8-2022

Using in vitro, in silico, and in-Classroom Techniques to Address the Gender Data Gap in Health Care

Kelsey Watts
kmwatts@clemson.edu

USING IN VITRO, IN SILICO, AND IN CLASSROOM TECHNIQUES TO ADDRESS THE GENDER DATA GAP IN HEALTH CARE

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

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by
Kelsey Watts
August 2022

Submitted to:
Dr. William Richardson, Committee Chair
Dr. Lisa Benson
Dr. Delphine Dean
Dr. Ann Foley
Dr. Jiro Nagatomi

ABSTRACT

Cardiovascular diseases (CVDs) are the leading cause of death worldwide in males (XY) and females (XX). Prior to menopause, females have a relative protection against serious cardiac pathologies compared to age-matched males. This phenomenon is widely attributed to the ovarian hormone estrogen. Unfortunately, hormone replacement therapy to maintain estrogen levels in postmenopausal females has overall adverse effects, and it is not recommended for long-term use or as a preventative measure for eCVDs. A major driver of CVDs, specifically heart failure, is cardiac fibrosis: the continued buildup of scar tissue that reduces the heart's ability to pump. There are currently no FDA-approved therapies to specifically target cardiac fibrosis, and the five-year survival rate for patients diagnosed with heart failure is typically under 50%.

Recent studies exhibit the potential of estrogen to decrease the fibrotic response of cardiac fibroblasts, the cells responsible for the progression of fibrosis. However, most of these studies were conducted on tissue culture plastic (TCP) and/or with pooled male and female neonate rat CFs, limiting their clinical relevance. The goal of this dissertation is to expand our understanding of the sex-specific signaling of estrogen within CFs using *in vitro* and *in silico* techniques to identify potential sex-specific dimorphisms in regulatory signaling that will allow for the creation of novel treatments of cardiac fibrosis that mimic estrogen's therapeutic abilities while negating its adverse systemic effects.

Biological sex impacts the presentation, prognosis, and severity of many conditions. Yet, females have been historically underrepresented in clinical trials and experimental studies, resulting in health inequities that disproportionately affect women.

Literature has shown that women are more likely to include female samples in their study design and report sex-disaggregated data. However, they have been consistently underrepresented in STEM fields. Increasing the number of female scientists will aid in shrinking the gender data gap, which will help elucidate our understanding of the sex-specific differences of various diseases and biological functions. In addition to my *in vitro* and *in silico* initiatives, I have developed in classroom techniques utilizing inclusive pedagogy strategies that specially target female students with an aim to increase their STEM self-efficacy and identity. These *in vitro*, *in silico*, and in classroom techniques are designed with the intention of fostering a more inclusive and equitable approach to healthcare.

ACKNOWLEDGMENTS

Throughout my time at Clemson and writing this dissertation I have received invaluable support from many individuals who made completing this work possible. First, I would like to thank my adviser Dr. Will Richardson, who gave me the freedom to explore my interests in grad school which helped me find my passion. His unwavering belief in me fueled my perseverance and confidence to become the researcher I am today. I would also like to thank my other committee members, Drs. Lisa Benson, Delphine Dean, Ann Foley, and Jiro Nagatomi. I would especially like to thank Dr. Lisa Benson for becoming a mentor as I began exploring engineering education research (EER) and inviting me to join the Peer Reviewer Training (PERT) research team. Being part of the PERT team has enhanced my EER skill set and introduced me to additional great EER mentors, including Drs. Gary Lichtenstein, Karin Jenson, and Becky Bates.

The members of the Systems Mechanobiology Lab: Jake Potter, Sam Coeyman, Brendyn Miller, Jonathan Heywood, Anamul Haque, and Drs. Jesse Rogers, Amir Yeganegi, and Patilee Tate. Special shout out to Dr. Jesse Rogers, whose graduate work on the cardiac fibroblast signaling network mirrored my own, for his indispensable training on experimental and computational techniques and continued support troubleshooting even after he had graduated. In addition, the many undergrads I had the pleasure of mentoring: Wesley Nichols, Rachel Emerson, Zoë Gold, Donald Hartsfield, Christian Herrera, Kerri Wong, and Jenni Forkin. Wesley Nichols's computational work this past semester especially helped me finish my dissertation on time.

I would also like to thank the American Heart Association which funded my predoctoral fellowship for this work. Additionally, Sara Hanks from Clemson's Emerging Scholars program for helping us find a platform to pilot the education modules we developed.

To my parents, Kay and Mike Watts, whose constant support throughout my life allowed me to go after my dreams. To my friends, especially Tori Barnhouse, who uniquely understood the journey the past nine years have been, from being random roommates the first year of undergrad to also recently completing her Ph.D. in Bioengineering. To my boyfriend, Adam Hosier, who provided constant love, support, and a much-needed distraction from the stress of completing a Ph.D. And lastly (but most importantly) to my number one fan/best friend/supporter, my wiener dog Curie. Most of this dissertation was written with her asleep in my lap, which not only inspired me to write for longer because she was too cute to move but also, as her eponym suggests, was a constant reminder of the drive to become a female scientist who breaks barriers and leads the way for other women in STEM.

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CHAPTER ONE

INTRODUCTION

1.1 Study Significance

Cardiovascular disease is the leading cause of death in both males (XY) and females (XX). However, it has been widely observed that females have a relative protection against serious cardiac problems prior to menopause, likely due to the ovarian hormone estrogen. One of the leading contributors to heart failure is cardiac fibrosis, a continued buildup of extracellular matrix driven by cardiac fibroblasts (CFs) in response to both biochemical and biomechanical stimuli. There are currently no therapies approved to specifically target cardiac fibrosis, but there have been many studies that exhibit the potential of estrogen to decrease the fibrotic response of CFs. Notably, hormone replacement therapy (HRT) has been correlated to a decrease in mortality due to fibrotic-induced heart failure in postmenopausal females. Unfortunately, studies also show that HRT can elevate other risk factors, including cancer and stroke, and is currently not recommended for long-term use.

Recent literature has elucidated the role of estrogen in CF signaling in responses to biochemical stimuli; however, the majority of these studies were conducted on tissue culture plastic (TCP) and/or with pooled male and female neonate rat CFs. The goal of this dissertation is to expand our understanding of the sex-specific signaling of estrogen within CFs using *in vitro* and *in silico* techniques to identify potential sex-specific treatment recommendations for cardiac fibrosis that mimic estrogen's therapeutic sec abilities while negating its adverse systemic effects.

An additional aim of this dissertation is to increase the female-specific data in the literature related to cardiovascular diseases (CVDs). Biological sex is known to impact the presentation, prognosis, and severity of many conditions, including CVDs. Unfortunately,

females have been historically underrepresented in clinical trials and experimental studies. This has resulted in many health inequities that disproportionately affect women, including adverse drug responses occurring at double the rate in females than in males. As there is a push to develop artificial intelligence that enables patient-specific diagnostic and treatment recommendations through precision medicine, it is imperative to increase the amount of female data in the literature to have robust sex-disaggregated data sets to train and validate these algorithms.

Literature has shown that female-specific data is more likely to be considered and reported if either the first or last author of a manuscript is female. Increasing the number of female scientists will aid in expediting the understanding of female-specific data in the literature and our overall understanding of the sex-specific differences of various diseases and biological functions. In addition to my *in vitro* and *in silico* initiatives, I have also developed in classroom techniques that specially target females who have been historically underrepresented in STEM with an aim to increase their self-efficacy of computational methods and STEM identity. The *in vitro*, *in silico*, and in classroom techniques outlined below are designed with the intention of fostering a more inclusive and equitable approach to healthcare.

1.2 Specific Aims

Aim 1: Test the Effects of Sex and 17 β-Estradiol on Cardiac Fibroblast Morphology and Signaling Activities In Vitro

Several studies have demonstrated estrogen's cardioprotective abilities in decreasing the fibrotic response of cardiac fibroblasts (CFs). However, the majority of these studies are not sex-specific, and those at the cellular level utilize tissue culture plastic, a substrate with a much higher stiffness than physiological conditions. Understanding the intrinsic

differences between male and female CFs under more physiologically "healthy" conditions will help to elucidate the divergences in their complex signaling networks. We aimed to do this by conducting a sex-disaggregated analysis of changes in cellular morphology and relative levels of profibrotic signaling proteins in CFs cultured on 8 kPa stiffness plates with and without 17 β-estradiol (E2). Cyclic immunofluorescent analysis indicated that there was a negligible change in cellular morphology due to sex and E2 treatment and that the differences between male and female CFs occur at a biochemical rather than structural level. Several proteins corresponding to profibrotic activity had various sex-specific responses with and without E2 treatment. Single-cell correlation analysis exhibited varied protein-protein interactions across experimental conditions. These findings demonstrate the need for further research into the dimorphisms of male and female CFs to develop better tailored sex-informed prevention and treatment interventions for cardiac fibrosis.

Aim 2: Screen for Sex-Specific Drug Effects in a Fibroblast Network Model Integrated with Estrogen Signaling

A previously developed large-scale signaling network model (SNM) of cardiac fibroblasts was updated to include estrogen signaling. Male, female, and averaged pooled SNMs were developed by varying the weighting of estrogen stimulation of its three primary receptors. These models were validated against over peer-reviewed studies and found to be 77% accurate in matching simulation predictions to experimental outcomes in the literature. Additionally, sex-specific drug screens of 36 unique drug targets in the model were conducted with three experimental conditions: male, female post-menopausal, and female pre-menopausal. The cardioprotective effect of estrogen in the female pre-menopausal condition was evident; however, sex-specific differences between the male and female post-menopausal screens were much more subtle. Several regulatory pathways were identified that warrant further study in understanding the divergences in male and female cardiac

fibroblasts signaling. Additionally, we recommend the inclusion of more patient-specific parameters (i.e., genomic and transcriptomic data) in future model advancement to enhance its ability to make sex-specific predictions.

Aim 3: Development of Systems Biology Education Modules Utilizing Inclusive Pedagogy Strategies

There has been a persistent gender and racial gap in STEM. Recruitment and retention of these historically marginalized individuals are crucial to ensuring that there are diverse design teams as a way to combat inequities in healthcare. Five systems biology education modules were developed with inclusive pedagogy strategies designed to foster STEM identity and self-efficacy regardless of previous computational experience. Each module lesson plan is grounded in active learning techniques and consists of an unplugged activity, a model tutorial, and an open-ended model advancement exercise. Modules are focused on various biological and disease phenomena (e.g., tumor growth, viral spread, allergic reaction, gene regulation, and the menstrual cycle). The modules are available on the lab GitHub and will be piloted with the summer 2022 cohort of Clemson's Emerging Scholars program. During the piloting, we aim to assess student reception of the modules and investigate the use of representative problem statements (i.e., female students asked to model the menstrual cycle) on STEM identity, self-efficacy, and team dynamics.

1.3 Sex and Gender Terminology

Health disparities due to sex and gender are often integrally related. In this manuscript, sex refers to biological differences due to chromosomal genetic makeup (i.e., male=XY and female=XX). Gender refers to the societal and behavioral factors that influence a human's identity and actions (i.e., man/men= identify using he/him pronouns and woman/women= identify using she/her pronouns). Sex and gender will often be

referred to as a dichotomy throughout this manuscript. Still, we also acknowledge that for many patients, they are a spectrum that can result in additional complexities related to health care.

CHAPTER TWO

A REVIEW OF THE LITERATURE

2.1 Heart failure in cardiovascular diseases

2.1.1 Current trends and statistics

Cardiovascular diseases (CVDs) are consistently the world's leading cause of death, accounting for 1/3 of deaths worldwide[1]. In the United States (U.S.), an estimated 659,000 people die of CVDs each year[2]. CVDs include a multitude of pathologies, including stroke, coronary artery disease, arrhythmia, high blood pressure, heart failure, and myocardial infarction (MI). Stroke and MI are typically the deadliest of these pathologies as their sudden onset can result in death within hours without medical treatment. However, as knowledge of warning signs has increased over the past several decades in the U.S., there have been substantial increases in patient survival rates, especially for a MI[3,4]. If patients are hospitalized and receive treatment for an initial MI, survival rates are generally reported at or above 90%[5].

However, the long-term survival remains low, with 1-year survival rates dropping by about 10% and consistently declining in subsequent years due to the development and progression of heart failure (HF)- a pathologic condition in which the heart is not able to adequately supply blood to the rest of the body[5,6]. Currently, the five-year survival rate of HF is most often reported as 50% or less[7]. HF presently afflicts about 6.2 million Americans and was considered a cause of death in 13.4% of all deaths in 2018[8]. Due to these continued increases in short-term survival rate after an initial MI coupled with rising rates of CVDs, rates of HF are also expected to continue to increase by 43% by 2030[9].

2.1.2 Cardiac fibrosis disease progression and treatment

HF occurs after an initial MI due to the deposition of collagens and other extracellular matrix (ECM) proteins that are necessary to maintain structural stability in the infarct region. However, even after scar tissue has formed in the infarct area, this collagen deposition can continue uncontrolled resulting in excessive cardiac fibrosis. Fibrosis is defined as the development of fibrous connective tissue or "scar tissue" in regions that intrude on healthy tissue[10]. It most often occurs in the lungs (i.e., cystic fibrosis and pulmonary fibrosis), the liver (cirrhosis), the kidneys (renal fibrosis), and the heart (cardiac fibrosis)[11]. When it occurs in the heart, it can result in reduced ejection fraction because of left ventricle thickening and arrhythmia due to interruptions in electrical signaling due to the excess scar tissue[12]. As conditions become more pathologic, the heart must work harder to pump blood to the rest of the body, exacerbating fibrotic conditions.

There are currently no FDA-approved treatments to directly target cardiac fibrosis[13]. Patients diagnosed with HF are often prescribed a cocktail of angiotensin-converting enzyme (ACE) inhibitors and beta-blockers which can lower blood pressure and help control cardiac signaling[14]. Although these drugs can mitigate HF symptoms, which can slow the progression of fibrosis, they will not reverse or completely stop continued fibrosis. This is especially troubling because HF can be difficult to diagnose. Although patients who have suffered an initial MI are routinely put on preventive treatments to slow the progression of HF, not all HF results from a MI. Coronary artery disease (CAD), high blood pressure, viral-induced myocarditis, and congenital heart defects are also common causes of HF. A diagnosis of HF usually requires a multitude of tests, including an electrocardiogram (ECG), blood test, chest X-Ray, and stress test that are often not conducted until the disease has advanced to a stage where symptoms are interfering with a patient's everyday life.

It can be challenging to predict which patients are most at risk of developing severe HF. Common risk factors include smoking and alcohol abuse, diabetes, a diagnosis of another cardiac pathology, and obesity. However, HF due to congenital heart defects and viral-induced myocarditis can remain undetected for decades before diagnosis. Recently, viral-induced myocarditis has become an area of increased interest due to the COVID-19 pandemic[15]. Initial research has exhibited a fraction of patients develop inflamed cardiac tissue after infection with COVID-19[16]. However, it is currently impossible to predict which patients are the most at risk for this complication and what lasting effects this may have.

There is a clinical need to develop new diagnostic and treatment mechanisms for cardiac fibrosis. In addition to the millions of patients worldwide suffering from the disease, HF's healthcare-related costs typically surpass $30 billion annually. This number is expected to double by 2030 if interventions are not taken[17]. Many researchers are focused on developing novel treatments and more accurate diagnostic capabilities. Still, remedies remain elusive due to the complexity of the disease, including the various biochemical and biomechanical factors that fuel it. In addition, it is believed that biological sex likely plays a role in the development of cardiac fibrosis. Yet, minimal research has focused on the sex-specific hormonal and genetic mechanisms that contribute to the development of cardiac fibrosis.

2.2 The effect of biological sex on cardiovascular disease

2.2.1 Historical context

Biological sex and gender can significantly impact the presentation, diagnosis, and treatment of many diseases, including CVDs. The most notable instance of this is the misconception that chest pain is the hallmark symptom of a heart attack. While this is true for males and even some females, it is more likely for females to experience more subtle

symptoms such as chest pressure, neck pain, and nausea[18]. This phenomenon, named the Yentl Syndrome, has resulted in misdiagnosis and ineffective treatment of heart attacks in female patients due to their different symptoms than male patients[19]. This misunderstanding has resulted from decades of underrepresentation of female data in research and clinical trials, which has compounded into a poor understanding of the differences between male and female cardiovascular health, resulting in inaccuracies in the diagnosis and treatment of CVDs that disproportionally affect women.

Until recently, heart disease was misconstrued as a "man's disease." This is best illustrated by the media, which often portrayed and targeted men as the most likely victims of a heart attack[20,21]. As CVDs are also the leading cause of death in women, this is inaccurate and a harmful serotype to women's health. It is true, however, that females typically develop CVDs 7-10 years later in life than males[22]. This is primarily thought to be due to the cardioprotective role of the ovarian hormone estrogen[23]. Premenopausal females have higher estrogen levels, which reduces their risk of developing cardiac pathologies. However, once they have undergone menopause and the resulting decrease in estrogen production, this cardioprotection subsides. Because of this, hormone replacement therapy (HRT) which maintains estrogen levels in menopausal females, was believed to be the solution for a brief time[24]. However, after randomized clinical trials in 2002 by the Women's Health Initiative (WHI), HRT was found to elevate the risk of stroke, cancers, and even heart attack and therefore is not recommended for long-term use to prevent CVDs[25].

Clinically, there have been many sex-specific findings related to cardiac fibrosis. Females are typically observed to have less fibrotic tissue than age-matched males in healthy and diseased contexts[23]. In a longitudinal study of patients aged 45-84 who enrolled

without CVDs, it was found that focal myocardial scarring was 5xs higher in males versus females by the ten-year mark. In addition, these patients typically presented with increased concentricity and preserved ejection in females and greater left ventricular dilation and reduced ejection fraction in males, indicating a sex-specific correlation in fibrotic remodeling[26]. These trends in a sex-specific correlation of fibrotic remodeling are also observed in other organs which can undergo pathologic fibrosis, such as the lungs, liver, and kidneys[11]. Just as estrogen is believed to impact overall cardiovascular health, several studies have reported that post-menopausal females have increased fibrosis markers compared to males, indicating that gonadal hormones likely play a role in these sex-specific trends related to fibrosis[11,23,27].

