The role of the tissue microenvironment in resistance and susceptibility of the rat mammary gland to carcinogenesis

A thesis submitted by

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ABSTRACT

N-nitrosomethylurea (NMU) administration to rats of several susceptible strains leads to rapid, reproducible, tumor production. The Copenhagen (COP) rat strain, however, is completely resistant to NMU carcinogenesis. The cause of this resistance is yet unknown. Using tissue recombination experiments, our lab has shown that the mammary gland stroma is the “gatekeeper” for tumorigenesis; also, NMU-exposed stroma transformed non-exposed mammary epithelial cells (MECs) while non-exposed stroma combined with exposed MECs produced normal ductal structures. Here, I hypothesize that the mammary tumor-resistant phenotype of COP rats is due to stromal-mediated stabilization of tissue-wide disruptions following carcinogenic exposure while susceptible strains fail to stabilize such disruptions.

In order to generate a surrogate model for tissue recombinations, I proposed a three-dimensional (3D) culture model that recapitulates the biochemical and biophysical forces required for the formation of normal structures found in the mammary gland. I detail the characterization of an epithelial cell line for use in hormone-responsive 3D culture. Co-culture of human MECs with rat fibroblasts in floating collagen gels results in the formation of multicellular structures. NMU treated fibroblasts from susceptible Wistar-Furth (WF) animals are involved in the formation of highly variable structures with low biological quality, a measure of an epithelial structures similarity to a duct-like structure found in the intact mammary gland. Expression data derived from COP and WF cleared fat pads following NMU exposure is analyzed.

I propose to study stromal factors involved in COP mammary tumor resistance and further, to compare stromal-epithelial interactions between resistant COP rats and Wistar-Furth rats, an NMU-susceptible rat strain. This work will give new insights into

the understanding of COP resistance and broaden our understanding of stromal contributions to induction or inhibition of carcinogenesis in both of these rat strains. Those insights, in turn, will better inform us about carcinogenesis in humans. Theoretical challenges to the current prevailing theory of carcinogenesis are given and used to explain the thesis findings as well as put into context how altered development influences tumor susceptibility. The outcomes of the experiments detailed here are important for furthering our understanding of the process of tumorigenesis, provide a possible explanation for tumor remission without clinical intervention and, more broadly, aim to widen the focus of treatment beyond the breast epithelial compartment.

DEDICATION

This thesis is dedicated to the memory of Patrick McDonough. Patrick suffered through months of chemotherapy and surgeries while cancer refused to leave his liver. The process wreaked havoc on his body but it never interfered with his kindness or sense of humor. Our final day together, in the Massachusetts General Hospital Cancer Center where my cancer research experience had begun, we watched a movie and didn't say a word about disease or surgeries. Sometimes it is easy to forget the connection between the lab bench and the hospital bed but my frustration while watching Patrick struggle through sickness *and* treatment reminded me how important that connection is. I hope my little tiny contribution to the field will advance our understanding of cancer and maybe, someday, help us survive it.

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TABLE OF CONTENTS

Title Page..i

Abstract..ii

Dedication..iv

Acknowledgements..v

Table of Contents..viii

List of Tables..xii

List of Figures..xiii

List of Copyrighted Materials Produced by the Author..xiv

List of Abbreviations..xv

Chapter 1: Introduction..1
- 1.1 Introduction to Theoretical Development..1
  - 1.1.1 The Big Picture..1
  - 1.1.2 Theories in Biology..3
  - 1.1.3 Theoretical Framework..7
- 1.2 Theories of Cancer..8
  - 1.2.1 The Somatic Mutation Theory..8
  - 1.2.2 Evidence Challenges the Premises Adopted by the SMT..10
    - 1.2.2.1 Mutation Status Does Not Correlate with Disease..10
    - 1.2.2.2 Foreign Body Carcinogenesis..12
  - 1.2.3 Introduction to the Tissue Organization Field Theory of Carcinogenesis..13
- 1.3 Breast Cancer..18
  - 1.3.1 Risk Assessment..19
    - 1.3.1.1 Lifestyle factors..20
    - 1.3.1.2 Reproductive History..20
    - 1.3.1.3 *In Utero* exposures..21
  - 1.3.2 Breast Cancer Hormone Dependency..22
  - 1.3.3 Neoplastic Remission..23
  - 1.3.4 Conclusions..24
- 1.4 Role of Stroma in Mammary Gland Development and Carcinogenesis..24
  - 1.4.1 Mammary Gland Development..24
    - 1.4.1.1 Fetal Development..27
    - 1.4.1.2 Development during puberty and pregnancy..28
    - 1.4.1.3 Involution..29
  - 1.4.2 The Role of the Stroma in Cancer..30
    - 1.4.2.1 Induction of Tumors by Manipulation of the Stroma..31
    - 1.4.2.2 Normalization of the Neoplastic Phenotype by the Stroma..33
- 1.5 Introduction to the Copenhagen Rat..34
- 1.6 Why 3D Culture?..37
  - 1.6.1 3D Cellular Growth..38

1.6.1.1 Collagen..38
1.6.1.2 Reconstituted Basement Membrane.................................39
1.6.2 Growth of Mammary Epithelial Cells in 3D....................................40
1.6.3 Cancer Cell Phenotype in 3D...41
1.6.4 Stromal/Epithelial Cell Co-culture in 3D..42
1.7 Thesis, Hypothesis and Aims...44
1.7.1 Specific Aim 1...45
1.7.2 Specific Aim 2...46
1.7.3 Specific Aim 3...47
1.7.4 Concluding Statement..48

Chapter 2: Environmental Endocrine Disruptors and their Effects on the Human Male Reproductive System...49
2.1 Introduction...50
2.1.1 What is an EDC? 51
2.1.2 How do EDCs Exert Their Effects?...51
2.2 EDCs in Development and Cancer..55
2.3 EDCs in Prostatic Development and Cancer...59
2.3.1 Prostate Gland Development...59
2.3.2 Prostate Cancer..60
2.3.3 Effects of EDCs on Human Prostate Glands...................................61
2.3.4 Effects of EDCs on Rodent Prostate Glands...................................63
2.4 EDCs in Testicular Development and Cancer..64
2.4.1 Testicular Development...64
2.4.2 Testicular Dysgenesis Syndrome...65
2.4.3 EDCs in Testicular Development and Cancer.................................66
2.4.4 Are EDCs linked to TC and TDS in Humans?.................................67
2.4.5 Animal Studies Linking EDCs to TDS andTC.................................69
2.5 EDCs in Male Mammary Gland Development and Cancer.............................70
2.5.1 Male Mammary Gland Development...71
2.5.2 Cancer and Associated Diseases of the Male Breast........................71
2.5.3 EDCs and Human MBC Cases..72
2.5.4 EDCs Linked to Conditions Associated with MBC in Humans........73
2.5.5 EDCs and MBC Associations – Evidence from Animal Studies.......73
2.6 Critical Analysis of EDC Research...75
2.7 Conclusions...78

Chapter 3: Investigating the Role of Stromal Fibroblasts in Copenhagen Rat Mammary Gland Tumor Resistance...80
3.1 Introduction...81
3.1.1 Potential Explanations of COP Mammary Cancer Resistance..........82
3.2 Materials and Methods...84
3.2.1 Cell Maintenance...84
3.2.2 Animals...84
3.2.3 Isolation of rat mammary fibroblasts and epithelial cells................85
3.2.4 Renal subcapsular recombination...86
3.2.5 3D co-culture in mixed gels...87
3.2.6 SAMA analysis..87
3.2.7 RNA isolation for microarray...87
3.2.8 Microarray analysis...88

3.3 Results..88
3.3.1 Kidney capsule recombination..88
3.3.2 3D culture..92
3.3.3 SAMA identifies differences in MCF10A and MCF10A+RMF..........93
3.3.4 COP fibroblasts alter MCF10A morphology....................................94
3.3.5 Exposure status of WF fibroblasts alters morphology......................96
3.3.6 Microarray Quality Control..97
3.3.7 WF and COP glands have distinct gene expression patterns...........98
3.3.8 Differential gene expression analysis..100
3.3.9 Associations with processes and diseases reveal strain differences..102
3.4 Discussion..105
3.4.1 Tissue recombinations..105
3.4.2 3D co-culture gels..105
3.4.3 Microarray findings...109

Chapter 4: Characterization of MCF-12A cell phenotype, response to estrogens, and growth in 3D..112
4.1 Background..113
4.2 Methods..115
4.2.1 Cell maintenance..115
4.2.2 Immunocytochemistry..116
4.2.3 Single cell cloning..118
4.2.4 Dose-response to estradiol..118
4.2.5 Estrogen-regulated gene induction assays.....................................118
4.2.6 3D cell culture..119
4.2.7 Analysis of epithelial structures..120
4.2.8 Statistical analysis...121
4.3 Results..121
4.3.1 Description of parental cells..121
4.3.2 Single cell cloning observations...122
4.3.3 Epithelial, myoepithelial, and mesenchymal marker expression in parental MCF12A cells...123
4.3.4 MCF12A cells proliferate equally with or without E2....................124
4.3.5 MCF12A cells lack expression of ER and its transcriptional targets..125
4.3.6 MCF12A cells form ducts and acini in 3D.....................................126
4.3.7 MCF12A morphogenesis varies due to cell-subtype dynamics.......128

4.4 Discussion..129
4.4.1 Morphology and cell-type markers..130
4.4.2 Estrogenicity..131
4.4.3 Growth in 3D..133
4.4.4 SAMA as a tool to analyze epithelial structures grown in 3D..... ...133
4.5 Conclusions..135

Chapter 5: Discussion..136
5.1 Outcomes of specific aims..136
5.2 Comments on the COP project...141
5.3 Project synergy..142
5.4 My journey to a PhD...143

5.5 Normal vs. Disease..147
5.6 Concluding Comments..148

Chapter 6: Bibliography..152

LIST OF TABLES

1.1 Results of recombinations by Maffini et. al...32
3.1 Tissue Recombination experimental groups...86
3.2. Characterization of mammary structures found in transplanted grafts....................89
4.1 Cell culture media preparations...116
4.2 List of antibodies used for immunohistochemistry...117
4.3 Estrogen responsive gene induction assay primer sequences..................................120

LIST OF FIGURES

3.1 Immunohistochemistry for Ki67, ER alpha and CK..90
3.2 Identification of myoepithelial cells by immunohistochemistry for SMA..................91
3.3 PAS staining showing the presence or absence of basement membrane....................92
3.4 Morphometric analysis of MCF10A structures grown alone and with RMFs.............94
3.5 Morphometric analysis of MCF10A structures grown with COP fibroblasts..............95
3.6 Morphometric analysis of MCF10A structures grown with WF fibroblasts................97
3.7 Principal component analysis of processed expression data......................................98
3.8 Heat map of ~5,000 genes reveals distinctly different expression patterns between COP and WF glands...99
3.9 Top 8 altered disease and process ontologies in COP and WF following NMU exposure..104
4.1 MCF12A cells grow as a heterogeneous population...122
4.2 MCF12A cells express epithelial and mesenchymal markers....................................124
4.3 MCF12A cells do not respond to stimulation by estradiol...126
4.4 3D growth of MCF12A cells in rat-tail collagen after 14 days...................................127
4.5 Contraction of collagen gels after 14 days in culture...128
4.6 Non-epithelial-like subpopulations alter the morphology of epithelial-like MCF-12A cells...129

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Sweeney, M. F., Hasan, N., Soto, A. M., & Sonnenschein, C. (2015). Environmental Endocrine Disruptors: Effects on the human male reproductive system. *Reviews in Endocrine & Metabolic Disorders*, *16*(4), 341–357. http://doi.org/10.1007/s11154-016-9337-4

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LIST OF ABBREVIATIONS

AGD	anogenital distance
AR	androgen receptor
BC	breast cancer
BM	basement membrane
BPA	bisphenol A
BPH	benign prostatic hyperplasia
CIS	carcinoma *in situ*
COP	Copenhagen rat
DBP	dibutyl phthalate
DCE	t-1,2-dichloroethylene
DDT	dichlorodiphenyltrichloroethane
DEHP	diethylhexyl phthalate
DES	diethylstilbestrol
DMEM	Dulbecco's modified Eagle's media
ECM	extracellular matrix
EDC	endocrine disrupting chemical
ER	estrogen receptor
GWAS	genome wide association study
IDP	intraductal hyperplasia
INSL3	insulin-like growth factor 3
MBC	male breast cancer
MEC	mammary epithelial cell
MEHP	mono(2-ethylhexyl) phthalate
MG	mammary gland
miR	micro-RNA
NMDR	non-monotonic dose response
NMU	n-nitrosomethylurea
PC	prostate cancer
PCB	polychlorinated biphenyls
PCE	tetrachloroethylene
PGC	primordial germ cells
PR	prolactin receptor
rBM	recombinant basement membrane
RMF	reduction mammoplasty fibroblasts
SMT	somatic mutation theory of carcinogenesis
TC	testicular cancer
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCE	trichloroethylene
TDS	testicular dysgenesis syndrome
TEB	terminal end bud
TGCT	testicular germ cell tumors
TOFT	tissue organization field theory of carcinogenesis
VEGF	vascular endothelial growth factor
VEH	vehicle
WF	Wistar-Furth rat strain

CHAPTER 1: INTRODUCTION

"The concern of science is to formulate theories to discover patterns of relations among vast kinds of phenomena in such a way that a small number of principles can explain a large number of propositions concerning these phenomena." - Ayala [1]

1.1 Introduction to Theoretical Development

1.1.1 The Big Picture

Humans and animals interpret their environment through their senses. Integration and reasoning in humans help in making decisions based on these sensations. In this regard, we observe the universe through an imperfect corporeal window. Although the interpretation of the environment is crucial for our survival, the world is not always the way it appears for us to be. Optical illusions, for example, exploit biological limitations of our retinal rod and cones and interfere with our ability to make sense of what we see by breaking our vision perception "rules." Similarly, common sense is a shared set of "rules" that aid in circumventing extended interpretation of sensations. However, common sense is biased, flawed, and can often be entirely wrong.

Common sense arises from our immediate experience of the world around us and is not useful to the development of scientific ideas. Scientific thought arose out of natural philosophy as humans started to ask questions about the nature of the world around them. Our conceptual understanding of these elements should be devoid of inherent biases like those imposed by common sense (i.e., the use of terms like "sunrise" and "sunset" in common language despite proof of a heliocentric universe). Because we can only experience the world around us subjectively, we need theories to understand the world objectively. Theories, in place of common sense, "provide organizing principles and construct objectivity by framing observations and experiments[2]." Theories are

crucial because they answer what it is that we can observe and how we can frame the experience of those observations.

The history of theoretical development in math and physics has provided us with examples of how theories drive scientific progress. Boyle and Newton proposed Aether theories to explain light's simultaneous wave- and particle-like behaviors. Although the existence of Aether was deemed unlikely by experiments starting with the Michelson-Morley Experiment, those experiments performed in the intervening years generated vast amounts of information regarding the motion of planets and the behavior of light. Similarly, the theory of phlogistons, chemicals believed to be present in combustible materials, led to brilliant experiments exploring the nature of elements and the usefulness of electricity. These theories, though eventually proven wrong, framed the observations and experimental design of their practitioners by providing a means to organize experimental results and construct objectivity.

Theories help to frame observations until those observations no longer support the theory. Accretion of unexpected experimental results that are contrary to an existing theory provide an opportunity for reassessment. Scientists determine what parts of the theory are inaccurate by revising their postulates based on the new evidence. Once a theory is found to be wrong, the intervening time before the establishment of a revised theory is often accompanied by the abandonment or reassessment of old tools and the development of new ones. Suddenly, things that appeared one way, such as the movement of a pendulum, are viewed completely differently, as if they are seen with new eyes [3]. This "paradigm shift" is a purely human experience; the phenomena have not changed but the objectivity with which they are viewed is forever changed.

While a “wrong” theory can prove to be quite powerful in leading to our collective understanding, a vague theory presents a real problem to scientific progress. In 1964, Richard Feynman said “…you cannot prove a *vague* theory wrong. If the guess that you make is poorly expressed and rather vague, and the method that you use for figuring out the consequences is a little vague…then you see that this theory is good, because it cannot be proved wrong!” For this reason, mathematicians and physicists spend a great deal of time working in the theoretical realm. A vague theory applied to a rocket launch or particle accelerator would result in huge losses and potentially dangerous consequences.

1.1.2 Theories in Biology

So, why has biology been so slow to adapt to the lessons from mathematics and physics? If biology takes place in the same universal realm as physics, should it not benefit from similar scientific principles? Aristotle implied that nature adapts organs to their functions, not functions to organs [4]. Teleological thinking such as this seems to involve backwards causation; the function of a process is its cause. Despite efforts to reduce the use of teleological language in biological education (“the kidney’s function is to filter waste,”) goal-oriented descriptions abound in biology. Forms of determinism, the belief that all events are completely determined by external antecedent events, have long burdened biology (and medicine) throughout its history. Especially during the ancient and Middle ages the world was viewed as being a product of a creation that exists unchanging. Animals and humans were considered to be static forms that were directed by fate. Genetic determinism posits that everything in biology can be reduced to genes because genes are the only means to store transmissible information [5]. Dawkins and other biologists championed the idea that development is simply an unfolding of a genetic program, and, in effect, that organisms are limited in form and function to only

minor variations of the genes they are born with, isolated from outside influences. However, variability does not only arise from genes; it abounds in nature and is crucial to the theory of evolution. The concept of univocal gene-phenotype relationships denies the complexity of organisms, their history, and the effects the environment on their growth and behavior.

A most powerful metaphor which can still be seen in biology and medicine textbooks today was developed by Descartes; it proposed that an organism operates as a machine. Descartes implied that an organism could be understood by studying its parts, in the same way that the inner workings of a clock can be understood by dismantling its gears. When the sequence of DNA base pairing was interpreted as a form of information storage, the possibility that biology could be reduced to chemistry arose. The bottom-up assumption here is that the more we understand about the functions of genes, the better we will understand the function of organisms as a whole. Immanuel Kant disagreed with the Cartesian view of the biological world. Kant's organicist view was that no part of the body could be properly understood apart from its relationship with the organism as a whole. Examples of this necessity occur at all levels of biological organization: from the interactions of cells in a tissue to reciprocal interactions between and organism and its environment. Thus, causation in biological systems is not limited to bottom-up, but it is also top-down and in every possible direction.

Physicists abandoned a reductionist program early in the 20th century, favoring theoretical unification over theory reduction. In this context, classical mechanics may be able to explain the behavior of ball bearings and planets; however, Newtonian mechanics does not apply when describing subatomic particle behavior; a different theory, quantum mechanics applies to phenomena at the scale of molecules, atoms and subatomic

particles, while mainstream biology never abandoned it, hoping for the day that biology will be reduced to chemistry. The Stanford Encyclopedia defines biological reductionism as "the idea that biological systems are most fruitfully investigated at the lowest possible level, and that experimental studies should be aimed at uncovering molecular and biochemical causes [6]." In other words molecular biology *should*, in principle, be able to explain all biological phenomena. Given the many biological breakthroughs during the past century is it fair to say that reductionist approaches to biology have been inadequate to explain relational phenomena in biology. There is no question that reductionism has been a powerful approach driving our understanding of biological chemistry and some biological phenomena, for example, muscle contraction. However, a reductionist approach is incomplete for several reasons. First, the lower level elements (genes, for example) do not completely determine a phenotype; the occurrence of polyphenism illustrates this fact. Second, the signal transduction network which presumably conveys "orders" to cells from the internal milieu, is not as specific as it needs to be according to the current view. For example, the same signal transduction network is employed for synthesizing hemoglobin in response to erythropoietin as is needed for synthesizing milk in response to prolactin [7]; in both cases, the specificity of the response resides on the organ, tissue and cell type involved, not in the hormone, its receptor and/or the signal transduction network. This specificity, instead, is attained through the process of organogenesis. Third, the reductionist myth of simplicity underestimates how important interactions are in complex systems, whether they be at the level of proteins or higher level ones involving the organism and its organs. Further, reductionist approaches largely disregard the properties of complex systems and view unexpected disturbances to a measured "norm" as noise. "Noise" found in complex data sets represents biological variation and has biological meaning, it is not a measurement error as implied by calling it "noise."

Biology is rife with vague theories with undefined domains. Instead of rigorous principles, it often uses metaphors such as program, information and signal. These precise mathematical concepts which have been rigorously formalized (using plenty of equations), are used in biology as mere metaphors. Short-hand talk about information, signals, and software abounds in the modern biological lexicon. Even “code” is not the same when referring to mathematical information (clear-cut symbols, precise strings 0s and 1s) and biological code (at best, 3D chemistry of pairing A-T when referring to DNA, and at worst the absolutely loose metaphor of epigenetic codes). If signals have meaning, where does such meaning arise from? Adrenal glands do not release aldosterone with the intention of lowering plasma sodium. Nature does not contain information; information and meaning arises from our observations and measurement of nature [8].

Abstraction is required for our understanding and communication of scientific ideas. Prediction and manipulation would be impossible without generalizing and grouping of entities. This requirement becomes destructive, however, when the “historical process of abstraction is forgotten”[9]. This is even more critical when the concepts in question are metaphors (ie, a figure of speech in which a word or phrase is applied to an object or action to which it is not literally applicable); the practitioners easily forget the metaphorical quality of these notions and accept them instead as real entities (reification). The molecular biology revolution has misappropriated precise theoretical concepts taken from the mathematical theories of information, and used them metaphorically (that is, without their precise mathematical meaning and equational rigor) to incorrectly describe cells as computers run by programs. Such reified metaphors are used extensively as biological explanations. Indeed, because a theory based on metaphors is inherently vague, all questions and answers asked within its framework

appear to be correct, thus making it appear to be a good theory. Hence, the lack of fit between the theoretical concept and the experimental result is not taken as an incongruence to be analyzed. Instead, complexity is claimed, and the lack of fit is never dealt with.

1.1.3 Theoretical Framework

This introductory discourse is relevant to the subject matter of the thesis I have chosen to consider in order to qualify for my degree of Doctor in Philosophy. In the following pages I will narrate first which are the current theories of carcinogenesis and later I'll try to accommodate the data already collected by others and my own to a satisfactory explanation of how mammary gland neoplasia develop or fail to develop in neoplasia-susceptible and -resistant rat strains.

My thesis aims at understanding why females of the inbred rat Copenhagen (COP) strain are highly resistant to the formation of mammary gland tumors. The COP story is especially interesting because, given that in the current state of cancer research it does not fit within existing ideas of how cancer is initiated and progresses. Without a specific, non-vague theory of cancer, researchers have studied COP within theoretical "safe areas." This means that COP resistance is the result of gene influences unique to COP, that the animals have more adept immunosurveillance, that precancerous lesions fail to promote angiogenesis, or that resistance to cancer is, in fact, what is being studied. The results of experiments designed under these premises have done little to further our understanding of why and how these animals manage to avoid mammary tumor formation. The failures of these experiments to explain COP resistance to mammary carcinogenesis do, however, allow for the opportunity to ask why current methodologies are unable to shed light on complex traits. Further, why if we continue to utilize

scientific thought, based on theoretical testing and course-corrected postulates, has a growing body of evidence contradicting the basic premises of cancer theory failed to change the field's conceptual framework and experimental approaches?

Cancer is a disease of multicellular organisms. Therefore, we need to ask questions about cancer within a framework of a theory of organisms. In doing so we allow for the opportunity to ask questions about what it is we are observing and how those observations support or contradict the theory of the organism as a whole. In the next section I will discuss two contemporary theories of cancer and explain why an organicist perspective, one more focused on the interaction of parts within biological systems than the understanding of the parts themselves, is more effective in explaining carcinogenesis.

1.2 Theories of cancer

A majority of textbooks, biologists, and clinicians define cancer as a (1) cell-based disease (2) caused by genetic mutations. These two premises are the basis of the Somatic Mutation Theory (SMT). Under this theory, it is assumed that mutated cells and their progeny express an acquired set of behaviors and characteristics specific to cancer. That the default state of cells in multicellular organisms is quiescence is implied by the SMT but rarely stated directly by its proponents. In order to move from this quiescent state, a so-called mother cell must receive a signal from external sources that "induces" it to enter and complete the cycle at the end of which two "daughter" cells are generated.

1.2.1 The Somatic Mutation Theory

According to the SMT, cancer occurs at the cellular level of biological organization. In two articles published a decade apart [10, 11], Hanahan and Weinberg reduced the complexity of cancer to a small number of principles that they considered as its

hallmarks.. In the first hallmarks of 2000, the authors presented an animation of a single cell with a multitude of signal transduction pathways inside it. The authors defined signal transduction as “integrated circuits in complexity and finesse, where transistors are replaced by proteins.” They claimed that the cancer cell is a “reprogrammed” version of its former self while highlighting functionally altered genes. The use of this type of metaphorical language to describe biological systems has numerous pitfalls, which were addressed above. It is a simplified explanation for what is more likely vast complex of non-binary instantaneous interactions among chemicals and proteins [12]. According to Hanahan and Weinberg, through “mechanistic strategies” cancer cells acquire unique properties including apoptotic evasion, self-sufficiency from growth signals, insensitivity to anti-growth signals, sustained angiogenesis, limitless replicative potential, and tissue invasion/metastasis [10]. The 2011 version of the hallmarks added metabolic “reprogramming” and immunosurveillance avoidance as well as other course corrections to account for additional findings made during the intervening decade [11].

The authors claim that their perspective is non-reductionist, yet they continued to support the idea of breaking down a system to its components and studying each part individually. Emergence, to them, arises solely from interacting transduction pathways, this is causality perceived as being from bottom-up.

The hallmarks of cancer were considered especially important in light of the human genome project, completed in 2003. An extensive list of pathways and cellular behaviors was compiled and researchers could access the complete sequence of the entire human genome. From this perspective, cures for cancer required the genetic identification of cancer genes and the pharmaceutical targeting of previously unknown nodes in signal transduction cascades using the hallmarks as a guide. As the genome of humans and

mice acquired higher resolution, the links between the academic research community and the molecular biology industry intensified.

Determining the extent to which the SMT framework influences current working models of cancer biology is difficult to gauge. As of this writing the two hallmark papers have been cited almost 60,000 times. However, of the very data collected using molecular biology technology challenges the fundamental premises of SMT.

1.2.2 Evidence Challenges the Premises Adopted by the SMT

1.2.2.1 Mutation status does not correlate with disease

Cancers can be classified into two main groups [13]. The first, **sporadic cancers**, represent over 95% of cancer cases and lack obvious genetic origins. The second, **inborn errors of development**, mostly affect younger patients. Within this group there are two subtypes: 1) **inherited inborn errors of development** form when a mutation is present in the parental gametes and is therefore present in every cell of the patient during the entirety of their development and 2) **induced inborn errors of development** arise from improper morphogenesis *in utero* due to exposure to carcinogens or exogenous hormones. Inherited inborn errors of development (i.e. those caused by germ line genetic abnormalities) can produce aberrant phenotypes. However, it is unclear how those mutations generate those phenotypes. This statement is valid for mutations carried by both normal as well as mutated cells. For example, a mutation in a gene affecting purine metabolism in Lesch-Nyhan syndrome patients causes behavioral and anatomical aberrant phenotypes but it is unknown how and what other factors are responsible for the overall phenotype [14].

Abnormalities at the single gene and chromosomal levels are unrelated to the organization or function of a tissue after organogenesis is complete. Highly malignant teratocarcinomas have been shown to contain normal teeth and mature elements of all three germ cell layers, an indication that neither mutations nor tumor status hinders the development of organized tissues [15]. Additionally, some tumor-associated cells do not contain mutations. For example, many common cancers often lack mutations in the 518 protein kinase genes that have been claimed to be required for driving tumor growth [16]. Also, two subtypes (A & B) of posterior fossa ependymomas lack mutations while type A shows only epigenetic changes compared to healthy tissue [17, 18]. Neuroblastomas are frequently shown to have regional deletions in chromosomes 1 and 11, however the corresponding regions on paired chromosomes lack mutations in so-called tumor-suppressor genes [19]. Conversely, mutations are commonly found in functionally and histologically "normal" tissues. Extratumoral interstitial cells in the colon often contain mutations in *C-KIT*, a common marker for tumors of the gastrointestinal stroma [20]. At the chromosomal level, one study looked at loss of heterozygosity at 14 polymorphic loci in normal terminal duct lobular units, adenoses, hyperplasias, and apocrine metaplasias and found genetic lesions at similar rates in both healthy and neoplastic tissues [21]. Alterations in genes considered responsible for tumor growth under SMT can be seen in pathologies other than cancer. *TP53*, thought to primarily function as a tumor suppressor in multicellular animals, is also mutated in noncancerous tissue diseases such as ulcerative colitis [22], atherosclerosis [23] , and rheumatoid arthritis [24] .. Enhanced and disorganized cellular growth (metaplasia) is common to each of these conditions.

Conditions that affect genomic stability are not always associated with an increased cancer risk. Patients suffering from Cockayne Syndrome have high mutation frequencies

in their cells but have never been reported to develop cancer despite their increased photosensitivity [25]. Individuals with congenital defects in their DNA repair genes are hyper-sensitive to cancers in only a subset of tissues. In a study of 4 different syndromes marked by faulty DNA repair, those affected individuals showed 1000-fold increases in rare leukemias, but were less likely to develop other cancers compared to the general population [26].The number of mutations found in tumors is consistent with the basal mutation rate seen in normal tissues, despite speculation that genomic instability is a major cause of tumor progression [27].

Instances of tissue abnormalities shown to arise prior to mutagenic lesions would directly contradict the SMT's proposed model of carcinogenesis. A growing body of evidence suggests that many of the most common putative genetic drivers for cancer, including *BRAF, RAS, EGFR, HER2,* and *PIK3CA* can also be found in benign and premalignant conditions, with even their malignant equivalents containing a *decreased* incidence of mutations in these genes [28]. Regardless of explanations for mutations in "non-driver" genes, such as non-coding regions, that occur both before and during tumor growth, the existence of these gene mutations in otherwise non-cancerous tissues indicates the need to reevaluate the weight given to mutations in cancer biology.

1.2.2.2 Foreign body carcinogenesis

Implanting otherwise inert foreign bodies into mouse and rat tissues can induce carcinogenesis [29]. Implantation of large, polymer films induces carcinogenesis in rat tissues while the same films fail to cause tumors when implanted in powdered form [30]. Pores (>0.22 um) made into otherwise carcinogenic films significantly reduced the carcinogenic activity of polymer foreign bodies [31]. In other words, large implanted materials form a barrier that interferes with normal tissue structure and function; these

local disruptions may trigger tumor formation. Foreign body-associated tumors are often classified as poorly differentiated sarcomas or fibrosarcomas [32], i.e., tumors types linked to stromal cell components. Attempts to describe this form of carcinogenesis have eluded explanation by the SMT program.

Mesothelioma resulting from asbestos exposure is one of the most well known environmental-associated cancers. Despite decades of methodological and conceptual advances in molecular biology, explanations of asbestos-induced cancers remain undefined [33]. There has been some debate as to whether or not asbestos fibers are mutagenic *in vivo* [34]*;* however, longer fibers are up to 500 times more likely to be associated with tumors than shorter ones [35] and it is unlikely that these fibers shed mutagenic chemicals. These observations represent further examples of carcinogenesis where genetic explanations are lacking.

That tumors arise from a single "cell-of-origin" is based on the similarity of genetic alterations identified within the tumor cells [36]. This line of thinking persists even though more recent findings show that breast, colon, and ovary tumors are polyclonal [37].

1.2.3 Introduction to the Tissue Organization Field Theory of Carcinogenesis (TOFT)

The TOFT is an alternative, organicist-inspired theory of carcinogenesis {Soto, 2011 #366}. In contrast to the SMT, that TOFT pertains to the tissue level of biological organization; it focuses on the importance of organization as crucial to the function of multicellular organisms and that of their cells. There are a number of concepts, derived from physics, which must be described to fully understand how TOFT is different from and incompatible with SMT. For one, physical objects are generic, biological ones are

specific. Additionally, biological systems are historical: they arise over time and over the course of their history (ontogeny and phylogeny) develop irreversible levels of complexity. This line of thinking is implicit in the cell theory - all life comes from life and cannot be generated spontaneously. Biological systems are remarkable for their emergent properties. Emergence is when a "new circumstance enables new outcomes, which are not predicted (anticipated) by the description of the system." [38] The ways organisms negotiate their external environment result from these emergent properties.

