETTAN-Brewer and W. Ladiges. *BMC Cancer.* **2011**, 11, 191.
- [20] S. E. Schriener, N. J. Linford, G. M. Martin, P. Treuting, C. E. Ogburn, M. Emond, P.

- E. Coskun, W. Ladiges, N. Wolf, H. V. Remmen, D. C. Wallace and P. S. Rabinovitch. *Science*. **2005**, 308, 1909-1911.
- [21] M. J. Brouillette, P. S. Ramakrishnan, V. M. Wagner, E. E. Sauter, B. J. Journot, T. O. Mckinley and J. A. Martin. *Biomech. Model Mechanobiol.* **2013**, 13, 565-572.
- [22] J. C. Tung, J. M. Barnes, S. R. Desai, C. Sistrunk, M. W. Conklin, P. Schedin, K. W. Eliceiri, P. J. Keely, V. L. Seewaldt and V. M. Weaver. *Free Radic. Biol. Med.* **2015**, 79, 269-80.
- [23] E. Tzima, M. A. D. Pozo, W. B. Kiosses, S. A. Mohamed, S. Li, S. Chien and M. A. Schwartz. *The EMBO J.* **2002**, 21, 6791-6800.
- [24] S. Chien. *Am. J. Physiol. Heart Circ. Physiol.* **2007**, 292, H1209-24.
- [25] K. Y. Lo, Y. Zhu, H. F. Tsai and Y. S. Sun. *Biomicrofluidics*. **2013**, 7, 64108.
- [26] S. N. Ghadiali and D. P. Gaver. *Respir. Physiol. Neurobiol.* **2008**, 163, 232-43.
- [27] G. W. D. Keulenaer, D. C. Chappell, N. Ishizaka, R. M. Nerem, R. W. Alexander and K. K. Griendling. *Shear stress and endothelial redox state*. **1998**, 1094-1101.
- [28] D. L. Holliday, K. T. Brouillette, A. Markert, L. A. Gordon and J. L. Jones. *Breast Cancer Res.* **2009**, 11, R3.
- [29] S. Y. Jeong, J. H. Lee, Y. Shin, S. Chung and H. J. Kuh. *PLoS One*. **2016**, 11, e0159013.
- [30] K. Alessandri, M. Feyeux, B. Gurchenkov, C. Delgado, A. Trushko, K. H. Krause, D. Vignjevic, P. Nassoy and A. Roux. *Lab Chip*. **2016**, 16, 1593-604.