1.2.2 Estrogen mitigates cardiac fibrosis

Even though estrogen is not currently approved as a cardioprotective agent, there have been several notable instances clinically and in the lab since the 2002 WHI trials where it has shown evidence in reducing the severity of HF. In a retrospective 2003 study by Lindenfield et al., it was found that female patients who took beta-blockers in conjunction with HRT showed a significant reduction in mortality in females with nonischemic HF compared to those just taking beta blockers[28]. Currently, researchers hypothesize that the timing of HRT may play a role in its efficacy. Recent studies have shown that when females take HRT earlier in life, closer to the time they undergo menopause, it effectively reduces CVDs[29,30].

These findings are supported by a multitude of *in vivo* and *in vitro* studies that report decreases in HF markers after estrogen treatment. For example, Pedram et al. demonstrated reductions in angiotensin II (AngII) induced increases in collagens and alpha-smooth muscle actin (α-SMA) upon treatment of 17-β estradiol (E2). This was confirmed via an *ex*

vivo analysis of AngII and E2 treated ovariectomized (OVX) mice. Mice who had received E2 treatment had hearts that were notability smaller than the hearts of just AngII treated mice, indicating preserved left ventricle wall thickness and lower markers of fibrosis in histology in the E2 treated mice[31]. Additionally, Iorga et al. found that estrogen therapy restored ejection fraction in male and female mice with pressure-overloaded induced HF[32]. These findings demonstrate the need for further study of estrogen's role in CVDs to possibly find a way to mimic estrogen's therapeutic abilities while mitigating its harmful effects.

2.2.3 Other sex-specific biologically relevant influences

Hormonal differences between male and female hearts are often not observed before puberty. There are only slight differences in male and female overall body size at birth and until their preteen years. However, after puberty, on average, males grow larger than females; these differences in body proportions are also reflected in heart mass, with adult female hearts being $1/4^{th}$ smaller than adult male hearts[33]. Because of their different heart sizes, there are functional differences between male and female hearts, such as rhythms and rates. For example, males typically have a resting heart rate of 70-72 beats per minute (BPM), and females have one of 78-82 BMP to attain similar blood volume outputs[34].

However, not all of the physiological differences between male and female hearts and CVDs are believed to be solely due to hormones. Recent literature has also suggested genetic differences that persist regardless of gonadal hormones are also likely contributing to the dimorphisms in CVDs in males and females. At the macro level, male and female hearts often have different ratios of the cell types which make up the heart (e.g., myocytes, fibroblasts, smooth muscle cells, endothelial cells, valve cells, and macrophages)[35]. It is currently unclear how these different ratios may contribute to physiological differences that

affect cardiovascular health. At the microlevel, each cell is believed to have a sex associated with the chromosomal genotype (i.e., male cells= XY and female cells= XX). Gene ontology within the multiple cell types of the heart has also shown differences in the expressed genes between male and female cells, indicating that there are intrinsic sex differences that could contribute to the sex-specific differences in CVDs[35]. For example, in a recent study by Aguado et al., it was found that genes that escape X-chromosome inactivation were responsible for regulating some of the sex-specific differences observed in aortic valve stenosis, a pathology similar to cardiac fibrosis[36]. More research needs to be conducted to fully understand how these genetic differences combined with hormonal differences affect CVDs.

2.2.4 Sex-disaggregated data reporting

Much of this current lack of understanding of the sex-specific differences in CVDs can be attributed to the historical lack of representation of female data in clinical trials and the literature. We also want to acknowledge that although we are focused on the gender gap, this lack of representation is only further compounded if considering factors such as race, ethnicity, and socioeconomic background. Although current guidelines by the Food and Drug Administration (FDA), National Institutes of Health (NIH), and American Heart Association (AHA) require female samples/participants in experimental design, this is a recent development. Males, typically Caucasian, have been formally and informally considered the "norm" for medical research for centuries[37,38]. This has been exacerbated by systemic issues such as medical distrust and the misconception that female hormones are unpredictable and can skew experimental results. From 1977 to 1993, the FDA actively excluded women of childbearing age from participating in clinical trials in response to fetal malformations caused by thalidomide[39]. Additionally, it was not until 1998 that the FDA

required gender-based reporting of their phase II drug applications[40]. And although the NIH publicly supported the inclusion of women in clinical research since the 1990s, it was not until 2016 that the NIH implemented a guideline requiring the use of both sexes in vertebrate animal research[41]. These decades lacking female representation have resulted in many clinical trials and published literature skewed toward male data. Even in studies that use male and female samples, unless they are specifically investigating the effect of estrogen or sex, most published literature on *in vivo* and *in vitro* studies do not report results in a sex-disaggregated manner. This lack of representative data has resulted in many health disparities affecting women. The first FDA-approved artificial heart was designed only for a male build[42]. Additionally, adverse drug reactions occur at double the rate in females compared to males[43].

2.2.5 Experimental models to study sex-specific effects

Part of the reason for this lack of sex-disaggregated data in the literature is a result of poor experimental models to study hormonal-based differences. As humans and whales are the only mammals to undergo menopause, there are no good animal models to completely replicate the gradual onset of menopause and its implications for women's health[44]. As I briefly discussed earlier, it is believed that premenopausal females have a relative cardioprotection compared to age-matched men, so this is especially relevant to CVDs. In an *in vitro* setting, researchers typically treat cells with 17-beta estradiol to evaluate hormonal sex differences[23,45–48]. Few studies investigate how other sex hormones, including testosterone and progesterone, can also modulate sex-specific disease responses. Many commercially available human fibroblast cell lines are pooled and/or do not report the cell sex. For *in vivo* and *ex vivo* studies focused on hormonal differences, ovariectomized (OVX) mice or rats are often used[49]. However, this model is more representative of sudden

menopause (i.e., a hysterectomy) rather than the gradual perimenopausal state females are in for several years. Knockout animal lines have also been developed to study the specific effect of a particular estrogen receptor[50].

These models are limited in scope as they primarily study hormonal differences; it is also essential to discern intrinsic biological differences. Much of the current literature on HF either uses pooled neonatal cells, male cells, or cell sex is entirely unreported. There needs to be a shift in reporting practices to fully capture which sex-specific disease responses are due to intrinsic biological differences, which are due to hormones, and which are due to a combination. This will first require a greater understanding of the complex signaling pathways in cardiac fibroblasts.

2.3 Cardiac Fibroblast Signaling

2.3.1 Biochemical signaling

Cardiac fibroblasts are the cells responsible for the extracellular matrix (ECM) deposition in the heart. In a healthy myocardium, they contribute to the biomechanical, biochemical, and electrical homeostasis of the heart primarily through their regulation of collagen turnover. In a pathologic environment, such as immediately after a myocardial infarction (MI), they maintain structural stability by rapidly secreting collagens and other ECM proteins to create scar tissue to patch together the infarct region. However, this ECM deposition can continue uncontrolled resulting in fibrosis due to the disruptions in normal cell biochemical and biomechanical signaling.

The primary biochemical pathways involved in cardiac fibroblast signaling include inflammatory cytokines, growth factors, and paracrine signaling. Upon cardiac injury, immune cells induce a pro-inflammatory response by releasing cytokines such as tumor necrosis factor-α (TNFα), interleukin 1(IL-1), and interleukin 6 (IL-6), which can induce

the secretion of metalloproteinases (MMPs) to clear necrotic tissue[51,52]. Latent transforming growth factor-beta (TGFβ) is present in the ECM and can be activated and induce proliferation and ECM deposition, causing a significant downstream response in pathways involving SMAD3 and MAPK[53]. Collagen 1 and Collagen III (col-I and col-III) are secreted by fibroblasts as the main component of the infarct scar. Hormonal agonists angiotensin II (AngII), norepinephrine (NE), natriuretic peptides (NPs), and endothelin-1 (ET-1) are also activated during the post-inflammatory response and can continue to alter ECM related gene expression during the remodeling phase of the scar creation[54].

2.3.2 Biomechanical signaling

Like most cells, cardiac fibroblasts respond not only to chemical stimuli but also to mechanical stimuli. In a post-MI environment, the heart undergoes structural changes that affect the strain, stress, pressure, and stiffness that cardiac fibroblasts experience. These biochemical changes can cause intracellular responses via mechanotransduction. A cardiac fibroblast can sense changes to its biomechanical microenvironment through receptors on the plasma membrane, cytoskeleton, and nucleus and convert these to a biochemical response. Integrins form focal adhesion complexes on the plasma membrane that connects to the intracellular actin skeleton and can directly respond to biomechanical cues. For example, focal adhesion kinase (FAK) responds to increases in stiffness to activate the MAPK pathway[55]. In the cytoskeleton, increases in mechanical stress can cause the transformation of G-actin, which is typically present in low tension environments, to F-actin[56]. This releases myocardin-related transcription factors (MRTF) and allows them to enter the nucleus, where it can alter gene expression. Linker of the nucleoskeleton and cytoskeleton (LINC) is primarily responsible for the direct nucleus responses to

mechanotransduction by connecting the cytoskeleton to the nucleus and controlling chromatin packaging[55].

Understanding a cardiac fibroblast's response to its mechanical environment is fundamental. The majority of *in vitro* research investigating cardiac fibroblasts is conducted on tissue culture plastic (TCP), which has a stiffness magnitudes higher than *in vivo* stiffness, even a fibrotic one. The acknowledgment of mechanical stimulation as an essential consideration in cardiac fibroblast research is becoming more accepted, and softer polydimethylsiloxane (PDMS) and polyacrylamide (PA) substrates are becoming more widely used. However, the default is often still TCP which can make translating more complex signaling mechanisms of cardiac fibroblast signaling to an *in vivo* context difficult.

2.3.3 Estrogen signaling

One such complexity is estrogen signaling within cardiac fibroblasts. I mentioned briefly earlier (section *2.2.5 Experimental models to study sex-specific effects*) that most estrogen signaling studies within a cardiac fibroblast context were conducted with neonatal cells. However, this pools male and female cells together, so a sex-disaggregated analysis is impossible. Compounding this, nearly all of the few studies that conducted a sex-disaggregated analysis are done on TCP, so it is difficult to fully ascertain how male and female cardiac fibroblasts are inherently different under physiological conditions[23].

Like other biomolecules, estrogen is integrated into the cell via receptors located on the plasma membrane. Male and female cardiac fibroblasts are known to have different levels of the three primary estrogen receptors: estrogen receptor alpha (ER-α), estrogen receptor beta (ER-β), and G-protein coupled estrogen receptor (GPR30)[57,58]. This translates to divergences in intracellular signaling pathways that impact the progression of fibrosis in a sex-specific manner. Westphal et al. found that treating with an ER-α agonist reduced

signs of fibrosis in mice who had undergone transverse aortic contraction in female OVX mice[59]. Additionally, it was found that ER-α downregulates collagen I and III in female rat cardiac fibroblasts upon estrogen treatment, while ER-β can upregulate collagen I and III in male rat cardiac fibroblasts upon estrogen treatment[45].

Several studies have begun to elucidate the mechanism of the pathways downstream of these estrogen receptors in cardiac fibroblasts. For example, Pedram et al. showed that estrogen treatment competitively inhibited angiotensin II and endothelin 1 profibrotic activity by blocking TGFB induced SMAD activation downstream of ER-β[31]. GPR30 was found to attenuate cardiac fibroblasts proliferation via inhibiting Cyclin Beta 1 and CDK1 pathways[60].

Medzikovic et al. reviewed the current understanding of estrogen signaling within cardiac fibroblasts (see Figure 2.1)[23]. Although this review includes many downstream pathways and some sex-disaggregated results, no biomechanical cues are included in the schematic. Studies investigating estrogen's involvement in biomechanical pathways have, to our knowledge, only been published in regards to osteoporosis or other bone contexts[61–63]. Future studies must consider how biomechanical cues may affect estrogen signaling pathways within cardiac fibroblasts to fully leverage estrogen's cardioprotective abilities while mitigating its harmful effects.

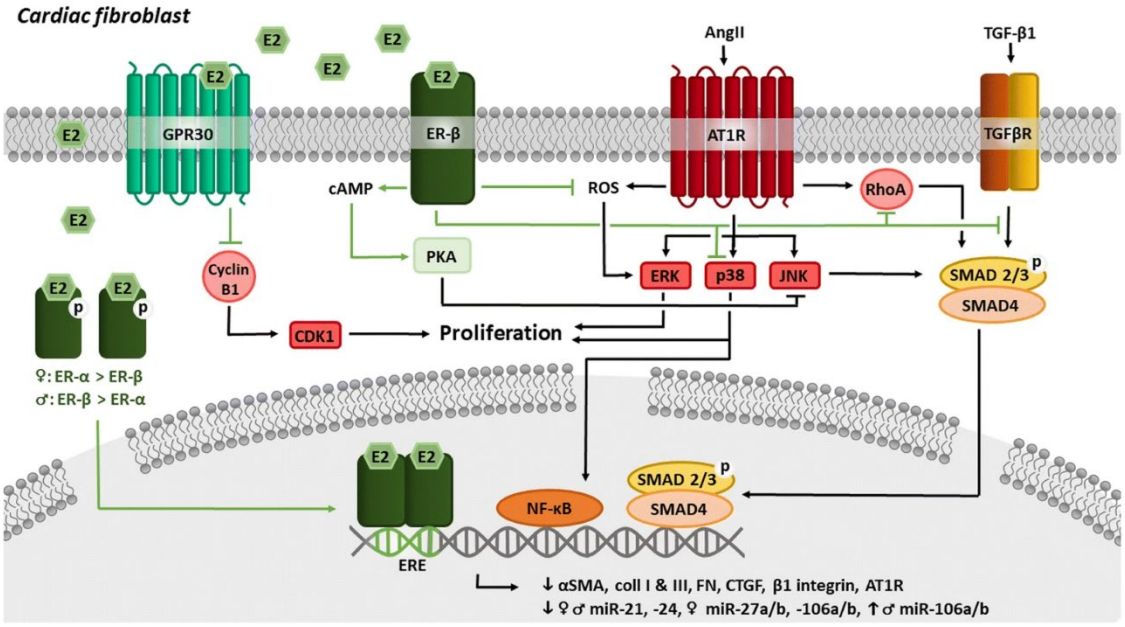

Figure 2.1 Current understanding of downstream estrogen signaling within cardiac fibroblasts.

2.4 Systems Modeling

2.4.1 Systems modeling of cardiac fibroblasts

One way to facilitate the understanding of complex signaling pathways is using computational models. *In silico* experiments are generally cheaper and faster than trial and error wet-lab experiments alone. In particular, signaling network models can be a handy tool for understanding complex systems-level biology. Such models have been around for decades and have provided essential insights further validated *in vitro*/*in vivo*[64,65]. Recently, computational models have been applied to identify potential new drug targets and diagnostic tools.

A computational signaling network model of cardiac fibroblasts was developed and published previously by Ziegler and Richardson et al. and included 10 signaling pathways comprised of 91 nodes and 134 reactions[66]. This model was validated and predicted to match the input/outputs of 80% of 82 independent published experiments. The model functions via a series of logic-based ODE representing different activation and intracellular

inhibitory reactions through AND and OR gates. The first iteration of this model only included one mechanical input and did not include mechanotransduction reactions. Our lab (Rogers et al.) updated the model to include mechanosensitive pathways (109 nodes and 174 reactions)[54,67]. One major limitation of this updated model is that it is not sex-specific and consists of no gonadal hormones, both of which are essential considerations in the disease progression of cardiac fibrosis (see section *2.3.3 Estrogen signaling*).

2.4.2 Sex-specific systems modeling

Sex-specific computational models have been created in a variety of disease contexts. Ahmeed et al. created a sex-specific model of blood pressure regulation[68]. Chen et al. developed a sex-specific model of renal activity to study nitric oxide bioavailability[69]. However, to our knowledge, no sex-specific disease models have been created to investigate cardiac fibrosis. There has been a push to make patient-specific models through precision medicine techniques. Our lab's cardiac fibroblast model has been used to make personalized patient predictions after integrating transcriptomic data from patient sera[70]. However, a concern with this technique is that the data used to create and validate these seemingly gender-neutral patient-specific models is often skewed toward male data. As discussed previously (section *2.2.4 Sex-disaggregated data reporting*), female data have been historically lacking in clinical and experimental research. The papers used to validate the current cardiac fibroblast model were 30% male and 0% female (70% were pooled neonates or unreported). These models will likely be more accurate for male patients than female patients unless female data is intentionally incorporated to train and validate the models.

2.4.3 Systems modeling for drug discovery

Many signaling network models are designed to aid in drug discovery, making it imperative that female data be considered during their design and validation. Currently,

adverse drug responses (ADRs) occur at double the rate in females compared to males, most likely due to their underrepresentation in clinical trials[43]. In a review of 9 significant heart failure drug trials, women made up only 24% of the patients enrolled in the trials[71]. Interestingly, literature has suggested that many ADRs occur in a sex-specific manner. In a study that investigated the pharmacokinetics (PK) of 86 FDA-approved drugs, it was found that PK values were correlated to sex, and 96% of drugs with female-biased PKs were associated with higher risks of ADRs[72]. These ADRs typically occur because of differences in drug absorption, metabolism, distribution, and elimination between males and females. For example, Warfarin protein binding is known to be influenced by estrogen and testosterone[71]. Despite higher plasma levels in females, beta-blockers were found to be less effective at lowering heart rate in females compared to males[71].

Unfortunately, many drug trials do not report sex-disaggregated data on ADRs, so an understanding of the sex-specific risks is only beginning to be understood. A recent study that investigated the sex-specific ADR reporting on common HF medications (e.g., angiotensin-converting enzyme inhibitors, β-blockers, angiotensin II receptor blockers, mineralocorticoid receptor antagonists, ivabradine, and digoxin), of the 155 records investigated only 7% reported ADR separately for males and females[43]. Much of this data will be very difficult to reproduce retroactively without additional clinical trials. However, sex-specific drug screens using *in silico* models could hasten this process by identifying drugs on the market that are most influenced by biological sex.