Laws of conservation describe symmetries in the physical world; the concept of symmetries is exemplified by the image of a scale held in stasis by equal weights on both sides. Biological systems, however, continually break symmetries. Broken symmetries that occur at all levels of biological organization are responsible for variability and diversity not seen in physics or, for example, simple chemical reactions. Indeed, the ontogenetic and phylogenetic trajectories of living things have "cascades of symmetry breaking" [39] built into them and continue to do so throughout the lifetime of each and every organism. Organisms are unique entities in the universe and thus must be approached differently than inert objects. For these reasons, reductionist approaches to complex biological situations fail to take into account the new circumstances and unpredictable outcomes that arise from organized complexity. This is especially true of the case made by the SMT which attempts to describe carcinogenesis at the molecular level and assumes congruence.

TOFT relies on two premises to explain carcinogenesis: 1) the default state of all cells is proliferation with variation and motility and 2) carcinogenesis is a tissue-based disease [40] [41]. The first premise is a fundamental biological postulate akin to that of inertia in physics. The default state of an object is defined as what happens when nothing is done

to it. Therefore, the topic of interest to the fields of developmental biology and, in turn, carcinogenesis, are the forces that alter the default state of the cell. Single celled organisms divide exponentially when grown in milieus with sufficient nutrients and space. Millennia of selective pressure would have favored those cells that were able to proliferate the fastest when conditions were less than perfect [42]. When nutrients are scarce, yeast alter their gene expression profiles and enter a state of quiescence, a constraint imposed by extracellular factors [43]. RAS, a protein that becomes ligand-independent in some cancer cells, does not require a ligand in yeast but is involved ligand-independent responses to altered metabolism and stress [44]. Over time, probably through symbiotic relationships among unicellular organisms, multicellular organisms arose and imposed new intracellular constrictions on the resident cells. Instead of metazoan organisms developing an entirely new set of genes required to assure survival, it is plausible that those players currently seen as being pivotal to cell cycle progression (such as RAS) may have been actually involved in releasing a metaphorical "brake" responsible for slowed proliferation [45].

In line with this history, zygotic cells proliferate exponentially during early development [46]. Cell proliferation during early stages of development begins to slow down only after the formation of morphogenetic fields brought on by physical forces and chemical gradients. In this regard, morphogenesis is a process marked by the constant breaking of symmetries, where new shapes and behaviors are possible and driven by the cellular default state of proliferation with variation and motility plus physical constraints. These emergent properties of tissues develop concurrently with the development of the tissue field as a whole – they have an irreversible history built into their structure. Cells incorporated in many adult tissues are held in an induced state of non-proliferation. The majority of their transcription is dedicated to maintaining cellular structure and

function. When freed from these restraints generated by the tissue and the organism in which they reside when provided with the adequate nutrition, cells of multicellular organisms resume their default behaviors: that is, proliferation with variation and motility. The morphology, behavior, and gene expression of tissues during development are recapitulated during carcinogenesis. Within this context, cancer is "development gone awry." [47]

The second premise takes into account the history, organization, and emergent properties of tissues that are as important during carcinogenesis as well as in normal development. Like many other tissues, mammary gland development, which will be described at length in section 4, relies on reciprocal interactions between its epithelial cells, the extracellular matrix into which they grow and the subjacent complex stroma. Collagen fibers of the mammary gland stroma impose constraints on mammary epithelial cells, limiting their movement and proliferation and, in turn, the cells alter the biomechanical makeup of the abutting stroma. The resulting arboreal structure of the gland in adolescent life contains all the cell types, their proper distribution, and stromal interactions required for milk production in the event of pregnancy. These properties are not possible without the proper timing and organization of the cells within the gland. Further, those alterations made to the glands extracellular components during development impart new properties to the gland that are crucial for proper lactation and involution of the organ. The interactions between tissue compartments and the forces associated with them are crucial for tissue shape and function. [48]

The ongoing accrual of evidence associating stromal health with the function and organization of the associated epithelium has resulted in an expanded cancer perspective incorporating the wider tumor microenvironment. Relevant tissue recombinations has

provided insight into the importance of cellular interaction during development and cancer. Interactions between epithelial cells and the extracellular matrix are the primary interest of this thesis specifically because carcinogen-exposed stromal cells have been shown to cause carcinogenic growth of otherwise normal epithelial tissues. These experiments will be detailed at length in Section 4.

The taking into account of the two above-mentioned premises has put into perspective a number of findings that cannot be properly explained by the SMT. From the TOFT perspective, carcinogens act by disrupting the constrains imposed on cells by tissue organization, altering local forces in such a way that fields of cells react to them in a manner similar to what is seen during development. In addition to recombination studies revealing NMUs target in the mammary gland to be the stroma (see section 4.2.1), other studies have shown that irradiated mammary stroma induces carcinogenic growth of non-tumorigenic mammary epithelial cells [49]. Tumor associated prostate fibroblasts promote disorganized growth of normal prostate cells [50]. The opposite is true as well; healthy stroma is able to interact with tumor-derived epithelial cells in such a way that normal morphogenesis and function is restored despite the presence of so called “oncogenic mutations” [51] in the cells participating in this process. Stromal influences are not limited by species- or organ-specificity; the mouse mammary gland microenvironment can redirect human embryonic carcinoma cells to differentiate into functional mammary epithelial cells [52].

There are at least two challenges presented by changing the theoretical framework through which we view cancer: 1) a vast, integrated reassessment of existing literature spanning the last century for the sake of reinterpreting results that fail to “fit” within the existing dogma and 2) a new approach toward experimental design that attempts to take

into account as many levels of biological organization as necessary. In the next section I will discuss Breast Cancer pathophysiology, epidemiology, and risk factors while attempting to put those aspects of the disease into a perspective compatible with the TOFT.

1.3 Breast Cancer

Breast Cancer (BC) is not a disease limited to recent data [53] . The earliest medical documents describing BC go back to more than a millennium before the Christian era [54]. It was described by doctors in ancient Egypt as incurable. Hippocrates claimed an imbalance of four bodily humors resulted in the formation of tumors, a belief that prevailed for almost 1300 years. Ramazzini, the father of occupational medicine, reported in 1700 that breast cancer was common in aged nuns while it was rare in married women with children [55], thus establishing a nexus between reproductive status and the disease. Theories involving humors or lymph were disproven in 1838 when Muller showed that tumors consisted of cells [56]. His student, Rudolf Virchow confirmed (or possibly stole) Robert Remak's notion that all cells, including cancer cells, arise from other cells, and proposed that inflammation provoked tumor development [57].

In more recent times, the National Cancer Institute implemented in 1971 what President Nixon dubbed the "War on Cancer." [58] This national effort committed significant amounts of funds and manpower to acquire a better explanation of the cancer process and eventual cures for both solid and liquid tumors. However, despite the availability of novel surgical and chemical interventions, BC continues to be inadequately understood. Meanwhile, its incidence remains steady and is responsible for the death of women around the world. It is estimated that 40,000 American women died of BC in 2015 [59].

The implementation of the War on Cancer took advantage of the molecular biology revolution, itself started in 1953 when the structure of the DNA molecule was originally described in detail [60]. Soon after, researchers who joined the War on Cancer effort aimed at identifying genes associated with BC susceptibility and tumor behavior, with the hope that this knowledge would ultimately be applied in effective cancer treatments. Those initial hopes have become unfulfilled and these failures were attributed to the complexities associated with tumor heterogeneity and to the high cost of alleged targeted therapies [61]. Meanwhile, improved screening and advances in early-stage therapeutic interventions have been responsible for a decrease in BC-related deaths in countries in the Northern hemisphere and in developing countries [62].

1.3.1 Risk assessment

The incidence of BC will probably continue to rise over the next 20 years despite preventative measures [63]. Although populations with higher and lower incidences have been identified, women from all ethnic and socioeconomic backgrounds are susceptible to BC. Despite the current focus on genetic predisposition to BC, environmental and lifestyle factors significantly contribute to an individual's BC susceptibility. Preexisting highly penetrant somatic mutations in so-called "oncogenes" such as *BRCA1/2*, *PTEN*, *TP53*, and *ATM* are associated with between 5-10% of BC diagnosed in the US [64] while are extremely rare in the general population [65]. Regardless of this low association rate, marketing for genomic screening of women to determine their risk level to develop BC thrives. Following the discovery of *BRCA1/2*, genome-wide association studies (GWAS) have identified over 80 low- and moderate- penetrance genes [66]. Combined, these variants ultimately account for less than 5% of BC heritability. Implicitly, these data suggest that women who carry multiple these germplasm genomic lesions will be

diagnosed with BC while women who lack these lesions may still be develop BC at some point in their lives.

1.3.1.1 Lifestyle Factors

Lifestyle factors such as obesity, physical inactivity, and alcohol consumption play a role in the increased BC burden as well [67]. Women who gain weight prior to menopause or who are obese following menopause are at an increased risk of BC [68]. In addition to the detriments that obesity causes to overall health, it is likely that increased peripheral fat provides an additional source of aromatase activity that has similar effects on the mammary tissue as hormone replacement activity. While there is an obvious link between level of physical activity and obesity, women over 19 who exercised for the recommended period of time per week or more were shown to be 25% less likely to develop BC compared to inactive controls [69]. Exercise has been shown to modulate the inflammation-immune axis [70] and may help maintain tissue architecture following trauma. Women who consume 4-9 units of alcohol per week are 15% more likely develop BC compared to women who never drink [71]. This effect may be due to the toxic and carcinogenic nature of ethanol metabolite acetaldehyde [72] combined with ethanol's ability to significantly increase estrogens serum levels. These risk factors can be adequately explained by TOFT given that each of them involves aberrant tissue remodeling and inflammation.

1.3.1.2 Reproductive history

Regardless of geographic region or ethnicity, reproductive history is the most consistent risk factor affecting women's incidence of BC. Several factors influence increased BC incidence in women as a result of changes in their biological and reproductive timeline in industrial societies: earlier menarche, later first pregnancy, fewer pregnancies, shorter

periods of breastfeeding, and later menopause [67]. Hormone replacement therapy to counter the unwanted effects of menopause has also increased BC incidence in postmenopausal women. Early childbirth drastically decreases BC incidence and, conversely, non-parous women are comparatively more likely to develop BC later in life. Women who deliver prior to the age of 25 have a 36% reduced risk of developing ER/PR-positive tumors compared to those who gave birth later in life [73]. Younger women are generally less likely to develop BC compared to older women; therefore the protective effect of early pregnancy during premenopausal years is difficult to gauge.

While cycles of lactation and involution appear to protect the breast against tumor formation later in life, the microenvironment of post-lactational involuting mammary glands is highly inflammatory and features extensive cellular debris, matrix metalloproteinase activity [74], and increased deposition of collagen [75]. This inflammatory, wound-like microenvironment may persist beyond the completed involution, as women who have given birth within 5 years face an increased risk of developing BC, an effect that diminishes after 6 years [76]. The rigidity of breast composition can also influence a woman's BC risk. Women with a higher mammary tissue density have almost 5-fold greater risk of BC compared to women with less dense breast tissue [77]. The role of mammary rigidity in epithelial morphogenesis will be discussed in sections 4 & 6 of the Introduction.

1.3.1.3 In Utero exposures

Exposure to estrogens, endocrine disruptors, and carcinogens *in utero* has been linked to increased BC rates. A detailed description of how *in utero* exposure to endocrine disruptors predisposes male tissues to cancer later is described in the included manuscript "*Environmental endocrine disruptors: effects on the human male*

reproductive system." Briefly, TOFT posits that morphogenic fields, formed during early development, persist throughout life and are still responsible for tissue remodeling and regeneration in the adult. Exposure to exogenous noxae such as hormones during morphogenetic field formation permanently alters interactions between the mesenchyme and its associated parenchyma [14]. Together, these findings highlight the role of tissue remodeling in resistance and susceptibility to tumor formation. While many cancers appear later in life, the formation of a detectable neoplasm is the result of a long history of tissue interactions, some of which may have been disturbed since fetal development. Further, these findings give insight to the complexity of hormonal influences in the mammary gland, as they seem to at once predispose and protect the tissue.

1.3.2 Breast Cancer Hormone Dependency

ER is expressed by ~75% of BC. Reduction of ER activity or of its expression has been a primary aim of hormonal therapies in metastatic BC. Although endocrine therapies initially result in significant decreases in ER-positive tumor size, in many cases patients with ER-positive metastatic BC ultimately relapse due the growth of estrogen-independent tumors. In 20% of relapsing patients, *ESR1* gene gain-of-function mutations can be detected in the metastatic tissues [78]. These mutations are usually in the ligand-binding domain and allow for estrogen-independent receptor activity. It has also been shown that several estrogen responsive elements are hypermethylated in relapsing patients, further complicating hormone-based therapeutic strategies [79]. Progesterone Receptor (PR) is often co-expressed with ER in hormone sensitive BC [80]. PR expression is driven by estrogen stimulation and ER activity and is therefore used as a marker for sensitivity to ER-targeted approaches. Tumors lacking expression of ER, PR, and HER2 (triple negative) have the worst prognosis because they lack hormone receptor targets for treatment regimes. Without hormone-based attenuation these

tumors are fast growing, highly malignant, and require combined aggressive surgery and chemotherapy for survival. It is unclear what is responsible for the formation of ER-negative tumors. Young women and women from some racial groups, especially African-Americans, most frequently develop ER-negative tumors [81].

Non-malignant mammary epithelial cells implanted into irradiated cleared fat pads of mice, preferentially form ER-negative tumors [82]. Young women who were irradiated to treat their childhood cancers are more likely to develop ER-negative tumors [83] than unirradiated controls. Irradiation of the fat pad of mice has been claimed to be responsible for carcinogenesis; the hormone receptor status of the tumor may be dictated by the interaction of the altered stroma with undifferentiated epithelial cell precursors.

The treatment of metastatic BC has proven to be largely ineffective. Metastatic lesions are responsible for the majority of BC-associated deaths. The extended latency between the initial detection of the primary BC and the appearance of distal metastases implicates the tissue microenvironment in disease progression [84].

1.3.3 Neoplastic remission

Spontaneous remission of tumors is rarely reported and thus its occurrence is difficult to evaluate. However, Zahl (2008) utilized the high rate of screening compliance by women in Norway and Sweden to assess the differences in BC incidence between screened and unscreened patients [85]. Using epidemiological approaches, Zahl concludes that 27% of lesions identified during screening are latent tumors while the remaining 73% of lesions that appeared during an earlier screening failed to show up later, suggesting that they regressed spontaneously [86]. Over the course of an individual's lifetime, a number of

preneoplastic lesions may have arisen and resolved without medical intervention. Even though it is difficult to study, neoplasia do have the capacity to slow and to revert to become normal tissues. In this context, following NMU exposure, COP rats develop preneoplastic lesions consistent with a diagnosis of human intraductal proliferations (IDP) but such lesions disappear after several weeks. This is a powerful reason to assume that understanding the factors involved in preneoplastic lesion resolution may lead to novel interventions.

1.3.4 Conclusions

A major goal of current cancer biology should be to determine why some, but not all, women get BC. Thus it is would be informative to identify what makes the breast susceptible to cancer development. This is a complex task that would require a better understanding of the tumor itself and also the organization of the tissue, the process of carcinogenesis (literally "the beginning of a cancer"), and the roles of hormones and resident immune cells in tumor biology. In this thesis, I hope to at least begin to address the following questions: a) Is cancer resistance receptive to being defined in an empirical context? And b) will this empirical characterization bridge the gap required to execute a tangible translational effort? In the next section, I will cover mammary gland development and discuss the importance of stromal-epithelial interactions in the process. Using developmental features as a guide, I will then discuss how those interactions in tumor promotion, progression, and remodeling affect BC.

1.4 Role of Stroma in Mammary gland development and carcinogenesis

1.4.1 Mammary Gland Development

As described in the previous sections, there is a growing body of evidence implicating cancer as a tissue-based disease. Carcinogenesis is a problem of faulty tissue

organization during organogenesis [41, 47] and subsequent tissue remodeling. It is important, therefore, to better understand the development of the mammary gland to help emphasize similarities between developmental processes and those seen during mammary carcinogenesis. Here, I will describe the mammary gland's development from birth through puberty as well as the morphogenic events associated with lactation and involution. Mammary development has been elucidated mostly using rodent models. Similarities and differences between human and rodent development will be noted where necessary. This review will focus on female mammary gland development.

The mammary gland is the organ that defines the mammalian animal class. The adult human mammary gland is composed of approximately 10-20 ducts which branch out from the nipple, invading the associated fat-based supporting tissue to points as deep as the chest wall [87]. The gland consists of an epithelial ductal tree embedded in a connective stroma populated by adipocytes and fibroblasts. Non-cellular components of the stroma, most importantly fibrilar collagen, are primarily secreted by fibroblasts. The gland is innervated, vascularized and contains transient and resident immune cells.

The developmental history and structure of the mammary gland are vital to its ability to rapidly grow, produce milk, and return to a pre-pregnancy state following weaning. Mammary epithelial cells are characterized as being one of two main types: (1) luminal cells form a simple cuboidal epithelium of ducts and secretory alveoli that are encircled by a layer of (2) basal myoepithelial cells (Figure 1.1, right middle). Luminal cells form a continuous epithelial layer between the lumen and the single layer of myoepithelial cells. Basal cells contribute some proteins to the basal lamina, including collagen IV, the laminins, entactin and proteoglycans [88]. In the developing breast each duct ends in a terminal end bud (TEB) lined with highly proliferative cap cells that contribute to both

epithelial cell populations (Figure 1.1, right, top). All secondary branching occurs after puberty commences.

Stromal cells produce the majority of the mammary extracellular matrix. The ECM is rich in adhesion proteins including fibronectin, tenascin, laminin, clusterin, and as well as several MMPs [75]. Work by Kratochwil [89] and Sakakura [90] suggests that the ECM plays an inductive role in mammary gland development through their identification of paracrine factors that regulate epithelial morphogenesis. Tissue recombination and genetic models reveal just how dependent the development of the stroma and the epithelia are upon each other. Mammary epithelial cells combined with mesenchyme from submandibular salivary glands developed in a ductal pattern similar to that seen in the intact salivary gland [89]. Despite the mammary gland origin of the epithelium the salivary stoma is responsible for its shape. In mice, the presence of white adipose tissue is crucial for secondary branching of the mammary gland tree to occur [91]. Conversely, proper mesenchymal development is responsible for the suppression of hair follicle formation around the nipple and rudimentary bud [92] that would otherwise interfere with proper gland development and function. Biomechanical forces generated within the stroma are also important to morphogenesis [93] of multicellular structures within the mammary tissue.

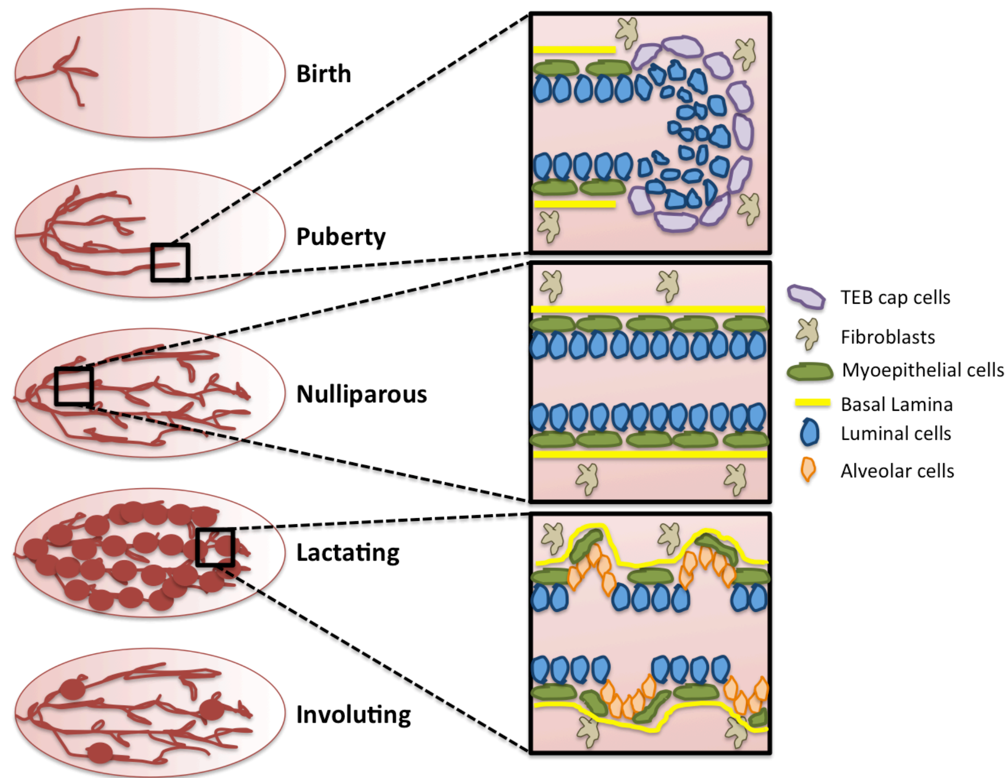

Figure 1.1 Mouse mammary gland development and associated anatomy. Illustration depicting the epithelial compartment invading into the female fat pad at different developmental stages. At birth (left, top) the gland consists of a rudimentary, non-branching tree. During puberty, TEB cap cells (right, top) advance into the stroma and differentiate providing epithelial and myoepithelial cells until the ducts reach the fat pad edge. Epithelial cell death results in lumen formation. Side branching occurs at this stage. Mammary ducts consist of a cuboidal epithelial cells surrounded by a single layer of myoepithelial cells (right, center) which secrete basal lamina. Stromal fibroblasts contribute additional ECM components. The lactating gland (left) is marked by milk producing alveoli surrounded by myoepithelial cells to aid in milk ejection (right, bottom). After weaning the gland undergoes widespread cell death and remodeling resulting in a gland similar to that seen pre-pregnancy with a small number of remaining alveolar structures (left, bottom). Nuclei omitted for simplicity.

1.4.1.1 Fetal development

The fetal mammary epithelial and stromal compartments develop through ongoing reciprocal interactions. By gestational week 6, mammary gland placodes form from epidermal cell populations. The establishment of mammary associated mesenchymal cells from mesoderm-derived cells is triggered by placode formation. The epithelial placodes continue to grow and eventually begin to bud by week 15 [94]. Surrounding the

invading epithelial bud, mesenchymal cells differentiate into fibroblasts, smooth muscle cells, endothelial cells, and adipocytes. This mammary fat pad precursor provides soluble factors to the epithelial branches as they begin to form from the epithelial buds. Distinct luminal and basal cell populations can be identified by week 20 accompanied by the formation of a true lumen and basement membrane production (Figure 1.1, left). The epithelial tree continues to expand into the fat pad and is capable of milk production, induced by circulating maternal lactogenic hormones. Following birth, any lactational activity ceases. Other than growth associated with increasing body size, mammary gland development is paused until the onset of puberty.

Hormone receptors are present only in the stroma in rodents during fetal mammary gland development. Human fetuses lack ER expression in the stroma and ER-alpha positive epithelial cells do not appear until gestational week 30 [95]. Early fetal morphogenesis is, however, hormone-independent as the ER-alpha knockout mammary gland exhibits normal fetal and prepubertal development but fails to develop further during puberty [96]. The ER-alpha knockout gland also lacks branching and lobulaoalveolar development. Exposure to exogenous hormones, including endocrine disruptors, has subtle effects on early glandular development however later life susceptibility to carcinogenesis is drastically increased [97].

1.4.1.2 Development during puberty and pregnancy

Increased levels of circulating estrogen and progesterone during puberty [98] cause ductal elongation and secondary branching of the mammary epithelial tree, respectively (Figure 1.1, left middle). Ductal elongation occurs through mitotic activity in the terminal end bud (TEB) and the area of duct immediately behind it. This high level of ovarian hormone-sensitive activity continues until the ducts reach the fat pad periphery at which

point the TEBs change morphology and display a dramatically decreased rate of cell division [99]. Stromal changes also result from puberty. Adipocyte content and fibrous tissue increase dramatically. Cycles of enhanced cell proliferation and restructuring of the gland continue unless a pregnancy occurs. From this post-pubertal stage until early menopause, the cycling of ovarian hormones causes the gradual accumulation of alveoli, which are superimposed along the ductal frame (Figure 1.1, right, bottom). Clusters of alveolar buds, called lobules, begin to form 1-2 years after the first menstrual cycle.

The hormones estrogen, progesterone, and prolactin cause rapid growth and remodeling of the gland. The number of mammary epithelial cells may increase as much as 8-12 times during this period [100]. The pregnant gland has higher levels of ductal branching and larger alveoli compared to the nonparous breast. Alveoli develop milk producing secretory epithelium, mediated by progesterone. The stroma of the lactating breast has fewer adipocytes and contains a unique composition of ECM. Once progesterone levels drop and prolactin levels incease, alveolar cells begin milk production. Ejection of the milk is aided by myoepithelial cell contraction.

1.4.1.3 Involution

Involution occurs shortly after weaning. Involution is marked by extensive epithelial cell death, mediated by an influx of macrophages, and a repopulation by adipocytes. The rodent mammary gland ECM contains high levels of proteolytic fibronectin and laminin as well as MMPs [75] and causes epithelial cell death *in vitro*. Isolated matrix from the mammary glands of involuting rats caused abnormal growth of normal MCF-12A cells and the invasive growth of MDA-MB-231 cells while growth on nulliparous matrix resulted in opposite effects [101]. Following complete involution, the gland is

histologically similar to its pre-pregnancy state its ECM composition, however, differs from that seen in the virgin gland (Figure 1.1, left, bottom) [102].

The involuting mammary gland stroma is marked by an increase in environmental cues that are pro-death and anti-survival [103]. Because cell death and remodeling are important aspects of COP resistance to NMU, the involuting mammary gland stroma as an interesting corollary to the process of lesion resolution, albeit on a smaller scale. Wound healing is marked by changes in ECM composition as well as activation of fibroblasts, endothelial cells, and immune cells. Despite these similarities, wound healing is also associated with tissue damage and necrosis; involution is comparatively orderly. Timed gene expression profiling of involuting mouse mammary glands revealed waves of cell death, debris clearing, metabolic changes, reestablishment of adhesion molecules, and indicators of ECM changes and morphogenesis [104]. It is possible that processes involved in controlled cell death, ECM remodeling, and return to the pre-pregnancy state are also involved in COP carcinogen resistance and corrupted in susceptible strains.

NMU is a potent alkylating agent yet it has an unexplained specificity for the bladder, salivary gland, and mammary gland. Susceptible mammary glands exposed to low doses of NMU have histologies that closely resemble pregnant or lactating glands [105]. Along with a desmoplastic stroma, the glands were marked by an increased number of acini and significant luminal debris caused by epithelial cell sloughing. Similar to the pregnant gland, acini were surrounded by limited collagen deposits. Interestingly, virgin glands exposed to NMU produce milk, as indicated by the expression of alpha-lactalbumin.

1.4.2 The role of the stroma in cancer

Just as the stroma was long thought to provide merely structural support for developing epithelial structures, alterations of the stroma were also thought to be secondary to the mutational events occurring in epithelial cells. Indeed, there are changes to the stroma at the organizational and molecular level. Tissue microenvironments associated with wound healing and inflammation correlate with tumor progression and promotion. Smoking [106], *Helicobacter pylori* infection [107] and inflammation of the prostate [108] are associated with disease progression in the lung, stomach, and prostate, respectively. The activity of cytokines and matrix macromolecules are interdependent: matrix components modulate the synthesis of cytokines as well as their associated cell surface receptors, whereas cytokines affect the expression of matrix components [109]. Cytokines including TNF-α, IL-6, TGF-β, and IL-10 are abundant in the tumor microenvironment [110] suggesting a high degree of matrix modulation during tumor progression. Myoepithelial cells secrete laminins, major components of the BM. A characteristic of early mammary tumors is the loss of myoepithelial cells, decreased BM thickness, and, in turn, reduced interaction of luminal cells with the BM resulting in loss of polarity [111].

1.4.2.1 Induction of tumors by manipulation of the stroma

Several examples of tumor induction via manipulation of the stroma were given in Section 2. Other stromal derived cues also influence epithelial behaviors. Mammary fibroblasts that overexpress hepatocyte growth factor promote tumor formation of histologically normal mammary epithelial cells [112]. Transgenic mice that over expressed the ECM degrading enzyme MMP3 developed mammary tumors at approx. 3-4 months of age [113]. In addition, the recombination of prostatic carcinoma-associated fibroblasts with nontumorigenic epithelial cells resulted in stimulated epithelial growth and altered histology [114].

In order to determine the target of carcinogens in the mammary gland Maffini et. al. [115] designed elegant rat mammary gland recombination experiments. The fat pads of 21-day old WF rats were cleared of epithelium and the animals were placed into one of 4 groups (Table 1.1). At 52 days of age groups 1 and 2 were given a single intraperitoneal injection of NMU (50 mg/kg) while groups 3 and 4 were injected with warm saline. Five days later the fat pads of each group were injected with 50,000 age-matched WF mammary epithelial cells. Epithelial cells used for groups 1 and 4 were treated with NMU *in vitro* 5 days prior to injection. Those cells transplanted into groups 2 and 3 were exposed to saline. As positive and negative controls (groups 5 and 6) intact virgin animals were injected with NMU (group 5) or saline (group 6). After 9 months, neoplastic lesions had formed in 76.9% and 75% of animals in groups 1 and 2 whereas groups 3 and 4 showed no signs of neoplastic lesions. 100% of animals in group 5 developed tumors while the negative control (group 6) had no incidences. Only in instances where the stroma was exposed to NMU did tumors form – regardless of the exposure status of the epithelial cells used. In addition, NMU-exposed epithelial cells failed to develop into tumors when implanted into an unexposed fat pad. Mammary glands removed from group 4 animals had ductal trees similar in complexity to those seen in group 3.

Table 1.1 Results of recombinations by Maffini et. al. [115]

	Fat Pad	**Fat Pad Exposure**	**Epithelium Exposure**	**Tumor Incidence**
Group 1	Cleared	NMU	VEH	10/13
Group 2	Cleared	NMU	NMU	6/8
Group 3	Cleared	VEH	NMU	0/10
Group 4	Cleared	VEH	VEH	0/6
Group 5	Intact	Positive Control (NMU, i.p.)		6/6
Group 6	Intact	Negative Control (VEH, i.p.)		0/6

The concept of the stroma as the target of mammary carcinogens is vital to this thesis. The beauty of the above experiments is that they concede two aspects of carcinogenesis: 1) the stroma can induce carcinogenic growth in otherwise normal epithelial cells and 2) a healthy stroma can attenuate carcinogenic growth potential of carcinogen-exposed epithelial cells. The results of these experiments show that the stroma must be considered when investigating carcinogenesis. As will be described in the next section, an explanation for COP resistance to NMU has long eluded epithelium-focused researchers indicating that the stroma may provide new insights into this phenotype.

1.4.2.2 Normalization of the neoplastic phenotype by the stroma

Additional experiments have also demonstrated the stroma's ability to revert cancer cells. Mouse mammary adenocarcinoma cells recombined with embryonic mesenchyme formed organized mammary tubules [116]. Normalization of neoplasia by the stroma is not unique to the mammary gland. The hepatic microenvironment can regulate differentiation of hepatocarcinoma cells, eliminating their tumorigenic potential [117]. Instances of tumor cell normalization by the stroma has also been reported in the testicle [118], and in different organisms including the newt [119] and hamster [120]. Tumor cell-microenvironmental interactions can also result in the development of entire organisms, as well. After eight years of *in vivo* propagation as ascites tumors, mouse teratocarcinoma cells injected into normal blastocysts participated in the normal development of mosaic mice containing markers from both the host and the teratocarcinoma cells [121]. The existence of tumor cell phenotype revision deserves great attention. If, instead of trying to kill tumor cells, the focus is on reverting them to

their non-neoplastic state, several clinical treatment hurdles including drug resistance, metastasis, and recurrence could be abrogated.

1.5 Introduction to the Copenhagen Rat

Rat mammary tumors closely resemble human breast cancer. Consequently, researchers studying BC have primarily been interested in quick and efficient generation of MG tumors by using tumor-susceptible rat strains. There are, however, several rat strains that are stubbornly resistant to spontaneous and induced MG tumors. One of them is the COP strain. COP rats form multiple preneoplastic lesions in their mammary glands 15 days after NMU exposure [122]. These lesions are indistinguishable from those lesions that go on to form *bona fide* tumors in WF animals. By 60 days post-exposure, COP mammary glands are tumor-free while similarly treated WF animals carry a significant tumor burden.

Little is known about the source of this resistance. Namely, no significant differences were found in epithelial cell proliferation or apoptosis rates, mutations in the Ha-*ras* gene, hormonal profiles, DNA repair, or immunological parameters that would explain different pathological responses to the same injury [123, 124].

Researchers have employed concepts from multiple different branches of biology to explore possible physiological reasons for strain-specific differences in carcinogenic responses. Blood vessel recruitment by early lesions was once considered a crucial step for tumor progression. To investigate the possibility that COP rats were able to block angiogenesis, Quan et. al. [125] administered vascular endothelial growth factor (VEGF) to COP rats two weeks after NMU administration in order to encourage preneoplastic lesion vascularization. Six months after NMU treatment the researchers saw no

difference in tumor burden between VEGF-treated animals and untreated controls. These results suggest that vascular recruitment by COP lesions does not play a determinant role in this resistant phenotype.