A collaborator recently conducted a large-scale drug screen with our cardiac fibroblast model[73]. This drug screen resulted in a few recommendations (i.e., Galunisertib) of potential drug targets for developing a therapeutic for cardiac fibrosis. However, this screen was not sex-specific. There are currently no sex-specific treatment recommendations

for the management or treatment of cardiac fibrosis. However, as disease presentation and progression of cardiac fibrosis can occur in a sex-specific manner, it is an essential consideration for future research.

2.5 Gender Gap in Education

Evidence has shown that papers are more likely to report sex-specific findings if the first or last author is female[74]. However, many science, technology, engineering, and math (STEM) fields remain predominately male-dominated. Women make up only about 15% of the engineering workforce[75]. And although this trend is less severe for life science related fields such as biomedical engineering, which is more female-dominated (47% of the workforce) than many other engineering disciplines, this is not the case for computational areas[76]. Women make up only 20% computational biology workforce[77]. Because of these trends, design teams are often heavily male-dominated, which can unintentionally lead to health disparities. For example, crash test and CPR dummies are typically a male build[78,79]. These disparities are further exacerbated when considering race and ethnicity. One way to mitigate these biases and make healthcare more equitable is to have diverse representative design teams.

Although many systemic issues can contribute to this gender/racial gap in the workforce, one contributor is the lack of women and other historically marginalized individuals choosing to major and graduate with a STEM degree. Although women outnumber men on the many college campuses, they make up only 36% of graduates with a STEM degree. Literature suggests that this is partly due to developing and maintaining a STEM identity[80].

STEM identity is one's ability to acknowledge their knowledge and contribution to STEM and has a sense of belonging in the field[81]. It can be influenced by many things such

as educational experiences, representative role models, parental and societal expectations, and STEM self-efficacy[82,83]. Self-efficacy is a well-established term used in education and psychology literature to describe how well one believes one can complete a certain task[84]. It can be influenced by experiences in the classroom, especially negative ones, as historically marginalized communities are typically more sensitive to failures. One way to mitigate the gender and racial gap in STEM is to recruit and retain more females and other marginalized individuals by creating more inclusive classroom environments.

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CHAPTER THREE

AIM ONE: TEST THE EFFECTS OF SEX AND 17 B-ESTRADIOL ON CARDIAC FIBROBLAST MORPHOLOGY AND SIGNALING ACTIVITIES IN VITRO

3.1 Introduction

The prevalence of heart failure (HF) continues to rise, currently afflicting over 6.2 million Americans in roughly equal proportions among men and women[1,2]. What is not equal is the diagnosis, prognosis, treatment, and overall understanding of HF on the basis of biological sex[2–5]. Female data are underrepresented in animal studies and clinical trials, so recommended treatment is not sex-specific, and adverse drug reactions occur at double the rate in females than in males[4]. Notably, premenopausal females have a relative protection against HF compared to age-matched males which subsides after menopause[2,6]. This phenomenon has been studied extensively and is largely thought to be because of the ovarian hormone estrogen[3,4,7]. Hormone replacement therapy (HRT) to maintain estrogen levels in postmenopausal females was even considered cardioprotective for several decades[8,9]. However, following randomized clinical studies, HRT was shown to have overall adverse trends, increasing the risk of stroke, breast cancer, and heart attack in postmenopausal females, and is not recommended for long-term use or as a preventive measure for cardiovascular diseases[6,8].

Although complete HRT is not a viable option to treat or prevent cardiac pathologies, 17-β estradiol (E2) has exhibited promise in reducing cardiac fibrosis - an accumulation of collagens and other extracellular matrix components that reduces pump and electrical function[7,10–12]. After an initial myocardial infarction, a fibrotic response is necessary to maintain structural stability but can continue uncontrolled resulting in chronic HF[13]. There are currently no FDA-approved therapeutics to specifically target and control

cardiac fibrosis[13]. In many *in vitro* studies, E2 treatment has been linked to a decreased fibrotic response of cardiac fibroblasts (CFs), indicating its potential as a therapeutic[10–12,14–16]. It is important to note, many of these studies were done with neonatal rat CFs, pooling male and female cells together, so sex-specific effects of estrogen treatment were, for the most part, not investigated. Understanding how estrogen interacts with male and female cells at the molecular level is imperative in order to leverage estrogen's therapeutic effects while minimizing potential adverse responses.

The few studies that do use sex-disaggregated analysis at the cellular level, nearly all used tissue culture plastic (TCP) as the experimental platform. TCP has a stiffness that is magnitudes higher than physiologic conditions, even a fibrotic environment. CFs are extremely sensitive to their microenvironment, and when cultured on stiff substrates, many proteins become activated due to mechanotransduction pathways that can make cells profibrotic[17–19]. Additionally, a recent *in vivo* study by the Pinto group demonstrated the sex dimorphic response of the regulation of several genes within CFs due to angiotensin II stimulation[20]. Furthering our understanding of the intrinsic differences between male and female CFs under physiologically "healthy" conditions is a necessary first step to understanding the divergence of their intricate signaling pathways related to fibrosis. A substrate stiffness of 8kPa was chosen for experiments because it is comparable to the stiffness of healthy myocardium and has been used to mimic a non-fibrotic environment in several other studies[21–25].

Expanding our knowledge of how estrogen interacts with both male and female CFs could aid in the discovery of novel treatment options for cardiac fibrosis that leverage estrogen's cardioprotective properties while mitigating its harmful effects. In this study, we used cyclic immunofluorescence to investigate potential morphological changes, cellular

localization, and activity levels of 12 proteins known to be heavily involved in estrogen and/or profibrotic signaling within CFs. This allowed for a sex-disaggregated analysis of not only each individual protein's response to estrogen but also single-cell cross-correlation analysis, which could uncover protein to protein crosstalk that could be potential sites to target for regulation of cardiac fibrosis.

3.2. Materials and Methods

3.2.1 Cell Isolation and Culture

Adult Sprague Dawley rats (n=8 male: 8 wks, 265 grams; and n=8 female: 12 wks, 255 grams) were euthanized, and hearts were removed and collected in Krebs-Henseleit buffer (Sigma, St. Louis, MO). All procedures were performed with approval from Clemson University's Institutional Animal Care and Use Committee. Ventricles were minced and digested to isolate CFs according to previously reported protocols[25,26]. Liberase TM (Roche, Indianapolis, IN) was used in each of the six successive enzymatic digestions at 37 C. Supernatants from each digestion were collected and centrifuged at 300 g and 4 C and resuspended in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) containing 10% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA), 100 U/mL penicillin G, 100 µg/mL streptomycin, and 1 ng/mL amphotericin B (all Sigma). Following isolation, cells were plated in T-25 culture flasks and incubated at 37 C and 5% CO2 for 4 h, after which media was changed and thereafter was changed every 72 h until the serum starvation.

3.2.2 Collagen Coated Culture Plates

Prior to cell plating, 8 kPa 24-well CytoSoft® plates (Advanced BioMatrix, San Diego, CA) were coated with Telocol-3 bovine collagen (Advanced BioMatrix). Collagen solution was made at a 1:30 ratio of Telocol-3 in Phosphate Buffered Saline (PBS, Sigma). 1 mL of solution was pipetted into each of the 24 wells and allowed to polymerize at room

temperature for 1 h. Excess solution was removed, and the wells were washed with PBS twice.

3.2.3 Estrogen Treatment

Male and female CFs were passaged one time (P1) with 0.25% trypsin (Fisher) at a 1:3 dilution before use in experiments. Once the CFs had reached ~75% confluence after the first passage, DMEM containing 10% FBS was removed, and flasks were washed with PBS. A 24 h serum starvation was started with phenol-free DMEM (Fisher) + 2 mM L-glutamine (Fisher) and 2.5% charcoal-stripped FBS (GE Health, Chicago, IL) incubated at 37 C and 5% CO2. After 24 h, CFs were passaged (P2) and plated onto the CytoSoft® plates at 10,000 cells/well. CFs were divided into 4 experimental groups across two conditions: male vs. female and with or without 17-β Estradiol (E2, Sigma). The 24-well plates allowed for two biological replicates with three technical replicates (wells) per experimental condition. All wells were filled with 1 mL of phenol-free DMEM + 2 mM L-glutamine and 10% charcoal-stripped FBS. E2 was dissolved in ethanol at 10 mM, and 10 nM of E2 was added to wells designated for E2 treatment. An ethanol vehicle control of 10 nM was used as a control for all non E2 treated wells. Plates were incubated at 37 C and 5% CO2 for 24 h. Following incubation, all wells were fixed with 4% paraformaldehyde (PFA, Sigma) for 30 mins and 99.9% methanol (Fisher) for 10 mins. Immediately after fixation, plates were filled with PBS, wrapped in parafilm, and stored at 4 C until use in cyclic immunofluorescence (CycIF).

3.2.4 Cyclic Immunofluorescence

Wells were washed with Odyssey blocking buffer (Fisher) for 1 h at room temperature on a rocker prior to antibody staining. Antibodies were purchased for the following proteins of interest: alpha-smooth muscle actin (α-SMA), filamentous actin (F-

Actin), mothers against decapentaplegic homolog 3 (SMAD3), myocardin-related transcription factor (MRTF), nuclear factor of activated T Cells (NFAT), nuclear factor kappa-light-chain-enhancer of activated B Cells (NF-kB), phosphorylated extracellular signal-regulated kinase (p-ERK), phosphorylated focal adhesion kinase (p-FAK), phosphorylated jun n-terminal kinase (p-JNK), phosphorylated protein kinase B (p-Akt), phosphorylated p38 mitogen-activated protein kinase (p-p38), and rho-associated protein kinase (ROCK). Each antibody was individually optimized to determine unique staining dilutions and microscope gain, exposure, and light settings. Appendix A Table 1 outlines where each antibody was purchased, Alexa Fluor conjugation, staining dilution, and microscope settings for each protein of interest. The order of CycIF and protocol were determined according to published recommendations[27].

Four consecutive rounds of CycIF were conducted with three proteins of interest each round: (1) p-p38, NFAT, SMAD3, (2) MRTF, ROCK1, NF-kB, (3) p-JNK, p-Akt, α-SMA, and (4) F-Actin, p-ERK, p-FAK (Figure 3.1). Primary and Alexa Fluor conjugated antibodies were applied and rocked overnight at 4 C. A secondary mouse-anti-rabbit IgG PE-Cy7 antibody for SMAD3, NF-kB, α-SMAD, and p-FAK was applied for 1 h at room temperature while rocking. A Hoechst nuclear stain was rocked for ten minutes at room temperature for each Cyc-IF round. All wells were washed four times with PBS between staining and imaging. Alexa Fluor light cubes GFP, TxRed, Cy7, and DAPI, were used for rounds 1 and 4 of CycIF; RFP, Cy-5, Cy-7, and DAPI light cubes were used for rounds 2 and 3. The ThermoFisher Fluorescence Spectra Viewer was used to ensure minimal spectra overlap between channels[28]. An EVOS fluorescent microscope at 10x objective was used to take ten images per well, and beacons were saved to return to that position in consecutive CycIF rounds. Following each round of imaging, fluorophore inactivation was achieved by

treating with 4.5% H_2O_2 (Fisher) in PBS plus 25 mM NaOH (Sigma) for 2 h under an LED light. Inactivation was confirmed visually with the EVOS before moving on to the next round of CycIF. Wells were washed with PBS four times after destaining and before the next round of CycIF. All images were saved as 8-bit TIFF files, which were imported into CellProfiler™ for post-image processing[29].

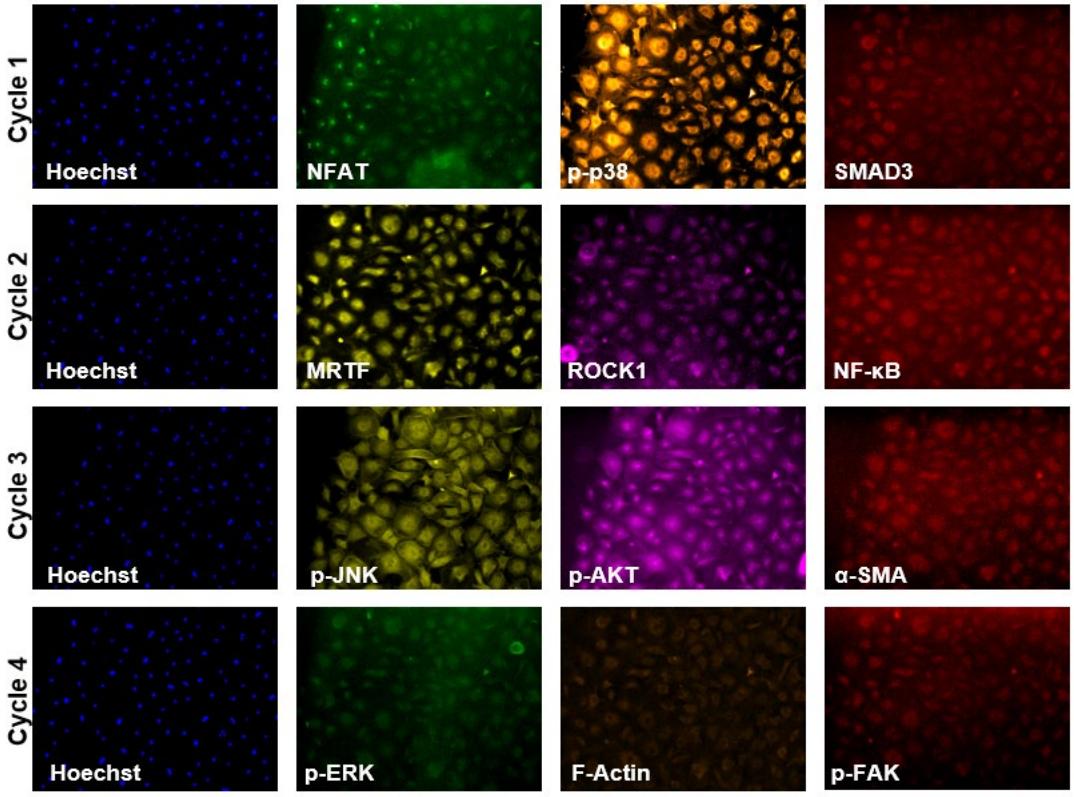

Figure 3.1. An example of a set of images for each of the proteins of interest for four rounds of CycIF. GFP, TxRed, and Cy7 (green, orange, and red) light cubes were used in rounds 1 and 4. RFP, Cy5, and Cy7 (yellow, pink, and red) light cubes were used in rounds 2 and 3. A Hoechst nuclear stain and DAPI light cube (blue) were used for all rounds.

3.2.5 Post-Image Processing

In CellProfiler™, the lower quartile intensity background was subtracted from each image. Images from consecutive rounds of CycIF were aligned with each other to account for small changes in the field of view that occurred over multiple rounds. The Hoechst nuclear stain images were used to identify Primary Objects (the nuclei), which were then used to identify Secondary Objects (cellular outlines) (Figure 3.2.a). Morphological

measurements of the total cell area, nucleus area and location, and minor and major axis lengths were measured for each cell. To account for errors in automated cell identification, an upper bound of 10000 μm^2 and a lower bound of 1000 μm^2 was set for acceptable cell areas. Integrated, mean, and median intensities were also recorded for each image channel (protein).

3.2.6 Statistical Analysis

Nearly 20,000 cells (~5,000/experimental condition) were identified across the images taken from the 8 male and female biological replicates and used to analyze morphological and protein-level data. Cell density was calculated per image across experimental conditions, and there was no significant difference in cell viability in regards to sex or estrogen treatment ($p>0.05$, 3.2.b). The median cell/nucleus area and elongation for each biological replicate were determined per experimental condition. To account for variability in fluorescent intensity among biological and technical replicates, normalization was conducted by dividing the channel (protein) intensity in each cell by the median of that channel intensity from all the cells on the entire plate (1 plate = 2 male and 2 female biological replicates). This allowed for comparison of relative protein levels across experimental conditions. MATLAB's anova2 function was used to run a two-way ANOVA to determine if a statistically significant ($\alpha=0.05$) difference existed between or within groups of sex (male vs. female) and estrogen treatment (baseline and +E2). Box and whisker plots which show the median, 25^{th}, and 75^{th} percentiles of the 8 biological replicates per experimental condition were generated. When there were instances of statistical significance it is denoted on the graph, and all p-values are reported in Appendix A Table 2. Single-cell correlation coefficients for each protein-protein and protein-morphology interaction were also calculated using MATLAB's built-in corrcoef function.

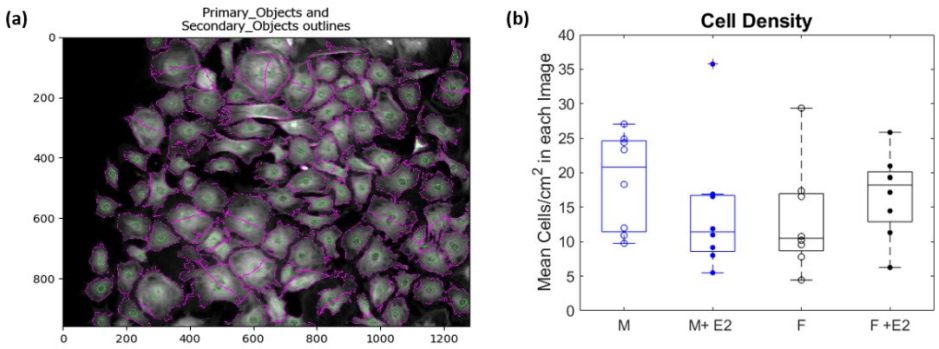

Figure 3.2. Sample CellProfiler™ outlines of nuclei (green) and cells (purple) (a). A two-way ANOVA was used to determine if there was any significant interaction (α=0.05) between sex (male=blue & female=black) and estrogen treatment (baseline=open dots to represent median of biological replicates and +E2=closed dots) on cell viability. No significant difference existed within groups or interaction between groups for the mean cells/cm^2 for each image (b).