Another potential cause of COP resistance to MG carcinogenesis has been the ability of COP immune cells to better invade the neoplastic microenvironment and clear away tumorigenic cells. To address this possibility, Korkola et al [126] crossed COP rats with athymic nude rats whose offspring were then interbred to create F2 animals with partial COP backgrounds. By comparing the NMU response of nude and non-nude littermates the authors asked whether T-cells were involved in the remodeling of preneoplastic lesions. No significant differences in tumor number between the two groups of littermates were reported. Similar doses of NMU in age-matched susceptible Buffalo strain rats produced tumors in 70% of animals. These experiments show that, at least in the case of T-cells, immune surveillance is not responsible for COP resistance. Given that these cell types are crucial for proper epithelial development [127] and mast cells [125] are known to invade COP tissue following NMU exposure, it is possible that other immune cells play a role in MG stroma homeostasis and lesion resolution.

Genetic tools having ever-increasing resolution have been applied extensively to COP and COP hybrid animals to assess the role genes play in tumor remission; this strategy was unfruitful as well. Gould (1986) showed that carcinogen susceptibility was a dominant trait but that this characteristic could not be attributed to a single locus [128]. However, using similar techniques, others suggested that the suppression may be due to a single gene [129]. The group later went on to admit that some degree of the COP resistance resides outside of the mammary epithelial cells and that low penetrance of the gene may be responsible for the inconsistencies in their analysis [130].

Over the next decade linkage analyses claimed to identify several loci responsible for COP resistance. They were suppressor 1 [131]; mcs5, mcs6, mcs8, suppressor 7 [132]; mcs2 [133]; and mcs30 [134]. Despite the insistence that there exists a single dominant gene within the COP mammary epithelium, later studies have been unable to identify such a gene regardless of advances in positional cloning or higher resolution genome sequencing. Many of these regions are largely devoid of known genes [135] but may contain non-coding elements responsible for epigenetic modulation of gene expression. Quan and Lu [136] looked at expression profiles of COP mammary epithelial cells and found that interleukin-2 receptor alpha and claudin-6 were preferentially expressed, a finding inconsistent with the single gene hypothesis.

Summarily, COP rats are unable to generate mammary neoplasia following exposure to a carcinogen that often produces mammary tumors in 80-100% of susceptible rat strains. For these reasons, COP rats are an excellent model for: 1) viewing the earliest stages of tumor formation 2) observing how preneoplastic cells interact with the surrounding stroma and form normal structures despite suffering oncogenic lesions; 3) potentially observing the process of neoplastic remission, and 4) comparing these data with, and draw conclusions from, the evidence collected for decades using tumor-susceptible rat strains.

Based on Dr. Maffini's findings detailed in the previous section, our lab decided to employ a similar tissue-based approach to further understanding on the stroma's role in COP resistance. The experimental design and findings of this project are detailed in the manuscript "*Investigating the role of stromal fibroblasts in Copenhagen rat mammary gland tumor resistance*."

1.6 Why 3D culture?

Advances in cell isolation techniques, media formulation, and lab equipment have allowed for the long-term culture of animal and human-derived cells on non-biological surfaces. Cells growing in two dimensions (2D) are amenable to manipulation and observations that would be impossible *in vivo*. However, cells grown in 2D are strikingly different from their *in vivo* counterparts in their morphology, behavior, and gene expression profiles [137].

From morula formation onward, nearly every metazoan cell exists in a three-dimensional (3D) environment where it is in constant contact with other cells and acellular components. Forces that push and pull on cells during their proliferation and remodeling are crucial to their organization into tissues as well as their differentiation [138]. Germ layer formation from seemingly identical cells allows for the formation of rudimentary organs. As organogenesis progresses, cells interact with each other as well as their basement membrane. Mammary gland epithelial cell differentiation, polarization, and survival are all dependent on cell-cell and cell-ECM interactions [139]. For these reasons, cellular behavior must be studied in three dimensions in order to properly understand multicellular organism development and, therefore, carcinogenesis, as carcinogenesis is development gone awry.

The discrepancies between findings from 2D culture models and their subsequent validation in animal models, especially in drug screening and genetic manipulation [140], have been widely reported. In order to recapitulate the forces and micro-environmental interactions present in intact tissues, while preserving the extent of manipulations permissible in 2D cultures, 3D cell culture systems have been developed

over the years where primary cells, immortalized cells, or established cells lines are suspended in or on synthetic biomaterials, tissue-derived ECM or single protein matrices (e.g. – collagen, laminin, fibronectin, etc.).

1.6.1 3D cellular Growth

Growth in 3D results in behaviors that are not seen when those same cells are grown in 2D. As seen in tissues, and described in detail above, these behaviors are dependent on molecular gradients and biophysical stress provided by the surrounding microenvironment [141]. Molecular gradients include chemicals and proteins shared over cell-cell junctions, autocrine/paracrine communication, matrix composition, and metabolic factors such as pH, nutrient availability, changes in electrical steady-state [142], and oxygen concentration; biophysical stresses include rigidity and mechanical forces within the tissue including the matrix. An ideal 3D culture system includes aspects of each of these physiological features. In our lab, we have utilized a collagen-based system in which cell populations are seeded within a floating gel matrix which provides a 3D-context in which a matrix is readily manipulated by cells while meeting all metabolic requirements for cell growth [143]. This 3D model is amenable to long-term growth, treatment with exogenous chemicals including hormones, and real-time morphological observation.

1.6.1.1 Collagen

Type I collagen is the most abundant protein found in the mammary gland ECM. Both collagen density and organization are important factors for the organization of epithelial structures. For example, high mammographic density, a risk factor for breast cancer, results from tissue that has greater proportions of collagen in the stroma and less fat compared to low-density glands. Epithelial cells seeded into collagen type 1 matrix

migrate using ROCK-dependent movement [144]. Interestingly, experimental blockade of all extracellular proteases including MMPs, *in vitro* had no effect on cellular invasion. Cell-directed manipulation and movement are important advantages of collagen-based systems over inert matrices such a silk. Epithelial cells and fibroblasts are able to manipulate collagen fibers, forming thick, organized fibers from disorganized fields of thin fibers. Matrix manipulations are necessary for epithelial cells to organize into structures. MCF10A and T47D cells grown in type I collagen gels left attached to the edges of the well ("attached") formed disorganized sheets of cells [145]. When gels that were detached from the edges of the well ("floating"), those same cells formed acini and ducts, suggesting a role for biomechanical forces in morphogenesis [146]. When collagen fibers are aligned, tubular structures grow following the direction of the fibers. Acini form when fibers are organized randomly or radially. The propensity for acinar or ductal structures [93, 147] formation can be affected *in vitro* by altering collagen concentration or matrix composition. In addition, a cell's location within a gel influences [148] its behavior through differences in collagen organization and local tensile forces.

1.6.1.2 Reconstituted Basement Membrane

Another commonly used matrix for 3D cultures is reconstituted basement membrane (rBM or Matrigel) derived from Engelbreth-Holm-Swarm tumor cells [149]. rBM is composed of laminin A and B chains, collagen IV, entactin, and heparin sulfate proteoglycan. Mixed gels (collagen plus rBM) can be used to overcome some of the limitations of culture conditions in rBM alone [147]. After two weeks in culture, MCF10A cells grown in mixed gels (1 mg/ml bovine collagen 1 + >5% Matrigel) form ducts and acini [93] accompanied by the secretion of basement membrane, indicated by the presence of Laminin V. MCF10A cells grown in collagen alone do not secrete basement membrane. Therefore, biochemical and biophysical cues in mixed gels play important

roles in epithelial phenotype. In addition, higher concentrations of rBM in mixed gels promote apical polarization of MCF10A cells, an important step in lumen formation. Altogether, manipulation of gel composition using both rBM and collagen allows for the generation of epithelial structures more akin to those seen *in vivo*.

1.6.2 Growth of mammary epithelial cells in 3D

Ducts and alveoli are the most common epithelial structures found in fully developed, non-lactating human breast. Mammary epithelial cells grown on rBM form acini. Altering matrix composition promotes both normal and tumor-derived cells to form ducts as well as acini. The addition of rBM to bovine collagen gels decreases ductal elongation by MCF10A cells [150]; MCF10A cells grown in 30-40% Matrigel only formed acinar structures. Mixed gels contract less than collagen alone gels but the formation of acini or ducts does not depend on the bulk stiffness of the gel. Instead, fiber organization is the major determinant of ductal shape. rBM interferes with collagen fiber organization. Gels containing 50% rBM display decreased collagen fiber anisotropy resulting in abundant acini. Conversely, in gels with 0% rBM, collagen fibers have high anisotropy and are associated with the formation of ductal structures. Together, these results support the notion that alteration of the matrix biochemical composition and mechanical properties have considerable effects on cellular behavior and the genesis of epithelial shape.

Another benefit of the collagen gel 3D model is the ability to investigate the role of different hormones on epithelial morphogenesis. When estrogen receptor alpha, progesterone receptor, and Prolactin receptor positive T47D cells were treated with estradiol alone, or estradiol plus promegestone (a progesterone analog), or estradiol plus prolactin, they formed complex epithelial structures mimicking the effect of these

hormones *in vivo,* namely, included duct elongation, branching and budding, respectively [151]. Furthermore, the collagen matrix surrounding the structures was altered in a hormone-specific pattern. The organized hormone-dependent morphogenesis of T47D cells is an example of cancer-derived cells behaving similarly to normal cells. The identification of a normal hormone-responsive cell line to study in a 3D context was a major aim of this thesis. MCF-12A cells appeared to be an ideal candidate for this purpose; however, their status has been misrepresented in the literature. MCF-12As have been reported to be ER-alpha positive and respond to stimulation by estrogens, however, in our hands, MCF-12A do not alter their proliferation or gene expression behaviors in response to estradiol. The characterization of MCF-12A cells ER responsiveness and growth in 3D is detailed in the manuscript "*Characterization of MCF-12A cell phenotype, response to estrogens, and growth in 3D.*"

1.6.3 Cancer cell phenotype in 3D

Normal and breast cancer cells are indistinguishable when grown in 2D. Only when grown in 3D is it likely to distinguish non-malignant cells from their malignant counterparts [152]. Malignant cells derived from primary tumors or established cells lines do not undergo growth arrest, form lumena, polarize correctly or deposit BM when grown in rBM. In these models, normalization or enhancement of malignant cellular behaviors is made possible by the alteration of the associated microenvironment. Non-invasive MCF7 cells grown on recombinant basement membrane formed large, loose aggregate of cells. In contrast, those same cells, as well as invasive MDA-MB-231, form growth-arrested polarized acini when grown on rBM and concurrently treated with inhibitors of cell adhesion molecules [153].

Morphogenesis of relevant epithelial cell structures require appropriate micro-environmental interactions in 3D conditions. Cells established from neoplasms retain the ability to form normal structures despite their cancer-associated historicity. Conversely, cells derived from normal tissues form disorganized tumor-like structures when grown in 3D cultures mimicking the proinflammatory microenvironment seen during wound healing and involution [101]. Tumorigenic MCF7 cells grown in mixed gels organized into large structures and form lumena surrounded by sialomucin-expressing polarized cells [154]. Such findings are consistent with results from *in vivo* recombinations where NMU-treated epithelial cells grown in a non-exposed fat pad developed normal structures. These traits further emphasize the need for 3D culture models as surrogates when studying normal and abnormal epithelial morphogenesis in a tissue-like context.

1.6.4 Stromal/epithelial cell co-culture in 3D

Stromal fibroblasts are responsible for production of ECM components. Resident fibroblast populations also function in immune response modulation and homeostatic tissue maintenance [155]. Higher numbers of fibroblasts are found in the tissue abutting neoplasias and wounds and are therefore believed to be responsible for the increased collagen deposition surrounding tumors [156] [157]. Interest in tumor-associated fibroblasts' contributions to tumor promotion has been concurrent with the use of 3D culture systems. The inhibitory effect of rBM on MCF10A ductal elongation can be overcome by coculture with normal mammary fibroblasts [150]. Epithelial cells and fibroblasts alter collagen organization [158], a major determinant of acinar or ductal structure formation. Normal breast fibroblasts increase the number and size of MCF10A ductal structures when grown in rat-tail collagen [148]. MCF10A cell tubulogenesis was associated with thick collagen fibers aligned with the direction of ductal elongation.

Like epithelial cells, fibroblasts grown in 3D behave differently than their 2D counterparts. 3D culture of fibroblasts in mixed gels resulted in increased HGF, MMP-14, and COX2 secretion and decreased CXCL12 transcript expression compared to fibroblasts grown in 2D. These secretions are characteristic of an activated stroma like that seen following injury [159] and resulted in more aggressive growth of MCF10-DCIS cells grown in 3D [160]. However, fibroblasts can also contribute to tumor cell normalization. Reduction mammoplasty fibroblasts (RMFs) induced cell polarization, decreased cell death, and increased the number of round and elongated multicellular structures formed by MCF7 cells when grown in collagen gels [154]. Human mammary epithelial 1-7-HB2 cells underwent branching morphogenesis when co-cultured with foreskin, breast, and lung fibroblasts, as well as mouse-derived fibroblastic cells in 3D collagen gels [161]. These authors attempted to exclude fibroblast-mediated biophysical influences by treating the seeded gels with fibroblast-conditioned media and attributed the branching morphology to soluble factors secreted by the fibroblasts

Interactions in a co-culture mixed gel happen in multiple dimensions. Epithelial cells, fibroblasts, the composition of the ECM, as well as forces generated by collagen fiber organization are all interrelated; changes to each of these components alter the microenvironment and, in turn, change aspects of other system elements. Floating gels can also be maintained for several weeks. Like living tissues, 3D gels show historicity and break symmetries as they develop.

Together, these elegant 3D experiments shed light on the interactions required for normal and abnormal development while corroborating *in vivo* observations. They also prompt further investigation into the use of epithelial/fibroblasts coculture 3D models

for the study of tumor biology. In conclusion, mixed gels provide biochemical and biophysical cues necessary for the development of biologically relevant epithelial structures. In combination with co-culture techniques, 3D collagen gels represent an appealing tool for investigating cell-cell and cell-ECM interactions in both normal and disease contexts. For these reasons, I propose to use mixed gels containing rat fibroblasts and human epithelial cells to identify factors involved in COP resistance to NMU carcinogenesis.

1.7 Thesis, hypothesis and aims

The majority of breast tumors are epithelial in origin; therefore, most research has focused on fast and efficient production of epithelial-derived tumors for study *in vivo* and *in vitro*. Tumor resistance has been described in several rat strains but the origin of this resistance is poorly understood. The neoplasia-resistance of COP rats to carcinogen exposure has yet to be adequately described. A pilot project performed several years ago in our lab has implicated the stroma of COP rats as the source of tumor resistance. This project consisted on the recombination of fibroblasts from fat pads of resistant and susceptible strains exposed to NMU or vehicle with unexposed epithelial cells. Subsequently, the fate of this tissue recombinant was tested under the kidney capsule of SCID mice. Based on these experiments, I propose to examine differences in COP rat mammary stroma and their role in carcinogenic resistance when compared to the NMU-sensitive WF rat strain. My thesis project is aimed at explaining whether epithelial-stromal interactions are responsible for tissue-wide stabilization and suppression of neoplastic lesions.

Aim 1) To characterize MCF-12A cells and determine their behavior in a 3D culture model. **Aim 2)** To determine differences in morphology of MCF-12A structures grown in

the presence of COP and WF fibroblasts exposed to NMU. **Aim 3)** Use microarray data to determine whether or not differences in gene expression profiles exist between NMU-exposed WF and COP stroma that may contribute to resistance.

1.7.1 Specific Aim 1: MCF12A cells develop normal, branching, ductal structures and acini in a 3D culture model.

To explore this hypothesis, I propose to develop a 3D model using MCF-12A cells, which were reported to be ER-alpha positive. To verify ER-alpha expression, I will perform qPCR with RNA extracted from cells collected under normal culture conditions. ER-alpha positive MCF7 cells proliferate in the presence of estradiol and fail to proliferate when cultured in estrogen-free media. This feature will be tested in MCF-12A cells to verify receptor functionality. Verification of both receptor expression and function will allow for additional manipulations of MCF-12A cells once seeded in the 3D culture environment. The behavior of MCF-12A in combined collagen-Matrigel environments has yet to be determined.

In a 3D culture environment, breast epithelial cell lines grow in markedly different patterns depending on the biomechanical properties of the ECM. MCF-12A form round colonies in Matrigel-only culture conditions used by Kenny et. al. [162]. We expect to see these structures as well as lumenized ductal structures recapitulated in our culture model containing both rat-tail Collagen-I and Matrigel. MCF-12A cells are expected to proliferate and to be gene expression-responsive to mammogenic hormones. Colonies are expected to express markers for cell adhesion and produce at least a partial basal lamina.

1.7.2 Specific Aim 2: To determine differences in morphology of MCF-12A structures grown in the presence of COP and WF fibroblasts exposed to NMU.

I propose to use fibroblasts derived from the cleared fat pads of tumor-susceptible WF rats and tumor-resistant COP rats both treated with NMU or vehicle. These cells will be used as stromal components in our 3D breast model. In this model, extracellular matrix (ECM) consists of 1 mg/mL rat-tail collagen 1 and 5% vol/vol Matrigel. I hypothesize that vehicle-treated WF and COP fibroblasts will promote normal morphogenesis. In contrast, I argue that NMU-treated WF fibroblasts will form disorganized, grape-like or stellate, epithelial structures. Finally, NMU-exposed COP fibroblasts are hypothesized to overcome NMU insult and behave as vehicle-treated fibroblasts by promoting normal ductal morphogenesis.

Experiments performed in our laboratory using the WF strain have shown that the stroma is responsible both for tumor suppression and for promotion [115] [163] thus, healthy stroma should induce epithelial cells to form normal lumenized structures. Conversely, WF fibroblasts treated with NMU are hypothesized to promote morphogenesis of disorganized acinar epithelial structures when recombined with normal epithelial cells. Given that an explanation of the COP resistance phenomenon based on studies centered on the epithelium has remained elusive, we hypothesize that it is the stroma that attenuates tumor susceptibility. Thus, we expect to see increased number of organized acinar and ductal structures of MCF12a cells in the presence of NMU-treated COP fibroblasts compared with those co-cultured with NMU-treated WF fibroblasts.

1.7.3 Specific Aim 3: Use microarray data to determine whether or not differences in gene expression profiles exist between NMU-exposed WF and COP stroma cells that may contribute to resistance.

Preliminary work was done in our lab to identify differential gene expression in COP and WF rat mammary fat pads following NMU injection. I will reanalyze these data taking advantage of recent advances in computational analysis of large data sets that allow for greater resolution in tissue-wide gene expression changes.

At 17 days of age, the 4th and 5th abdominal mammary glands of WF and COP rats were cleared of epithelium (CFP). Animals were placed into four groups: Group 1 WF/Vehicle (VEH), Group 2 WF/NMU, Group 3 COP/VEH, Group 4 COP/NMU. At 50 days of age, rats were given a single intraperitoneal injection of 50 mg/kg NMU or VEH. CFPs were dissected from three animals per group at 5, 15, and 60 days post-injection. Our previous work has found that three repeats were sufficient for statistical purposes. The rationale for these time-points was as follows: 1) when vehicle-treated WF epithelial cells were injected into the CFPs of WF animals 5 days after NMU treatment, the resulting recombinations gave rise to neoplastic lesions in over 75% of animals. And 2) Korkola et al. [122] reported that both COP and WF had similar numbers of pre-neoplastic lesions at 15 days after NMU injection and were completely resolved in COP animals after 60 days.

Gene Ontology and Integrated Pathway analyses will be performed to identify cellular components, molecular functions and biological processes associated with resistance and susceptibility.

VEH-treated COP stroma from all three time-points will be considered as normal tissue control. Comparable stroma of COP-VEH and WF-VEH glands at all time points are expected to provide information regarding expression differences in a healthy resistant gland and those which are unable to correct carcinogenic instabilities. We expect to see the widest differences in expression between the stromal compartment in WF and COP following NMU treatment. Because the mammary stroma is responsible for deposition of the majority of ECM proteins, expression of fibronectin, tenascin, laminin, clusterin, MMPs, which have been shown to vary during tissue remodeling and other components, will be of particular interest. Furthermore, comparisons of differential gene expression between WF and COP animals, exhibiting tumor progression and stabilization/normalization, respectively, will give insight into the dynamic carcinogen-induced response expected to exist within the tissue. Preliminary microarray results from CFP implicate changes in expression of genes responsible for cell adhesion, ion transport, metabolism, and MAP kinase activity. Changes in gene expression that are specific to the stromal compartment could potentially lead to novel sources of neoplastic resistance and stabilization.

1.7.4 Concluding Statement

I hypothesize that the tumor-resistant phenotype of COP rats is due to stromal-mediated normalization of tissue-wide disruptions in the mammary gland that develop following carcinogenic exposure. The outcomes of the experiments detailed here are important for furthering our understanding of the process of tumorigenesis, provide explanations for autonomous tumor remission and, more broadly, aim at widening the focus of treatment beyond the breast epithelial compartment.

CHAPTER 2: ENVIRONMENTAL ENDOCRINE DISRUPTORS AND THEIR EFFECTS ON THE HUMAN MALE REPRODUCTIVE SYSTEM [1]

[1] Sweeney, M. F., Hasan, N., Soto, A. M., & Sonnenschein, C. (2015). Environmental Endocrine Disruptors: Effects on the human male reproductive system. *Reviews in Endocrine & Metabolic Disorders*, *16*(4), 341–357. http://doi.org/10.1007/s11154-016-9337-4

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2.1 Introduction

Between 1950 and 2000, the incidence of several conditions deleteriously affecting male sexual organs increased annually. Epidemiological studies showed that, in most areas of the industrialized world, incidence rates of prostate and testicular cancer [164], maldescended testes and anatomical malformations of the male genitalia increased [165], while sperm quality steadily declined [166]. In the past 25 years, explanations have been offered to justify these trends and better interpret the scientific data collected in this field. For instance, Sharpe and Skakkebaek proposed the "estrogen hypothesis," wherein exposure to increased levels of estrogen *in utero* was responsible for these changes drawing connections between altered development and diseases of adulthood [167]. Others suggested a connection between the increased presence of environmental chemicals which affected pregnant women and, directly or indirectly, their conceptus during early development [168].

Normal development and maintenance of the prostate gland and testes are heavily dependent on the regulation of both locally acting and circulating hormones; the same is true for postnatal development and maintenance of the mammary gland (MG). Knowledge regarding the complex development of these organs as well as the nature of their interactions with endocrine disrupting chemicals (EDCs) has increased substantially. These findings suggest that EDCs have the potential to alter cell and tissue behavior over both short- and long-time frames. Importantly, it has been proposed that exposure to EDCs during a man's lifetime, i.e. from fetus to adult, is responsible for an increased predisposition toward developing endocrine-related cancers later in life. This "EDC hypothesis" was derived from the broader perspective originally offered at the 1991 Wingspread Conference, where biologists met to discuss the implications of EDCs in the environment, their effects on wildlife, and their impact on the future of human health

[168, 169]. In this review, we discuss the current literature regarding the roles of EDCs in sexual development and carcinogenesis in men, how the two may be linked, and attempt to address some of the contradictions and controversies in the field.

2.1.1 What is an EDC?

In the Statement of Principles released by The Endocrine Society, an EDC is defined as "an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action [170]."

2.1.2 How do EDCs exert their effects?

While the biological function of an EDC is disruption of normal hormonal action, the processes by which this is achieved vary widely. Hormones interact with their specific receptors, mostly nuclear, and exert their effects on cells by activating or repressing target genes [171]. Similarly, EDCs also interact with hormone receptors specific for the hormones that they mimic. For example, estrogen-mimics like bisphenol A (BPA) and diethylstilbestrol (DES) act by exerting their effects mainly through the estrogen receptor (ER) alpha, while BPA can also exert its action through ER-beta [172]. EDCs can also exert their effects through different nuclear receptors such as members of the peroxisome-proliferator activated receptor (PPAR) family present in reproductive tissues [173]. Following phthalate exposure, adult mice have shown developmental effects attributed to direct activation of PPARs, especially PPAR-alpha and PPAR-gamma [174].

Of note, EDCs are not strictly "specific" in their binding to hormonal receptors, as several EDCs have been found to have multiple hormonal activities. Dichlorodiphenyltrichloroethane (DDT), for example, is categorized as an estrogen agonist, while one of its metabolites is an anti-androgen [175]. BPA, which has estrogenic

activity, is also a thyroid hormone antagonist and has been shown to bind prostatic androgen receptors in men afflicted by castration-resistant prostate tumors [170, 176, 177]. Estrogens from exogenous sources bind the nuclear ER, cell membrane ERs, estrogen-binding protein GPR30, and plasma carrier proteins, albeit with a lower binding affinity than the endogenous estrogens [178-180]. Additionally, chemical structural analysis of a candidate EDC is an insufficient predictor of its potential hormone receptor targets [181]. It is also important to note that given the dynamic physiology of exposed organisms, these chemicals affect their perceived "primary target," which in turn can secrete other hormones or factors that act on "secondary target tissues" and so on. Xenoestrogens exert their activity in additive and synergistic manners when present in low doses [178]; the effects of EDCs do not have a linear relationship with the dose of exposure, a topic that is discussed in the next section.

EDCs also interfere with hormone synthesis and metabolism either directly or indirectly [168]. Thiophosphates inhibit p450 enzymes that are involved in the metabolism of estrone and testosterone in the liver [182, 183]. EDCs can also affect hormone receptor expression, e.g. perinatal exposure to BPA showed dysregulation of steroid receptors and co-regulators in the rat testes, and interestingly, this latter phenotype was passed down for generations [184]. *In utero* exposure to diethylhexyl phthalate (DEHP) resulted in decreased mineralocorticoid receptor mRNA and protein expression in adult interstitial Leydig cells of Sprague-Dawley rats [185].

Besides directly interfering with hormonal pathways, EDCs can affect the epigenetic landscape of cells in target tissues. For example, exposure to BPA [186], cadmium [187], vinclozolin [188] and DEHP [189] altered DNA methylation patterns in both prostate and testicular cells. Histone methylation patterns can also be affected, as seen in testes

exposed to vinclozolin and dibutyl-phthalate (DBP) [190]. EDCs directly or indirectly affect gene expression profiles and transcriptomes on a large scale. For instance, *in utero* exposure to vinclozolin altered the expression of 576 genes in embryonic rat testes [191]. Also, the testes of CD-1 mice exposed to mono(2-ethylhexyl) phthalate (MEHP), and zearalenone, a fungicide, showed distinct gene expression signatures [192]. Given that epigenetic patterns can be heritable, it is not surprising that the effects of EDCs on transcriptomes, DNA methylation patterns and histone modifications have been observed in successive generations [184, 193, 194].

The evidence that EDCs alter development is further supported by findings that EDCs can affect small non-coding RNAs that are implicated in development. In particular, microRNAs (miR) are involved in proper differentiation of primordial germ cells (PGCs) and have been shown to be dysregulated in testicular germ cell tumors [195]. Alterations in miR expression have been observed in mouse Sertoli cells that were exposed to nonyl-phenol [196]. Other EDCs, such as BPA and DDT have also been linked to altered miR expression in estrogen-responsive human breast cancer MCF-7 cells and in placental cell lines, respectively [195].

Epithelial-stromal interactions are necessary for development of the MG [197] and the prostate [198]; disruption of these interactions have been well characterized in these tissues during tumorigenesis [115, 198-200]. While effects of EDCs are mostly manifested in the epithelial compartment of target tissues, EDCs can also exert their effects through the stromal compartment. For example, prenatal exposure to BPA alters the differentiation pattern of periductal stromal cells in the rat ventral prostate [201]. *In utero* exposure to BPA accelerates fat pad maturation and increases the density of collagen fibers around epithelial structures during embryonic development of the mouse

MG with a concomitant delayed lumen formation in the epithelium [202]. Transcriptomal analyses of BPA-exposed embryonic mammary epithelium and periductal stroma showed alterations in apoptosis genes in the former and focal adhesion and adipogenesis genes in the latter [203]. In the adult mouse MG, *in utero* exposure to BPA altered the DNA synthesis rate in stromal cells [204]. Similarly, inhibition of the prostatic epithelial bud during embryonic development in mice by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is mediated through the mesenchyme rather than the epithelium [205]. TCDD also mediates its inhibitory effects on pregnancy-associated MG development in mice by acting on both the stroma and the epithelium [206]. DES exposed hamster seminal vesicles showed altered stromal cell organization and epithelial dysplasia in both neonates and adults [207] and perinatal BPA exposure altered the prostatic stroma [201].

Epithelial morphogenesis requires changes in cell shape, a phenomenon that involves biomechanical forces [208]. The role of biomechanical cues in tissue development is only recently being explored in different tissue systems such as the developing lung [209, 210], heart [211], kidney [210] and the MG [93, 210]. Hormones can also provide biomechanical cues; for example, estrogen has been shown to affect the biomechanical properties of cells isolated from the cornea [212], endothelium [213] and the anterior cruciate ligament [214]. In addition, treatment of estrogen-responsive human breast cancer T47D cells in collagen type I matrix with mammogenic hormones (estradiol, progesterone and prolactin) resulted in differential collagen organization patterns around epithelial structures [151, 215]. Although the hypothesis that EDCs change the biomechanical properties of target tissues has yet to be tested, there is evidence that EDCs affect structural protein expression in the extracellular environment. For example, BPA has been shown to affect collagen fiber organization around epithelial structures

[202], as well as expression of tenascin and filamin b proteins in the periductal stroma [203] in the embryonic mouse MG. Neonatal hamster seminal vesicles exposed to DES showed altered expression of E-cadherin and beta-catenin proteins [207]. Malignant transformation of normal tissue is associated with changes in biomechanical properties as seen in the MG [157, 216, 217] and the prostate gland [218, 219].

Together, these findings highlight both the short- and long-term effects of EDCs on tissues. While the phenotypes of affected tissues might be different, it is clear that EDCs act in a systemic manner, a conclusion also supported by reports that EDCs can trigger inflammatory responses in several target tissues [220].

The effects of embryonic and fetal exposure to EDCs are best understood from the perspective of ecological developmental biology (Eco-Devo), because this discipline studies the environmental determination of phenotypes, a phenomenon known since the late 19th century [221]. Eco-devo also further supports the Barker hypothesis whereby some adult diseases have fetal origins [184]. The timing of exposure to EDCs can influence the severity of consequential effects; those effects observed after fetal exposure occur at significantly lower doses than those reported to produce effects in adults (example in section 5.4).

2.2 EDCs in development and cancer

The activity and long-term effects of individual EDCs have been studied in the context of endocrine action, especially during early development. Thus, it becomes difficult for an EDC to fit the classical definition of a carcinogen under the current *zeitgeist*. Under the tenets of the currently prevailing theory of carcinogenesis, namely the Somatic Mutation Theory (SMT), carcinogenesis is a two-step process that occurs within a single normal

cell, which, after accumulating somatic mutations, proliferates uncontrollably, and gives rise to a tumor [222]. As discussed above, beyond their cell-specific effects, EDCs can have tissue-centered effects. While some EDCs can generate mutations, most are not mutagenic *per se* [184]. However, evidence points to correlations between EDC exposure and the development of cancers in endocrine target tissues [223]. Therefore, on the one hand, empirical evidence requires an alternative theory of carcinogenesis to comprehensibly explain the available data, while, on the other, the same evidence demands attention on how to evaluate a chemical's toxicity and carcinogenicity by regulatory agencies.

The connection between EDCs and tumorigenesis is better understood under two alternative key premises. They are 1) proliferation and motility is the default state of all cells, and 2) carcinogenesis is a tissue-based process [64]. These are the premises adopted by the Tissue Organization Field Theory (TOFT) of carcinogenesis. More explicitly, TOFT posits that the morphogenetic tissue units are the target of carcinogens, and that carcinogenesis occurs when the reciprocal communication between the stroma and the epithelium of those morphogenetic units is disrupted [41].

The case of DES, administered to pregnant mothers *en masse* beginning in the 1940s to prevent miscarriage [224], is a prime example that explains how an EDC effectively acts as a carcinogen under the premises of TOFT. Young women who had been exposed to DES *in utero* developed a rare cancer, namely, clear cell carcinoma of the vagina. Later, this same exposure resulted in increased incidence of breast cancer at the age of prevalence [224, 225]. Rodents exposed to DES *in utero* showed abnormal development of the female reproductive tract in adulthood [223]. Experimental data suggests that exposure to DES, as well as other synthetic estrogens altered the reciprocal stromal-

epithelial interactions needed for proper development, thus increasing the tissues' proclivity towards a neoplastic state as posited by TOFT [203, 223].

These findings, along with emerging epidemiological and experimental data, lend to the conclusion that cancer is "development gone awry" [13, 180, 223]. The xenoestrogen BPA represents a more recent model of how an EDC can cause cancer - BPA affects MG development in female rats exposed to it *in utero* and perinatally in a number of ways such as by accelerating fat pad maturation, changing the stromal composition, inducing ductal elongation, inhibiting lumen formation and altering the pattern of gene expression of both stroma and epithelium during fetal development. The exposed MG exhibits increased sensitivity to estrogen and progesterone, and thus continues to develop abnormally in response to normal levels of ovarian hormones. All these changes, when taken in the context of hormonal action during puberty, disrupt the MG environment. As a result, an increased incidence of preneoplastic conditions in the MG is observed when sexual maturity is reached [180, 223].

Human and animal studies, as well as *in vitro* experiments, have shown that sex hormones have differential effects on target tissues; in the same tissue, a hormone may act to induce cell proliferation at low concentrations, and inhibit it at higher concentrations (shut-off) – a phenomenon that was termed the direct negative hypothesis of control of cell proliferation [226]. Both estrogen and testosterone induce the proliferation of their respective target cells *in vitro* at low concentrations and inhibit it at higher doses and therefore support the above-referred hypothesis [227, 228]. Interestingly, the androgen-induced shut-off was mediated by the *APRIN* gene, that is only expressed at high doses of androgens and not at the low doses, both *in vitro* and *in vivo* [229]. Similar observations were made *in vivo* in the rat prostate [230] and, in the

case of estrogens, the uterus [231], and the mouse MG [232]. These findings support the biphasic pattern of androgen therapy that favors intermittent androgen suppression approaches for prostate cancer (PC) treatment in clinical settings [233-235]. EDCs exhibit a similar dose response in reproductive tissues; male CF-1 mice exposed to DES or estradiol *in utero* showed an inverted-U-dose-response relationship with their prostate weight in adulthood [236]. Similarly, female CD-1 mice exposed *in utero* to either DES or methoxychlor (organochlorine pesticide), followed up by an estradiol challenge in adulthood, showed increased uterine weight at lower doses of DES or methoxychlor compared to those exposed to higher doses of both EDCs [237], thus confirming the non-monotonic dose response (NMDR) of EDCs.

These findings illuminate two seemingly conflicting aspects regarding EDC's effects, namely, 1) receptor affinity to many EDCs is much lower than those of endogenous hormones and 2) mid-to-high doses of EDCs do not always result in obvious effects nor are dose-effect relationships always consistent throughout life stages. Over the past decade, increasing evidence accumulated supporting the deleterious "low-dose" effects of chemicals, such as BPA [203, 238, 239]. The NMDR nature of EDCs is a direct result of the dual activity of hormones under normal conditions. Out of 148 papers describing NMDR-like chemical action across a wide-range of doses, 82 examples of moderate-to-high plausibility of non-monotony have been identified [240]. Explanations for NMDR include: differential activation of discrete receptors at varying concentrations, receptor desensitization, receptor dimerization incompatibility, dose-dependent differential gene expression, and cell-specific negative feedback regulation [240, 241]. These factors must be considered in both tissue-dependent and developmental contexts when investigating the action of candidate EDCs.

2.3 EDCs in Prostatic Development and Cancer

The prostate gland relies on circulating hormones for normal developmental morphogenesis and functionality in men during the reproductive years and beyond. Reciprocal interactions between the urogenital sinus epithelium and its associated mesenchyme regulate prostatic differentiation and morphogenesis; however, the presence of exogenous hormones or reductions of systemic hormone levels disrupt this sensitive balance [242]. In the former circumstance, an increased risk of PC has been documented; that is, chronic exposure of humans or rodents to high levels of estrogen leads to increased risk of PC [243, 244]. In contrast, castration in early life results in dramatic decreases in the levels of circulating androgens, prostate size and, consequently, PC [245].

2.3.1 Prostate gland development

The prostate gland develops from endodermal origins while the seminal vesicles are derived from the mesonephric ducts and are of mesodermal origin [246]. Prostatic development and function are highly dependent on androgen receptor activity. Loss of the androgen receptor (AR) in rodents or in humans leads to failure of the prostate to develop [247, 248]. Conversely, exposure of female urogenital sinus tissues *ex vivo* to androgens leads to the formation of prostatic buds [249]. Androgenic steroids trigger both the initiation of prostate development from the urogenital sinus and the masculinization of the Wolffian ducts to form the epididymis, seminal vesicles, and vas deferens [250]. Unlike the multi-lobular rodent prostate, the human prostate is a single, dense organ divided into three distinct zones, each defined histologically based on its ductal organization. Androgen dependent expansion of both stromal and epithelial compartments of the prostate occurs throughout sexual maturation with the gland

growing from 1 g at birth to 20 g following puberty [251]. The expression of 5-alpha reductase by the prostatic mesenchyme is necessary for local conversion of androgens to testosterone, which, together with local conversion of androgens to estrogen by aromatase, play important roles in cell proliferation and morphogenesis [252].

The prostatic stroma expresses ER-alpha while prostate epithelial cells express ER-beta [253]. Expression of the ERs varies throughout life and their function in adult tissue is not fully understood. ER-alpha is expressed homogenously in the prostatic stroma where it seems to be indirectly responsible for the hyperplasia and squamous metaplasia reported in the epithelium following estrogenic exposure [254]. Complete loss of ER-alpha activity does not result in gross changes in prostate development or function in rodents. In rodents, ER-beta expression is low at birth and gradually increases until puberty, while in the human urogenital sinus, it is maintained during morphogenesis and cellular differentiation before drastically decreasing in early puberty. This low-level expression continues throughout men's reproductive life but may increase in response to androgenic activities associated with benign prostatic hyperplasia (BPH) and PC [255]. Excessive *in utero* exposure to estrogens may be responsible for the estrogenization of the prostate leading to permanent alterations of hormone receptor expression and function [256]. African-American men have a two-fold higher risk of developing PC and higher levels of circulating estradiol compared to Caucasian Americans [257]. In addition to heredity and lifestyle differences, exposure to elevated levels of maternal estrogen in the womb may be a major factor in these men's higher incidence of PC [258].

2.3.2 Prostate Cancer

In 2012, PC resulted in the death of an estimated 307,500 men worldwide, making it the fifth most common cancer-type mortality in men [164]. Following an increased incidence

of PC in the three previous decades, the number of patients with PC has stabilized, a pattern primarily associated with early detection [259]. The prostatic zones differ in their respective cancer susceptibility; the inner zone is the most common site of BPH while the outer zone is the most likely site of PCs. Differences in developmental patterns between the prostate and adjacent accessory glands may be responsible for the profound differences in their cancer susceptibility, as only about 50 cases of seminal vesicle adenocarcinoma have been reported [260]. Most prostate tumors are dependent on circulating androgens for growth and, accordingly, early interventions are aimed at lowering androgen serum levels [261]. Despite initial reductions seen in tumor size following castration or androgen-attenuating interventions, advanced PC continues to be dependent on androgens through increased AR and/or 5-alpha reductase expression [261]. In some patients, anti-androgen treatments may later act as AR agonists in advanced cancers [262]. Later in life, the aromatization of testosterone to estradiol by peripheral adipose tissue, coupled with decreasing testosterone synthesis, is believed to be, at least partially, responsible for the increased occurrence (~50%) of BHP and PC in men by the age of 60 [263].

2.3.3 Effects of EDCs on Human Prostate Glands

Driscoll and Taylor performed a histological analysis of tissues derived from infant autopsies and found that all infant boys in the study exposed to DES *in utero* presented prostatic utricles and dilated ducts with squamous metaplasia while low exposure-risk controls appeared normal [264]. Recently, it was found that young men with histopathologically verified PC had higher serum levels of BPA than healthy controls. In fact, BPA levels were a more relevant indicator of PC than prostate-specific antigen, which was shown to be a less reliable marker [265].

On the experimental front, administration of genistein, a phytoestrogen commonly present in soy-based foods, dietary supplements and infant formulas, to athymic mice implanted with human prostate PC3-M cells reduced metaplasia by 96% [266]. While this may suggest that some estrogens directly attenuate prostatic hyperplasia and metastasis, it is equally possible that genistein triggers a negative feedback response through the pituitary gland resulting in reduced androgen synthesis. Finally, using prostate epithelial cells from healthy donors, Prins et al. found that BPA increased the expression of genes associated with self-renewal and maintenance of non-differentiated phenotypes [267]. These cells were grown under the mouse kidney capsule and the resulting epithelial structures were exposed to an estrogen/testosterone mix. The exposed, grafted, tissues developed neoplasia, implying that the prostate epithelial cells were targets of BPA.

Polychlorinated biphenyls (PCBs) are highly lipid-soluble compounds that leach continually into ecosystems from waste, despite not having been commercially manufactured since the late 1970s [268]. Occupational and environmental studies have provided strong evidence for an association between lifetime exposure to PCBs and the prevalence of PC [269, 270]. Teenage boys born with high levels of PCBs in their cord blood where shown to have significantly lower levels of both luteinizing hormone and testosterone, indicating an interaction of PCBs with the hypothalamic-pituitary-gonadal axis [271]. Various pesticides have been shown to induce endocrine disruption and exposure to them has been found to correlate with increased risk of PC [169]. Organochlorine pesticides have a half-life of up to several years, accumulate in adipose tissue and exposure may result in continuous endocrine disturbances that increase PC risk through both direct exposure or indirectly through maternal transfer through the placenta [272]. Pesticide applicators who came in contact with organophosphates

fonofos, malathion, terbufos or the organochlorine Aldrin showed an increased risk of aggressive PC [273].

2.3.4 Effects of EDCs on Rodent Prostate Glands

High serum levels of estrogen have been implicated in an increased risk of PC in humans; however, rodents required increased levels of both androgens and estrogen to promote PC formation [274]. Early life exposure of neonatal rats to BPA enhances the prostate's susceptibility to estrogen-induced hyperplasia following exposure [275]. Similarly, Sprague-Dawley rats exposed to low-dose BPA (25 ug/kg/day) *in utero* showed a dramatic increase in hyperplastic lesions in the ventral prostate at PND180 [276]. Epigenomic analysis of the rat prostates exposed to environmentally relevant doses of BPA revealed alteration in the methylation status of several genes associated with preneoplastic lesions [275].

In rats exposed to mixtures of anti-androgens or a mixture of 13 well-characterized endocrine disruptors, the ventral prostate epithelium was hypertrophic and displayed cribriform patterns in late adulthood despite a normal prostate weight observed at PND55 [277]. The anti-androgenic EDC vinclozolin caused atrophy of the prostate in adult rats while low-dose exposure during gestation resulted in irreversible decreases in prostate size [278]. Metabolites of methoxychlor have both estrogenic and anti-androgenic properties. Administration of methoxyclor to pregnant mice led to male offspring with permanently enlarged prostates [238]. In the same study, male mice exposed to high levels of estrogen or DES *in utero* presented with smaller prostates than controls. Despite these findings, methoxyclor's effects in developing and adult humans have been given limited additional attention as an EDC. Complex mixtures of PCBs were reported as *bona fide* rodent carcinogens and were shown to demonstrate both

estrogenic and anti-androgenic activity *in vitro* [279]. Following exposure to PCBs, male rodents showed reduced ventral prostate weight, possibly resulting from their dual action on sex hormone receptors in the prostate [280]. Aroclor-1254, a mixture of 60 PCBs believed to be relevant in the evaluation of environmental exposures in humans, altered the expression of gap-junction proteins [281], ultimately disrupting cell-cell communication [282].

2.4 EDCs in Testicular Development and Cancer

Testes are the site of male gametogenesis and where most of the production of testosterone, the principal androgen in males, takes place. In turn, testicular development and function require proper hormonal control and, therefore, are sensitive to disruption by exogenous hormones [242]. The most common risk factor for testicular cancer (TC) is improper testicular development [283], further strengthening the notion that cancers represent "development gone awry", a notion that is implicit in the TOFT, and made explicit in a later publication [47]. In addition, by interfering with normal testicular development, EDCs may increase the risk of neoplastic development.

2.4.1 Testicular Development

Early in human development, the male and female gonads are sexually naive. The *SRY* gene on the Y chromosome, responsible for testicular organogenesis and the formation of testicular cords from pools of germ cells, is inactive before the 7th week of gestation [284]. Sertoli cells produce anti-Müllerian hormone responsible for suppressing the formation of the uterus and associated structures from mesonephric ducts. Once the testicular capsule, the tunica albuginea, is formed, the connection between the testicle and its adjoining supporting tissue is severed. Leydig cells within the testicle begin secreting testosterone, which is responsible for the masculinization of the Wolffian ducts

into the vasa deferentia, seminal vesicles, and epididymides as well as the morphogenesis of the penis and scrotum [285]. Along with Insulin-like growth factor 3 (INSL3), testosterone is crucial for testicular descent from the abdomen into the scrotum via the inguinal canals [286]. After the testicles complete their descent, Sertoli cells differentiate and polarize, eventually forming a true lumen during early puberty. While testicular development is not completely understood, it is clear that hormones play a pivotal role in the proper formation and descent of these organs from their earliest developmental stages. Interruption of hormonal function within the developing tissues have been shown to lead to undescended testicles (cryptorchidism) [268], improper positioning of the urethral opening (hypospadia) and sterility [287].

2.4.2 Testicular Dysgenesis Syndrome (TDS)

Based on epidemiological, clinical and laboratory findings, Skakkebaek et al described the Testicular Dysgenesis Syndrome (TDS) consisting of varying degrees of cryptorchidism, hypospadias, and impaired spermatogenesis. These features are closely associated with TC susceptibility and it was claimed that these features result from altered prenatal testicular development [288]. This hypothesis fits well within the TOFT in the sense that, as mentioned above, neoplasia is "development gone awry"; TDS qualifies as a representative example of an *inborn induced error of development* [13]. Human TDS may be further exacerbated by exposure to EDCs during childhood and adult life [242].

Alteration of the hormonal milieu during gestation or perinatal life by exposure to exogenous estrogens or anti-androgens resulted in the malformations described in TDS as well as Leydig cell tumors and teratomas [268]. Several male CD-1 mouse offspring exposed to DES through daily prenatal injections (100ug/kg/d) became infertile and 15

of 24 males had noticeable testicular abnormalities including intra-abdominal testis at 9-10 months of age [289]. In a trans-generational study, twenty percent of F2 male CD-1 offspring borne from mice exposed *in utero* to DES presented with exposed urethral flaps, analogous to hypospadias, compared to 0% from oil-exposed controls, implicating parental exposure as a source of TDS characteristics [290]. BALB/c mice exposed to DES by means of a subcutaneous pellet displayed interstitial cell tumors, marked by hyperplastic Leydig cells, after 180 days of treatment [291].

2.4.3 EDCs in Testicular Development and Cancer

TC is the most common type of malignancy in men aged 15-40 years in industrialized countries and is the most frequent cause of death from solid tumors in this age group. Approximately 95-98% of all TCs are germ cell tumors (TGCTs), and 1-5% of TC result from hyperplasia of testicular somatic cells (Sertoli and Leydig cells). TGCTs consist of a diverse group of neoplasms, based on the different anatomical locations within testis where they appear. Histopathologically, they have been classified into two main categories - seminomas, which have features similar to those of PGCs and non-seminomas, which include mixed germ cell tumors (the most common), embryonal carcinomas, teratomas, choriocarcinomas and yolk sac tumors [195, 292]. The incidence of TC has increased worldwide over the last 40 years, with an incidence peak in young adults [292, 293]. The most consistently identified risk factor associated with TC is cryptorchidism, which increases men's risk of developing TC by almost 5-fold [283].

TC consists of carcinoma *in situ* (CIS) cells that supposedly derive from PGCs that did not differentiate into spermatogonia *in utero*. This interpretation is supported by findings that human TGCTs show similar gene expression profiles and DNA methylation patterns to PGCs and embryonic stem cells [195]. In fact, the only mouse model to

develop experimentally-induced TC, the 129/SvJ strain, have tumors arising from PGC populations [294]. There is also strong evidence that developmental arrest in the early germ cell lineage is necessary for neoplastic transformation of cells to CIS [292]. The findings that miRs involved in testicular development show aberrant expression in TGCTs also lend support to the link between TC and abnormal development of the testes [195]. This type of evidence is consistent with the notion that in addition to the classic *sporadic* cancers, inborn induced errors of development are responsible for the appearance of tumors or malformations in the initial decades of life in humans [13].

2.4.4 Are EDCs linked to TC and TDS in humans?

The TDS hypothesis posits that EDC exposures cause developmental disorders and TC (see above). EDCs can alter testicular development because they interfere with hormone action by mimicking and/or antagonizing hormones, and altering their production or metabolism. It has also been suggested that low and high androgen levels and/or excessive estrogen exposure during development can give rise to CIS in the testes [295]. In this regard, a meta-analysis of seven case control studies showed a statistically significant association between subfertility, possibly due to low sperm quality, and increased risk of TC [296].

By exploring the link between EDC exposure and TC, epidemiological data implied how EDC-specific effects can be associated with a carcinogenic outcome. However, in some cases, while a trend is obvious, it may not become statistically significant. For example, a meta-analysis of nine studies showed an increased risk of TC associated with prenatal exposure to DES, but this increase was not statistically significant [297]. Maternal exposure to DES during early pregnancy also increased the risk of factors linked with TC, such as male genital defects, cryptorchidism and impaired sperm quality [298]. A few

case control studies have found a positive association between blood levels of p,p'-DDE, a partial estrogen agonist and androgen antagonist and the primary metabolite of DDT, and risk of TC [292]. Korean newborn boys with hypospadias had significantly higher levels DEHP and n-nonylphenol in urine and BPA and phthalic acid in plasma compared to that of newborns without hypospadias [299]. Although higher levels of BPA were not initially detected in male newborns with undescended testes in France, a negative correlation between cord blood BPA concentration and INSL3 levels was observed recently [300].

Altogether, links between PCBs and TC remain uncertain. A Swedish hospital study noted no differences in PCB levels in men with or without TC; serum from mothers of men with TC, however, showed significantly increased concentrations of several PCB congeners when compared to healthy controls [301]. In contrast, a US study found an inverse correlation between PCB congeners and TC [302], whereas a Norwegian study found epidemiologic evidence that some PCB congeners (99 and 167) may be linked to TC risk [303]. Finally, a recent case control study (125 TC patients vs. 103 controls) concluded that serum concentration of PCBs and hexachlorobenzene were tied to a statistically significant increase in TC risk and lower semen quality [304].

The anogenital distance (AGD) is a parameter of proper masculinization of external genitalia, and, is considered a marker of testicular function. Swan et al. presented the first evidence that AGD was correlated with incomplete testicular descent in infants exposed prenatally to phthalates [305]. Chinese males whose mothers were occupationally exposed to BPA during pregnancy showed shorter AGD [306]. AGD has been confirmed to be a good readout for fetal androgen exposure in humans by several meta-analyses [307].

2.4.5 Animal Studies linking EDCs to TDS and TC

Currently there are no experimental models to study the effects of EDCs on TC, nor are there rodent models that develop TC spontaneously. These factors make it difficult to study TC in the context of endocrine disruption given that experimentally induced TGCTs through genetic manipulation in rodents do not reflect the mode of EDC action.

Although further investigation is required to establish a causative link between TC and EDCs, animal studies have linked EDC exposure to TC risk factors such as TDS. *In utero* exposure to phthalates, inhibitors of androgen synthesis, has been shown to induce characteristics of TDS in male rats, such as cryptorchidism, hypospadias, impaired spermatogenesis, and reduced male fertility, along with lower levels of testicular testosterone indicating dysfunctional fetal Leydig cells [308]. AGD in rodents has been shown to be a sensitive end-point for phthalates and anti-androgens such as flutamide and finasteride [285]. CD rats exposed to DBP *in utero* showed reduced AGD compared to controls, along with hypospadias and aberrant development of the male reproductive tract [309]. Embryonic mouse testes exposed *in utero* to DES showed a delay in testicular descent and alterations in Sertoli cells [310]. Exposure to zeranol (a non-steroidal estrogen used by cattle handlers in the U.S. and a metabolite of the fungicide zearalenone) *in utero* resulted in accelerated differentiation in mouse embryonic testes along with fetal Leydig cell hyperplasia [310]. Male Long-Evans and Holtzman rats, and Syrian hamsters exposed to TCDD perinatally showed dose-dependent developmental defects that ranged from reduced epididymis weights to reduced AGD, delayed testis descent and reduced sperm numbers and quality [311].

The effects of BPA on TC and testicular development remain suggestive but not yet definitive. A 1982 National Toxicology Program study reported an increased incidence of testicular interstitial cancers in mice and rats exposed through diet to doses of BPA similar to oral LD50 levels [312]. However, the controls of the study also showed an elevated baseline of tumor incidence when compared to controls in preceding studies. In contrast, other studies showed that dietary BPA exposure was insufficient to result in TC or aberrant lesions in the testes of Sprague-Dawley rats [313]. Long-Evans rats exposed to low doses of BPA perinatally showed an increase in the number of Leydig cells and altered androgen secretion, although the development of testicular neoplasias was unexplored [314]. *In utero* exposure to BPA did not result in changes in sperm production, seminiferous tubules, germ cell apoptosis and testosterone levels in C57Bl/6 mice [315]; but neonatal CD-1 and C3H/HeJ mice exposed to BPA orally showed aberrant meiotic recombination events in the testicular meiocytes [316]. Male rat pups exposed to BPA *in utero* showed a lower level of serum testosterone compared to non-exposed pups, although the decrease in serum testosterone did not change with various doses of BPA [317]. While an earlier study did not find any effects on AGD in Sprague-Dawley rats exposed to BPA perinatally [318], a more recent one showed that AGD was reduced in male Wistar-Furth rats, examined at PND4, when exposed to BPA *in utero* [319]. It should be noted that these studies were not designed to test whether BPA increased the risk of testicular carcinogenesis or the development of TDS.

2.5 EDCs in Male Mammary Gland Development and Cancer

The female rodent MG has been the model of choice to study the relation between endocrine disruptors and breast development and cancer. Combined with the rarity of male breast cancers (MBCs), there exists a paucity of knowledge on the effects of EDCs on the development of the male breast and its pathologies.

2.5.1 Male Mammary Gland Development

In humans, the male breast is mostly populated by adipose tissue with sparse ducts and periductal stroma. This contrasts with the female breast, which is predominantly comprised of ducts, glandular epithelium, and non-adipose stroma [320]. In mice, the male and female MGs develop similarly until embryonic day 13 (E13). The testosterone surge observed at E14 causes the mammary mesenchyme to condense around the epithelial stalk causing detachment of the glandular epithelium from the epidermis, which leads to a lack of nipples in males [320]. While this lack of nipples has created doubts as to whether the male mouse MG may be used as a representative model to study diseases of the male breast, studies that examined the effects of pre-pubertal exposure to hormones and pharmaceuticals on the male MG concluded that it is still amenable to such studies [321]. Rat MGs show distinct sexual dimorphism; histological analyses from different rat strains show the male MG epithelium to be squamous type with abundant vacuoles, highly eosinophilic cytoplasm and indistinct lumena that occasionally contain secreted materials. The male MG epithelium is also lacking in organization and has large and contiguous groups of alveoli, in contrast to the ductal organization observed in female rat MGs [322].

2.5.2 Cancer and associated diseases of the male breast

MBC is a rare form of cancer with a reported frequency of less than 1% in the population [323]. It is estimated that in 2015, 2,350 new cases of invasive breast cancer will be diagnosed in the U.S. male population, resulting in 440 mortalities [324]. Male breast cancer typically manifests itself around 70 years of age, with 40% of individuals advancing to stages III or IV [325]. MBCs are mostly ER positive (up to 90%), with ductal carcinoma being the most common subtype. Other subtypes such as lobular,

inflammatory, medullary, papillary and trabecular duct carcinomas have also been reported [323]. Multiple factors have been associated with development of breast cancer in men. These include inborn inherited errors of development [13] (e.g. *BRCA1/2* germline mutations, Klinefelter and Cowden syndrome), lifestyles (obesity, excessive alcohol intake, hormone manipulation), and occupational hazards (exhaust emissions, high temperatures). Diseases such as pituitary adenoma, testicular inflammation or damage, liver disease causing hyperestrogenism, as well as exposure of the chest to radiation have also been shown to increase risks for MBCs [323].

The most common condition of the male breast is gynecomastia, i.e. the benign enlargement of the breast due to proliferation of the ductal epithelium that affects 48-64% of boys around puberty; the condition is normally resolved spontaneously within 3 years [326]. Pseudo-gynecomastia, the benign enlargement of the breast due to excessive adipose tissue, is also observed in the male breast [321]. Recently, risk of male breast cancer was significantly associated with gynecomastia in the Male Breast Cancer Pooling Project, a consortium of 11 case-control and 10 cohort investigations involving 2405 case patients and 52013 control subjects [327].

2.5.3 EDCs and human MBC cases

While there are clear examples of EDC exposure linked to breast cancer in women [223, 328], there is no direct evidence of EDCs causing MBCs. In a small number of cases, patients having no family history of breast cancer when treated with DES for PC later developed bilateral gynecomastia and breast cancer [329]. A case-control study on the 71 reported cases of MBC in residents (1950-1985) of Camp Lejeune, North Carolina, suggested associations between vinyl chloride, tricholoroethylene (TCE) and tetrachloroethylene (PCE) found in the drinking water of Camp Lejeune residents and

MBC risk in that population. Exposure to vinyl chloride, TCE, PCE and t-1,2-dichloroethylene (DCE, also found in Camp Lejeune water) was also associated with earlier age onset of MBC [330].

2.5.4 EDCs linked to conditions associated with MBCs in humans

EDCs have been linked to higher incidences of gynecomastia in humans. Case studies suggest that exposures to environmental estrogens or estrogen mimics can contribute to the development of gynecomastia in adult males [331]. For example, 20% of male workers at an oral contraceptive plant in Puerto Rico reported cases of gynecomastia when they were exposed to aerosolized estrogen during the manufacturing process [332]. Environmental anti-androgens have also been associated with increased incidences of gynecomastia; phenothrin used as a delousing agent for treating bedding and clothing provided to Haitian refugees led to significantly higher rates of gynecomastia in that population [333]. A retrospective epidemiologic study of adult men who were administered low doses of DES during treatment of castration-resistant PC showed that 59% of them went on to develop gynecomastia [334]. Reports of pubertal gynecomastia have been linked to exposures to health care products with estrogenic or anti-estrogenic properties [335]. Additionally, a case-control study showed levels of two phthalates were higher in adolescent boys with gynecomastia compared to boys without this condition [336].

2.5.5 EDCs and MBCs associations – evidence from animal studies

Few studies have addressed the effects of endocrine disruptors on male MG development and MBC. Vandenberg et al. [321] showed that the mammary tissue of male CD-1 mice exposed prenatally to BPA displayed an age-dependent NMDR. At 3-4 months of age, animals exposed to 0.25 ug BPA/kg BW/d and 2.5 ug BPA/kg BW/d showed more

advanced gland development than controls, but 25 and 250 ug BPA/kg BW/d groups were indistinguishable from controls. More specifically, the 0.25 and 2.5 ug groups showed more epithelial tree branching points and 2.5 ug group also showed increased ductal area relative to controls. The 2.5 ug group was most severely affected presenting a 4.5-fold increase in branching points and 7.7 fold increase in ductal area, compared to controls. However, at 7-9 months age, the NMDR had shifted such that animals treated with 2.5 and 25 ug BPA/kg BW/d were the most affected. Glands from 25 ug BPA-exposed males had a 3.6-fold increase in branching points and 4.8-fold increase in ductal area. At 12-15 months age, 25 and 250 ug group animals had more branching points compared to controls, and although there was a trend of increase in ductal area, the difference was not significant. The significance of this data is highlighted at the end of section 2.1.

Male Sprague-Dawley rats exposed chronically (*in utero*, nursing and dietary) to genistein showed ductal and alveolar hyperplasia and hypertrophy in MGs, with significant effects observed at 25 ppm and above [337]. These effects were observed across generations where hyperplasias were sustained but did not increase in magnitude or result in neoplasias [338]. The administration of flutamide, primarily used to treat PC, and also present in pesticides, resulted in persistence of nipples and mammary epithelium in male Sprague-Dawley rats exposed *in utero* [339]. Pre-pubertal male Sprague-Dawley rats showed increased lateral budding in MGs when exposed to methoxychlor or genistein and methoxychlor *in utero* and through diet [340]. In adult male Sprague-Dawley rats, exposure to methoxychlor or a combination of genistein and methoxychlor resulted in increased longitudinal growth, density and size of the MGs, increased number of ductal branches and alveolar mass, and exhibited ductal hyperplasia [341].

2.6 Critical Analysis of EDC Research

Extensive evidence obtained from epidemiological studies in human populations have identified DES and DDT as carcinogens in women; animal studies have presented ample evidence to suspect BPA as a carcinogen for the prostate and MGs [328]. However, as the Endocrine Society has recently asserted [342], more research is required to establish the link between defects of the male reproductive system, including cancers, and EDCs, such as the ones discussed above. Currently, there exists a public controversy surrounding the effects of EDCs that originates from confounding results described across multiple studies. In this section, we will describe the sources of such confounding data and whether a connection between EDCs and carcinogenesis and defects of the male reproductive system can be verified.

A major source of confounding data arises from choice of models to study the link between EDCs and developmental defects and carcinogenesis. Models for hormone-induced carcinogenesis were originally developed to obtain high tumor yields with a short latency period; this goal was achieved by treating animals with supra-physiological levels of hormones with or without additional chemical carcinogens [343]. Therefore, these models do not accurately represent how EDCs may affect hormonal action in human populations. Studies on carcinogens have traditionally used a linear dose scale, a method that largely masks those effects seen at low doses [344]. This may explain why EDCs, which may not have significant effects at high doses due to their NMDR, had been ignored as potential carcinogens in early studies. Similarly, traditional toxicological studies that operate under the premise that larger doses should have greater effects also failed to describe the low dose effects of EDCs [241]. Given that the regulation of a chemical is based on the no-observed-adverse-effect-level, which is extrapolated from

the LD50 doses, it is not surprising that the low dose effects of EDCs were ignored given their NMDR nature.

While these comments may address why EDCs and their carcinogenic potential and low dose effects were not discovered earlier, it does not resolve the contradictory results observed in practice. This issue can be partially resolved by taking into consideration the model's sensitivity to candidate EDCs. For example, *in vitro* assays to detect the estrogenicity (E-SCREEN) and androgenicity (A-SCREEN) of environmental chemicals employ cell lines growing in 2D conditions [345]; however, the characteristics of these cell lines differ based on the source they are obtained from and, depending on laboratory practices, can give differing results. For instance, a comparative analysis of MCF-7 cell lines obtained from four different sources showed only one line of MCF-7 cells to be estrogen responsive and suitable for use to screen for xenoestrogens [346]. Rodent models have long been used to study the effects of EDCs in the laboratory. However, different strains of laboratory mice and rats do not show the same sensitivity to EDCs. For example, while Wistar-Furth rats exposed to 250 ug/kg BW/day of BPA show a shorter AGD and other developmental defects [319], Sprague-Dawley rats exposed up to 40 mg/kg BW/day of BPA did not show any changes in AGD [318]; studies have suggested that the Charles River Sprague-Dawley rat strain is relatively insensitive to estrogen and therefore, cannot be used to detect low dose effects of BPA [347]. Different inbred mouse strains also exhibit different sensitivity to EDCs, as seen in the case of BPA [313].

The study design, such as administration route, window and duration of exposure and assessment of endpoints can also influence the outcome of a study [239, 348-350]. For example, oral gavage is widely used by regulatory bodies in risk assessment and hazard

identification studies; however, this method of chemical administration does not mimic human dietary exposure to BPA and other EDCs and can affect the endocrine system by inducing stress in animal subjects [239]. Another study that used Long-Evans rats to study low dose effects of BPA lacked proper positive controls [349]. This highlights the need for novel guidelines for research on EDCs.

Research on endocrine disruptors is based on the principles of endocrinology. On the one hand, hormonal regulation is conserved among mammals; thus, rodent studies, for the most part, teach us about the human. On the other hand, there are species, strains, and individual differences that vary from quantitative differences (higher or lower doses are needed to achieve the same effect) to qualitative ones (no effect). For example, using a fetal testis assay (FeTA) culture system, it was found that phthalates do not alter testosterone production or INSL3 expression in human fetal testes while similar concentrations reduced testosterone production in rat fetal testes [351], however, they do affect the production of testosterone in adult testes [352]. Human fetal testes were shown to be more sensitive to BPA compared to rat and mice testes in an *in vitro* organotypic culture system. While BPA reduced testosterone production in all 3 species at high doses, only human testes were affected at low doses. Additionally, BPA treatment reduced *INSL3* mRNA levels in human testes only [173]. The knowledge gap existing between rodent experimentation and human epidemiology can only be bridged by selecting models that more closely mimic human biology.