3.3 Results

3.3.1 Sex-disaggregated analysis of CF morphology

Microscopic image analysis demonstrated no change in cell area across experimental conditions ($p>0.05$, Figure 3.3.a). Likewise, cell elongation ($p>0.05$, Figure 3.3.b), which was calculated by determining each cell's aspect ratio (major/minor axis), was also not affected by sex or estrogen treatment. Nuclear area and aspect ratio were observed and determined not to be dependent on sex or estrogen treatment ($p>0.05$, Figure 3.3.c-d). F-Actin and α-SMA's relative protein concentrations ($p>0.05$, Figure 3.3.e-f) did not vary among experimental conditions, indicating that under physiological conditions, the structure and morphological presentation of male and female CFs do not differ significantly.

3.3.2 Relative levels of fibrotic related signaling proteins

Relative protein levels were determined by comparing normalized median cell intensities for each protein of interest. p-ERK had a statistically significant interaction between sex and E2 treatment, with the female baseline being higher than all other experimental conditions ($p<0.05$, Figure 3.4.a). p-p38 and ROCK1 were found to be statistically different due to sex, with male cells having higher levels of both p-p38 and

ROCK1 in the baseline and E2 treated cells compared to female cells with or without E2 ($p<0.05$, Figure 3.4b-c). p-FAK showed a statistically significant downregulation of the relative levels of p-FAK in both male and female cells when E2 was present ($p<0.05$, Figure 2.4.d). No statistically significant change existed across experimental conditions for the relative protein levels of p-JNK and p-Akt ($p>0.05$, Figure 3.4e-h).

3.3.3 Nuclear localization of mechanosensitive proteins

Many profibrotic proteins in CFs are in their most activated form when they have translocated to the nucleus, which allows them to act as transcription factors to influence gene regulation. In our study, MRTF, NFAT, NF-κB, and SMAD3 are all most activated in the nucleus. Therefore, instead of measuring their total cell intensity, we calculated the ratio of the intensity within the nucleus vs. the cytoplasm (normalized mean nuclear intensity/ normalized mean cytoplasm intensity). While MRTF, NFAT, NF-κB, and SMAD3 all had ratios greater than 1 for each experimental condition indicating that more was present in the nucleus than in the cytoplasm, only the levels of NFAT were different across experimental groups ($p>0.05$, Figure 3.5.a-c). Male cells had NFAT levels in the nucleus that were higher than both the baseline and E2 treated female cells ($p<0.05$, Figure 3.5.d).

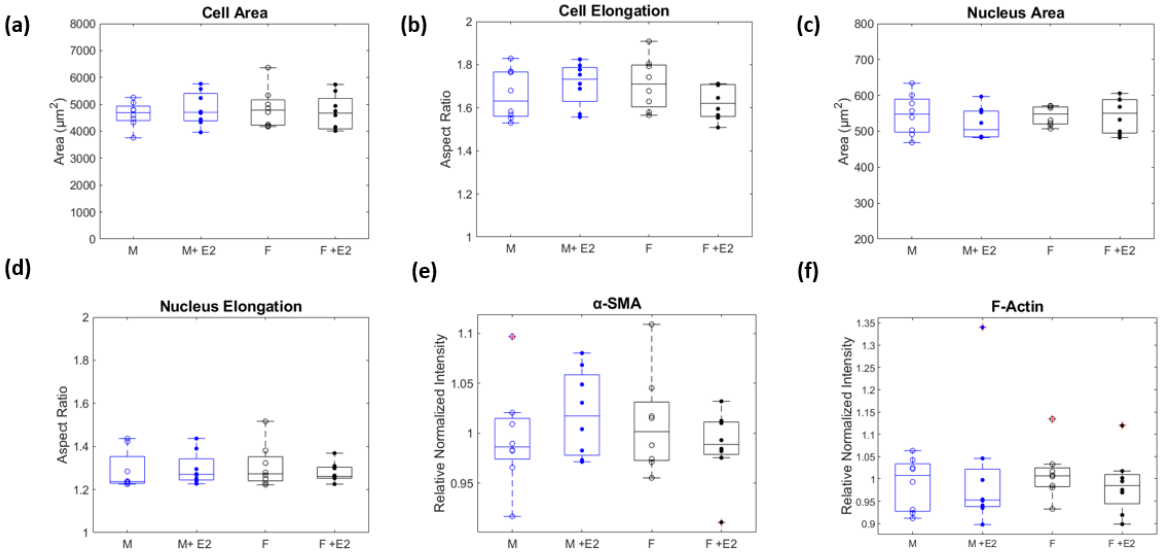

Figure 3.3. A two-way ANOVA was used to determine if there was any significant interaction (α=0.05) between sex and estrogen on morphological factors. No significant difference existed within groups or interaction between groups for total cell area and elongation (a and b), nor nucleus area and elongation (c and d). F-Actin and α-SMA relative protein levels did not significantly change between experimental conditions (e and f).

3.3.4 Correlation analysis of protein-protein interactions

An advantage of cyclic-IF analysis for protein quantification is that it enables single-cell measurements, which can be tested for protein-protein and protein-morphology relationships. The Pearson's correlation coefficients of the normalized relative protein-protein levels and protein-morphology interactions were calculated along with their corresponding p-values. These data were used to create dot plots (Figure 3.6), which allow for the comparison of changes in protein-protein/protein-morphology interactions between experimental conditions. The most striking difference is that there was a much stronger correlation of protein-protein interactions for female CFs treated with estrogen (indicated by large orange and yellow dots) than for male CFs treated with estrogen. Similarly, male CFs without E2 demonstrated several strong and significant correlations, which were dampened in the presence of E2. Female CFs experienced similarly correlated relationships with and without E2 treatment.

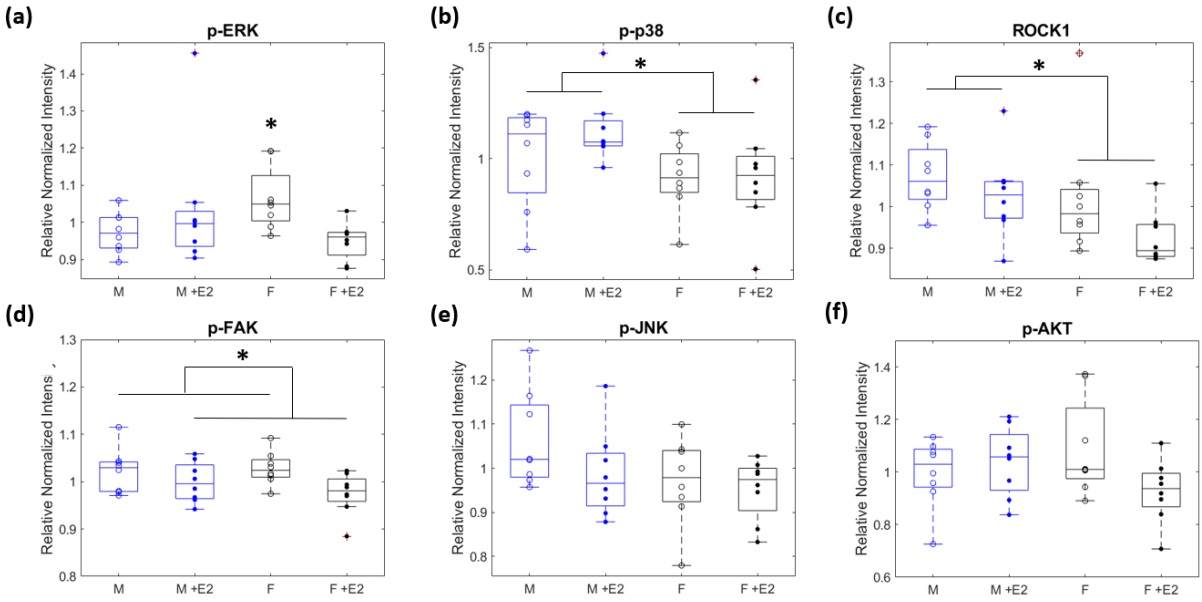

Figure 3.4. A two-way ANOVA was used to determine if any significant interaction (α=0.05) existed between sex and estrogen treatment on the normalized median intensity of profibrotic proteins. p-ERK showed a significant interaction between sex and E2 treatment ($p<0.05$, a). The median intensity of ROCK1 and p-p38 was significantly different due to biological sex ($p<0.05$, b and c). E2 treatment caused a significant difference in median intensity for p-FAK ($p<0.05$, d). No significant interactions between or within groups existed for p-JNK or p-AKT ($p>0.05$, e and f).

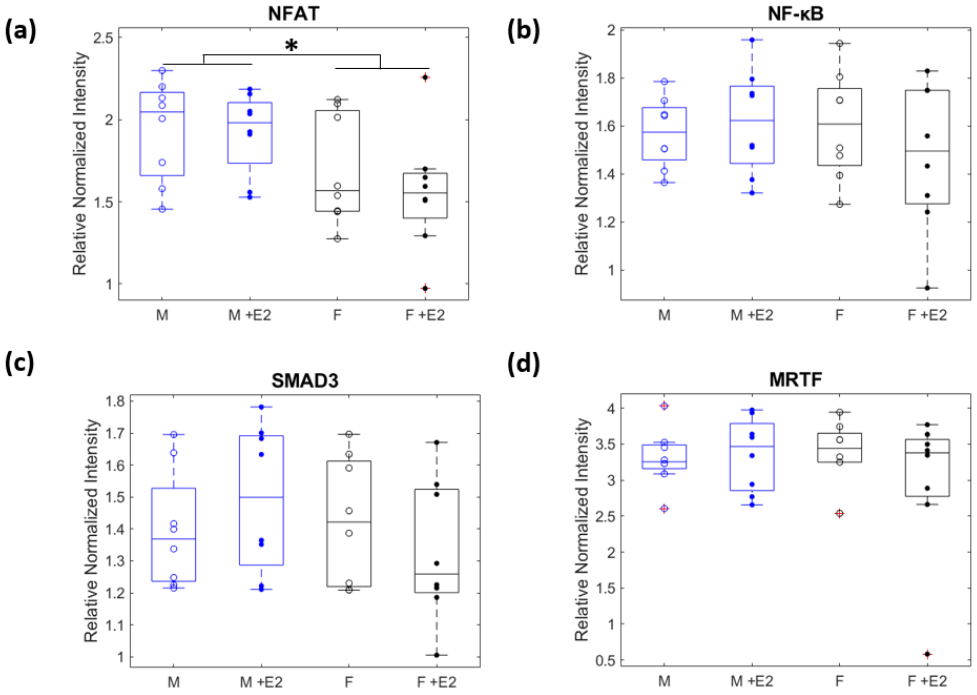

Figure 3.5. A two-way ANOVA was used to determine if any significant interactions existed between sex and estrogen treatment of translocation of profibrotic proteins to the nucleus. The median nucleus: cytoplasm ratio of NFAT was significantly different due to biological sex ($p<0.05$, a). There were no significant interactions between or within groups for NF-κB, SMAD3, or MRTF ($p>0.05$, b-d).

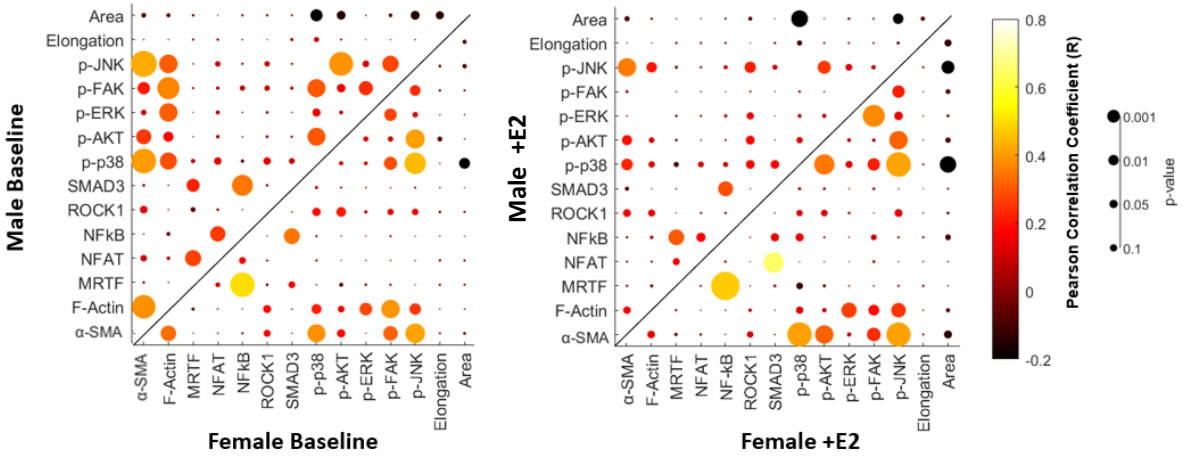

Figure 3.6. Dot plots of correlation coefficients and their corresponding p-values for analysis of protein-protein and protein-morphology interactions.

3.4. Discussion

Although many studies note the phenotypic differences between male and female cardiac fibroblasts, very few have investigated if these phenotypic changes result in

observable morphological differences in cell size and elongation. At a macro level, male and female morphology are dimorphic, with male hearts and their components, including the left ventricle, often being larger than female hearts from the same species[30,31]. As fibrosis progresses, CFs undergo morphological changes, elongating and covering a larger area due to interactions with their changing microenvironment[24]. This can also cause nuclear morphologic changes mediated by LINC[19]. To fully understand the differences in how male and female CFs interact with and respond to changes in their mechanochemical environment during fibrosis progression, it is necessary to know if any morphological differences are present in physiologically "healthy" environments. Our results indicate that on a stiffness that mimics physiological conditions, there are no changes in cell and nuclear morphology due to sex and estrogen treatment. This indicates that while male and female cells may be phenotypically different at an intracellular level, these changes are more likely to present biochemically rather than structurally. Our finding of no significant difference in α-SMA and F-Actin relative protein levels due to sex and estrogen treatment also supports this theory, as elevated α-SMA and F-Actin levels are both indicative of increased cell contractility which can cause changes in cell morphology[32].

Of the 12 proteins investigated, NFAT, p-p38, and ROCK1 were found to be more elevated in male cells than female cells regardless of E2 treatment. Each of these proteins is typically more elevated in a profibrotic environment[19]. This indicates that even in a physiologically "healthy" environment, male CFs may be more sensitive to chemical changes and prone to fibrotic behavior than female CFs. Sex disaggregated literature of the behavior of these proteins in relation to fibrosis in CFs is extremely sparse. One *in vivo* mouse study found that female mice underwent p-38- induced ventricular hypertrophy and mortality at a slower rate than male mice[33]. Future research should investigate potential

intrinsic differences of NFAT, p-p38, ROCK1, and other downstream proteins in male and female CFs to clarify the divergence of male and female signaling pathways. This could support development of sex-specific prevention and treatment methods for cardiac fibrosis. CFs are very susceptible to changes in their microenvironment. A major way they sense and translate these signals within the cell is through integrins and adhesion receptors on the cell membrane. One highly studied CF adhesion receptor is focal adhesion kinase (FAK), which can be activated (p-FAK) by interactions with the extracellular matrix[24]. In numerous studies, FAK inhibition has been shown to stop adverse cardiac remodeling [34,35]. Our results showed that upon treatment with E2, both male and female CFs had reduced expression of p-FAK, indicating its promise as a potential regulation pathway that mimics estrogen's cardioprotective effect. To our knowledge, no other studies investigate the effect of estrogen on FAK in cardiac fibroblasts. However, there are a few studies that demonstrate how E2 treatment can actually activate FAK in breast cancer cells[36,37]. The microenvironment of a breast cancer tumor is likely much stiffer than the 8kPa physiological like stiffness used in our study, so it is possible that there is a complex interaction of mechanical cues and hormone signaling, which affect FAK activation. FAK has many proteins downstream of it, which are also considered profibrotic factors, so FAK's pathways are a promising source of potential regulation if more research is conducted to understand its response to combined estrogen treatment and mechanical stimulus.

Not all of our proteins of interest had statistically significant differences between experimental conditions (SMAD3, NF-κB, p-JNK, and p-Akt). This finding was slightly surprising in regards to SMAD3 and p-JNK, because of past literature that cites the ability of estrogen to downregulate SMAD3 and p-JNK activity in CFs[7]. These contradictory findings are not unusual - a recent review of the limited research of sex differences and

estrogen signaling in CFs notes additional discrepancies among various other peer-reviewed studies [7]. There are many differences in experimental setup, including *in vivo* vs. *in vitro* design, pooled male and female cells vs. sex-disaggregated analysis, and neonatal vs. adult cells. Our study adds an additional variable, substrate stiffness. Nearly all previous *in vitro* studies of sex or estrogen signaling in CFs were done on TCP, which has an unrealistically high stiffness (>1000 fold stiffer than myocardium). It is imperative to conduct further sex/E2 focused studies within CFs controlling for individual variables before it is possible to synthesize the results from multiple studies into a broader understanding of sex-specific and estrogen-induced signaling in CFs.

In our study, the only protein of interest that had a statistically significant interaction between sex and E2 treatment was p-ERK. Baseline levels of p-ERK in female CFs were higher than in any other experimental condition; however, upon E2 treatment, female CFs had levels similar to male CFs. There was a negligible difference between male baseline and male +E2 relative protein levels of p-ERK. We hypothesize that this difference among experimental conditions may related be to β-Adrenergic receptors (β-ARs), which are believed to increase fibrotic activity through ERK(1/2) related pathways[38]. Many studies have observed crosstalk in β-ARs and estrogen signaling[39]. Additionally, a recent study outlined the sex dimorphic response in CFs due to β-AR stimulation[40]. As β-blockers are already an FDA-approved treatment for many cardiovascular pathologies, including high blood pressure and heart failure, this connection offers a promising avenue of potential regulation of uncontrolled fibrosis that warrants further investigation.