While independent research groups have confirmed the low-dose effects of EDCs, the public debate on this issue is partly fueled on by industry influence. This has become evident, especially in the case of BPA, one of the highest volume chemicals synthesized in the world [350]. Recently, the Food & Drug Administration claimed that low-dose effects

of BPA are not amenable to study due to ubiquitous contamination issues [353], a move that marked an apparent end to the debate. However, vom Saal et al. [354] and others [239, 355] were able to show that studying low-dose effects of BPA is perfectly possible without any contamination issues. This emphasizes that the Good Laboratory Practices paradigm exercised by the regulatory bodies is not a reliable indicator of a study's quality when gauging public health risks for endocrine disruptors in a lab setting [350].

We have presented here a summary of the ever-growing body of information on EDCs and their potential and actual deleterious effects on the human male reproductive system. A fair assessment of this subject is challenged by its biological complexity, limitations of animal models and, of course, economic interests. We have discussed how manipulation of systemic hormone synthesis, residential reduction or aromatization of circulating hormones, and receptor function or expression in rodents results in distinct changes in organ structure, function, and development. The safety of candidate EDCs, and those which have yet to be investigated, deserves further questioning in the proper experimental context.

2.7 Conclusions

This review highlights the carcinogenic properties of EDCs on the male genital tract and breast. However, animal studies for the most part address a single chemical and a single window of exposure, whereas in the "real world" humans and wildlife are exposed to a mixture of EDCs that act jointly and contextually. As humans continue to release chemicals into the environment without proper evaluation of the consequences of these decisions, science is ill-prepared for tackling this newly created situation. Most chemicals are not traceable, for lack of sensitive assays, and their effects cannot be studied using classical methods because unexposed controls do not exist, either as a result of direct

exposures or transgenerational effects. In addition, the concept of exposome (i.e. tracking all exposures throughout life), created to bridge this gap, has yet to be properly described, developed and tested.

Regardless of whether or not this concept will materialize as a useful tool, the quandary still remains that in order to understand the problem we created novel experimental approaches will have to be implemented. These new methods should integrate the effects of different doses of structurally different chemicals that for the most part show non-monotonic dose-response curves with the fact that EDCs act at different ages on different target tissues. Would mathematical modeling and computer simulations help to arrive at more definitive answers? While this question awaits resolution, the existing body of evidence justifies the application of the precautionary principle. Therefore, preventive measures should be effectively adopted by those who are genuinely concerned with and are responsible for the public's health to reduce exposure to EDCs.

CHAPTER 3: INVESTIGATING THE ROLE OF STROMAL FIBROBLASTS IN COPENHAGEN RAT MAMMARY GLAND TUMOR RESISTANCE [2]

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3.1 Introduction

The mammary gland of the rat is comparable to that of humans in both histopathology and hormone responsiveness; its tumors originate from ductal regions, closely resembling those found in human breast cancer [356]. The gland's functional epithelium and surrounding connective tissue are in constant communication with each other throughout embryonic development, puberty, and adulthood [197]. While it was long thought that the stroma played merely a structural role, it has become apparent that its roles are far more integral to the glands form and function. Tissue recombination studies have shown that the stroma dictates epithelial morphogenesis even when the two tissues are derived from different organs.

The stroma also plays a major role in carcinogenesis. We have previously shown that the stroma of WF rats is responsible for its susceptibility to the chemical carcinogen n-NMU [115]. Regardless of the exposure status of the transplanted epithelium, tumors only formed in instances where the stroma was exposed to NMU. These findings have two implications: 1) the stroma is the target of NMU-based mammary carcinogenesis and 2) a non-exposed mammary stroma is able to guide the normal morphogenesis of epithelial cells that would otherwise be considered tumorigenic. If the stroma is responsible for susceptibly to mammary tumors, the potential that it may also be responsible for carcinogenic resistance becomes compelling.

COP rats are resistant to both spontaneous and induced mammary gland carcinogenesis. Between two and three weeks after susceptible rat strains are exposed to NMU, their mammary glands are characterized by IDP, a desmoplastic stroma, a marked inflammatory cell invasion, and luminal debris [357]. IDPs are consistent predictors of ductal carcinomas in situ (DCIS) and, eventually, adenocarcinomas. Following a single

exposure to NMU, both resistant and susceptible rat strains, such as WF, develop multiple hyperproliferative IDP lesions throughout their mammary glands. These lesions progress to palpable tumors in WF. In contrast, COP glands undergo complete resolution between 15 and 60 days post-exposure, implicating this time period as a crucial window in the resolution process.

COP mammary glands can develop tumors if a "two-hit" method of carcinogen exposure is used. Neonatal animals (2-3 days old) are injected i.p. with 30 mg/kg of NMU and then again via tail vein injection following puberty at 50 days of age [358]. Before one year of age, 30% of animals that received a single neonatal dose presented with mammary tumors while 80% of animals exposed as both neonates and adults developed mammary tumors. These rates are significantly lower than Sprague-Dawley or WF strains. The ability to predispose the mammary gland during early development reiterates the idea that carcinogen resistance is acquired during development. Also, the administration of perphenazine, which transiently increases serum levels of prolactin, also increased COP susceptibility to NMU by ~5% in pubescent females [134] . However, COP animals typically have circulating levels of estrogen, progesterone, and prolactin similar to what is seen in susceptible strains [359], so it is unlikely that resistance can be ascribed simply to hormonal sensitivity. NMU mammary carcinogenesis can only be brought about in COP glands that were damaged during development or are undergoing aberrant remodeling. NMU mammary carcinogenesis can only be brought about in COP glands that were concurrently undergoing inflammation or aberrant remodeling.

3.1.1 Potential explanations for Copenhagen mammary cancer resistance

NMU is a direct-acting carcinogen in that it requires no metabolic activation to bestow its carcinogenic properties. Although NMU has a short half-life following preparation, it

is a potent alkylating agent and forms DNA-adducts prior to hepatic clearance [360]. To investigate the possibility that WF rats may be more vulnerable to NMU-induced point mutations in the *Ha-ras* gene; the evidence, however, showed that both COP and WF lesions express mutated Ha-ras protein at comparable levels [124]. This finding is in line with other studies that have shown *Ha-ras* mutations in the healthy mammary tissue of both strains [361]. The potential roles of reduced angiogenesis [125] or increased immunosurveillance [126] have also been considered. These tests have failed to answer the question of how the same injury could lead to different pathological responses.

While these approaches were unable to fully explain carcinogenic resistance in COP rats, there has been a concerted effort by geneticists to determine which genes are present in the COP genome that may be responsible for the response. Gould [128] showed that carcinogen susceptibility was a dominant trait but that this characteristic could not be attributed to a single locus. Using similar techniques, others showed that the suppression may be due to a single gene [129]. Despite the insistence that there exists a single, dominant gene within the COP mammary epithelium, later studies have been unable to identify this gene regardless of advances in positional cloning or higher resolution genome sequencing. Many of these regions are largely devoid of known genes [135] but may contain non-coding elements responsible for epigenetic modulation of gene expression.

Here, we describe how stromal-epithelial interactions within the COP mammary gland prevent carcinogenesis. Because previous approaches have been unable to address COP tumor resistance, we will attempt to describe the cancer resistance phenotype under the premise that cancer develops as a tissue-based disease in which reciprocal tissue interactions play a central role.

3.2 Materials and Methods

3.2.1 Cell maintenance

MCF10A (CRL-10317) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) at passage 110. As indicated by the ATCC catalog, cells were grown in Dulbecco's Modified Eagle's/F12 medium (DMEM/F12, 1:1) containing 5% equine serum (ES), 20 ng/mL epidermal growth factor, 0.5 μg/mL hydrocortisone, 0.1 μg/mL cholera toxin, and 10 μg/mL insulin. MCF10A cells were passaged at <80% confluence, using a 0.25% (w/v) trypsin-0.03% (w/v) EDTA solution; media was replaced every 2-3 days. Primary rat fibroblasts were maintained in stromal media (Phenol-red free Dulbecco's Modified Eagle's/F12 medium (DMEM/F12, 1:1), 10% fetal bovine serum, and 50 ug/mL gentamycin). All cells were maintained at 37°C with humidity and 6% CO_2.

3.2.2 Animals

Wistar-Furth and Copenhagen rats were purchased from Harlan and housed with food and water *ad libitum*. SCID mice were purchased from Charles River. Animals were maintained on a 14:10 hours light:dark cycle and housed in accordance with the Guidelines for the Care and Use of Animals and the Tufts-New England Medical Center Institutional Animal Care and Use Committee.

To generate cleared fat pad-derived fibroblasts and glands for microarray analysis, the 4th and 5th inguinal mammary gland epithelium was surgically removed from PND14 animals according to DeOme [362]. The excised epithelium was whole-mounted and observed microscopically to assure that the ductal tree was removed in its entirety and that only a small portion of the fat pad remained attached to it.

3.2.3 Isolation of rat mammary fibroblasts and mammary epithelial cells

At PND50, rats from each group were randomized and injected intra-peritoneally with either vehicle or 50mg/kg NMU. Five days later, the animals were culled, and their cleared fat pads were removed and processed for fibroblast isolation. The fibroblasts were maintained in 75 cm^2 flasks (Corning) in stromal media.

Age-matched, intact, virgin COP and WF rats were euthanized for mammary epithelial cell isolation. Briefly, the 4th and 5th inguinal mammary glands were excised, minced and digested in phenol red-free DMEM with 0.15% collagenase III added at 37°C for 2 hours with agitation. The dissociated tissue was centrifuged and the resultant pellet was treated with 0.05% pronase for 30 minutes at 37°C with agitation before being filtered through a 530 µm-pore Nitex® filter (Sefar America) The filtrate was centrifuged at 100 ***g*** for 3 minutes then resuspended in 1-2 ml of serum-free medium containing phenol red-free DMEM/F12 plus 10 µg/ml insulin, 1 µg/ml progesterone, 10 ng/ml EGF, 1 µg/ml prolactin, 1 mg/ml BSA, 1 µg/ml hydrocortisone, 5 µg/ml human transferrin, 0.88 µg/ml ascorbic acid and 50 µg/ml gentamicin [363]. This cell suspension was layered over a pre-made Percoll gradient and centrifuged for 20 minutes at 800 ***g***. The layer containing single cells and organoids was recovered and pelleted before being resuspended in serum free media and plated on Matrigel-coated (100 µg/cm^2) 6-well plates (Becton Dickinson). This layer was sufficient for cell attachment but inadequate for three-dimensional growth. Any remaining non-epithelial cells were successfully removed by treating the plates with a 0.025% trypsin and 0.01% EDTA solution.

3.2.4 Renal Subcapsular Tissue Recombination

COP and WF fibroblasts exposed to vehicle alone were considered to be negative controls for normal morphogenesis while WF fibroblasts exposed to NMU are considered to be the positive control for tumorigenesis. According to the groups in Table 3.1, cell types from both strains were counted and mixed before being resuspended in 10 uL of collagen type 1 and grafted under the kidney capsule of SCID mice. 80% of the grafts were recovered 2 months later, fixed and paraffin embedded. Each graft was sectioned in its entirety; then, every fifth section was stained with Hematoxylin and Eosin and those containing recombinants were selected. PAS staining was performed to identify the presence of basement membrane. Following antigen-retrieval with 0.01 M sodium citrate (pH 6), optimized immunohistochemistry techniques were for the detection of cytokeratin (CK), ER alpha, alpha- smooth muscle actin (SMA) and the proliferation marker Ki67.

Table 3.1 Tissue recombination experimental groups

	Fib. source	**Fat Pad treatment**	**MEC source**
Group 1	COP	NMU	COP
Group 2	COP	VEH	COP
Group 3	COP	NMU	WF
Group 4	COP	VEH	WF
Group 5	WF	NMU	WF
Group 6	WF	VEH	WF
Group 7	WF	NMU	COP
Group 8	WF	VEH	COP

3.2.5 3D co-culture in mixed gels

3D hydrogels were formulated as described before [93, 151]. Briefly, rat-tail type I collagen (Corning) was diluted to 1 mg/ml using 10X PBS, 1N Sodium Hydroxide in water). 10% Matrigel was added to the gel solution and solution was stored on ice until plating. Cells were trypsinized, lysed, and counted using a Coulter Counter (Beckman Coulter). 60,000 MCF10A cells/gel and 20,000 fibroblasts/gel were spun at 1200 rpm for 3 minutes. Resulting pellet was resuspended in collagen+Matrigel solution and 1.5 ml of gel solution was seeded in each well of a 12-well plate treated to remove any static electric effects. Gels were allowed to solidify for 30 mins at 37C before adding 1.5 mL of 1:1 MCF10A:RMF media and detached from the well walls. Both fibroblasts and epithelial cells were tested individually for growth in 1:1 media with no apparent negative effects. Gels were maintained for 7 days with media replaced every 2-3 days. After 7 days in culture, gels were harvested, fixed and processed for whole mounts as described previously [364].

3.2.6 SAMA analysis

A region of interest on each gel was visualized with a confocal microscope as previously described [365].The Software for Automated Morphological Analysis (SAMA) was then used to quantify five geometric parameters (Biological Quality, Sphericity, Volume, Elongation, and Flatness, described in Paulose [366]) of the resultant MCF10A structures and perform statistical tests to determine significance.

3.2.7 RNA isolation for Microarray

COP and WF rat fat pads were cleared of epithelium at 14 days of age as described above. The animals from each strain here divided into two groups and treated with NMU or vehicle at 50 days of age. The glands were harvested 5, 15, and 60 days later. RNA was

extracted, quantified and hybridized to GeneChip Rat Genome 230 2.0 Arrays (Affymetrix) and scanned by the Tufts University Core Facility. RNA yields varied from 241 ug/mL to 1679 ug/mL.

3.2.8 Microarray Analysis

Raw intensity files were normalized and background adjusted using an RMA algorithm built into the Affymetrix Console. Raw data from 36 microarray chips were uploaded to Array Analysis (Arrayanalysis.org) for Quality Control. Principal component analyses were performed using the bioconductor package biomaRt [367]. Non-supervised hierarchical clustering were run using Cluster (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) to filter the gene set to 5,000 genes based on expression intensity and dynamic range. Clustered data was imaged using Treeview (http://jtreeview.sourceforge.net/). Gene sets derived from filtered data were analyzed using David (david.ncifcrf.gov) and Integrated Pathway Analysis (Qiagen) in parallel.

3.3 Results

3.3.1 Kidney capsule recombination

In pursuit of a more tissue-based rationale to carcinogenesis, our lab utilized the fibroblasts of isolated from NMU- or vehicle-treated 50-day-old COP and WF rats recombined with mammary epithelial cells derived from the same strains. These findings suggest that the tumor resistance observed in COP rats maybe due to the properties of their mammary fibroblasts. Tissue recombinations between rat strains cannot be used due to their histoincompatibility. Therefore, cells were combined and grafted under the kidney capsule of immunocompromised SCID mice.

The types of epithelial structures obtained in each recombinant group are described in detail in Table 3.2. All epithelial structures were both cytokeratin and estrogen receptor positive, indicating their epithelial and mammary origin, respectively (figure 3.1). Preneoplastic lesions showed poorly defined borders, discontinuous SMA staining (figure 3.2) loss of basement membrane and cell polarity, and presence of multiple lumina (figures 3.2 and 3.3) Moreover, the ducts were frequently filled with cells and epithelial cells were heterogeneous in shape and size. However, structures consistent with preneoplastic lesions and hyperplastic ducts seen *in vivo* in WF rats (luminal debris, poorly defined borders, and heterogeneous cell sizes and shapes) were observed only in grafts using NMU-exposed WF fibroblasts (figure 3.2, group 5).

Table 3.2 Characterization of mammary structures found in transplanted grafts

Group	# of transplants	Transplants with mammary structures (%)	Histopathology of epithelial structures # of transplants (%)		
			Ducts	Organized	Disorganized
COP NMU COP EPI	9	7 (77.7)	7	3 (43)	2 (29)
COP NMU WF EPI	8	7 (87.5)	6	4 (57)	3 (43)
COP VEH COP EPI	11	8 (72.7)	7	5 (63)	1 (13)
COP VEH WF EPI	12	11 (91.6)	10	8 (73)	5 (45)
WF NMU COP EPI	10	10 (100)	7	6 (60)	8 (80)
WF NMU WF EPI	9	7 (77.7)	5	0 (0)	7 (100)
WF VEH COP EPI	10	10 (100)	3	6 (100)	2 (33)
WF VEH WF EPI	9	7 (77.7)	6	3 (43)	3 (43)

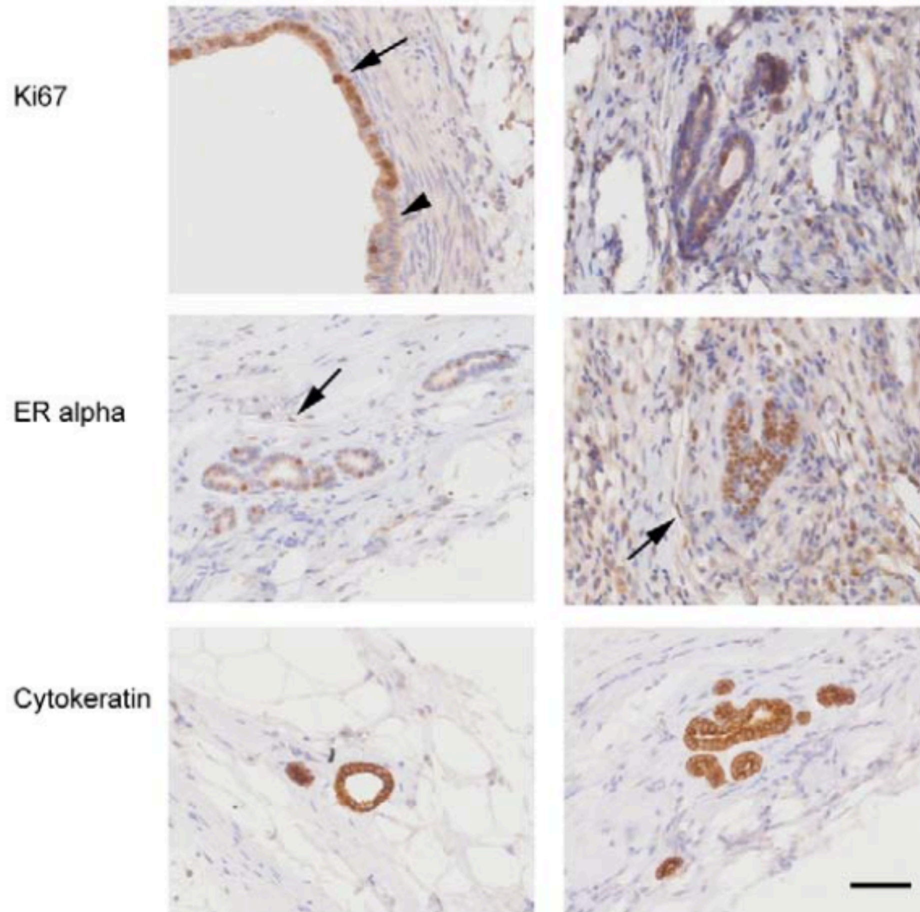

Figure 3.1 Immunohistochemistry for Ki67, ER alpha and CK.
Sections are representative of the different experimental groups. Positive cells are stained brown. Ki67 and ER: nuclear staining (arrow). ER alpha was also expressed in the stromal cells (arrow). CK: cytoplasmic staining. Counterstaining: hematoxylin (blue, arrowhead). Scale bar: 50um.

Normal mammary ducts were formed using any combination of stroma and epithelial cells. Recombinants of COP mammary fibroblasts exposed to NMU and either COP or WF MEC developed normal ducts and well defined clusters of MEC. The exposure of WF MF to NMU induces abnormal ductal structures. Both COP and WF MEC developed ductal hyperplasias, preneoplastic lesions and disorganized structures similar to those observed in invasive carcinomas, when combined with the tumor-susceptible NMU-exposed fibroblasts.

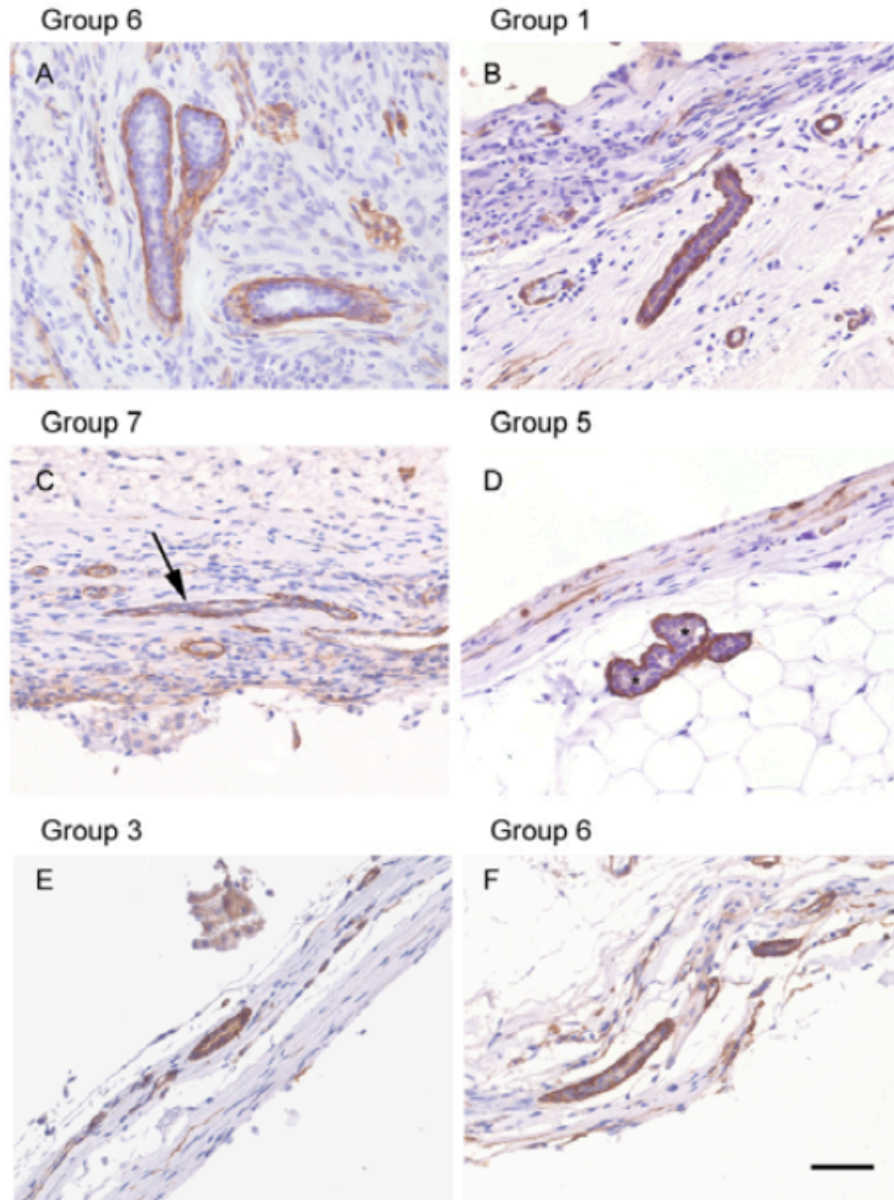

Figure 3.2 Identification of myoepithelial cells by immunohistochemistry for SMA. Myoepithelial cells form a continuous layer below the luminal cells in the majority of epithelial structures. Group 7 panel is an example of a discontinuous myoepithelial layer (arrow). Group 5 panel shows multiple lumina (asterisks). Counterstaining: hematoxylin. Scale bar: 50um

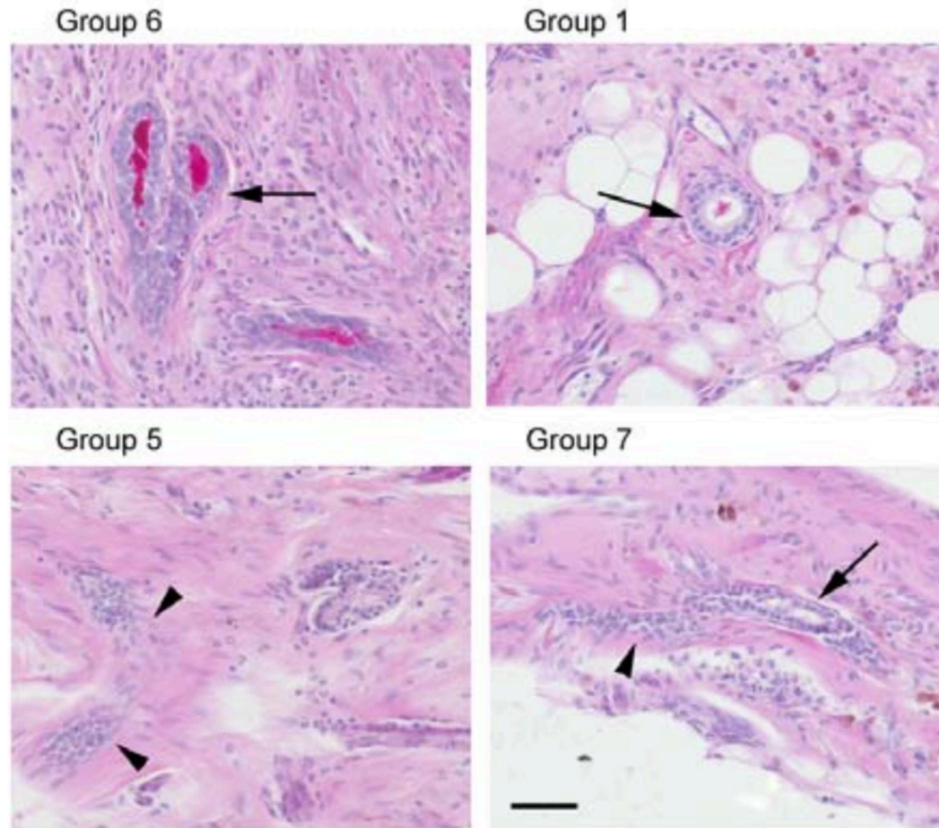

Figure 3.3 PAS staining showing the presence (arrow) or absence (arrowhead) of basement membrane. Lack of basement membrane is a common feature in Groups 5 and 7. Scale bar: 50um

3.3.2 3D culture

To address the technical, temporal, and financial obstacles of multispecies renal capsular recombinations, we developed an *in vitro* surrogate model of the rat mammary gland using a collagen-based 3D culture model containing COP and WF rat fibroblasts and MCF10A cells. We first validated the system by growing MCF10A cells in mixed gels alone and with RMFs. RMF co-culture altered every parameter of interest including increased elongation and biological quality of the structures formed.

3.3.3 SAMA identifies differences in MCF10A and MCF10A+RMF

Co-culture with RMFs resulted in altered MCF10A morphogenesis. Principle component analysis PCA shows that MCF10A structures grown in the absence of RMFs are distinct from those grown in co-culture (Figure 3.4, right). MCF10A cells grown with RMFs formed significantly larger (p=0.0007), longer (p=<0.005), and flatter (p=0.0013) structures than those formed by MCF10A cells alone, which primarily formed spheroid structures (Figure 3.4, left). Compared to structures grown with RMFs, MCF10A cells had lower biological quality (a measure of similarity to the duct-like structures of the intact mammary gland) (p=0.013) and higher sphericity (p=0.00012).

These findings are consistent with the epithelial structures described by Krause et al. grown under similar conditions [154].

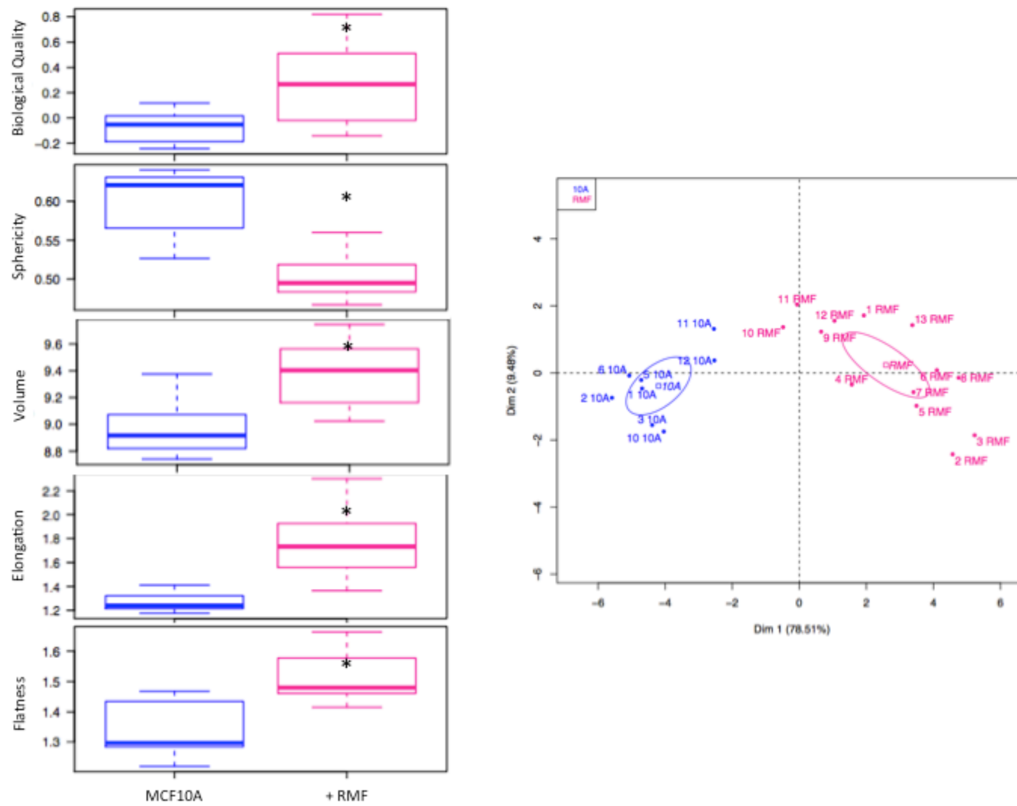

Figure 3.4 Morphometric analysis of MCF10A structures grown alone and with RMFs. MCF10A cells grown in mixed gels form smaller, more spherical structures compared to their longer, flatter, more duct-like counterparts grown in coculture with RMFs (left). Principle component analysis reveals difference between MCF10A-alone and MCF10A+RMF gels and a wider degree of variation between RMF-containing gels (right). Asterisks denote significant parameters

3.3.4 COP fibroblasts alter MCF10A morphology

When grown in the presence of NMU-treated COP fibroblasts, MCF10A cells formed rounded structures similar to those seen in MCF10A alone gels. COP fibroblasts treated with vehicle caused MCF10A cells to form what appear to be larger elongated and globular structures in addition to spheroid structures however, there are also a high number of smaller, spheroid structures found in these gels.

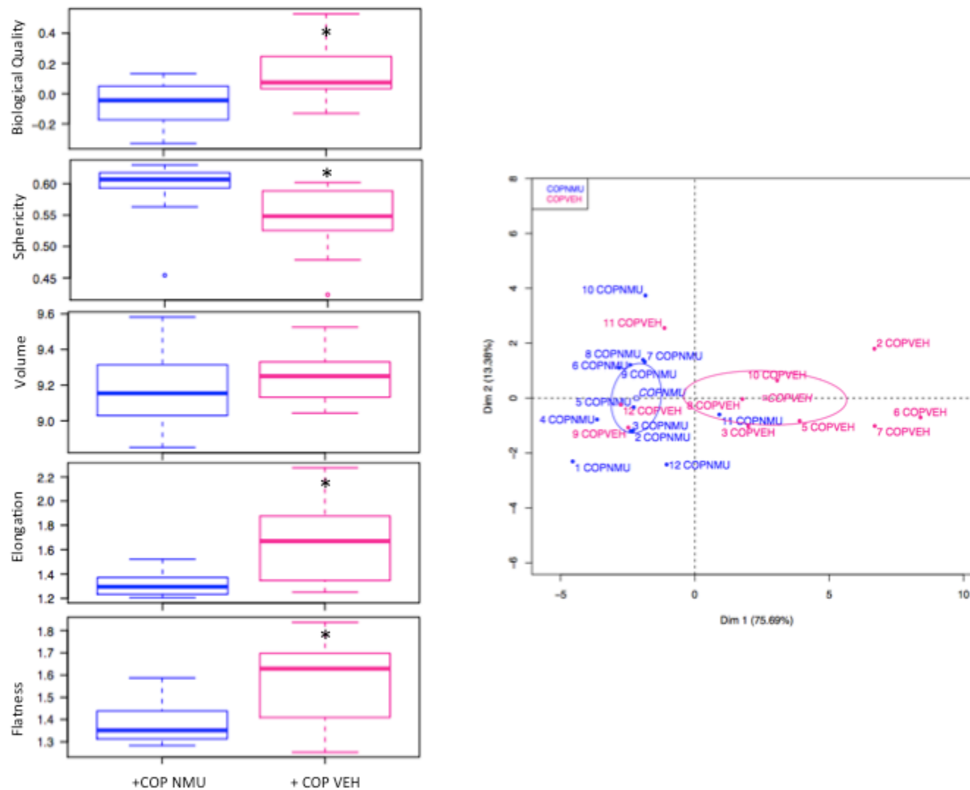

Fig 3.5 Morphometric analysis of MCF10A structures grown with COP fibroblasts. MCF10A cells form more spherical structures when grown with NMU-treated COP fibroblasts compared to their longer, flatter, more duct-like counterparts grown in coculture with vehicle treated fibroblasts (left). Principle component analysis comparing MCF10A+COPNMU and MCF10A+COPVEH gels (right). Asterisks denote significant parameters.

Figure 3.5, left, shows the distribution of epithelial structures that developed under the influence of COP fibroblasts according to several morphometric parameters. On average, structures formed by MCF10A cells grown in COP fibroblast co-cultures were the same volume regardless of fibroblast exposure status (p=0.31). MCF10A structures grown with vehicle-treated COP fibroblasts were longer (p=0.0044) and flatter (p=0.021) than those combined with NMU-exposed fibroblasts. Consistent with Figure 3.5, MCF10A organization in the presence of vehicle-treated COP fibroblasts were less spherical. The PCA analysis (Figure 5, right) shows that, while MCF10A structures in these COP fibroblast-containing gels were more similar to each other than those analyzed in Figure

3.4, the structures found within the gels are generally different between the two treatment groups.

3.3.5 Exposure status of WF fibroblasts alters morphology

After 7 days in culture with NMU-exposed WF fibroblasts, MCF10A cells organized into spherical structures with few elongated structures interspersed throughout. Epithelial structures formed in the presence of VEH-treated WF fibroblasts were generally similar to those grown with NMU-treated WF fibroblasts but had lower biological quality (Figure 3.6, top left). MCF10A structures grown in the presence of NMU-treated WF fibroblasts were more variable than those seen in any other co-culture replicates.

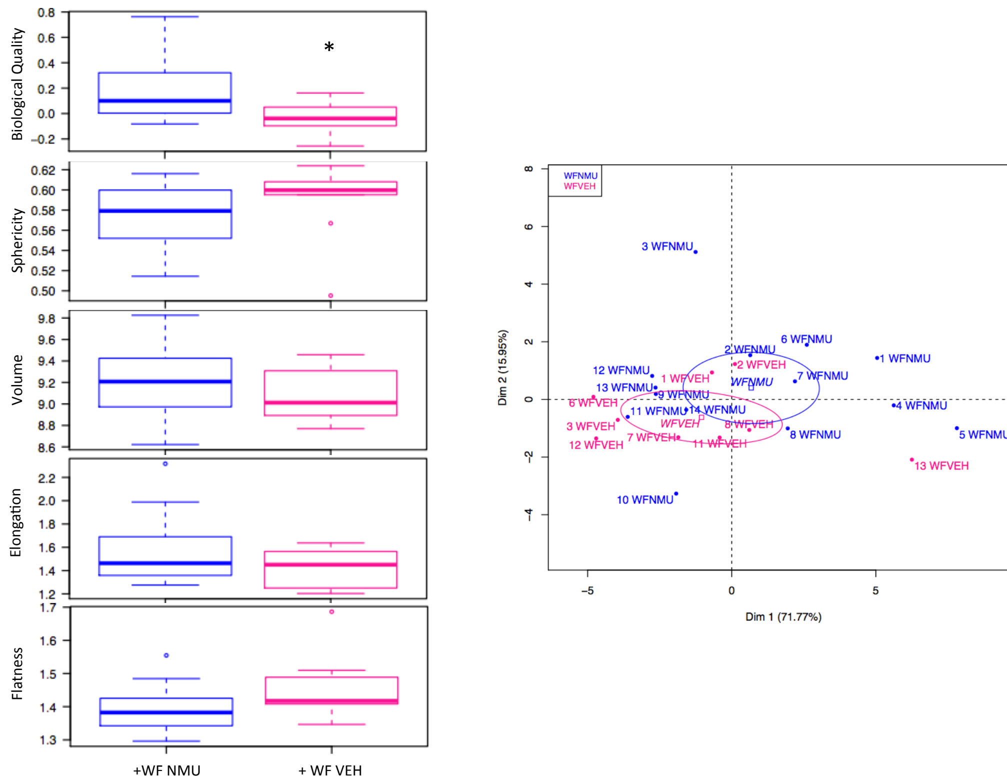

Fig 3.6 Morphometric analysis of MCF10A structures grown with WF fibroblasts. MCF10A cells form similar structures when grown with WF fibroblasts regardless of treatment (left). Principle component analysis comparing MCF10A+COPNMU and MCF10A+COPVEH gels (right). Asterisks denote significant parameters

3.3.6 Microarray Quality control

The 3':5' ratio is a measure of the amount of RNA degradation present in each sample generated by comparing the number of starting sequences to the number of polyA tails. For all chips, this ratio was close to 1 (max 1.17). Spike-in hybridization controls are used to verify the transcript binding and dynamic range of each chip. These non-mammalian gene products are added in quantities such that transcript concentrations follow the rule bioB<bioC<bioD<creX. One sample (WF Veh 15 B) did not properly hybridize bioB, however, the quantities of bioB added are so small that detection is not crucial for all samples. However, all samples followed the rule bioC<bioD<creX indicating sufficient sensitivity for the purpose of this study. All but two sample background intensities fell

within the optimal range required for detecting low-level expression. The remaining samples (COP NMU 15 A, COP Veh 60 C) had sufficient intensity for reliable background subtractions.

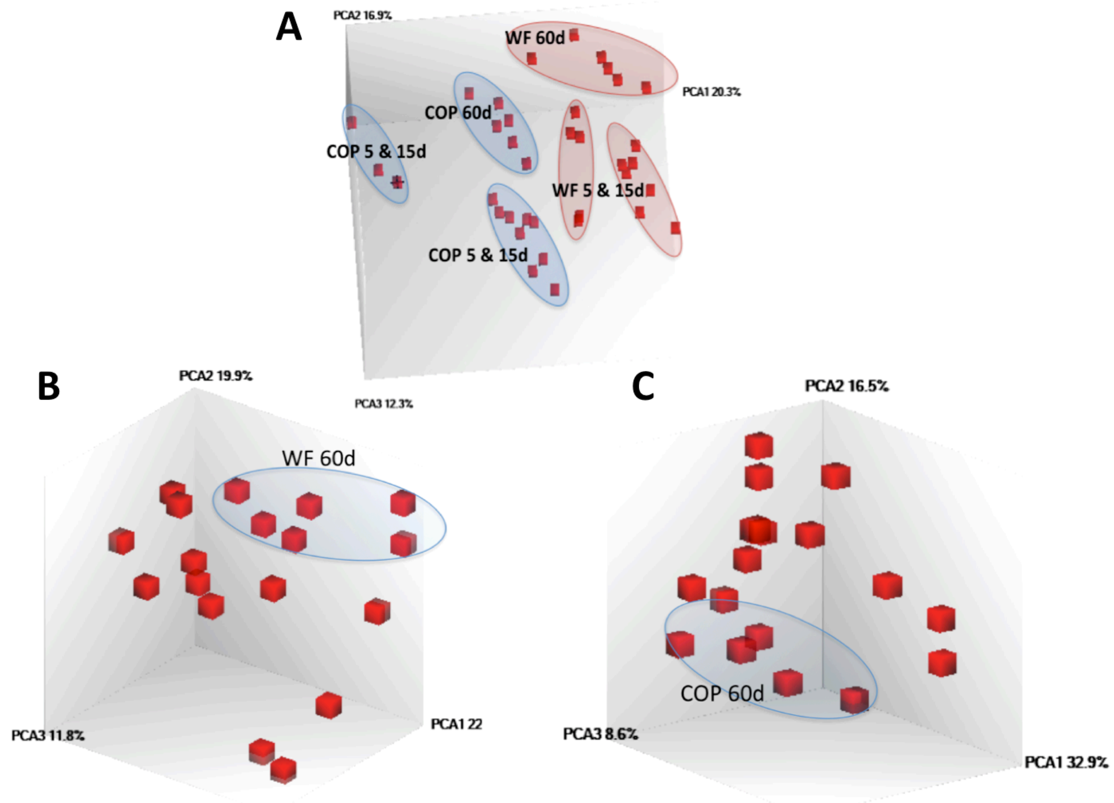

Figure 3.7 Principal Component Analysis of Processed Expression Data. WF and COP glands segregate into two principal components (A). 60 day glands from both WF (B) and COP (C) glands are highly similar while 5 and 15 day glands show high variability. Note that treatment identities are not shown.

3.3.7 WF and COP glands have distinct gene expression patterns

When analyzed using PCA, COP and WF glands segregate into distinct, isolated clusters (Figure 3.7A). The 60-day post-exposure glands from both strains cluster together regardless of treatment. Day 5 and 15 glands are more similar to each other, based on the proximity of their grouping. PCA of each strain independently reveals the high level of variability between replicates. While 60-day WF glands cluster closely together, 5 and 15-

day WF glands are spread throughout the space (Figure 3.7B). The same highly variable distribution is seen in COP glands (Figure 3.7C).

To further assess the differences in gene expression between the strains, the dataset, containing 30,000+ genes was filtered using Cluster to identify genes that were highly expressed and had a wide range of expression values between all glands. Non-supervised clustering of the top ~5,000 genes reveals clear inter-strain differences in expression (Figure 3.8). Replicates failed to cluster properly based on treatment, implying an unexpectedly wide-range of variation between individuals, even those treated with vehicle. 60 day animals from both strains did successfully cluster together.

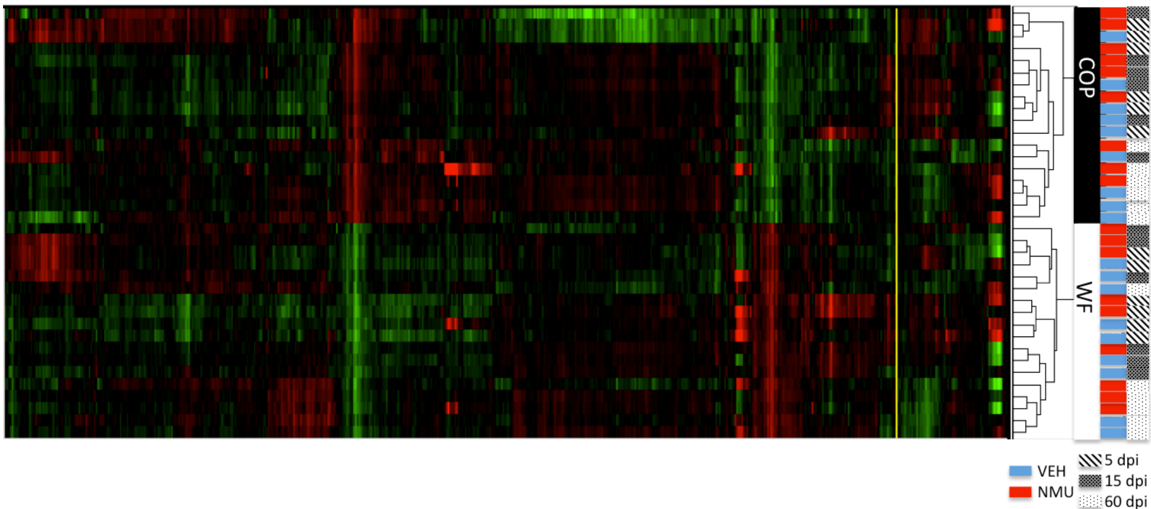

Figure 3.8 Heat map of ~5,000 genes reveals distinctly different expression patterns between COP and WF glands. Unsupervised clustering (right) of individual glands (color coded by strain, treatment, and age was performed using Cluster.

3.3.8 Differential gene expression analysis

To identify individual genes that were significantly altered by NMU exposure, differential gene analyses were performed.

Wistar-Furth, NMU compared to Wistar-Furth, Vehicle - Five days after treatment, three genes are disregulated in the Wistar-Furth mammary fat pad. MRGPRG (+3.76) is a G-coupled protein receptor associated with the development and/or function of nociceptors. LOC100134871 (-2.3) is a beta globin minor gene implicated in binding of oxygen. After 15 days on Cyp1a1 (+8.82), a member of the cytochrome P450 superfamily, is altered following NMU exposure. Sixty days after NMU exposure, there are no genes significantly upregulated in WF animals. However, of the 45 genes downregulated, several cluster together under muscle contraction, ion binding and cytoskeletal ontologies.

Copenhagen, NMU compared to Copenhagen Vehicle - Five days after treatment, Gria2 (+2.25), a glutamate receptor, and Mcpt10 (+2.04), a mast-cell protease are upregulated. Casein kappa (-3.39), casein alpha (-6.68), and alpha-lactalbumin (-6.91) are downregulated. These genes are usually associated only with the epithelial compartment. Epcam (-2.67), often overexpressed in breast cancers, is downregulated in Copenhagen rats 5 days after NMU exposure. After 15 days, Gpam (-2.13) a regulator of cellular triacylglycerol and phospholipid levels is slightly downregulated while three loci, currently unidentified, are slightly up regulated. Sixty days following exposure there is no significant differences between NMU and Vehicle treated animals.

Copenhagen, Vehicle compared to Copenhagen, Vehicle over time - In vehicle injected control animals, alterations of gene expression are indicative of normal physiological changes in the cleared fat pad during development. COX8B (-3.31) the last component of the mitochondrial electron transport chain is upregulated at 15 days. Zeb2 (-2.01) and Egr1 (-2.53) are both developmentally important zinc-finger proteins. Thrombospondin 2 was identified twice (-2.79, -3.22) and, while little is known about its role in the stroma, it has been implicated as required for prostate homeostasis. From 15 to 60 days thrombospondin 2 is downregulated (-3.28, -3.72) though no major ontologies are identified.

Wistar-Furth, Vehicle compared to Wistar-Furth, Vehicle over time - From 5 to 15 days post-vehicle treatment, binding (23 genes), specifically of calcium, nucleic acids and proteins, catalytic activity (21 genes), and structural molecule activity (17 genes) categories are highly represented. These groups, taken together, imply developmental alterations in the mechanical properties of the fat pad as well as a reduction of muscle-like complexes. 20% (14 genes) of DE genes are associated with cytoskeletal protein class designations. Expression of Fibrillin 2 (+3.17) an important component for the formation of elastic fibers decreases between these time points, as well. From 15 to 60 days, expression of genes associated with fatty acid metabolism, Elovl6 (+5.97), Acly (+2.49), Acaca (+2.18), Pnpla3 (+3.37), and Fasn (+2.93) decreases.

Copenhagen, NMU treated compared to Wistar-Furth, NMU treated 5 days post-treatment - 231 genes are differentially expressed between COP and WF rats five days after treatment. COP glands show up-regulation of signal peptides and immunoglobulin-like genes. Enrichments for genes associated with Wound healing (PLAA, C6, EPHX2, C4BPA, STAT3, SOD2, H19) and Epithelial Development (MIB1, C6,

KRT8, PTK7, ZEB2, LIG4, H19) are down regulated in COP compared to NMU-exposed WF animals.

Copenhagen, NMU treated compared to Wistar-Furth, NMU treated 15 days-post treatment - 281 genes are differentially expressed when comparing COP NMU and WF NMU rats after 15 days. COP show significant up regulation of genes associated with cytoskeleton contraction (MAEA, ACTA1, TNNC2, MYH1, MYL1, KIF15, LDB3, MYLPF, MYOZ1, RDX, DPYSL2, TPM2, TPM1, TNNI2, TNNT3, NEFH) and calcium binding (CDH7, TNNC2, PVALB, CAPN13, MYL1, ATP2A1, MYLPF, ACTN3, CAPN3, CASQ1) while genes known to be involved in lipid biosynthesis (SCD1, DGAT2, FASN, ACACA, ELOVL6, PNPLA3, PC) are down regulated

Copenhagen, NMU treated compared to Wistar-Furth, NMU treated 60 days post-treatment - 60 days after NMU exposure COP glands have increased expression of immune response genes (FCER1A, RT1-A2, CCL11, AGTR1B, CXCL13, TAP2, RT1-CE5, RT1-S3, RT1-EC2, TRAF6, RT1.AA, RT1-BB). However, COP glands express cell adhesion transcripts (CADM1, RT1-S3, NRXN1, RT1-EC2, RT1-N1, RT1-BB) and ECM transcripts FMOD, OMD, ALB, OLFML2A, LAMC1, GPC1) lower than similarly treated WF.

3.3.9 Associations with processes and diseases reveal strain differences

To accommodate the wide variability within the microarray findings, Integrated Pathway Analysis was performed to identify genes with less significant differences between strains or treatment groups. While these findings would require more intense investigation to show their applicability to changes in the gland, they do provide some insight as to global adjustments made to the glands following a carcinogenic insult. Figure 3.9 shows the top 8 disregulated ontological groups in NMU-treated rats from each strain. Five days after

NMU exposure COP rats show altered expression of genes associated with Reproductive system development, cancer, injury, cell morphology and inflammation than do WF. 10 days later, expression of genes correlated with injury and reproductive system disease are altered in both WF and COP while COP also alter the expression of genes releated to cell movement and function, tissue development, and cancer. At 60 days after NMU exposure the WF gland is striking in its dysregulation of genes associated with injury, cancer and muscle development.

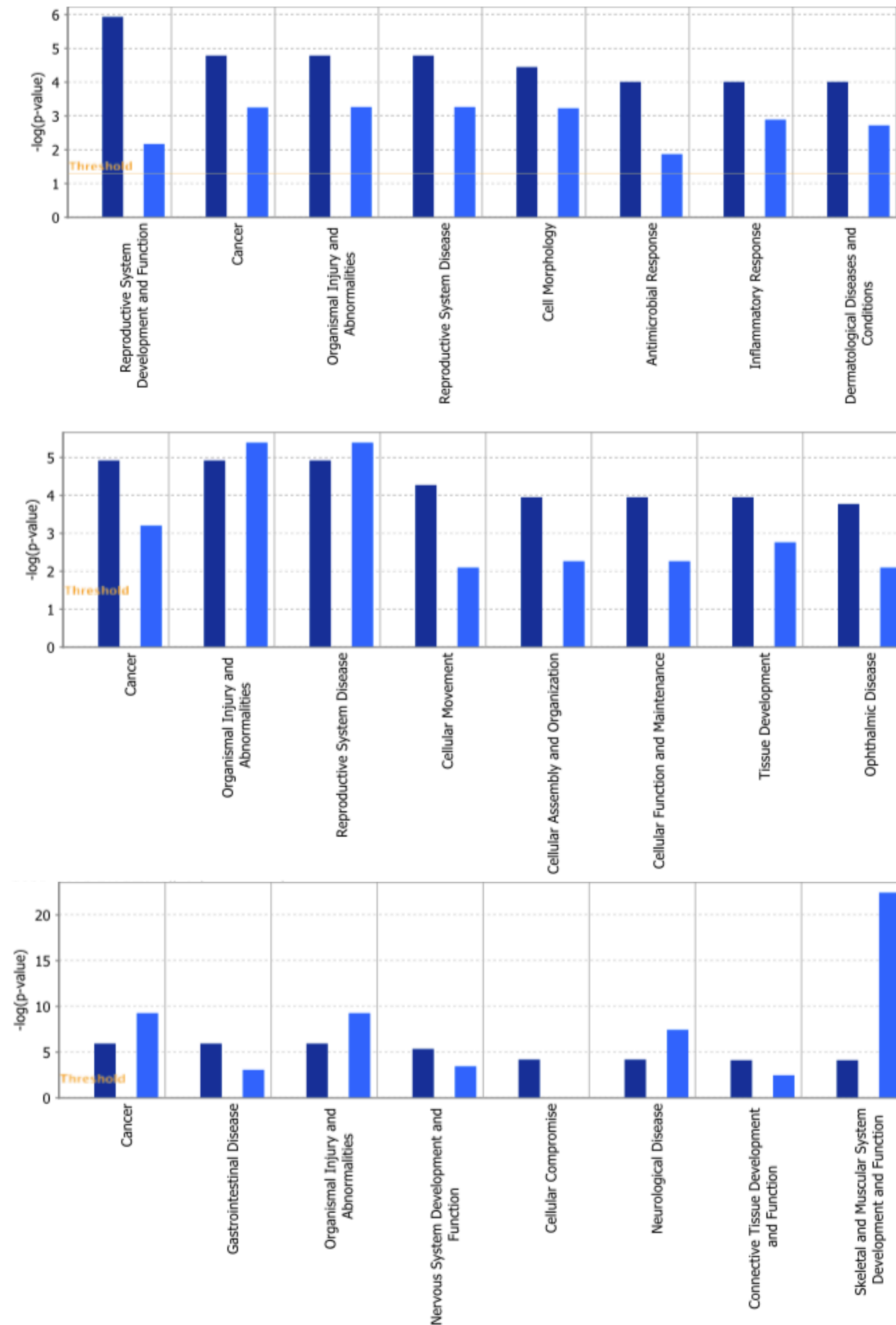

Figure 3.9 Top 8 altered disease and process ontologies in COP and WF following NMU exposure. Correlation with defined gene sets linked to known diseases and processes by COP (dark blue) and WF (light blue) animals at 5 (top) 15 (middle) and 60 (bottom) days after exposure to NMU.

3.4 Discussion

Given that an explanation of COP resistance has remained elusive, we hypothesize that it is the stroma, not the epithelium, which attenuates tumor resistance. Based on our previous studies, we expected the stroma to be responsible both for tumor suppression (in COP rats) and for promotion (in WF rats).

3.4.1 Tissue recombinations

Subcapsular renal recombinations of fibroblasts with epithelial cells revealed that WF fibroblasts exposed to NMU do play a role in the formation of disorganized epithelial structures similar to what is seen *in vivo* (Figure 3.1). Among the most common structures we observed were cross sections of ducts embedded in a fat pad. This is a very important finding because the presence of white fat may be due to the differentiation of a population of fibroblasts into adipocytes. The observation may also be related to changes we see in the expression of genes related to lipid biosynthesis by WF after NMU treatment.

Due to the short duration of the experiment (2 months) and small size of the grafts, no macroscopic tumors were identified in any of the samples. Although we could not diagnose these structures as frank carcinomas we assume, based on their histology that they would develop into tumors. These findings show that the tumor resistant phenotype observed in COP rats resides in the mammary stroma.

3.4.2 3D co-culture gels

In order to expand upon the subscapular recombinations, we developed a surrogate *in vitro* model. By combining rat mammary fibroblasts with well-characterized human mammary epithelial MCF-10A cells, we created a model that was amendable to real-time

observation and manipulations otherwise impossible *in vivo*. As most breast cancers are of ductal origin [368] model systems that successfully develop both acinar and ductal structures are ideal for the study of mammary carcinogenesis. We have attempted to modify the floating mixed gel model for use as an *in vitro* surrogate model of the rat mammary gland in which to study fibroblast-mediated epithelial morphogenesis. A floating collagen gel has several advantages over other culture systems because it features numerous biophysical and biochemical contextual cues found in the intact mammary gland. Collagen, the most abundant protein in the mammary fat pad, is amenable to manipulation by both fibroblasts and epithelial cells. Changes to collagen fiber organization and thickness impart novel biophysical forces to the gel microenvironment. The addition of Matrigel adds various extracellular matrix proteins, including laminins and fibronectins, that further influence mammary epithelial morphogenesis and fibroblast migration.

We expected to see an increased number of organized acinar and ductal MCF10A structures in the presence of NMU-treated COP fibroblasts when compared with those co-cultured with NMU-treated WF fibroblasts. Similar to what was seen in the subcapsular recombinations, all fibroblast/epithelial cell recombinations formed normal acinar structures. Vehicle-treated COP fibroblast co-culture resulted in more elongated, flatter, less spherical structures than fibroblasts treated with NMU. This may imply that untreated COP fibroblasts act in a similar fashion to RMFs when cultured in 3D. Co-culture with NMU-treated COP fibroblasts resulted in more spherical MCF10A epithelial structures reminiscent of those grown in mixed gels without RMFs. One possible explanation for this finding is that COP fibroblasts may respond to carcinogenic insult by altering the mammary gland microenvironment in such a way that favors acinar growth

over elongation thereby reducing the promiscuous morphogenesis associated with preneoplastic lesion formation.

WF fibroblasts from both treatment groups are associated with MCF10A structures that are widely variable. It is possible that the WF mammary gland is more susceptible to carcinogenic insult because the gland itself undergoes altered development that predisposes it to carcinogenesis. The similarities between the treated and untreated fibroblasts of each strain, compared to what is seen in the case of RMF co-cultures may be explained by a number of possibilities. While rat-tail collagen makes up the majority of protein in the gels, it is possible that Matrigel interferes with the fibroblasts secretion of other components that may modulate epithelial development. Also, the combined collagen/Matrigel matrix may supersede the fibroblast-based influences and instead cause MCF10A cells to act primarily as readout of the mixed gel microenvironment.

The presence of multiple lumena were a major indicator of abnormal, disorganized epithelial growth in the initial recombinations. However, the number of lumen in each gel area was insufficient for statistical analysis. The short time period of the culture was chosen to reduce overcrowding of the cells but may have been too short for the MCF10A structures to form lumena. Formation of lumen by MCF10a cells grown in rat-tail collagen has been reported after 2 weeks in culture [369]. Future co-culture experiments would benefit from additional modulation of seeding density to accommodate longer incubation times and, therefore, more developed morphogenesis.

Cells (both epithelial and fibroblast) do not remain evenly distributed throughout the gel during the course of the experiment. Along with the heterogeneous mixture of cells and cell deposits this reorganization also causes forces to be unevenly distributed throughout

the gel. We along, with others, have shown that local modifications to collagen fiber thickness and orientation alter the growth of epithelial cells. In order to accommodate this feature of the gels, we attempted to hold the region of interest the same across all gels. It is clear, however, that this area is not always representative of the structures found in the gel as a whole, making it difficult to assess the true extent of fibroblast/epithelial cell interactions. This is not a difficulty that is unique to 3D culture, as even in susceptible WF glands there is preneoplastic resolution occurs at several sites following NMU insult, adding a spatial component to following tumor progression. This is an obstacle that must be overcome in future iterations of these investigations.

Rat mammary tumors are similar to human breast cancers in their development, histology, and hormone sensitivity. Given the mammary gland microenvironments complex interaction with tumor biology, surrogate models that recapitulate the tumor/stromal interface are increasingly attractive. The behavior of fibroblasts in 3D is much different than their 2D counterparts in both gene expression and morphogenesis (fibroblasts grown in 2D display a pseudoapicobasal polarity that does not exist in the animal). Therefore further use of primary rat fibroblasts in 3D culture models such as the one described here may potentially be valuable to our understanding of human mammary carcinogenesis.

It is possible that the human-derived epithelial cells are less sensitive to rat fibroblast influence. We were unable to identify previous research that employed a hybrid rat/human culture system in 3D. Attempts to propagate rat epithelial cells in culture have been met with difficulty. There are many examples, however, of the rodent mammary gland microenvironment's ability to direct the proper differentiation and

morphogenesis of human epithelial cells [52, 370]. The gland is also amenable to population by human stromal components [371].

3.4.3 Microarray findings

Unsupervised hierarchical clustering revealed that COP and WF mammary fat pads have distinct signatures of gene expression. Interestingly, treatment was an ineffective predictor of gene expression patterning in both strains. Age did have some effect on the distribution of individual glands. It is possible that the untreated gland undergoes few changes in the 10 days between the first two time points. These similarities alone may account for the correct clustering of 60-day glands together.

High variability within the microarray samples made multiple testing corrections difficult. Variability may be due to the nutrient status of the rat at the time of sacrifice (as indicated by alterations in lipid metabolism and ion exchange) or the animal's estrous phase. It is also possible that the effect of NMU itself results in a wide range of random gene expression changes in the gland as well as systemically. One could speculate that COP rats have a more "stable" stroma based on the limited number of genes that significantly change expression following NMU exposure (11 across all time points) COP rats respond to NMU exposure of gene expression profiles associated with cancer, development, and wound healing at 5 and 15 days.

Assessment of WF gene expression following NMU exposure was statistically inadequate (only 45 statistically down-regulaed genes after 60 days) because of a high level of variation between treated animals. This is consistent with difficulties in predicting cancer susceptibility in humans. However, the inability of WF animals to express genes in pattern similar to COP increases their tumor susceptibility to 100%

At low doses (10 or 20 mg/kg) NMU exposure results in increased luminal debris, an increased number of acinar structures, and secretion into the lumen, features that are usually associated with pregnancy and lactation [105]. Genes associated with milk production and prolactin signaling were disregulated in animals treated with NMU: NMU-treated COP rats showed significant decreases in the expression of Csn3 (-3.39), Csn1s1 (-6.86), and Lalba (-6.91) compared to vehicle 5 days after treatment. Interestingly, WF glands display a pregnancy-like histology following exposure to NMU [372] and perphenazine pretreatment increases COP NMU-susceptibility. Suppression of prolactin receptor activity following DMBA in susceptible Lewis rats reduced the formation of mammary dysplasia [373]. The role of prolactin signaling in the initiation and progression of mammary cancer [374] has been investigated previously, however, it is curious that markers of milk production were identified in the stroma.

The COP stroma appears to undergo a remodeling and healing process following carcinogen exposure. This is relevant because many of the gene sets disregulated in WF animals are associated with wound healing and actomyosin contraction. These findings imply a reduction in contractile capabilities of the mammary stroma following NMU exposure, despite reports of increased stromal fibrosis abutting lesions in other models. Genes that were significantly up- and down- regulated in WF compared to COP animals indicate that the susceptible strain responds to NMU in a manner that may preclude the stabilization of the gland. Deregulation of genes associated with cancer, development, and contraction at 60 days imply that the gland undergoes extensive remodeling following NMU exposure. Unlike the spike in tissue remodeling genes seen at 15 days by COP glands, this extended injury of the WF gland may aid in the progression of neoplastic lesions to *bone fide* tumors.

As stroma and epithelium are in constant communication during development and carcinogenesis, those gene products involved in resistant and susceptible phenotypes can only be made completely clear by using intact animals. Difficult, yet necessary future experiments should include the isolation of stroma abutting preneoplastic lesions from intact WF and COP glands tissues at multiple time points. Current sequencing technology may allow for detailed transcriptomal changes within the stroma following NMU exposure. Transcriptomal changes may not, however, fully reveal what occurs during preneoplastic lesion resolution.

It is clear from the current study as well as those before it that, despite being a consistent happening with a relatively simple outcome, COP resistance to tumors is an extremely complex puzzle to solve. The use of tools from scalpels to molecules has failed to accurately explain how a gland can recover from a major carcinogenic insult. It must be said however, the goal of reducing the complex events leading to tumor formation to the role of few genes ("tumor suppressor genes" or "oncogenes") expressed in the epithelial cells would be the equivalent of placing a blindfolded person in a maze. We must consider the extraordinary plasticity and influence of the stroma in shaping epithelial phenotype. This is a concept totally accepted in the field of developmental biology; the time has come to re-evaluate the way we study carcinogenesis.

CHAPTER 4: CHARACTERIZATION OF MCF-12A CELL PHENOTYPE, RESPONSE TO ESTROGENS, AND GROWTH IN 3D [3]

[3] Sweeney, M. F., Sonnenschein, C., & Soto, A. M. (2018). Characterization of MCF-12A cell phenotype, response to estrogens, and growth in 3D. *Cancer Cell International*, *18*, 43. http://doi.org/10.1186/s12935-018-0534-y

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4.1 Background

Three-dimensional (3D) culture of breast epithelial cells has become a widely accepted and highly relevant tool for the examination of mammary gland biology [375, 376]. Immortalized breast epithelial cells in a 3D context have contributed to a deeper understanding of breast morphogenesis including the role of its microenvironment. In this latter regard, the composition of extracellular matrix [75, 154, 369], its stiffness [93, 216], as well as collagen fiber density and organization [93, 369] are all relevant factors responsible for the behavior of individual cells, their intercellular communication, and their organization into complex multicellular structures.