Limitations of our study include that it was simply an *in vitro* monolayer culture analysis with a serum starvation used to induce the baseline lack of estrogen condition. In the future, enhanced *in vitro* platforms that utilize (1) a coculture of the multiple cell types

present in the myocardium, (2) a 3D cell culture platform such as hydrogels, (3) and applying cyclic stretch could all be used to better mimic a healthy cardiac environment[25,41–46]. We also want to acknowledge that α-SMA expression remained elevated in our cells during the short experimental period. This is likely due to mechanical memory wherein cells become activated during initial plating on TCP and then maintain some of that activity even after reseeding onto softer substrates[47]. Future studies will either be conducted for a longer time course or cultured on softer substrates immediately after cell isolation. Additionally, an *in vivo* study with OVX mice could be used to truly mimic the changes in estrogen levels due to menopause and other differences that are difficult to capture with an *in vitro* platform. We also chose to use immunofluorescence to capture any potential morphological and nuclear translocation of profibrotic factors intrinsic to male and female CFs with and without estrogen treatment. Our analysis indicated that no significant structural differences existed between male and female CFs on a physiologically similar stiffness of 8kPa, and only NFAT expressed different levels of translocation to the nucleus among experimental conditions. In future research, we would recommend that analysis could be done with methods that could allow for a more robust signaling analysis, such as flow cytometry, western blotting, or RNA-seq.

A more robust data set would provide the opportunity to conduct *in silico* experiments, which could further elucidate our understanding of the complex signaling networks of CFs. Our study was primarily focused on how mechanically activated signaling pathways in CFs are impacted by estrogen and biological sex. There are other profibrotic and proinflammatory pathways (i.e., DAMPs) in CFs independent of mechanical stimulation that may be affected by biological sex and/or estrogen, which warrant study[48]. In addition, the downstream response to estrogen stimulation can be affected by the

presence of estrogen receptors (ER-α, ER-β, and GPR30)[49,50]. Future studies should utilize a sex-disaggregated analysis to uncover possible differences in estrogen receptor expression and regulation under physiologically "healthy" conditions. A computational approach will facilitate the synthesis of findings from many independent experiments into a network of the complex interactions of cardiac fibroblast signaling.

3.5. Conclusions

Our results support existing literature that cites male and female CFs are sexually dimorphic, even under physiologically "healthy" conditions, and should be treated as such when designing experiments to allow for sex-disaggregated analysis to determine how biological sex may be affecting response to treatment interventions. Future research could be directed toward uncovering the complex signaling interactions related to biological sex, E2, and profibrotic signaling pathways. One way to hasten this investigation could be through the use of sex-specific computational disease models. Existing disease models such as the signaling network model of cardiac fibroblasts' response to mechano-chemo signaling could be improved by incorporating biological sex and hormone pathways[51,52]. Large-scale sex-specific network modeling could greatly accelerate the pace and reduce the costs of identifying important interactions involved in the regulation of fibrosis rather than trial and error experiments alone.

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CHAPTER FOUR

AIM TWO: SCREEN FOR SEX-SPECIFIC DRUG EFFECTS IN A FIBROBLAST NETWORK MODEL INTEGRATED WITH ESTROGEN SIGNALING

4.1 Introduction

Heart failure (HF) currently afflicts roughly 6.2 million Americans and has a five-year survival rate of only 50%[1]. Cardiac fibrosis, the uncontrolled accumulation of ECM proteins, can exacerbate the progression of HF[2–4]. This deposition of ECM proteins is crucial to a patient's initial survival of myocardial infarction (MI) to form the scar tissue that maintains the structural stability of the infarct region[5]. However, when it continues uncontrolled, it becomes pathologic by reducing ejection fraction via thickening of the left ventricle wall and causing a greater chance of arrhythmia due to disruptions in electrical stimulus caused by collagen buildup[6,7].

The current treatment regimen for patients suffering from HF includes drugs such as angiotensin-converting enzyme (ACE) inhibitors and beta-blockers to reduce their blood pressure and slow their heart rate, which reduces stress on the heart in an attempt to slow the progression of HF[8]. However, no current FDA-approved treatments directly target cardiac fibrosis[9]. Ongoing research focuses on developing a drug to directly inhibit cardiac fibrosis or even reverse it[10]. Despite gains in understanding the complex signaling networks of cardiac fibroblasts- the drivers of cardiac fibrosis- like most drug development, it is expensive, slow going, and often found to have low efficacy rates upon clinical trials[11].

The use of the computational models to conduct *in silico* experiments has been used in a variety of ways to accelerate drug discovery[12–15]. Currently, these models are limited in scope and, at best, can narrow down a pool of potential drug candidates for further study. As the field of precision medicine grows, it is likely that the accuracy and predictive power

of these models will also improve. However, in order to fully leverage the predictive power of these models, it is imperative that the data used to create them is robust. Unfortunately, experimental studies and clinical trials are historically skewed to male data. For several decades women of childbearing age were banned from participating in clinical trials[16]. Even after this ban was reversed in the early 90s, it was not until 2016 that the NIH required the use of both male and female animals in preclinical studies[17]. This has resulted in several FDA-approved drugs having double the adverse drug response (ADR) rate in women.

Biological sex is an essential factor when considering cardiovascular health[18–21]. Most notably, premenopausal females are less likely to suffer from MI compared to age-matched men-primarily thought to be due to the cardioprotective role of the ovarian hormone estrogen (E2)[19]. However, these discrepancies in cardiovascular health are not just the result of hormones; there has also been research to show that genetic sexual dimorphisms can also contribute to cardiovascular disease presentation and severity[22]. Additionally, the literature suggests that many ADRs in both males and females occur in a sex-specific manner[23]. Including sex hormones into computational models could help correct for the dearth of sex-disaggregated studies available in the literature and make precision medicine more accurate for patients.

In this study, we aim to make a previously published signaling network model of cardiac fibroblasts sex-specific by incorporating E2. We then conducted a sex disaggregated drug screen to analyze divergences in drug response between males and females.

4.2 Methods

4.2.1 Integration of estrogen into cardiac fibroblasts signaling network

A previously published signaling network model (SNM) of cardiac fibroblast was expanded to include the ovarian hormone estrogen (E2). The previous model was created

via a manual literature search of ~300 papers and included 109 nodes (i.e., proteins, integrins, cellular receptors, and transcription factors) and 174 edges (reactions)[24,25]. Specifically, this model was comprised of 11 biochemical and biomechanical inputs, including transforming growth factor beta (TGFβ), angiotensin II (AngII), endothelin 1 (ET 1), and tension; downstream reactions of these inputs culminated in 22 cellular outputs, including alpha-smooth muscle actin (α-SMA), procollagen I & III (proCI and proCIII), several pro-matrix metalloproteinases and tissue inhibitors of metalloproteinases (proMMPs and TIMPs), and proliferation. New nodes and/or reactions were added to the SNM if two independent studies reported activation of inhibition of another node downstream of estrogen signaling. At least one of these two papers used for model advancement was from experiments conducted with cardiac fibroblasts; the second paper, if not from cardiac fibroblasts, typically reported results from experiments from other cardiac or fibroblast cell types. Nearly all of the papers used for model updates used neonate rat cardiac fibroblasts, pooling male and female cells together, as their cell type.

As previously described, the reactions of the SNM are governed by logic-based ODEs modeled as a system of Hill equations to capture node activity level[24–26]. Logical NOT, AND, and OR gates were used for inhibitory and complex signaling interactions by applying logical operations: $f_{INHIB}(x) = 1 - f(x)$, $f_{and}(x_1, x_2) = f(x_1)f(x_2)$, and $f_{or}(x_1,x_2) = f(x_1)+f(x_2) - f(x_1)f(x_2)$. Differential equations were constructed using the open-source software Netflux (https://github.com/saucermanlab/Netflux) for MATLAB (Mathworks, Natwick, MA)[27]. Cytoscape was used to create all SNM visualizations included in this paper[28]. All additional validation, perturbation, and drug screen simulations were conducted using MATLAB; finalized codes used in the analysis will be made available on SysMechBioLab GitHub (https://github.com/SysMechBioLab).

4.2.2 Sex-Specific Model Creation

Sex-specific models were created by varying the weighting of the three estrogen receptors added to the model to mimic the physiological difference of varied expression and activity levels of estrogen receptors in male and female cells[29]. The reactions of E2 to the three estrogen receptors were weighted as 0.5 (50% of maximum stimulation) and 1 (100% of maximum stimulation in the male and female SNM respectively. Additionally, a pooled SNM with a weighting of 0.75 for E2 stimulation of the three receptors was created to validate studies conducted with pooled male and female neonate cells.

4.2.3 Model Validation

Previous model validation of the cardiac fibroblast SNM conducted with 47 independent papers of direct measurement of model intermediate and output nodes found the SNM to be 81.8% accurate in predicting experimental activity levels of input-outputs (i.e., AngII treatment on proCIoutput) and input-intermediates (i.e., AngII treatment out ERK activity level) found in the literature[24]. In total these 47 papers accounted for 120 perturbations of input-outputs/intermediates validated by comparing literature experimental results to model predictions according to their change in activity level as 'Upregulation' (Δ Activity $\geq$5%), 'Downregulation (Δ Activity $\leq$ -5%), or 'No Change' (-5% < Δ Activity < 5%). Of these 120 perturbation experiments, 29 were counted in pooled neonates, 46 in male cells, and 41 cell sex was unreported; no paper used in prior validation reported results in a sex-disaggregated manner than included female data, and validation was not done in a sex-desegregated manner.

To conduct a sex-desegregated validation of the updated model, 6 new papers were added to the validation set to account for estrogen and female-specific data. Each of the papers used for validation measured direct output secretion or intermediate signaling

response (i.e., ELISA, Western Blot, or PCR) to a single input stimulus in fibroblasts. Validation perturbations were grouped by experiments reporting results in pooled neonates, male, and female cells. Prediction accuracy was calculated using the same Δ Activity bounds described above using either the pooled, male, or female SNM. Model simulation predictions were generated in MATLAB as previously described (simulating basal conditions for 80 hrs, followed by simulating single input stimuli (w=0.9) for 240 hr, tension weight= 0.6)[24]. Ideal EC_{50} and n values were determined for each SNM through multiple perturbations (Appendix C).

As the validation set was comprised of studies conducted on TCP, an additional validation was conducted to compare model predictions to the results of my Aim 1 study conducted on 8 kPa gels with both male and female cells. All parameters were kept consistent with the above validation simulation, except a tension weight of 0.1 was used instead of 0.6 to mimic the lower stiffness.

Additional validation was conducted to determine the accuracy of estrogen involvement in signaling pathways by comparing a combination of simultaneous treatments to experimental results. A tension weight of 0.6 and dosing weights of 0.9 for AngII, ET 1, and E2 were simulated to compare experimental results from Pedram et al. that reported E2 effect on AngII and ET 1 stimulation on α-SMA, fibronectin, proCI, and proCII in pooled neonate cardiac fibroblasts[30]. A negative control simulation of input weights of 0.1 was generated by simulating basal conditions for 80 hrs, followed by simulating the treatment conditions (AngII, ET-1, AngII + E2, ET-1 +E2, and E2) for an additional 240 hrs.

4.2.4 Network Perturbation Analysis

A network perturbation analysis was conducted in MATLAB as previously described to identify influential signaling nodes under different estrogen conditions[24]. The

input reaction weight of estrogen was attenuated, and all other input weights were kept at 0.1 for 80 hrs to simulate basal conditions. A Y_{max}=0.1 knockdown of individual nodes for 240 hrs was used to identify sensitive nodes by calculating Δ Activity for each node as the sum of all knockdown simulation activity subtracted by basal conditions. Three perturbation conditions were conducted: 1) male (male SNM, E2 input w=0.25), 2) female post-menopausal (female SNM, E2 input w=0.25), and 3) female pre-menopausal (female SNM, E2 input w=0.9). Heat maps of the total knockdown effect on the SNM nodes were created, as well as heat maps of the top ten influential and sensitive nods.

4.2.5 Drug Screen

A drug screen was applied to the cardiac fibroblast model as previously described[12]. In this prior study, 121 drugs were identified from DrugBank to connect with nodes in the model, totaling 36 unique drug-target interactions[12,31]. A sex-specific drug screen was conducted using the same three experimental conditions used for the perturbation analysis (male, female post-menopausal, and female pre-menopausal). A static application of drug administration (w=0.85) on individual and paired profibrotic stimuli (w=0.6) for each of the three experimental conditions. The effect of these simulations on proCI, EDFAN, α-SMA, and MMPs was measured.

4.3 Results

4.3.1 Integration of estrogen into cardiac fibroblasts signaling network

We expanded the model to integrate E2 into the previously published model of cardiac fibroblast signaling via a manual literature search of over 20 peer-reviewed manuscripts to add 23 nodes and 29 edges (Figure 4.1). In addition, 6 already present reactions were altered to include direct estrogen receptor inhibition activity. The nodes added include E2 and its three primary receptors: estrogen receptor alpha (ERα), estrogen

receptor beta (ERβ), and g-protein coupled receptor (GPR30)[29,32]. Additionally, cyclin beta 1 (Cyclinβ1) and cyclin-dependent kinase 1 (CDK1) were added downstream of GPR30 as they were not already included in the fibroblast SNM[33]. The total network now consists of 132 nodes and 203 edges. The complete model, including all species (nodes), reactions, and default parameter settings, can be found in Appendix B. In summary default parameter settings included: reaction weights (w) as normalized activity levels between 0-1, Hill coefficient (n)=1.25, EC50=0.6, y_{int}=0, y_{max}=1, and time constant (τ)= 1, 0.1, or 10 for signaling reactions, transcription reactions, and translation reactions respectively.

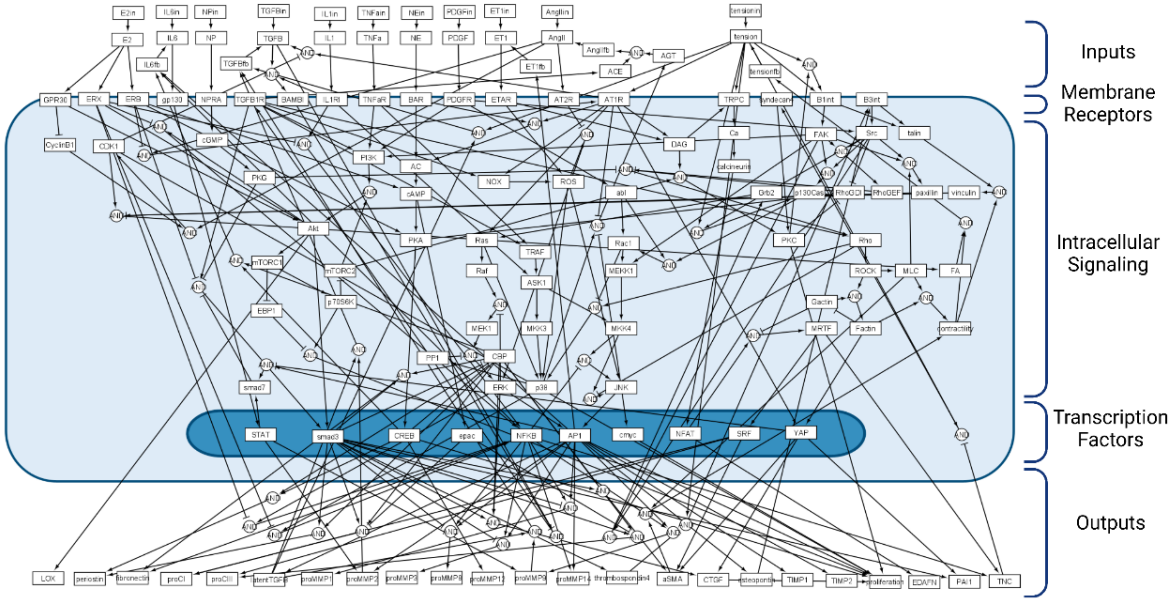

Figure 4.1. The updated SNM integrated with estrogen (E2) and its three receptors.

4.3.2 Model accuracy remains high after integration of E2

Accuracy of the model was maintained after the integration of E2 in the SNM. When compared with 53 independent studies, 77% (94/122 simulations) of model predictions matched results in the literature. Model predictions of estrogen treatment specifically were 88% (15/17 simulations) accurate across experimental conditions. To assess if model accuracy was influenced by sex, a sex-disaggregated analysis was

conducted (Figs. 4.2 and 4.3) using the sex-specific SNMs for model simulations compared with sex-disaggregated reporting in the literature. Model predictions were the most accurate for the male SNM (81%, 46/57 simulations) and least accurate for the female SNM (67%, 2/3 simulations). The pooled SNM was 74% accurate (46/62 simulations). It is important to note that the female SNM model validation was only conducted with 3 simulations and compared with 2 papers. This is because very few papers report female-specific data for cardiac fibroblasts.

For the most part, E2 stimulation without any other profibrotic stimulation (besides tension) has relatively little effect on cellular signaling activity, which the model accurately predicted. However, the model was accurate in predicting the divergent effect of estrogen treatment on male and female proCI and proCIII production, with E2 treatment increasing collagen production in males and decreasing it in females[34]. Additionally, the inclusion of E2 into the model did not impact the accuracy of model predictions for other major cellular inputs, including AngII and TGFB. The lower accuracy of the model in predicting interleukin 1 (IL1), interleukin 6 (IL6), and neutrophil elastase (NE) effect on cellular outputs is consistent with the previously published SNM's limitations[35].