From puberty onward, mammary gland structure and function are dependent on circulating hormones. Curiously, however, most mammary epithelial morphogenesis has been studied using hormone receptor-negative cell lines, such as MCF-10A. During the last decades, a number of immortalized mouse and tumorigenic human-derived mammary epithelial cell lines that express estrogen receptor alpha (ER) and display hormone sensitivity have been established and used for such a purpose; they include HC11 (mouse), MCF7, BT-474 ZR-75-B, MDA-MB-361 and T47D [377, 378]. Cells of the latter one have recently been shown to form normal-like structures in 3D culture in response to mammotropic hormones. Exposure to estradiol alone resulted in the formation of multicellular structures while further addition of promegestone (progesterone analog) or prolactin resulted in flattened branching structures and budding structures, respectively [379]. While searching for a model that closely resembles the human mammary tissue, we chose to test MCF-12A cells, a human mammary cell line allegedly reported to be non-tumorigenic and estrogen-responsive.

MCF-12A cells originally established at the Michigan Cancer Foundation are non-tumorigenic human cells that were derived from the reduction mammoplasty of a postmenopausal 63-year-old nulliparous woman [380]. Tissues excised from the donor's

breast revealed non-malignant fibrocystic disease containing intraductal hyperplasia abutted by dense stroma. After they were dissociated, these cells were plated for long-term culture in DMEM/H containing <0.06 mM calcium, later supplemented with 5% Chelex-treated equine serum and maintained for 1717 days. At this point, "passages one to 15 [were] exposed to 45° C for as long as 72 hours" due to an incubator malfunction. As a result of this event, most cells died. The few surviving cells were expanded and over the next two months sublines MCF-12A (adherent cells) and MCF-12F (floating cells) were maintained separately and became "established." (Pauley RJ, et al. Immortal human mammary epithelial cell sublines. US Patent 5,206,165 dated Apr 27 1993)

During their initial characterization, MCF-12A cells were implanted subcutaneously in athymic mice, some of which were also implanted with pellets containing 17β-estradiol (E2) but no tumors developed at the inoculation sites in either groups of mice [380]. Given their origin and this outcome, the cell line was labeled as "normal." Like MCF-10A cells, and unlike tumorigenic MCF7 cells, MCF-12A cells were also considered as ER-negative and non-tumorigenic. Later, Zeillinger [381] described the expression of ER transcripts in MCF-12A as "extremely weak" and Subik [382] was not able to identify any ER positive MCF-12A cells via immunohistochemistry. At least three other studies done using MCF-12A cells described them as ER negative [383-385] [14-16]. Notwithstanding, literature identifying MCF-12A cells as ER-positive gradually accrued [386-395]. In sum, diverse groups using PCR, western blots, and immunostaining have come to conflicting conclusions regarding the ER status of these cells.

Marchese et al [396] grew MCF-12A cells in a Matrigel-based 3D model that resulted in the formation of acini and went on to show alterations in lumen formation following treatment with a variety of different estrogens. Western blots purportedly show the expression of ER and ER-beta proteins by MCF-12A, and MCF7 cells. In addition,

estradiol was reported to induce progesterone receptor (PGR) and pS2 in these cells; however, MCF-10A cells were used as a control in this experiment instead of the estrogen-responsive MCF7 cells. This more recent report suggested that MCF-12A cells were a desirable tool for the study of hormone-mediated epithelial morphogenesis.

In order to test the worthiness of the MCF-12A call line to study hormone-mediated epithelial morphogenesis, we ran experiments from which we conclude that a) that MCF-12A cells are not responsive to estrogens, b) the heterogeneous morphology of MCF-12A cells is due to the expansion of tightly growing epithelial colonies which gradually release populations of motile cells that generate morphologically distinct subclones, and c) when grown in a rat-tail collagen type I matrix, MCF-12A cells produce acini and ducts, resembling those seen in the human mammary gland. Implications of these data are further discussed below.

4.2 Methods

4.2.1 Cell Maintenance

MCF-12A (CRL-10782) and MCF-10A (CRL-10317) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) at passage 58. As indicated by the ATCC catalog, cells were grown in Dulbecco's Modified Eagle's/F12 medium (DMEM/F12, 1:1) containing 5% equine serum (ES), 20 ng/mL epidermal growth factor, 0.5 μg/mL hydrocortisone, 0.1 μg/mL cholera toxin, and 10 μg/mL insulin. MCF-12A cells were passaged at <70% confluence, using a 0.25% (w/v) trypsin-0.03% (w/v) EDTA solution; media was replaced every 2-3 days. In our lab, MCF7 cells were grown in DMEM supplemented with 5% fetal bovine serum (FBS). For experiments intended to explore estrogenic effects in 2D culture we used phenol red-free DMEM/F12 (1:1) containing serum stripped to remove endogenous estrogens. Serum was stripped using 5% charcoal-0.5% Dextran T70 (CD) at 37° C for 1h. Stripped fetal bovine (CD-FBS) or

equine (CD-ES) serum was used for MCF7 and MCF-12A, respectively. Media formulations are listed in Table 4.1. All cells were maintained at 37°C with humidity and 6% CO_2.

Table 4.1 Cell Culture Media Preparations

Name	*Base Media*	*Serum*	*Additives*
MCF-12A/MCF-10A	DMEM/F12, 1:1	5% Equine	20 ng/mL EGF, 0.5 μg/mL hydrocortisone, 0.1 μg/mL cholera toxin, 10 μg/mL insulin
MCF7	DMEM	5% Fetal Bovine	
Rinse	DMEM/F12, 1:1, phenol red-free		
CD-FBS	DMEM/F12, 1:1, phenol red-free	5% Charcoal-Dextran Stripped Fetal Bovine	
CD-ES	DMEM/F12, 1:1, phenol red-free	5% Charcoal-Dextran Stripped Equine	20 ng/mL EGF, 0.5 μg/mL hydrocortisone, 0.1 μg/mL cholera toxin, 10 μg/mL insulin

4.2.2 Immunocytochemistry

Twenty thousand cells were plated and cultured on Nunc Thermonox plastic coverslips (Thermo fisher) in a 24-well plate for 72 hours, washed twice with phosphate-buffered saline (PBS), and fixed in 4% formalin at room temperature for 15 min. Coverslips were then washed twice with PBS and cells were permeabilized with 0.1% Triton X-100 at RT for 15 minutes. The coverslips were washed in PBS and then blocked with 1:20 goat serum in 1.5% milk for 60 min at RT. Primary antibodies were diluted in BC-11 (0.02M

$NaPO_4H_2$, 0.15M NaCl, 0.02% Sodium Azide, 1% BSA in distilled water) buffer and diluted antibody (Table 4.2) was pipetted onto parafilm before the inverted coverslips were placed on top and incubated overnight in a humid chamber at 4° C. Coverslips were washed twice with PBS, incubated for 1 hr with appropriate peroxidase-conjugated secondary antibody diluted in 1% Bovine Serum Albumin (BSA), and visualized with 3,3'-diaminobenzadine (DAB; Sigma) after washing with PBS. Finally, coverslips were washed twice with PBS, counter stained with Harris' hematoxylin, dehydrated, and mounted on glass coverslips with Permount (Fisher scientific).

Table 4.2 List of Antibodies Used for Immunocytochemistry.

Primary Antibody	*Source*	*Dilution*
Mouse anti-E-Cadherin	Novocastra	1:75
Mouse anti-vimentin	Novocastra	1:50
Mouse anti-pankeratin	Sigma	1:500
Rabbit anti-Smooth muscle actin	Abcam	1:400
Mouse anti-p63	Santa-Cruz	1:200
Mouse anti-beta-catenin	BD	1:500
Secondary Antibody		
Goat anti-Mouse Peroxidase	Pierce	1:1000
Goat anti-Rabbit Peroxidase	Pierce	1:1000

4.2.3 Single Cell Cloning

MCF-12A cells were trypsinized and spun down at 1,200 rpm for 3 min on a bench-top centrifuge and the resulting cell pellet was resuspended in 5 ml of medium. Cells were then counted using a Beckman Coulter Z1 particle counter and diluted to 10 cells/ml. The diluted cell suspension was plated at 100 µl per well in 96-well plates and visually scored for single cell containing wells 24-hours later. Wells were assessed for different cell types and eventually expanded serially in 24-, 12- and 6- well plates.

4.2.4 Dose-response to Estradiol (E2)

E2 (Sigma) was diluted in ethanol at a concentration of 1 mM and stored at -20°C. MCF-12A cells between passages 61- 66 and MCF7 cells between passages 116-131 were used. Cells were plated at a density of 25,000 cells per well in 12-well plates. The next day, cells were rinsed with phenol red-free DMEM:F12 which was then replaced with phenol red-free medium containing 5% CD-FBS (MCF7) or CD-ES (MCF-12A) containing five concentrations of E2 (1pM, 0.01 nM, 0.1 nM, 1 nM, 10 nM). After 5 days, cells were fixed with ice-cold 10% tricholoracetic acid and stained with sulforhodamine B (SRB). Extra dye was rinsed with 1% acetic acid then retrieved using basic Tris buffer (pH 10.5) and read at 515 nm absorbance [397].

4.2.5 Estrogen-regulated gene induction assays

MCF-12A and MCF7 cells were counted and then plated in 6-well plates at a density of 300,000 cells per well. After 24 hours, medium was removed and cells were rinsed once with rinse medium and then incubated in CD-ES or CD-FBS for an additional 24 hours. E2, diluted to 0.1 nM, 1 nM, or 10 nM in applicable media (Table 4.1), was added to cells and cells were incubated at 37°C for 48 hours. Cells were then rinsed once with sterile PBS before being lysed according to the Qiagen RNeasy protocol. RNA was isolated and

quantified with a Nanodrop photospectrometer and then 2 μg of RNA was used to prepare cDNA libraries using Superscript reverse transcriptase (Invitrogen). Estrogen-responsive transcripts were quantified via qRT-PCR using a SYBR green master mix (Bio-Rad) in an iQ5 thermo cycler. Fold change induction was calculated with the Bio-Rad software (version 2.1) and normalized to the expression of *RPL19* transcripts. Primer sequences are shown in Table 4.3.

4.2.6 3D cell culture

3D cultures were generated as previously described [148]. Briefly, a 1 mg/ml rat-tail collagen type I solution (Corning) was made according to the manufacturer's "alternate gelation procedure" and stored on ice prior to use. Cells were detached with trypsin, pelleted at 1,200 rpm x 3 min and then resuspended in 10 ml of MCF-12A medium and counted. 75,000 cells were seeded per gel per 1.5 ml of collagen solution in a 12-well plate. After 30 min at 37°C, 2 ml of MCF-12A medium was added to each well and the gel was detached from the edges of the well using a sterile pipette tip. Culture medium was changed every 2-3 days and gels were harvested after 14 days. Gels were processed for paraffin embedding for histological analysis and whole mount microscopy as described in [151]. Gel diameter was measured using Axiovision (Zeiss) imaging software.

Table 4.3 Estrogen Responsive Gene Induction Assay Primer Sequences.

Gene	*Forward Primer*	*Reverse Primer*
L19	5'TAGTCTGGC TTCAGCTTCCTC3'	5'TCTGCAACAT CCAGCTACCC3'
Estrogen Receptor Alpha	5'TAAATGCTG CCATGTTCCAA3'	5'CCTGTGAGAGAA CAGAAACTGG3'
Amphiregulin	5'GTGGTGCTGTCGCT CTTGATACTC3'	5'TCAAATCCATCAGC ACTGTGGTC3'
Progesterone Receptors A/B	5'GAGGATAGCTCTGA GTCCGAGGA3'	5'TTTGCCCTTCAGAA GCGG3'

4.2.7 Analysis of epithelial structures

Whole mounted, carmine-stained gels were imaged at 200X with a LSM800 (Zeiss) confocal microscope. A region of interest was established 500 µm inward from the apex of each semicircular gel and maintained for all replicates. An area 120 µm thick was imaged using a HeNe 633 laser. The Zeiss software was used to create arrays of tiles 5X3 wide which were stitched together with a 20% overlap. Stitched images were then analyzed with the "Software for Automated Morphometric Analysis" (SAMA) [366] that allows for the unbiased, unsupervised analysis of physical attributes of each epithelial structure in the region of interest. Raw data produced by SAMA was filtered based on volume (1000 μm^3 cutoff) and analyzed using Prism Software.

4.2.8 Statistical analysis

One-way ANOVAs were performed to compare cell proliferative effects of estradiol on MCF7 and MCF12A cells. Dunnett 2-sided t-tests were applied to analyze differences in gene expression data. Students t-tests were used to compare gel contraction. Mann-Whitney non-parametric t-tests were used to analyze 3D morphometric data derived from SAMA.

4.3 Results

4.3.1 Description of parental cells

After receiving frozen stocks from ATCC, MCF-12A cells were expanded in their recommended media and passaged twice. Consistent with previous publications, the cells grew as a heterogeneous population [380, 398]. A subpopulation of MCF-12A cells from this initial stock grew as colonies of cobblestone-like cells (Figure 4.1a, black arrowhead). The epithelial cells were mononuclear with a well-defined nucleolus and nuclear size varied between cells within the epithelial plaques. Isolated spheroid and elongated fibroblast-like cells were observed beyond the perimeter of the epithelial colonies interspersed with domed cells (Figure 4.1a, white arrowhead).

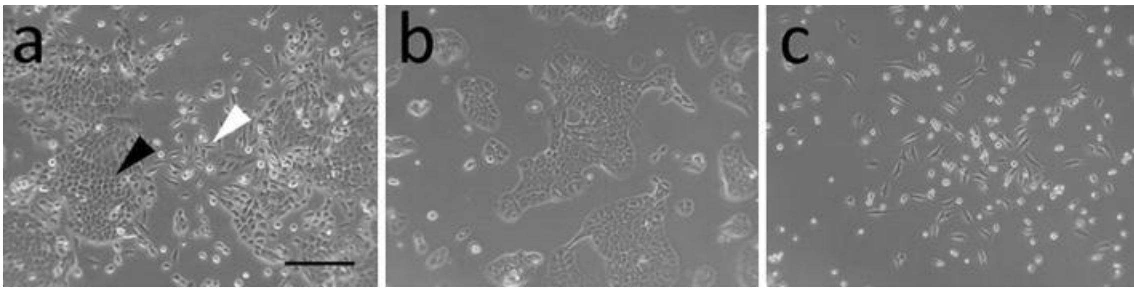

Figure 4.1 MCF-12A cells grow as a heterogeneous population.
Parental cells grow as epithelial plaques surrounded by single fibroblast-like and spherical cells (**a**). Single cell cloning lead to the isolation of epithelial-like colonies (**b**) and a mixed population consisting of both fibroblast-like and spheroid cells (**c**). (Scale bar = 250 μm)

4.3.2 Single cell cloning observations

Based on the assumption that there were two or three subpopulations of cells within MCF-12A parental cells, single-cell cloning was performed in order to isolate the varied morphological populations. Following expansion of single-cell colonies, cobblestone-like colonies (BF9) were isolated, further expanded and frozen. These colonies contained cells of homogeneous morphology and formed large plaques with well-defined borders (Figure 4.1b). After 5 passages, the edges of these colonies began to accumulate elongated cells that migrated away from the plaque. During subsequent passages, these spindle-shaped cells, which briefly appear spheroid following cell division, continued to multiply and populated the spaces in between epithelial plaques. After 10 passages the initial cobblestone-like clonal population reverted to a heterogeneous population resembling the parental cell line.

An additional subpopulation was selected containing only spheroid and elongated cells types (Figure 4.1c). Cells in this sub-population appeared highly motile and grew separately without forming colonies. These cloned cells failed to reestablish the cobblestone cell morphology seen in the parental line.

4.3.3 Epithelial, myoepithelial, and mesenchymal marker expression in parental MCF-12A cells

While several papers have alluded to the expression of some epithelial and/or mesenchymal markers by MCF-12A cells, a more thorough analysis of these cells studied under those conditions has yet to be published. MCF-12A cells did not adhere to acid-washed glass coverslips or coverslips coated with collagen or poly-lysine. Cells did, however, adhere to plastic coverslips. To investigate the cell states present in MCF-12A cultures, a panel of epithelial and mesenchymal markers was employed. MCF-10A cells were used as a positive control for epithelial cell markers. The epithelial sub-population of MCF-12A expressed E-cadherin at cell-cell junctions (Figure 4.2) while their elongated fibroblast-like counterparts failed to express E-cadherin (Figure 4.2). Expression of E-cadherin was more pronounced in the center of epithelial plaques and diminished in between peripheral cells. MCF-12A cells also expressed vimentin. Remarkably, cells at the epithelial plaque borders contained accumulations of vimentin radiating away from the plaques center, and vimentin was excluded from areas of cell-cell contact (Figure 4.2, arrowhead). Cells separated from epithelial plaques expressed vimentin throughout their cytoplasm. As expected, vimentin expression was absent from control MCF-10A cells.

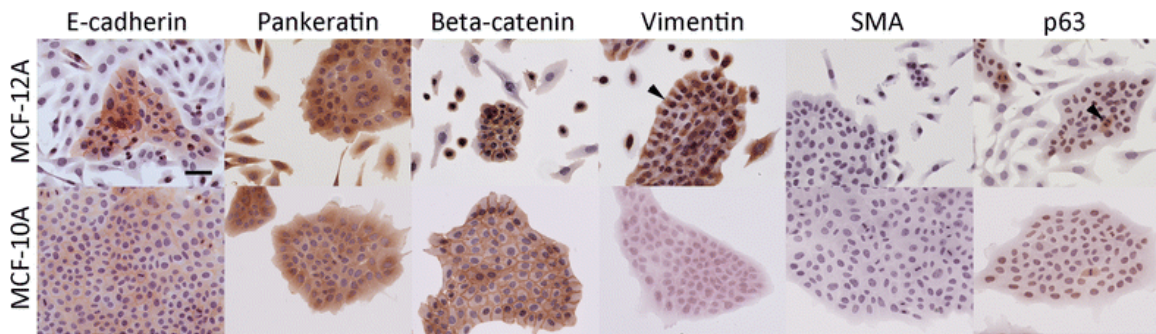

Figure 4.2 MCF-12A cells express epithelial and mesenchymal cell markers. E-cadherin is expressed throughout plaque-associated cells and highly localized to cell–cell junctions in the center of epithelial plaques. Localization at cell–cell junctions decreases at plaque edges. Cytokeratins are expressed in all MCF-12A subtypes. Beta-catenin is expressed by epithelial-like MCF-12A colonies with increased localization at their cell–cell junctions and dispersed throughout the cytoplasm of fibroblast-like and spheroid cells. Vimentin is expressed in the cytoplasm of all cells but concentrated in fan-like projections from peripheral cells. P63 is expressed in dividing cells but absent from others. MCF-10A cells used as a control for epithelial marker expression. Scale bar = 100 μm

Cytokeratins were expressed in all MCF-12A cells. Beta-catenin was expressed strongly at cell-cell interfaces, but not in the nuclei of cells within epithelial plaques formed by both MCF10A cells and MCF-12A cells. However, MCF-12A cells growing isolated from plaques contained beta-catenin throughout their cytoplasm and in their nucleus in some smaller cells. Despite their appearance, elongated fibroblast-like MCF-12A cells did not express the myoepithelial marker smooth muscle actin (Figure 4.2). Myoepithelial cell marker p63 was expressed in the nucleus of epithelial-like MCF-12A cells and in the cytoplasm of dividing cells within the epithelial plaques. This distribution of p63 expression is similar to that seen in colonies of MCF10A cells (Figure 4.2, lower right). MCF-12A cells growing away from epithelial plaques, however, lacked expression of p63 in both their nucleus and cytoplasm.

4.3.4 MCF-12A cells proliferate equally with or without E2

ER-positive MCF7 and T47D cells have been shown to exhibit a proliferative dose response to E2 when cultured in medium containing CD stripped serum-supplemented

medium, and are inhibited from proliferating by a serum-borne inhibitor [399]. This represents a standard case for studying the proliferative effects of E2 [400]. Thus, if MCF-12A cells were indeed ER-positive, enhanced proliferation of these cells in the presence of estrogens accompanied by quiescence in medium supplemented with CD-striped serum would indicate the functionality of the ER. However, as shown in Figure 4.3a, while MCF7 cells proliferated maximally in E-stripped serum supplemented with 0.1 nM E2 and did not proliferate under CD-FBS conditions, MCF-12A cells proliferated maximally regardless of whether the cells were exposed to CDFBS or to E2. These results indicated that MCF-12A cells were not influenced in their proliferative behavior by the presence of CD-FBS or of E2 in the culture medium.

4.3.5 MCF12A cells lack expression of ER and its transcriptional targets

The proliferative response by MCF7 in response to E2 was accompanied by a decrease in the expression of ER transcripts (Figure 4.3b). In CD-FBS media, MCF7 cells expressed ER transcripts ≥250-fold higher than MCF-12A cells when maintained in a comparable medium. Increasing concentrations of E2 resulted in decreased expression of ER transcripts by MCF7 cells but had no effect on the expression by MCF-12A cells. We next attempted to determine whether or not there was transcriptional activation by ER in MCF-12A. The expression of progesterone receptor (PGR) and amphiregulin (AREG) by MCF12A cells was unaffected by E2 and showed no estrogen-dependent induction (Figure 4.3c), unlike that observed in MCF7 cells (Figure 4.3d).

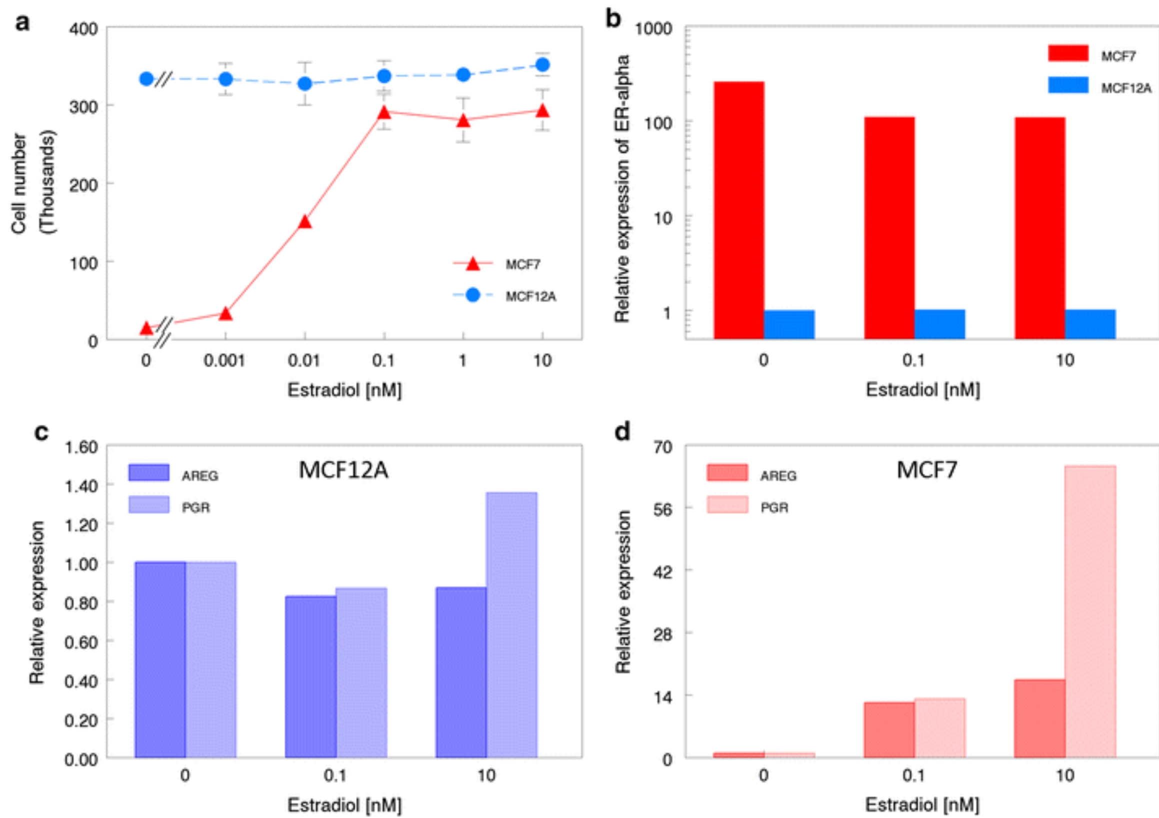

Figure 4.3 MCF-12A cells do not respond to stimulation by estradiol. MCF-12A cells proliferate maximally under all experimental conditions while MCF7 cell proliferation is inhibited in the absence of estradiol and stimulated at increasing concentrations (**a**). Relative to MCF7 cells, MCF-12A cells do not express estrogen receptor alpha (**b**). The induction of estrogen responsive genes amphiregulin (AREG) and progesterone receptor (PGR) is not seen in MCF-12A (**c**) cells while estrogen receptor alpha-positive MCF7 cells show expression following estradiol exposure (**d**). Note differences in y-axis scales to accommodate variation in expression differences

4.3.6 MCF12A cells form ducts and acini in 3D

To study MCF-12A morphogenesis in 3D, we employed an embedded; floating rat-tail collagen type I culture system. When examined 14 days after seeding, MCF-12A cells showed multiple phenotypes. The majority of them were solid spherical structures varying in size (Figure 4.4a). Acini with single-cell thick borders and cleared lumena were observed (Figure 4.4B). MCF-12A cells also formed branching, ductal structures (Figure 4.4c), of which some showed lumen (Figure 4.4d). Spherical clusters of

concentric cells (Figure 4.4c, arrow) were often seen within ductal structures. In addition to multicellular structures, spindle-shaped single cells grew throughout the gel.

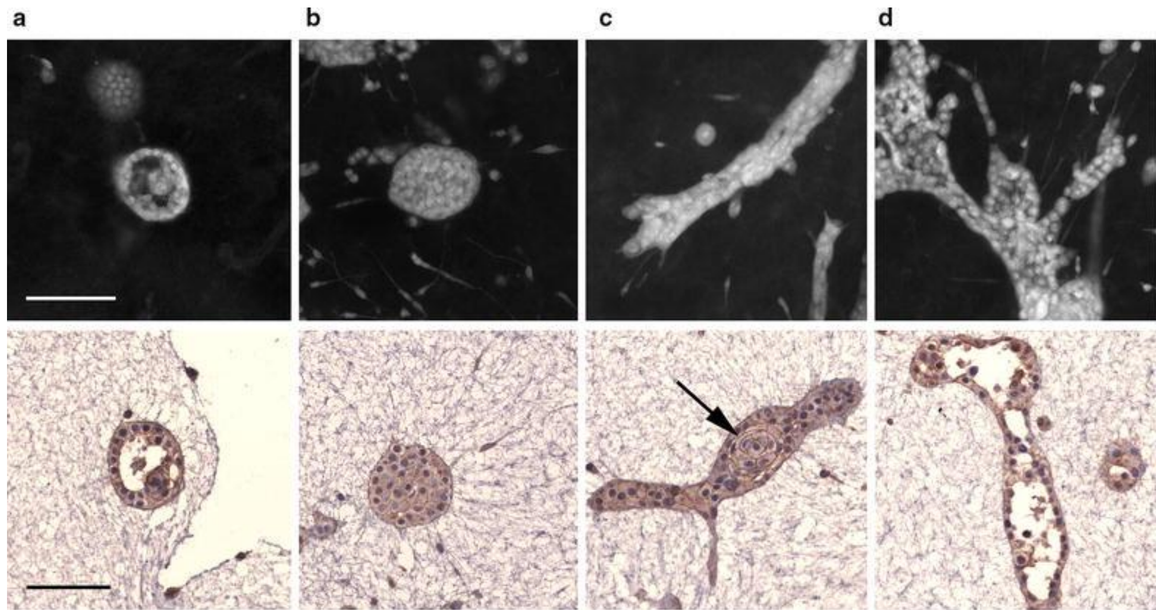

Figure 4.4 3D growth of MCF-12A cells in rat-tail collagen after 14 days. Confocal projections of select structures (top row) and E-cadherin stained cross sections of corresponding structures (bottom row). MCF-12A cells form hollow (**a**) and solid acini (**b**) as well as solid bifurcating ducts (**c**) and lumenized branching ducts (**d**). Spherical clusters of concentric cells are often seen within elongated ductal structures (black arrow). Scale bar = 100 μm

Addition of parental MCF-12A cells caused collagen gels to contract compared to acellular gels after 14 days (Figure 4.5). BF9 cells contracted gels to a similar degree.

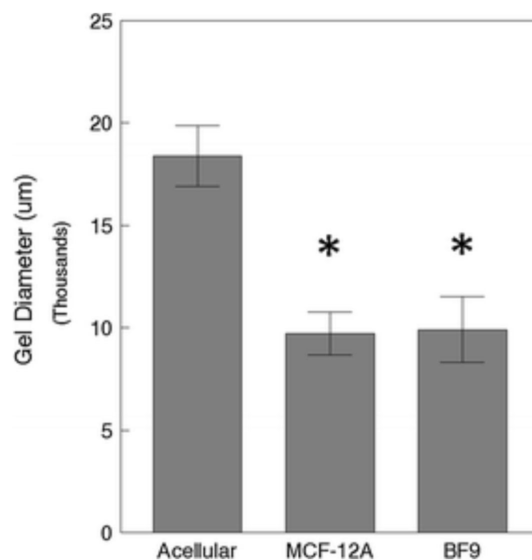

Figure 4.5 Contraction of collagen gels after 14 days in culture.
The addition of MCF-12A cells to rat-tail collagen gels results in a decreased gel diameter compared to acellular gels. MCF-12A-derived BF9 cells cause a similar extent of contraction. Asterisks signify gels that are significantly more contracted (p-value < 0.05) than acellular gels

4.3.7 MCF12A morphogenesis varies due to cell- subtype dynamics

Because of the unique 2D phenotypes of the two cellular subtypes isolated from MCF-12A parental cells, we asked whether or not the epithelial-like populations could form different biologically relevant structures in 3D. We were specifically interested in observing complex, multicellular epithelial structures. Therefore, the SAMA data was analyzed with filters in place to ignore single cells and non-relevant entities, such as sheets of cells or gel artifacts. All structures below 1000 um^3 were removed prior to statistical analysis. As shown in Figure 4.6, structures formed by BF9 cells are longer (p-value = <0.0001) and flatter (p-value = 0.0007) on average than those found in parental cell gels. Conversely, MCF-12A structures were more spherical structures on average (p-value = <0.0001). These parameters were also statistically significantly different at the 500 um^3 (except flatness) and 2000 um^3 cutoffs (data not shown).