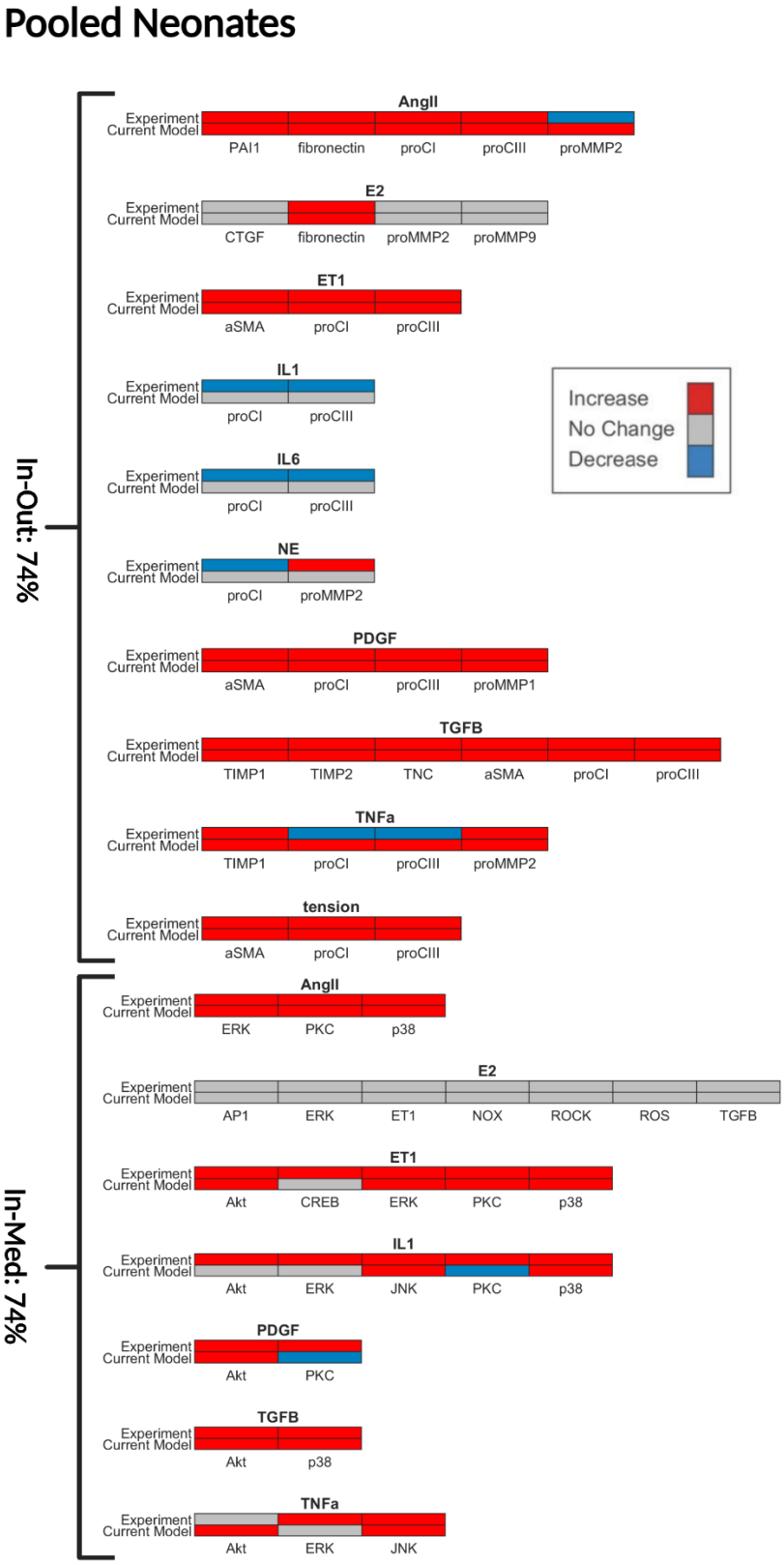

Figure 4.2. Model validation of the pooled neonate SNM created by altering weighting of estrogen stimulation of its three receptors.

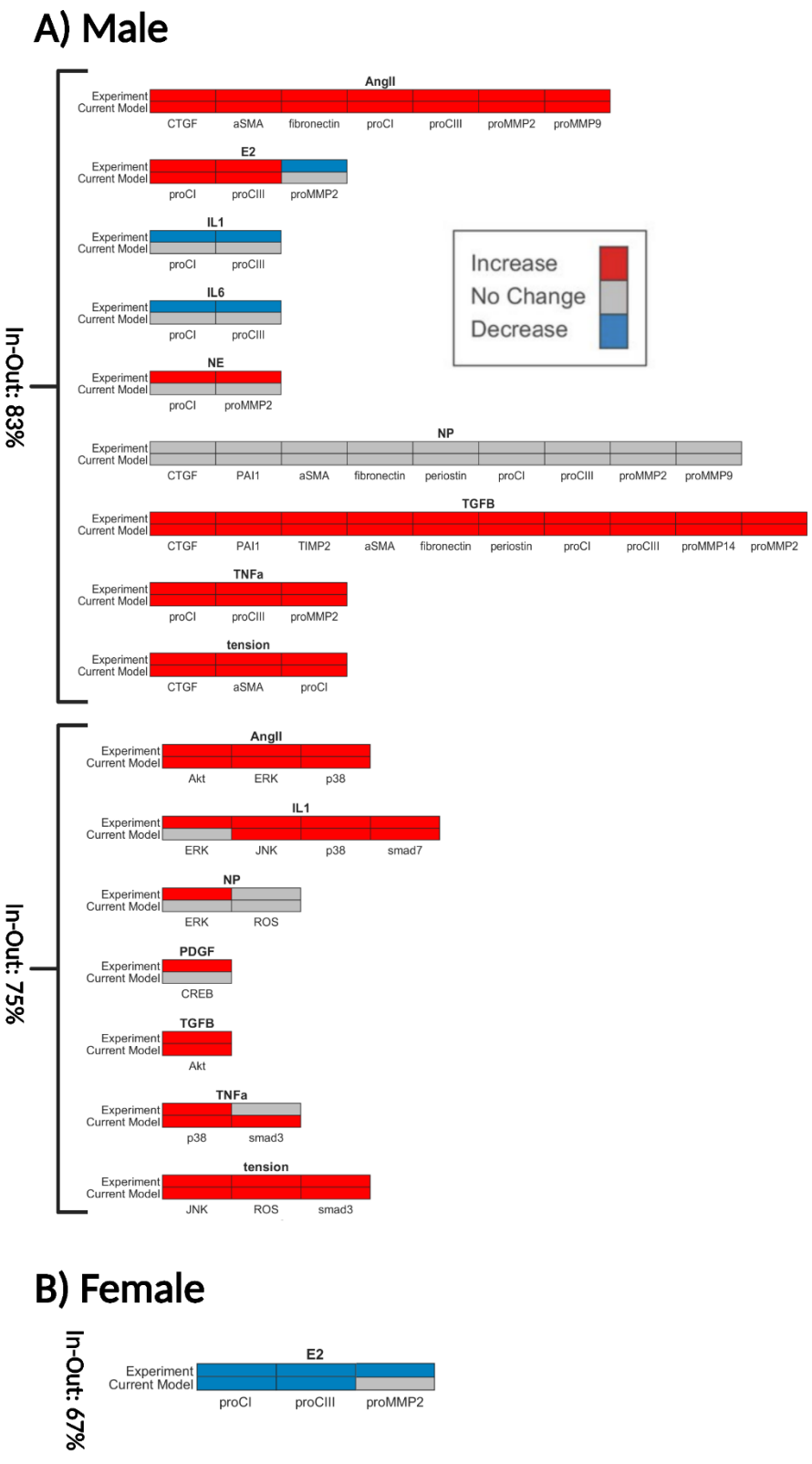

Figure 4.3. Model validation of male (A), and female (B) SNMs created by altering weighting of estrogen stimulation of its three receptors.

4.3.3 Model is accurate in predicting cellular outputs in varied experimental contexts

The experiments used in the validation set were nearly all on an analysis of a singular stimulus on cardiac fibroblasts plated on tissue culture plastic (TCP). To determine if the models were accurate in predicting outcomes in various experimental contexts, model predictions were validated against results from two additional peer-reviewed studies. The first, Watts et al., were the results from my first aim in which I measured the effect of estrogen on male and female cardiac fibroblasts plated on a soft stiffness of 8 kPa[36]. The male and female SNMs were used to conduct sex-specific predictions of E2 treatment on the 12 proteins of interest (Fig. 4.4). In total, the model was 79% accurate in matching the experimental findings from my first aim. These results, combined with the validation set above, provide evidence to support that E2 alone has a relatively limited effect on downstream signaling activities within cardiac fibroblasts. However, when E2 treatment is combined with fibrotic agonists effect on downstream signaling is more pronounced.

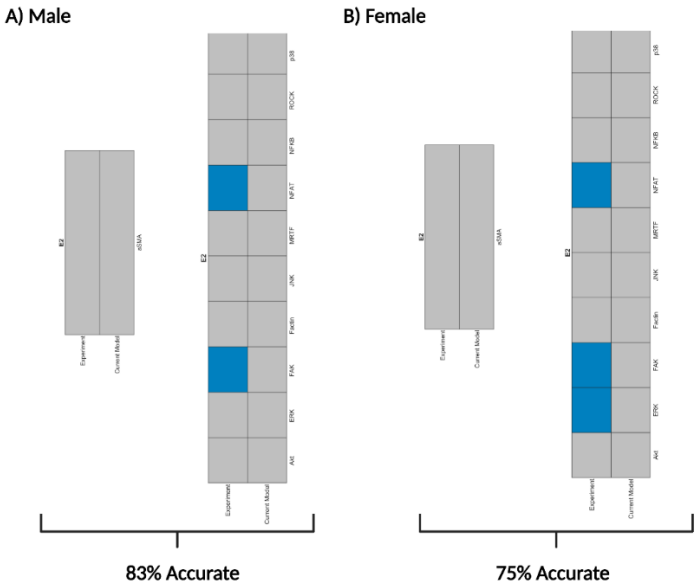

Figure 4.4. Model predictions compared to results of Watts et al. of the effect of estrogen treatment on male and female cardiac fibroblasts plated on an 8kPa substrate[36].

Additionally, the model was validated by a paper from Pedram et al. that investigated the effect of estrogen in conjunction with the fibrotic agonists AngII and ET 1 on pooled neonate cardiac fibroblasts (Fig 4.4)[30]. The model predictions were consistent with *in vitro* results of the study, accurately predicting an increase in α-SMA, fibronectin, collagen 1, and collagen II production when stimulated with AngII or ET 1. Likewise, when these fibrotic agonists are paired with E2, the model predicts a return to control level values of the measured outputs. E2 treatment alone also produced similar outcomes to control level predictions similar to experimental results. The effect of E2 on collagen 1 and collagen III was slightly more extreme in our model predictions than experimentally overserved outputs.

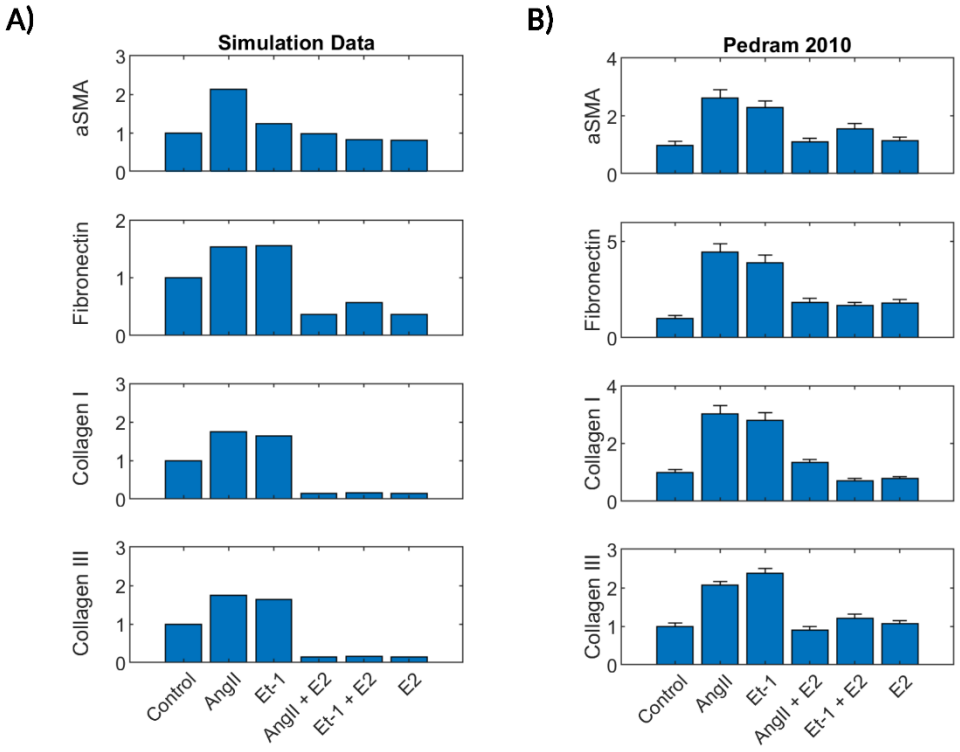

Figure 4.5. Model validation of the effect of estrogen (E2) in conjunction with fibrotic agonists. E2 maintained (or reduced) levels of the profibrotic cellular outputs in the model similar to *in vitro* findings[30].

4.3.3 Perturbation Analysis

The role of estrogen in cardiac fibroblast signaling is poorly understood, especially in a sex-disaggregated context. A perturbation analysis was conducted to computationally

uncover the downstream effect of estrogen treatment on a variety of experimental conditions (Fig. 4.5). These conditions had input weights of 0.6 of all 10 fibrotic agonists' model inputs. The male and female post-menopausal condition had an estrogen input weight of 0.25, and the female pre-menopausal condition had an estrogen input weight of 0.9. The top ten influential and sensitive nodes were also calculated for each condition. There were many shared top influential nodes among each condition (tensionin, AT1R, ROS, NOX, and AKT). Only the male SNM had P13K and NFKB as top influential nodes. In addition to the top influential nodes the female SNM simulations shared with the male SNM, the female conditions also shared E2 as expected. Only the female post-menopausal condition had CBP and GPR30 as top influential nodes. The female pre-menopausal condition has ERB, ERX, and PKA as unique top influential nodes. There were no shared top sensitive nodes among all experimental conditions, indicating many points of divergences in downstream signaling activity.

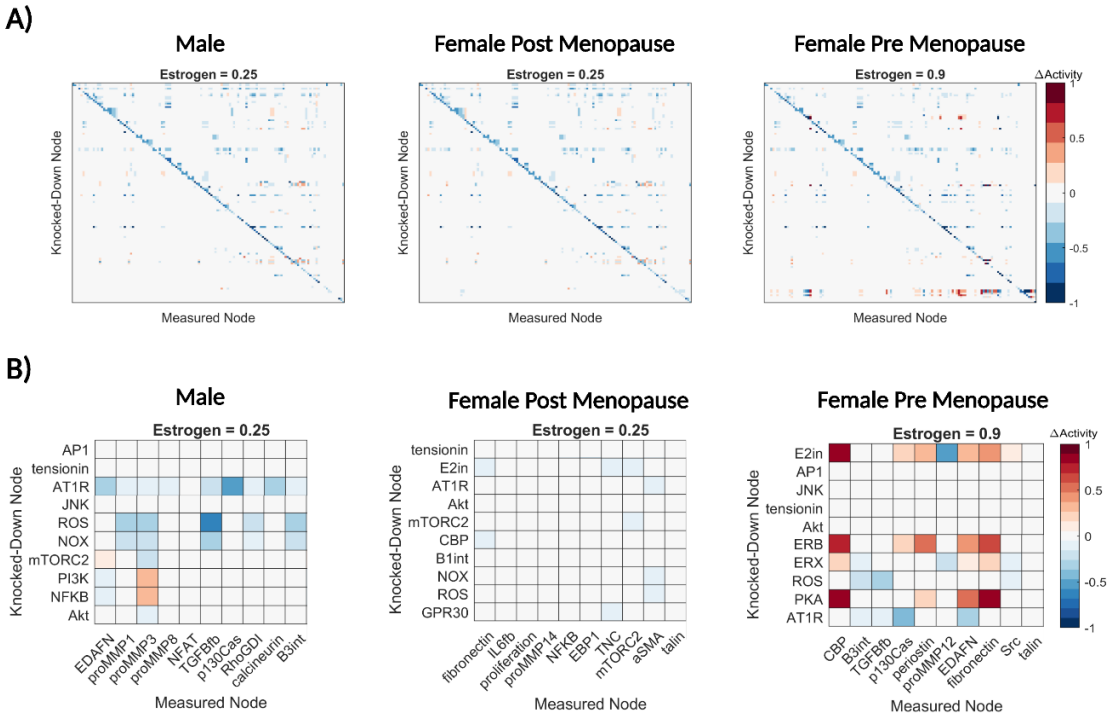

Figure 4.6. Perturbation analysis of downstream signaling activities due to estrogen treatment.

4.3.4 Drug Simulation

A sex-disaggregated drug screen was conducted by dosing with 36 unique drug targets (Appendix D) connected to nodes in the model (Fig. 4.7). The effect of the drugs on fibrotic factors was analyzed when stimulated with various input stimuli with the same experimental conditions used in the perturbation analysis. The most apparent impact is that in the female pre-menopausal condition, the drugs' effects on the model outputs of interests (proCI, proliferation, EDFAN, proMMPs 1, 2, and 9) were significantly reduced. This is likely because E2 is playing a cardioprotective role that is overshadowing the effect of the drugs. The differences between the male condition and the female post-menopausal conditions are much more subtle. In several cases (i.e., drugs targeting TNFα), it appears that the up and down regulatory intensity of the drugs are influenced in a sex-specific manner. There are only a few instances in which the drug has an entirely opposite effect in the male vs. female post-menopausal screen. A notable example is the effect of drugs targeting BAR and IL1RI on MMP1 that have been stimulated with TGFB and ET 1.

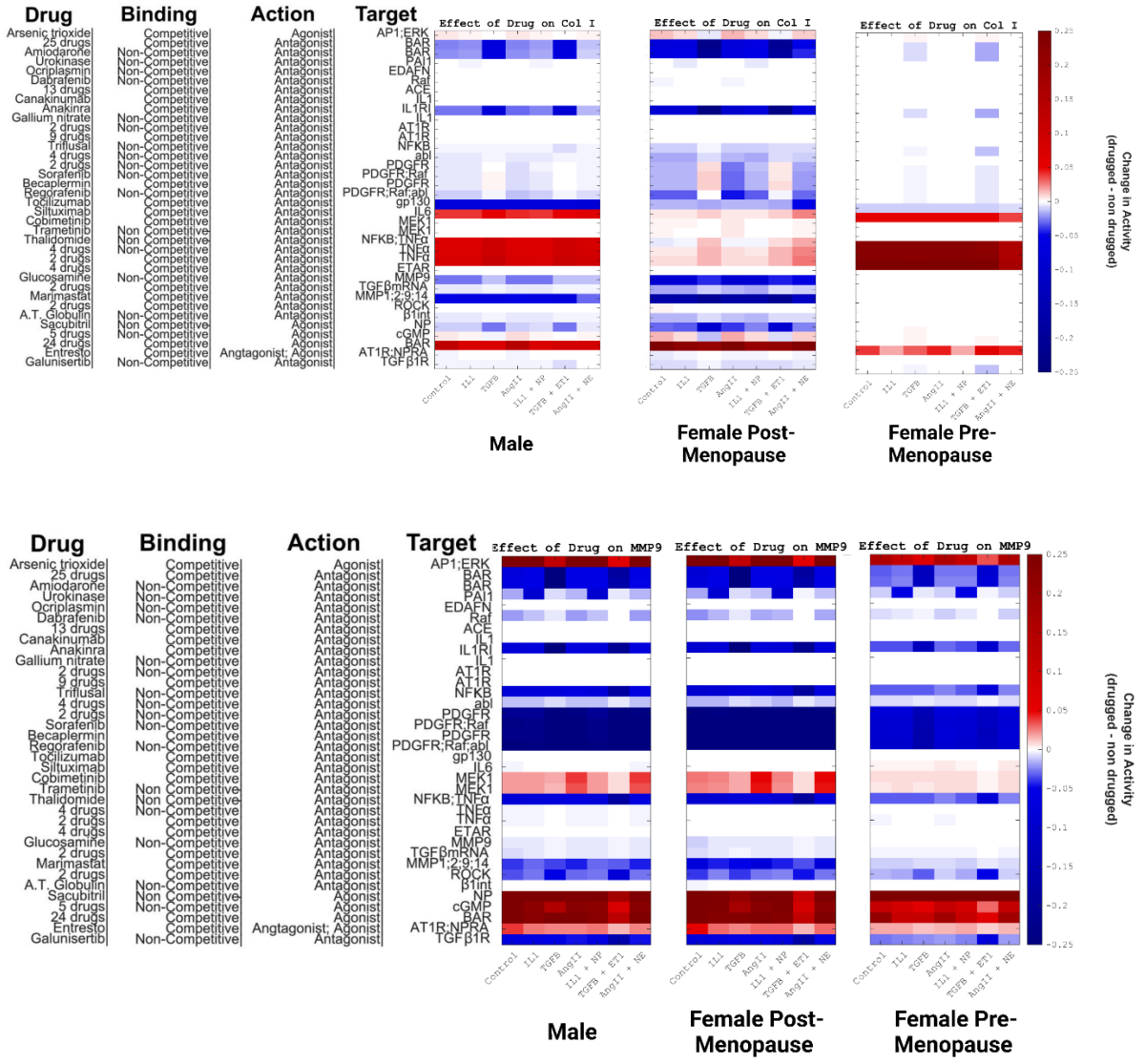

Figure 4.7. Sex-specific drug screen of 36 unique targets for male, female post-menopausal, and female pre-menopausal conditions.

4.4 Discussion

Developing a therapeutic to target and inhibit cardiac fibrosis has remained elusive due to the complex signaling mechanisms of cardiac fibroblasts, which can be influenced by biochemical factors, mechanical forces, and cellular sex. Very few studies investigate the combined effect of all three of these influences on cardiac fibroblast signaling. In this study, we aimed to develop sex-specific signaling network models (SNMs) of cardiac fibroblasts by integrating estrogen signaling into a previously published SNM of cardiac fibroblasts to

understand sex-specific divergences in fibrotic signaling networks. Additionally, we conducted a sex-specific drug screen to determine if we could make sex-specific treatment recommendations for cardiac fibrosis.

Integration of estrogen into the SNM did not negatively alter its previously high predictive power in simulating experiments from the literature. Unsurprisingly, the male SNM was the most accurate as the previously published versions of the model were built and validated using experiments from papers that were overwhelmingly conducted using only male cells[25,35]. It was difficult to fully validate the accuracy of the female SNM as almost no studies report the effect of fibrotic agonists on female cardiac fibroblasts outside the context of estrogen. Therefore, it was difficult to fully assess if the female SNM accurately predicted the effect of the other model inputs such as AngII and TGFB on outputs and intermediates. In the future, we recommend sex-disaggregated reporting to help fill in the gaps of our understanding of sex-specific fibrotic signaling.

The results of the perturbation analysis uncovered potential regulatory nodes that warrant further study. Two nodes that were in the top influencers of the male SNM and the female post menopause SNM were NOX and mTORC2. However, in the female pre-menopause SNM, these nodes do not make the list as top influencers, indicating that at higher estrogen levels, their effects are dampened. Neither NOX nor mTORC2 was directly involved in reactions that were added or adjusted when integrating estrogen into the model. Inhibition of NOX has been linked to decreases in collagen deposition[37]. Likewise, mTORC2 has been reported to play a role in cellular contractility[38]. Targeting NOX and mTORC2 could be a way to mimic the cardioprotective effect of E2 signaling.

The sex-disaggregated drug screen clearly exhibited the cardioprotective role of E2 in the female pre-menopause SNM. However, the differences between the male and female

post-menopause SNMs were much more subtle. Although there were slight differences in the intensity of the effect of the drugs in up or downregulating fibrotic factors in a sex-specific manner, the results did not provide enough evidence to elucidate mechanisms that account for the ADRs that are more likely to occur in females compared to men. It is possible that the integration of estrogen signaling alone was not enough to account for the sex-specific differences in fibrotic response to different therapeutics. Future studies could address this by integrating genomic or transcriptomic data into the model to account for additional differences due to biological sex in addition to gonadal hormones.

4.5 Limitations and Future Directions

Many of the limitations of the sex-specific models designed in this study are due to the lack of sex-specific data, particularly female data, available to build and validate the model with, as discussed above. An additional major limitation of the current models is their lack of quantitative data to infer input parameter values. Without these inputs, the models can only make semiquantitative predictions that can be qualitatively compared to experimental literature but have little clinical significance. Because of this constraint, the model was only validated with estrogen levels of high (pre-menopausal) and low (male and post-menopausal). However, like other hormones, estrogen levels can fluctuate from person to person and throughout life. These attenuations were not accounted for and would likely play a role in the downstream signaling response. Future studies could address this by testing with more levels of estrogen and integrating feedback loops for estrogen and its receptors.

4.6 Conclusion

We were able to successfully integrate estrogen signaling into a cardiac fibroblast SNM in order to make sex-specific models of cardiac fibrosis. These models were validated

to be ~80% accurate in predicting experimental outcomes from the literature, making it a valuable tool to further study the sex dimorphisms in cardiac fibroblasts signaling caused by estrogen. Estrogen's cardioprotective effects were evident in the drug screen of the pre-menopausal SNM. Nuanced differences between the male and female premenopausal conditions perturbation analysis and drug screen indicate several potential regulatory mechanisms that warrant further study. Future research should focus on incorporating more sex-specific data into the model itself and validation set to further enhance its capabilities in making sex-specific predictions related to cardiac fibrosis.

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CHAPTER FIVE

AIM THREE: DEVELOPMENT OF SYSTEMS BIOLOGY EDUCATION MODULES UTILIZING INCLUSIVE PEDAGOGY STRATEGIES

5.1 Introduction

In my first two aims, I investigated how biological sex affected the development of cardiac fibrosis and discussed the need for further research due to the poor understanding of sex as a biological variable. I intentionally framed my analysis around only biological sex and not gender because I was primarily focused on the cellular level. However, at the macro level, gender and biological sex can intersect to result in additional health disparities. The male body has been historically considered the "standard," and technologies are designed as one size fits all to accommodate it. This has resulted in surgical tools being developed for larger hands, crash test and CPR dummies having a male build, and the first FDA-approved artificial hearts being too large for the average female patient[1–3]. These biases are likely due partly to the lack of representation in the workforce, which also contributes to healthcare inequities due to race and ethnicity. For example, recently, software designed to identify skin cancer was found to only be accurate for those with white skin[4]. One way to mitigate these biases and make healthcare more equitable is to have diverse representative design teams.

Women and racial and ethnic minorities remain underrepresented in science, technology, engineering, and math (STEM). On average, women make up roughly 50% of the population and hold only a fraction of the engineering and computer workforce jobs (15% and 25%, respectively)[5]. Likewise, historically marginalized communities, including Blacks/African Americans, Hispanics/Latinos, and American Indians, collectively make up about 31% of the US workforce but only 20% of STEM jobs[5]. Not only are these gender

and racial/ethnic gaps leading to inequitable designs, but they are also limiting the ability of the US to compete in the global science and engineering market[6]. Having an increased number of women and racial and ethnic minorities enter the workforce would allow the US to keep pace with the growing number of STEM jobs and lead to more productive and creative teams that create equitable technology[7,8].

To achieve a more diverse STEM workforce, the recruitment and retention of these historically marginalized communities in postsecondary education will also need to increase. Although women outnumber men on college campuses, they are less likely to major and graduate with STEM degrees[9]. Likewise, Black and Latinx students who have declared a STEM major are more likely to change majors or drop out than their White peers[10]. These trends are often attributed to the formation of STEM identity, which can be influenced by numerous factors, including middle and high school preparation, societal expectations and stereotypes, university and workplace culture, representative role models, and STEM self-efficacy[11,12]. Many informal education and mentoring programs have been created to target underrepresented individuals in an attempt to counteract negative influences and increase STEM identity. However, trends have remained relatively stagnant or even dropped over recent years, indicating that more research needs to address the gender and racial/ethnic gap in STEM[13].

Recently the lens has turned to pedagogy and how creating a more inclusive classroom environment can foster STEM identity in previously marginalized communities[14]. It is well established that nearly all students benefit from a more active learning environment than traditional lectures[15,16]. These techniques allow students to learn by doing rather than passively listening. Literature has shown that in a classroom that utilizes active learning techniques, students are less likely to fail the course, which in turn,

can lead to higher rates of retention rates[17,18]. However, even in settings that utilize some active learning techniques such as flipped classrooms and problem-based learning, there can still be additional barriers that impact student performance. For example, in computational classes, previous coding experience can be a predictor of perceived self-efficacy throughout the course[19,20]. This affects those from under-resourced schools and others (like women and racial/ethnic minorities) who do not typically partake in such activities even if they were an option.

To address the growing need for computational skills in the workforce and higher education, computational thinking and literacy have become part of the core curriculum in recent years. Because the majority of K-12 teachers do not have a lot of previous coding experience, researchers and practitioners have been investigating the use of unplugged activities to facilitate the teaching of computational skills[21,22]. Unplugged activities facilitate the learning of computational thinking skills through hands-on or role-playing activities in lieu of technology or coding[22–24]. This technique has been shown to be effective in increasing the computational literacy of primary school students, but little research has been done on how this technique can also benefit older students with limited prior coding experience to increase their self-efficacy in computational methods[24]. Using unplugged activities in conjunction with traditional coding in computational settings could help level the playing field for previously marginalized communities.

Additionally, it is well established that representative role models can increase STEM self-efficacy for students from underrepresented populations. However, little research has been done on how representative problem statements (i.e., gender or race-specific) in problem-based learning could also increase self-efficacy. Framing problems as gender or race specific to target underrepresented student populations may provide them

with additional motivation and interest and increase their STEM self-efficacy and identity formation.

Problem-based learning is often paired with team-based learning in STEM classrooms as a powerful tool to increase motivation and overall learning gains[25,26]. However, this presents issues for marginalized communities. Stereotypes can be leveraged against them, and task allocation for the technical aspects of a project is not always equal[27]. We are interested in studying if representative problem statements (i.e., gender or race-specific) would also impact team dynamics such as task allocation, team interactions, team contributions, and having relevant knowledge, skills, and abilities by providing students from underrepresented populations with the additional agency to take the lead.

This work focuses on the development of computational biology education modules designed to promote an inclusive learning environment by (1) utilizing a hybrid of unplugged activities with coded simulation and (2) a female-oriented problem statement. Additionally, I will discuss plans to investigate these interventions' effects on STEM self-efficacy and team dynamics. Our research questions are:

1. What effect does pairing unplugged learning activities with traditional coding have on the computational literacy of high school students?
2. To what extent does a female-centered computational biology problem statement impact high school girls' STEM self-efficacy and identity?
3. How are team dynamics such as task allocation and contribution affected when working on a female-focused problem?

5.2 Module Development

5.2.1 Module Development

We have created five systems biology education modules for high school students focused on various physiological processes and diseases. These modules will be piloted in collaboration with Clemson's Emerging Scholars program in July 2022. All modules include three major components:

- **Unplugged Activities:** To advance computational thinking and ease students and teachers into modeling, the lesson plans start with a short active learning activity to provide a hands-on or role-playing approach to the phenomena being modeled (i.e., a modified card game to model uncontrolled tumor growth). Each lesson plan also includes recommended discussion questions to connect the unplugged activity with computational concepts.
- **Computational Model Tutorial:** Following the unplugged activity, students will work through a guided tutorial to model the biological or disease phenomena. This tutorial is designed to be conducted on a chrome book or similar device with the open-source software NetLogo (see Figure 5.1)[28]. NetLogo was chosen as the platform for model development because of its use of drag and drop coding paired with traditional script coding, which we believe is best for our target audience of high school students with limited previous coding experience.
- **Computational Model Advancement:** The last component of our educational modules is a deliverable in which students create their own virtual model by modifying the model they created in the tutorial. The lesson plan provides guided questions as suggestions for model improvement (e.g., What if the patient took a drug that inhibited X, Y, or Z?) and encourages students to develop their own questions. This last component allows educators to assess gains in their students' computational abilities.

Each of the five lesson plans created can be found in Appendix E. In addition to the tutorial and model advancement questions given to students, each lesson plan contains an overview page for instructors with learning objectives, definitions of applicable terms, a time to complete estimation, and a guide to the unplugged activity with suggested follow-up discussion questions. All modules are designed to be completed in 2-4 hours each. The unplugged activities are intended to be completed in a group setting. The coding part of each module could be completed individually or as a team. Because they are intended for students with minimal coding experience, the modules build off each other and are developed to be completed in sequence except for Modules 4 and 5, which cover very similar computational concepts. Students will only complete either Module 4 or Module 5 in our pilot study, but in theory, they could complete both if they wanted extra practice on independent module development. Completed NetLogo models can be found on the Richardson Systems Mechanobiology Lab GitHub: (https://github.com/SysMechBioLab). We plan to disseminate the modules publicly via journal publication after the completion of the pilot study.

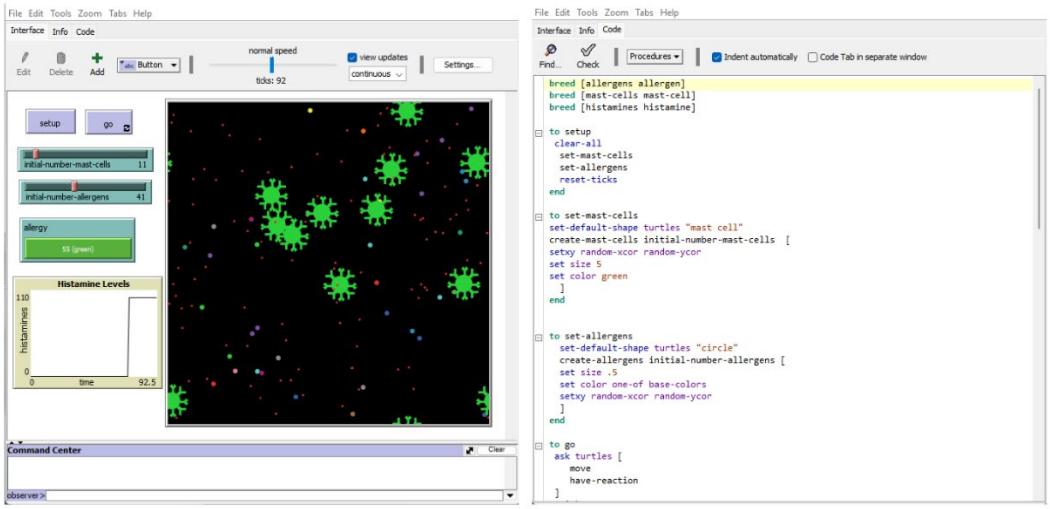

Figure 5.1. NetLogo interfaces with drag and drop buttons and coded script.

5.2.2 Modules Created

All of the modules created are centered around common biological phenomena. The first two modules build upon NetLogo's built-in Tumor and Virus models to ease students into the computational software. Modules 3-5 were independently created and require students to develop a model from scratch (with a guided tutorial). The five modules designed are detailed below and in Figure 5.2:

- **Tumor Growth:** This module employs a built-in NetLogo 'Tumor' model as a way to get students acclimated with the environment before asking them to code in it[29]. It simulates a growing tumor and intervention techniques to stop the growth. Biologically, students will observe the progression and treatment of a tumor from a cellular level. Computationally, students will gain experience in using a model to test predictions and identify model limitations. The unplugged activity tasks students with playing a modified game of UNO, which simulates the behavior of the model by showing how even if you are down to one card (i.e., a cancer cell), there is still the possibility of regrowth. The model tutorial guides students through interacting with the NetLogo interface. In the model advancement, students are asked to answer questions about the model's limitations and make suggestions for improvements.
- **Virus Prevention:** This module modifies the built-in NetLogo 'Virus' model to introduce additional parameters that model an intervention to slow the spread of a virus within a population[30]. Biologically, students will examine population dynamics and viral spread. Computationally, students will research and test the effect of input parameters on a model. The unplugged activity guides students through a role-playing game that uses logic comparable to the probability functions which guide the simulation. In the model tutorial, students are shown how to introduce a new variable to the model, 'mask compliance.' In the model advancement, students are

asked to search the literature to find potential input parameters to model the spread of a virus of their choosing.

- **Immune Reaction:** This module will guide students through a tutorial to code a NetLogo model from scratch that simulates an allergic reaction. Biologically, students will consider how different cells and molecules interact to elicit an immune response. Computationally, students will define rules and make assumptions to create an agent-based model. The unplugged activity asks students to design the rules for their own game, similar to how they will be defining rules for the simulation. The model tutorial guides students in setting up the input parameters and all of the agents needed for the model (e.g., mast cells, allergen, and histamines). However, the tutorial does not end with a completed model; the agents do not interact with each other. In the model advancement, students are tasked with defining rules for their model and coding them to finish the simulation.
- **Gene Regulation:** This module will guide students through a tutorial to code a NetLogo model from scratch that uses Boolean Logic to create a simulation of the lac operon genetic regulation system. Biologically, students will be exposed to a standard model of gene regulation. Computationally, students will define rules using Boolean logic and analyze model outputs to identify a system's emergent phenomena. The unplugged activity will lead students through a game of If and If Else Simon Says as a way to familiarize them with Boolean logic. The model tutorial will guide students through the beginning steps of creating a model with many interactive parts from scratch. As this simulation is more challenging than the other modules, students will work in teams during the model advancement to finish the simulation and identify the model's emergent phenomena.