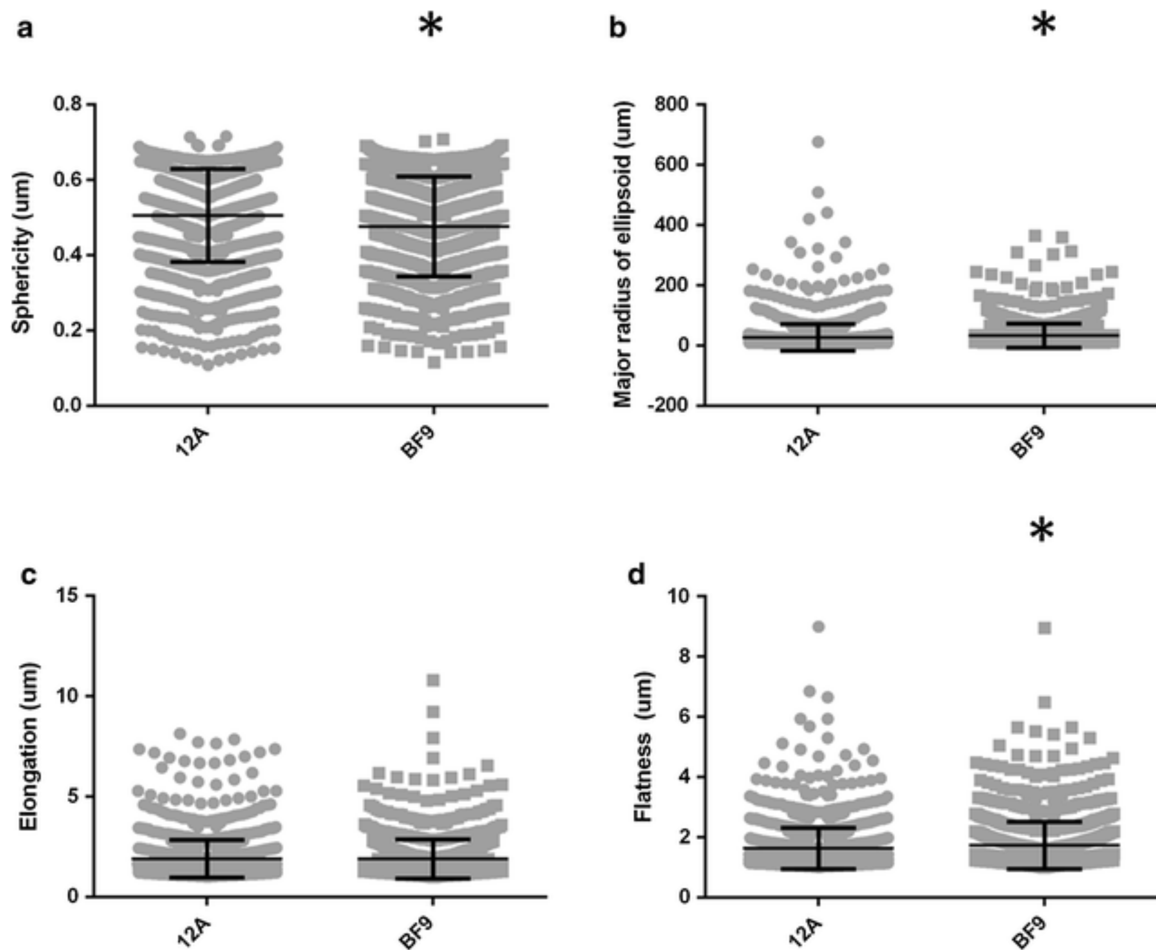

Figure 4.6 Non-epithelial-like subpopulations alter the morphology of epithelial-like MCF-12A cells. Structures formed by the heterogeneous parental MCF-12A cells are more spherical (**a**), have shorter major radii (**b**), and are flatter (**d**) than those formed by primarily epithelial subclone BF9. Elongation (**c**) is not significantly different between the parental cells and BF9 cells. Structures were pooled from all technical and biological replicates for analysis (MCF-12A n = 1537, BF9 n = 1099). Error bars show SD. Asterisks denote p values < 0.05

4.4 Discussion

In vitro studies on tissue morphogenesis affected by hormonal effects are limited by the few established culture models that use immortalized cell lines. At first, we were interested in employing the MCF-12A cells in our 3D culture model due to their normal, non-tumorigenic origin and their alleged ER-alpha expression. To our knowledge, currently, no “normal”, ER-positive established cell lines are available. Claims of estrogen-sensitivity made about MCF-12A cells appealed to us for the exploration of the

human mammary gland in both normal development and in pathological contexts, like carcinogenesis. It is acknowledged, however, that a given cell line often vary widely in hormone receptor content from one lab to the next [346, 401, 402]. Thus, before embarking in an extended study of the subject it was necessary to accurately characterize the cell line to be used in such a project.

4.4.1 Morphology and cell-type markers

Unlike MCF-10A cells, which form epithelial plaques with smooth, defined boarders in 2D cultures, MCF-12A cells underwent a process of phenotypic changes during their propagation. Namely, cells at the edge of plaques lost the plasma membrane localization of E-cadherin and Beta-catenin, altered their distribution of vimentin, and became highly motile. Morphologically, these cells appeared to be migrating away from the plaques due to their fan-like projections (Figure 4.2). A possible explanation for the dynamic morphologies seen in MCF-12A cells would have been the co-expression of e-cadherin and vimentin. An inverse relationship between e-cadherin and vimentin in intact tissues is considered to be an indicator of epithelial-mesenchymal switching [403]. When vimentin was overexpressed in MCF7 cells they adopted mesenchymal morphologies with vimentin localized within the cells in a pattern similar to that seen in the outermost MCF-12A cells [404]. The mechanical and biochemical constraints imposed upon MCF-12A cells growing near the center of epithelial plaques became reduced on cells residing in the plaque's periphery.

MCF-12A cells express gene products that are associated with both luminal and basal subtypes and have features of basal progenitor cells [162]. Dual expression of e-cadherin and vimentin has been linked to highly aggressive tumors in other tissues including those in the lung and neck [405, 406]. However, MCF-12A cells are considered non-

invasive [380]. Expression of p63 has been reported to be restricted to the basal layer of complex glands such as the mammary gland and prostate [407, 408]. Decreasing p63 expression in these cells was linked to a need to replenish cells composing the luminal compartment. The finding that p63 -/- mice lack stratified epithelium would support this notion [409]. The transient expression of p63 in dividing epithelial-like MCF-12A and MCF-10A cells is likely to affect the behavior of their daughter cells. However, non-epithelial MCF-12A cells do not express p63 (Figure 4.2), further bringing into question the cell type that MCF-12A cells more closely resemble *in vivo*.

4.4.2 Estrogenicity

Until now, the estrogen responsive profile of MCF-12A has remained ambiguous. In the original report, MCF-12A xenografts failed to grow in the presence of E2 and additional findings, mentioned but not shown by the authors, suggested that the cells may be ER-negative [380]. The expression of ER is not necessarily sufficient to describe a cell line as being responsive to estrogens. Nevertheless, published reports claimed these cells to be ER positive and have therefore used them as normal controls to genuine estrogen-responsive cancer cells. Mischaracterizations such as these lead to contradictory conclusions and may add to the increasingly poor reproducibility of biomedical studies [410]. By using multiple assays, here we have now shown that MCF-12A cells are unresponsive to E2 in culture conditions, both in regards to the control of cell proliferation as well as on the control of expression of estrogen-regulated genes.

Estrogen responsive cells remain quiescent in the presence of serum and absence E2 and proliferate in the presence of estrogenic compounds [411]. The proliferative effect of estrogen on estrogen target cells only becomes effective when serum supplemented to the basic cell culture medium is CD stripped; in this condition, cells enter proliferative quiescence due to the effect of the serum-borne inhibitor, albumin [399, 412]. Addition of physiological levels of estradiol neutralized the inhibitory effect of serum and thus restored the capacity of these cells to proliferate. Instead, MCF-12A cells proliferated equally well regardless of the media's estrogen content. This implies that MCF-12A cells are insensitive to CD-serum induced quiescence and thus are non-responsive to both the serum-borne inhibitor and to estradiol.

In order to explore whether the lack of proliferative response to E2 was due to absence of ER, we tested transcriptional activation resulting from estradiol-ER binding to estrogen responsive cells (MCF7) and compared it to that of MCF-12A cells. The binding of estrogenic chemicals to ER affected the induction and/or attenuation of different transcripts within MCF7 cells [413]. We used qRT-PCR to investigate the induction of estrogen-responsive genes in MCF7 cells by adding estradiol concentrations that resulted in maximal proliferative responses. While the expression of both amphiregulin and progesterone receptor was significantly increased in MCF7 cells treated with estradiol, MCF-12A fail to express either gene significantly following 48 hours of estradiol stimulation. These results imply that, in addition to the lack of a proliferative response by estrogens, exposure to estrogens also failed to induce specific gene transcription in MCF-12A cells. Moreover, relative to MCF7 cells, MCF-12A cells express levels of ER transcripts similar to those seen in ER-negative cell lines. The basal-like transcriptional profile of MCF-12A cells further suggests that these cells are ER negative, as only luminal cells express ER in tissues [414].

4.4.3 Growth in 3D

The formation of epithelial structures in 3D culture allows for the *in vitro* study and manipulation of the mammary epithelium. We have previously shown that some cell lines form both acini and ducts when grown in collagen with and without the addition of Matrigel [147, 148]. Herein, we examined the capacity of MCF-12A cells to form normal epithelial tissue structures. In a 3D context, the formation of lumena is a hallmark of normal mammary phenotype [154]. When grown on top of laminin-rich extracellular matrix for 4 days, MCF-12A cells formed rounded, non-lumenized structures [162]. Using a similar cell model, these early round structures were shown to form growth-arrested acini consisting of polarized cells after 16 days in culture [396]. After 14 days of being embedded in rat-tail type I collagen, in addition to lumenized acini and solid, round structures, MCF-12A cells in our growth conditions organized into large, lumenized, branching structures. The formation of duct-like structures by MCF-12A cells has previously been described only when grown on top of matrix derived from nulliparous rat mammary glands [101]. This implies that rat-tail type 1 collagen recapitulates the mammary gland environment found *in vivo* better than in several other 3D models currently in use. The rounded clusters of concentric cells (Figure 4.4c, arrow) never occurred outside of other structures implying that their formation is dependent on the interruption of interactions between those cells and the collagen matrix. MCF-10A cells grown in rat-tail type I collagen only form acini and short non-branching ducts without any "rosebud" structures.

4.4.4 SAMA as a tool to analyze epithelial structures grown in 3D

We employed the SAMA package for the unbiased, unsupervised analysis of MCF-12A structures formed in collagen gels. SAMA allows for unbiased and automated measurements of a host of biologically relevant physical parameters of epithelial

structures, an ability that is conveniently useful in analyzing large number of structures. One of the advantages of the SAMA software is the ability to filter the data based on both size and biological relevance of the structures analyzed. This software package enabled us to analyze hundreds of structures within a 3D space for geometric parameters that otherwise would have been difficult to perform. The variability of structural shapes formed in other areas of these gels may be due to the distribution of MCF-12A cells migrating toward the perimeter of each gel. The ability of MCF-12A to drastically contract the gels (Figure 4.5) may be responsible for stochastic forces in the gel microenvironment. Gel contraction of similar magnitude has been previously described when MCF-10A cells were grown in rat-tail collagen and resulted in a non-homogenous distribution of structures [148]. The finding that fibroid cell-depleted BF9 subclone contracted gels at a similar degree to that seen by the parental cell population implies that those non-epithelial-like cells do not contribute to gel contraction. If those cells were responsible for increased gel contraction, BF9 gels would be expected to contract to a degree intermediate between acellular and parental gels.

The results of SAMA confirm that MCF-12A parental cells form spherical, acinar and duct-like structures. Upon removal of the MCF-12A cells which grow away from the epithelial plaques in 2D, the remaining cells form longer, flatter structures than those produced by the heterogeneous parental cell line, on average. The differences seen between parental and BF9 gels are not simply due to the exclusion of individual spindle-shaped cells but, rather, imply an interaction between the two cell types in 3D which results in distinctly different structural compositions. Human fibroblasts have been shown to speed up the morphogenesis of epithelial structures from MCF-10A cells and increase the number and length of ducts formed when seeded jointly in collagen gels; this effect is likely due to fibroblast-mediated formation of thicker, parallel collagen

fibers [143, 369]. The single cell population of MCF-12A acts differently than fibroblasts, however; this effect might be due to fibroblasts interfering with the ability of the epithelial cells to alter collagen organization leaving thinner, less organized fibers favoring the morphogenesis of rounded acinar structures.

4.5 Conclusions

We have documented that MCF-12A cells are a non-tumorigenic, heterogeneous, estrogen receptor-negative cell line that expresses a combination of epithelial and mesenchymal markers. In floating rat-tail type I collagen gels, MCF-12A cells form complex, lumenized acini and ducts, the characteristics and distribution of which are altered by either the presence or absence of different subpopulations. Based on the time-dependent progressive population changes within the parental cell line and the complex interactions between these two cell types in 3D. MCF-12A cells appear unsuitable for use in epithelial morphogenesis studies when compared to MCF10A cells.

CHAPTER 5: DISCUSSION

5.1 Outcomes of Specific Aims

The overall aim of this thesis was to determine how COP rats overcome NMU carcinogenesis of the mammary gland. Based on previous tissue recombination experiments that showed the carcinogen exposure status of the stroma predicted susceptible strain tumor formation potential, we proposed that the COP stroma was responsible for the remodeling of preneoplastic lesions and, ultimately, its resistance to mammary gland tumors. The specific aims at the start of the project were to 1) characterize a novel "normal" hormone responsive cell line for use in 3D culture 2) to use the newly characterized cell line as a "read-out" of rat mammary fibroblast mediated epithelial morphogenesis 3) analyze existing microarray data derived from COP and WF cleared fat pads to determine changes in gene expression following NMU exposure.

We were initially interested in MCF12A cells after it was shown they responded to estrogens in 3D. There are several hormone responsive cell lines available for use in our lab but they are all tumorigenic. Identifying a non-cancer associated cell line for use in the study of hormone-mediated epithelial morphogenesis was an intriguing possibility. We have used hormone responsive cells extensively to determine the hormone-like properties of chemicals and to study the roles of hormones in cell proliferation.

There were several unusual things about MCF12A cells. First, after their original isolation they were exposed to high heat due to an incubator malfunction that killed the majority of cells. The remaining cells were expanded and patented. Second, like a similar cell line, MCF10A, MCF12A cells require a complex media containing growth factors to proliferate. Removal of EGF from the media resulted non-proliferation and cell death.

Third, MCF12A cells grow as a heterogeneous population and undergo apparent cell type transformations during normal handling. Short-term culture of isolated epithelial plaques results in the eventual reestablishment of round and fibroid-like cells seen in the parental cells. As epithelial plaques expand, cells at the periphery lose e-cadherin expression and redistribute vimentin. Over time, peripheral these cells become motile and move away from the plaque. If all epithelial-like cells are removed from culture the cells will remain as fibroid-like and spherical cells indefinitely. These findings imply that the distinction between cells being "cancer cells" or "normal" is insufficient to properly characterize cellular behaviors.

Despite MCF12A cells being described as ER-negative during their initial characterization they gradually became identified as such in the literature. I believe this arose for two reasons: 1) reliance on claims made in previous papers resulted in groups failing to verify the ER status of cells prior to their use and 2) if groups did verify receptor expression or activity they failed to properly interpret the results due to poor experimental design or lack of proper controls. Papers using cells for hormone activity purposes should show proof of receptor expression AND functionality with their data.

Characterization of MCF-12A in 3D cells was difficult due to the ongoing changes in population dynamics. Non-epithelial cells do not express markers for fibroblasts or myoepithelial cells but they do interfere with the morphogenesis of epithelial-like cells when grown in collagen gels. These influences resulted in changes to the complexity and distribution of epithelial structures within each gel. It is also possible that, over several passages the increasing number of fibroid cells would further alter morphogenesis making reproducibility an issue. For these reasons we chose not to incorporate MCF12A cells into the co-culture experiments planned for Aim 2, where fibroblast-mediated

influences on morphogenesis required reproducible formation of acini and ductal structures.

Aim 2 set out to use fibroblasts isolated from rat mammary glands in a 3D culture model to support the findings of renal subcapsular recombinations done previously. In those recombinations, mixtures of MECs, fibroblasts, and collagen formed structures similar to those seen in the intact mammary gland including adipose tissue and multicellular structures containing lumen and expressing basement membrane. Based on these promising findings, I had hoped to develop a more robust model that allowed for cheaper and faster production of more structures. Because of the *in vitro* nature of this model the gels would be amenable to further manipulations and characterization than the subcapsular recombinations.

MCF10A cells were selected as the epithelial read-out of fibroblast influence because they form both acinar and ductal structures when grown in mixed gels. It was important to grow both structures because once formed, growth arrested acini are relatively stable structures. Ducts, on the other hand, continue to grow and form lumen as they expand into the matrix. My rationale was that rat fibroblasts growing in rat-tail collagen and Matrigel would behave in a manner similar to how they grew in the tissue recombinations, manipulating collagen fibers and secreting ECM unique to the strain, age, and treatment animals from which they were derived. These cues would then be incorporated into the matrix and alter MCF10A morphogenesis of both acinar and ductal structures.

The MCF10A/rat fibroblast 3D culture model deserves further attention in the future study of fibroblast-epithelial cell interactions. If I had had access to more rat fibroblasts I

would have liked to do additional work adjusting the ratio of fibroblasts to epithelial cells. I suggest decreasing the total number of cells in the gel and extending the length of the experiment to encourage growth of larger structures, lumen formation, and deposition of BM. In the event that clearer gene expression results are obtained, gels such as these could be used to verify the expression and distribution of gene products associated with “normal” mammary structures and those with neoplastic phenotypes.

Aim 3 of the thesis involved the reassessment of microarray data generated using cleared fat pads from treated and non-treated rats. The goal of these experiments was to identify biological processes, functions, and disease-like patterns of gene expression that would explain differences between COP and WF as well as their response to NMU. The analysis revealed that COP and WF fat pads have wildly different gene expression patterns; they can be identified by expression signature alone. Further, COP gland gene expression changes little following NMU exposure, a finding that could be interpreted to mean that the gland is more “stable” and quickly overcomes carcinogenic insult. Compared to COP, WF response to NMU involves many more genes. These signatures include genes associated with injury and wound healing and persist for over two months after injury. It is possible that the enhanced response by WF glands aids in the promotion of preneoplastic lesions while the subtler COP response allows for the local epithelial-stromal organization to remodel the aberrant growth.

A major hurdle of the microarray analysis was variation between samples. There are number of possible sources for this variation. Reconstruction of the microarrays based on intensity data shows a few areas on multiple chips where physical imperfections (appearing as folds or dents) led to areas of high or low expression readings. These areas only account for small areas of the chips, however, so the impact on the final assessment

was probably limited. It is also possible that cleared fat pads are highly variable due to the complexity of their makeup. Even without epithelium, glands contain metabolically active adipocytes, fibroblasts, blood vessels, nerves, lymph, and a variety of immune cells. Not only is the makeup of the glands different from point-to-point they also change temporally. Finally, if we were to assume that the effects of NMU on the WF gland are truly random, then single gene changes in each rat would corrupt any attempts to analyze expression statistically. If each COP glands responded to NMU with minor adjustments it would explain the small number of differentially expressed genes. Similarly, if WF glands responded to NMU insult by altering a large number of random, non-stereotyped gene groups, the analysis would be tedious.

I spent years looking at the microarray data using multiple analysis platforms with multiple collaborators. It was never my intention to identify a single gene that was unique to COP and not to WF or visa versa. I had hoped to identify patterns of tissue behaviors that promoted tumor growth or lesion resolution in WF and COP, respectively. The question remains if there is something unique about COP glands that allow them to actively stop tumor formation OR if WF glands are simply prone to tumor formation due to their selective breeding. Based on my findings, it is more likely that the latter is true.

Were I to repeat these experiments, I would use more animals at fewer time points and pool the samples to account for the tissue complexity. In addition, modern sequencing techniques may be better for the quantification of transcripts especially of yet-to-be identified genes and non-coding products. Finally, a major theme of this thesis has been the association between development and cancer. For this reason, I would prefer to use intact glands, as opposed to epithelium-free cleared fat pads, in which the epithelium and the stroma had been altered during development due to the reciprocal interactions

of the two compartments. Perhaps the assessment was difficult because, without the potential to form tumors, NMU interacts with the stroma differently. Knowing full well how difficult expensive and time consuming laser microdissection can be I would recommend isolating preneoplastic lesions from the tissue, along with their associated stroma for future analysis. Further, because not all WF lesions go on to become tumors collection of a large number of lesions, as well as structurally healthy tissue as control, could potentially aid in identifying histology and gene expression patterns that could predict the outcome of some WF lesions.

5.2 Comments on the COP project

After injection into the intraperitoneal space, NMU reaches the bloodstream and thus reaching every cell in every organ. However, the strongest carcinogenic effect occurs on the mammary gland. If these mammary lesions are removed the animal will eventually develop lethal bladder tumors. How is it possible that otherwise non-specific carcinogens are capable of producing tumors in only a subset of tissues? Do all cells in the organism bear similar mutations?

The most widely accepted explanation for NMU carcinogenicity is its ability to mutate mammary epithelial *H-ras*. This linear explanation for its carcinogenesis fits well within the framework of SMT, however several studies contradict the idea. One group [415] used homologous recombination to insert a single copy of *H-ras* mutated at codon 12 into immortalized rat fibroblasts found that this mutant clone, when expressed at normal levels failed to transform the fibroblasts. The standard belief is that this single codon mutation imparts an irreversible functionality to the H-ras protein causing increased proliferation, survival, and differentiation through Raf, ERK, and RalGDS pathways. Another group found that mutant *h-ras* was insufficient for *in vivo* mouse mammary

carcinogenesis unless its expression was under the influence of highly active promoters [416] . Finally, and most relevant to the current discussion, is the finding that mutated *H-ras* was insufficient for the induction of mammary tumors in rats until after NMU exposure [417]. The authors conclude that it "seems unlikely that NMU promote[s] the outgrowth of mutants via a mutagenic mechanism" and that NMU likely induces carcinogenesis by acting as a hormone in already mutated cells – a conclusion that fits poorly within SMT dogma. However, this conclusion is strengthened by findings that the NMU-treated mammary glands display histological features similar to that of the pregnant gland. Is this because NMU acts like a hormone or because the tissue damage associated with NMU-exposure is similar to biochemical and biophysical changes associated with early pregnancy.

5.3 Project Synergy

What is the common theme behind the three manuscripts presented in this thesis? Endocrine disruption is a fascinating field because it impinges on multiple biological topics including development, cancer, and behavior but it also involves sociology and challenges with disseminating scientific knowledge. The main reasons there is any controversy surrounding EDCs is the complex nature of their interaction with tissue combined with special interests of powerful groups. The EDC story is a great example of how scientific findings can be criticized and misinterpreted based on expectations of the observer. In a similar manner, MCF12A cells have been used for over a decade as a hormone responsive normal cell because of data misinterpretation and misuse. Proper review of the literature should be sufficient to reveal discrepancies in ER-status of MCF12A cells and prompt immediate validation for researchers using them in their work. Their description as "normal" cells brings into question how we determine normal in biological terms. Are COP rats normal because they are resistant to tumors? Are WF

rats normal because they do get tumors? Altogether, my thesis and the projects I worked on while at Tufts University revealed to me the complexity of biological systems and how even basic interpretations are complicated by limitations of tools, time, and human perception of the world.

5.4 My journey to a PhD

During the summer prior to my last year of university, I received a letter from a chemistry professor who had come across some last minute funding for an organic chemistry synthesis pilot study and he was desperate for a worker. Dr. Cormier heard I may be interested in work and by the end of the week, after passing his test on molarity calculations and basic synthesis reactions, I was a chemist. The project was to synthesize a novel non-phosphate surfactant that could resist high heat and pressure for use in industrial drilling applications. Unfortunately for us, one of the intermediate products was too viscous to meet the synthesis requirements. But to me the feeling of interpreting an NMR plot and synthesizing a novel chemical was reward enough; it was one of the most rewarding experiences of my academic life.

Just weeks after completing my undergraduate work in biochemistry and cell biology I started work in a cardiac development lab at Children's Hospital Boston. The young PI had recently discovered a gene the expression of which was necessary for ensuring that cardiomyocytes divided in the correct orientation to generate a thick ventricular wall. Zebrafish "heart of glass" mutants have the same number of cardiomyocytes as their wild-type siblings but their ventricle is enormous, thin, and unable to generate the force required for proper blood flow. My job in the lab, in addition to maintaining the fish colony, was to identify other cardiac mutants, characterize their defects, and identify the gene responsible for the phenotype using positional cloning. This process, I later learned,

was my training in "classical genetics." Several of the gene mutations we studied were embryonic lethal in humans and others had non-cardiac consequences, such as the formation of cavernous cerebral malformations in adults [418]. I did not think it a possibility that defects in a single gene would cause unique problems in different tissues. However, I left my position at Children's Hospital thinking that I fully understood genetics, both theoretically and practically.

The way biology had been taught to me in high school and university had not only resulted in my having a career in biology, but I was also good at it. I was reliable and fast at the bench and I volunteered to participate in lab meetings, something that was unusual for technicians to do. At the time, my vision of developmental biology was that of an unfolding program; each gene had a role to play at certain times during organogenesis. A flawed or missing protein resulted in a predictable phenotype. In the case of our zebrafish colony, I used a top-down approach to discover Mendelian cardiac mutants and use positional cloning to find where the point mutation was. This line of thinking worked perfectly for my environment. The way I asked questions and designed experiments within the accepted framework was successful because the interpretation of the answers utilized the same premises.

After my time at Children's Hospital, as well as a failed round of graduate school applications, I was hired by the Cancer Center at Massachusetts General Hospital (MGH). Like Children's Hospital, MGH was one of the best research hospitals in the world and I had the opportunity to work shoulder-to-shoulder with some of the top head and neck surgeons in the country. My mentor, Jeff Settleman was a pioneer in the field of personalized medicine and was the director of a cancer cell drug-screening core. In the core, which I remember gazing into in awe, thousands of human derived cancer cell lines

were plated and fed into liquid handling robots where they were treated with a library of over 20,000 chemical compounds. After each cell line was shown to have sensitivity to a chemical it was rerun through the system and screened for combinatorial effects. It seemed like a brute force approach to medicine; knowing the tissue source and mutation status of each cell line and which drugs they were sensitive to sounded like a foolproof data generator. I imagined an atlas being built in which a doctor could follow a mutation based flow chart and determine a specialized, and successful, drug regime for each patient.

I had never wanted to get involved in the cancer field prior to my being hired by MGH. I had had limited interactions with the vascular biology department at Children's Hospital, specifically members of Judah Folkman's angiogenesis lab. From my perspective, at the time, cancer biology was a crowded and hyper-competitive field. I naïvely believed at the time, that, armed with the human genome and an army of medicinal chemists, the end of cancer was in sight and then what would all the cancer biologists be left to do? Weekly meetings and seminars covered everything from surgeries to chemotherapies to basic cell biology. It was a whole new lexicon for me. One thing I was surprised by was how integrative cancer biology was. I was exposed to the immune system, viruses, lung histology, hormones, colon anatomy, and metabolism.

Here, I experienced a shift from classical genetics into the new and expanding field of systems biology. My focus of biology shifted from the level of the gene and the occasional receptor-ligand interactions to giant networks of signal transduction pathways. This, I thought, must be what the *process* of biology looks like. It was a whole new level of complexity for me and I was excited by it. I worked with a post-doc who was interested in the concepts of oncogene addiction and synthetic lethality. He had determined that some

(~40%) colorectal tumor cell lines are "addicted" to mutated KRAS meaning that short hairpin knockdown of KRAS resulted in their death. Together we created a panel of over 50 colorectal cell lines and, based on their signaling and expression patterns were able to predict their KRAS addiction status and their sensitivity to pharmaceutical targeting. Using expression data we identified a downstream kinase that was upregulated in non-addicted colorectal cancer cell lines and induced apoptosis with a kinase inhibitor. The results were recapitulated in a xenograft model (albeit accompanied by severe liver toxicity) and the findings were published in Cell. At the time this was the first example of synthetic lethality exploitation of colorectal cancers.

Throughout this period my understanding of cancer remained true to the textbook concepts I had been taught with a few modifications concerning tumor heterogeneity and immune surveillance. I believed in the "lightning bolt" of genetic mutation within a single cell driving it to proliferate endlessly and form a tumor. It was clear to me that tumor growth caused by cellular modification made them at once dangerous and difficult to target. My review of the literature at the time was primarily concerned with studies similar to my own: identification of mutations, pharmaceutical targeting, and some form of *in vivo* validation. It felt like progress was being made. Caught up in the excitement of being published in Cell, I again left a job thinking I had come to understand biology and would continue to be successful at it. Trying to finish the last round of revisions to the Cell manuscript, I worked in lab at MGH until the night before my first day of classes at Tufts University.

Now I stand on the edge of a new limit of my understanding. I had hoped that a graduate career would be the final chapter in my biological education but what I came to understand is that I can never fully understand living things. My career in biology has

included exposure to many fields. I now realize that information and experimental findings are meaningless if you fail to think of them without a theoretical framework to help guide your objectivity. The reason I describe my background is because I believe it paints a fairer picture of my relationship with biology. I learned how chemicals interact and saw how I could change them. I tugged on genetic strings and watched a heart grow in front of my eyes. I was part of a team that figured out a way to kill tumors that wasn't a true cure. What I had failed to learn before graduate school is that these experimental snapshots I had experienced were not being integrated properly into a manner that allowed me to ask what exactly it was I was studying. Objectivity provided by theoretical frameworks shapes the way we interpret results. Those operating under imprecise theoretical frameworks, including doctors and scientists of the past, are not stupid but unable to put their findings into a broader perspective that reflects reality – such as the case with a definition of "healthy."

5.5 Normal vs. Disease

The philosopher and physician Georges Canguilhem observed a movement in medicine, following the introduction of physiological metrics [419], whereby the "normal" or average patient was held as the standard by which care was administered to those who were abnormal. In other words, sickness was a deviation from a physiological constant. For Canguilhem however the normality of living beings is an active process, rather than a steady state. Canguilhem asserts that healthy organisms do not present with "too-much" of an organ [420]: we are not bestowed with additional organs in reserve to help us adapt in the event of a major environmental change, but rather our organs are capable of a great deal of adaptive capacity in both their form and function.

One example how this perceived discrepancy between what is normal and abnormal can impact medical practice is illustrated by Austrian pathologist Paltauf's observations published in 1889 and 1890. He reported that enlarged thymi caused restricted blood flow through the aorta of infants resulting in "crib death," which would late become known as Sudden Infant Death Syndrome (SIDS). These early reports incited 820 publications regarding the ailment during the next 33 years. In order to reduce the size of the thymus, pediatricians recommended irradiation of the throat, a medical trend that continued until the 1950s. These radiation treatments had no effect on the frequency of SIDs deaths but resulted in an expansive increase in pediatric thyroid cancers.

The use of infants who had not died as a result of SIDS as controls was a major flaw in Paltauf's assertion. Infants used as controls likely presented with atrophied thymi caused by inflammation or malnutrition prior to death [421]. The thymi of SIDs victims were likely the first pathologically "normal" seen by an investigator. The prophylactic irradiation of children's necks to stop the uncontrolled growth of their thymus implies that a majority of physicians believed the organ was prone to expand to a fatal extreme, despite the fact that "thymic asthma" had been disproven half a century earlier.

5.6 Concluding comments

Over the last 50 years biomedical research has undergone several waves of intense interest in certain biological aspects and topics such as metabolism, growth factors, genetics, and angiogenesis. Whenever a biological field became fashionable, it soon became incorporated to the study of cancer. COP mammary carcinogenesis resistance was no exception. The resulting negative findings from such studies, however, have provided some useful clues about where to search and what questions to ask when explaining cancer resistance and susceptibility. Less reductionist approaches to

understanding biological interactions have led to an appreciation of tissue-level complexity such as epithelial-stromal interactions and their ability to stabilize following change. The application of these theoretical concepts will be crucial for understanding how tissues develop, function, and adapt to changes including tumor suppression and promotion.

The initiation and resolution of a neoplasia can be described as a disturbance in the tissue field followed by a period of readjustment resulting in a localized alteration of constraints after which the tissue continues to function under a new set of “rules”. Alternatively, in the case of tumor development, the new constrains formed following dysplasia induce modifications in cellular behaviors and metabolism thereby influencing neighboring cells and the creation of a tumor microenvironment with an organization unlike anything seen in the tissues of the intact organism.

That remission happens at all is a further indication that many tissues are capable of reverting the dysplastic organization of diagnosable tumors and their cells below a pathogenic threshold. Perhaps more telling is that many of the cells that once participated in the neoplasia are later reassigned a normal function within the tissue despite a the preservation in these cells of the genomic lesions which, according to the SMT are believed to have caused the cancer. The existence of individual tumor cells within a functional tissue stands in contradiction to the idea that once a cell is a tumor cell it will always BE a tumor cell. Perhaps remission is a more common occurrence than believed due to a combination of human longevity, the rate of tissue remodeling, and the size of early dysplastic regions. Indeed, this possibility is supported by observations made during autopsies and assessments of otherwise normal tissues (see above).

I propose that the genetic lesions present in tissues are a result of "normal" cellular activity, as they exist within multicellular organisms. Such lesions are insufficient to account for altered cellular behaviors due to external constrains, protein redundancy functional plasticity. In accordance with the framework of evolution, and often erroneously applied in the tumor biology field, the variation described by Darwin happens on all levels of organization including within the cell and within the tissue. Modifications to the tissue, whether they are from internal or external sources, elicit structural and functional responses. Variation is intrinsic to biological entities; the alterations that occur may be neither helpful nor harmful and may often result in no apparent changes at all. Variation, especially on large scales results in an increased of survival and continued variation. So it is within the dysplastic environment - cells change due to alterations in homeostasis and whether those changes result in tumor promotion or remission are dependent on the establishments of new local and global symmetries.

It has been shown under multiple circumstances that the mutational status of cells, when incorporated in an organized, functional tissue, is irrelevant. The current push to determine the genomic fingerprint of primary tumors and their metastases for targeting is flawed because modulation of those altered genes is likely insufficient for inducing reorganization. Even in cases where the vast majority of cells within the tumor are targeted for cell-specific death, without a planned intervention for the surviving cells' microenvironment the likelihood of eventual reoccurrence is high. Small molecules currently target less than 5% of the proteome and, pending major advances in structural biology that number is unlikely to increase substantially. Further, the thinking that molecular activators or inhibitors exist for the majority of human proteins is possibly overoptimistic. This thesis is a call for future solid tumors treatments to refocus the

strategy from the tumor cell killing toward tumor biology maintenance in a new steady state and/or tumor size constriction.

CHAPTER 6: BIBLIOGRAPHY

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