- **The Menstrual Cycle:** This module will guide students through a tutorial to code a NetLogo model from scratch that uses Boolean Logic to create a simulation of the menstrual cycle. Biologically, students will be exposed to the hormones involved in the menstrual cycle. Computationally, students will define rules using Boolean logic and analyze model outputs to identify a system's emergent phenomena. This module was designed intentionally to mirror module 4 and will be used to test the use of a female-focused problem statement vs. a generic problem statement on female self-efficacy and team dynamics. The unplugged activity, model tutorial, and model advancement utilize similar concepts, and the end simulations are guided by similar logic.

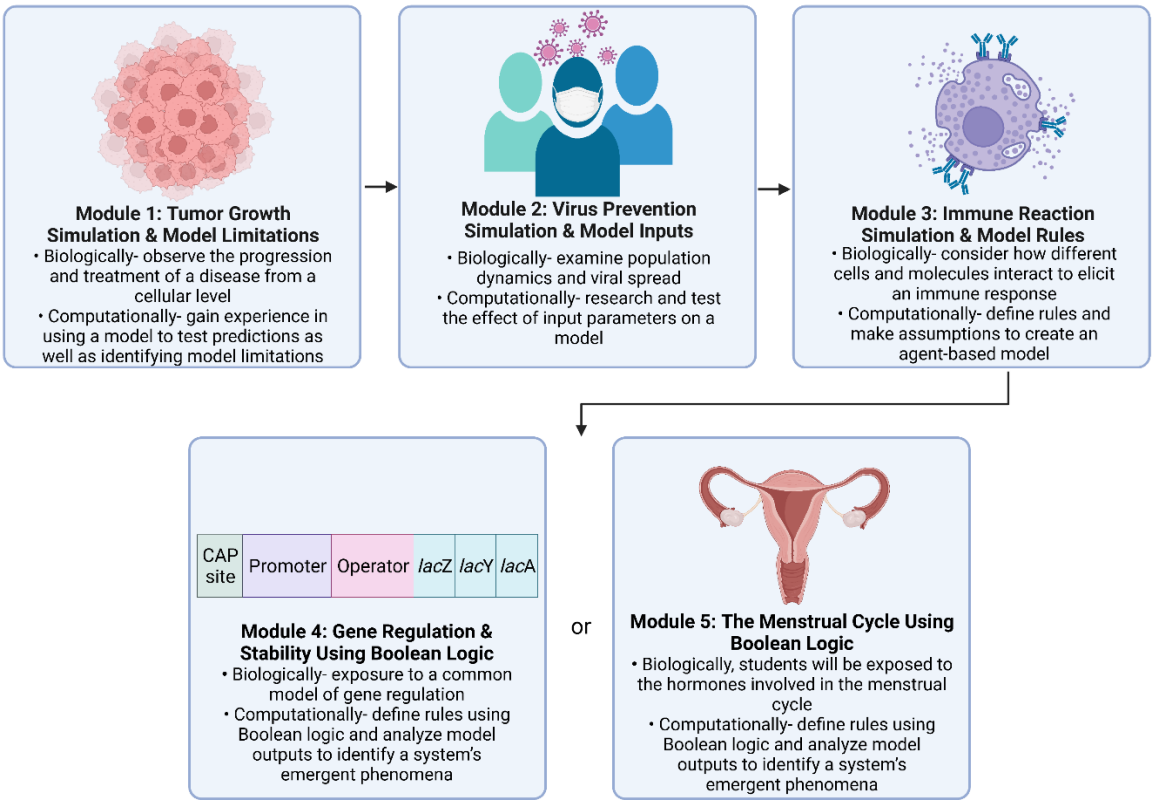

Figure 5.2. Five computational biology modules were created. All students will complete modules 1-3 in sequence and then complete modules 4 or 5.

5.3 Plan to Pilot

5.3.1 Clemson's Emerging Scholars Program

We plan to pilot the modules with the summer 2022 cohort of Clemson's Emerging Scholars program. This program invites rising high school sophomores-seniors from some of South Carolina's most under-resourced schools to Clemson's campus for several weeks to aid in college preparation. The program helps students prepare for their college applications and has them take classes to expose them to different skills to prepare them for college. I will be teaching a course on computational biology as part of this program from July 6- 16, 2022, using the learning modules developed.

The students accepted in the Emerging Scholars program are most likely to be female and or African American/Black, both of which are currently underrepresented in STEM, providing an ideal population to study how the inclusive learning techniques incorporated in the modules impact the self-efficacy team dynamics. We anticipate that there will be about 60 students in the cohort this summer that will be divided up into three classes of ~20 students. The course will be seven 1.5 hr sessions long per class. All research will be conducted in accordance with Clemson's IRB office and Protocol IRB2022-0059.

5.3.2 Quasi-Experimental Intervention

We will use a quasi-experimental approach to measure the effect of the modules on students assigned to groups who either complete module 4 or module [31]. All students will be asked to complete modules 1-3 individually. For the final module, students will be split up into a team of 4, with at least two female students per team. Half of the teams will be asked to complete Module 4: Gene Regulation and Stability Using Boolean Logic; the other half will be given Module 5: The Menstrual Cycle Using Boolean Logic. All teams may not be

used in the analysis if there is an uneven number of students or not enough male and female students.

5.3.3 Exit Survey

To measure any changes in self-efficacy, I will deploy an exit survey on the last day of the program to all students. This survey will ask students to rate their pre-and post-understanding of computational and biological concepts using a 1-10 Likert scale. The concepts they will be rating will be taken from the learning objectives of the lesson plans. I will conduct a sex-disaggregated analysis on whether there are similar self-efficacy changes for individuals on teams who completed the lac operon or the menstrual cycle modules.

Additionally, the exit survey will ask students to rate their satisfaction with various module components, including the unplugged module activities. There will also be open-ended questions to allow students to provide more depth to their answers. Results from the exit survey will be used to (1) evaluate students' overall satisfaction with the modules, (2) determine if students believed the unplugged activities affected student their perceived self-efficacy in completing computational tasks, and (3) determine if a female-focused problem affected student's perceived self-efficacy in completing computational tasks. The exit survey can be found in Appendix F.

5.3.4 CATME Survey

I will use the Purdue CATME survey to measure team dynamics, allowing students to rate their teammates on various facets, including contributions, interactions, knowledge, and skills[32]. This survey will be used to determine if a female-focused problem affected team dynamics. The Purdue CATME survey can be found in Appendix G.

5.3.5 Interviews

I will also conduct 5-10 follow-up interviews with students to ask them more in-depth questions about their satisfaction with the modules, perceived self-efficacy, and team dynamics. The sample interview protocol can be found in Figure 5.3.

Interview Protocol*:
1) What was your learning module? Why?
2) What was your least favorite learning module? Why?
3) Can you describe how your team communicated and worked together on the fourth activity?
4) Do you think having the problem be female focused made you more or less comfortable as a girl on the team?
5) In your own words how would you describe computational biology?
6) Do you have an idea of what you want to do after high school? If you plan to go to college, what do you think you want to major in?
7) How do you think the learning modules could be improved?
8) Would you recommend this class to you friends?
9) Have you had any prior experience coding?
10) Do you feel confident enough that you could code a basic biological process using NetLogo?
11) Did you prefer to work in teams or alone?
12) Can you describe your team dynamics, do you feel everyone participated equally? That everyone's voice was being heard?
13) Did anyone take the lead on your team?
14) Would you want to continue learning more about computational biology in the future?
15) Was there a concept or activity you struggled with the most to understand?

*Not all questions will be asked; questions will be chosen based on preliminary exit survey results.

Figure 5.3. Interview protocol that will be used with 5-10 students.

5.4 Conclusion

The learning modules developed allow for the teaching of computational techniques through the use of unplugged activities paired with coded simulation. We anticipate that this will enable students with limited prior computational experience to feel more comfortable and able to complete tasks, thereby increasing their self-efficacy. A significant limitation of our study is that while we hope these modules will improve recruitment and retention of underrepresented students to STEM degrees and careers, we do not currently have plans to run a longitudinal study to see if they have long-term effects. Additionally, we are only conducting a one-week course as part of the pilot study. Future studies could investigate the use of these pedagogies throughout a semester-long course and conduct follow-up studies on major retention and degree attainment to truly determine the long-term effect of these strategies.

These modules could be a useful tool for practitioners who teach computational courses at the high school or early college level who instruct students with varied coding experience. Additionally, our analysis of the use of a female problem statement on female self-efficacy and team dynamics will provide potential evidence of the efficacy of representative problem statements targeted at underrepresented student populations. We believe such problem statements could be developed to address a variety of contexts across STEM disciplines and other fields to foster an inclusive learning environment.

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CHAPTER SIX

CONCLUSIONS, LIMITATIONS, AND FUTURE DIRECTIONS

6.1 Summary of Findings

Multiple strategies were used to address the gender data gap in healthcare. In Aims 1 and 2, I discussed the lack of female data and understanding of estrogen signaling in relation to cardiac fibrosis. We found that the effects of sex and biological sex have a varied impact on profibrotic factors in cardiac fibroblast. Although morphologically we did not observe differences in a physiological stiffness of 8 kPa *in vitro*, different proteins reacted in various ways to estrogen treatment occasionally in a sex-specific manner. When done *in silico,* we were able to accurately integrate estrogen signaling into a previously created signaling network model of cardiac fibroblasts to develop sex-specific models. After perturbation analysis and drug screens with the sex-specific models, we were able to identify several regulatory pathways that warrant further study to elucidate their involvement in the sex dimorphisms of cardiovascular health. Finally, in Aim 3, five systems biology education modules were developed with inclusive pedagogy strategies, including unplugged activities and representative problem statements to target underrepresented individuals. The plan to pilot these modules to investigate the effect on STEM identity, self-efficacy, and team dynamics were also outlined. These *in vitro*, *in silico*, and in classroom strategies could be further advanced by addressing some of their significant limitations.

6.2 Study Limitations

6.2.1 Aim 1 Limitations

Limitations for Aim 1 include that it was an *in vitro* monolayer culture of fibroblasts, only treated with estrogen (E2), alpha-smooth muscle actin (α-SMA) remained

elevated, and that it was a limited analysis of protein-protein interactions. We used an *in vitro* monolayer culture of cells for the experimental platform because we were primarily interested in the effect of sex differences and E2 treatment on a physiological similar stiffness. However, in the future, to further mimic physiological conditions, a coculture of multiple cell types of the heart (i.e., cardiomyocytes) could be used[1]. Additionally, a 3-D culture platform such as spheroids or hydrogels could be developed if a more advanced analysis was desired[2,3]. Because the heart is an organ that dynamically beats, it could also be interesting to apply cyclic stretch or mechanical stimulus to mimic this phenomenon *in vitro* to investigate its effect on sex and E2[4]. Estrogen is one of many gonadal hormones in the body that play a role in cellular processes. In future experiments, other gonadal hormones such as progesterone and testosterone could be dosed in addition to estrogen when studying sex-specific effects. Additionally, in order to more accurately mimic menopause, an *in vivo* study with ovariectomized (OVX) mice could be conducted[5]. An unexpected result of Aim 1 was that α-SMA expression remained elevated even on 8 kPa stiffness plates. This was surprising as there are other studies that report minimal to no α-SMA expression on low stiffnesses[6]. Recent literature suggests that cells can maintain a mechanical memory after being plated on tissue culture plastic (TCP). In the future, we would recommend plating cells on softer substrates immediately after isolation when conducting stiffness analyses or for conducting experiments for more extended time points (as opposed to 24 hrs) to allow cells to get reacclimated to their new environment[7]. Finally, in Aim 1, we only conducted a limited analysis of protein-protein interactions. This limitation was addressed in Aim 2 with the fibroblast signaling network model.

6.2.2 Aim 2 Limitations

A significant limitation of the sex-specific SNMs of cardiac fibrosis developed in Aim 2 was that they have little physiological relevance in their current state. The models would need to be updated with more robust input parameters in order to have quantifiable outputs that could be used for diagnosis and treatment initiatives. Additionally, the female SNM needs to be further validated. There were insufficient female-specific data in the literature to provide a substantial validation set to ensure our confidence in the female SNM model's predictions. Finally, the results of the drug screen revealed very subtle differences between the male and female post-menopausal conditions. It is likely that estrogen signaling alone is not enough to account for the sex-specific trends in drug response and adverse reactions, and in the inclusion of other biologically relevant factors such as genomics, transcriptomics, or other hormones could added to future iterations of the model.

6.2.3 Aim 3 Limitations

A significant limitation of the learning modules developed in Aim 3 is that due to the COVID-19 pandemic, our plans were postponed until summer 2022, so we do not have any preliminary data on their efficacy and reception. Additionally, although our goal is that the inclusive pedological strategies utilized in the module lesson plans will increase the recruitment and retention of historically marginalized individuals to STEM fields, we do not plan to conduct a longitudinal study to truly investigate the long-term effect of such strategies. In the future, we would recommend integration of these strategies in a longer time frame (i.e., a semester-long course) with follow-up studies to directly investigate the effect on retention of underrepresented individuals.

6.3 Future Directions

As discussed throughout this manuscript, female data are widely underrepresented in the literature and clinical trials, which are significant contributors to healthcare inequities.

Although many funding agencies state they are now requiring the inclusion of female samples to receive funding, this is not always reflected in studies that continued to be published using only male samples. In the future, I believe journal editors and reviewers could have one of the most significant influences the to make inclusion of female and/or sex-disaggregated data an expectation, not just a bonus, of peer-reviewed studies. Additionally, bootstring representation of women in STEM could help to increase the studies which incorporate female samples into their studies.

The overarching goal of this dissertation was to address the gender data gap that exists in healthcare through various strategies in and out of the lab. However, there are many other contributors to healthcare inequities beyond the lack of female data. Race/ethnicity, socioeconomic status, sexual orientation, and gender identity are all also significantly understudied influences on the care patients receive. Additionally, although they were often treated as a dichotomy throughout this dissertation, for many individuals, sex and gender are on a spectrum that is not stagnant throughout life. These are all factors that need to be adequately investigated and addressed, especially as precision medicine techniques become more widely used to ensure that diagnostic and treatment interventions are accurate for all patients.

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APPENDICES

Appendix A

Table A1:

CycIF Round	Antibody	Manufacture	Alexa Flour	Dilution	Light	Exposure	Gain
1	Anti-p-p38 (E-1)	Santa Cruz (sc-166182 AF594)	594 (Tx-Red)	1:25	65	703	13.3
	Anti-NFATc4 (B-2)	Santa Cruz (sc-271597 AF488)	488 (GFP)	1:30	61	462	12.2
	Anti-Smad3 (38-Q)	Santa Cruz (sc-101154)	*	1:25	52	755	15.2
2	Anti-MRTF-A (G-8)	Santa Cruz (sc-390324 AF546)	546 (RFP)	1:25	52	582	6.6
	Anti- Rock-1 (G-6)	Santa Cruz (sc-17794 AF647)	674 (Cy5)	1:30	61	586	14.3
	Anti-NFκB p50 (E-10)	Santa Cruz (sc-8414)	*	1:25	58	908	15.4
3	Anti-p-JNK (G-7)	Santa Cruz (sc-6254 AF546)	546 (RFP)	1:50	42	151	7.4
	Anti-p-Akt1/2/3 (C-11)	Santa Cruz (sc-514032 AF647)	647 (Cy5)	1:30	61	586	14.3
	Anti-ASMA	abcam (ab5694)	*	1:250	61	911	14.4
4	Anti-p-ERK (E-4)	Santa Cruz (sc-7383 AF488)	488 (GFP)	1:30	58	391	14
	Texas Red®-X phal-loidin	Fisher (T7471)	594 (Tx-Red)	1:40	65	676	17.1
	Anti-p-FAK (Tyr397)	invitrogen (700255)	*	1:250	58	1140	15.4
1 - 4	*mouse-anti-rabbit IgG PE-Cy7	Santa Cruz (sc-516721)	750 (Cy7)	1:100	-	-	-
	Hoechst 33342	Fisher (H3570)	DAPI	1:1000	35	51	9

Table A2:

	p_{E2}	p_{sex}	p_{sex*E2}
Cell Density	0.87	0.58	0.15
Cell Area	0.89	0.81	0.43
Cell Elongation	0.62	0.67	0.07
Nucleus Area	0.45	0.53	0.43
Nucleus Elongation	0.72	0.97	0.49
α-SMA	0.94	0.57	0.16
F-Actin	0.92	0.97	0.49
P-ERK	0.49	0.94	**0.02***
P-p38	0.37	**0.04***	0.43
ROCK1	0.06	**0.048***	0.47
P-FAK	**0.01***	0.53	0.34
p-JNK	0.17	0.07	0.394
p-AKT	0.24	0.86	0.06
NFAT	0.52	**0.01***	0.62
NF-κB	0.63	0.50	0.30
SMAD3	0.99	0.38	0.20
MRTF	0.43	0.53	0.31

Appendix B

Species information

module	ID	name	Yinit	Ymax	tau
g-coupled	AngII	angiotensin II	0	1	1
g-coupled	AngIIin	ang II input	0	1	1
g-coupled	AngIIfb	ang II feedback	0	1	1
g-coupled	AT1R	angiotensin II receptor type 1	0	1	0.1
g-coupled	AGT	angiotensinogen	0	1	10
g-coupled	ACE	angiotensin converting enzym	0	1	0.1
g-coupled	NOX	NAD(P)H oxidase	0	1	0.1
g-coupled	ROS	reactive oxygen species	0	1	0.1
g-coupled	ET1	endothelin 1	0	1	1
g-coupled	ET1in	endothelin 1 input	0	1	1
g-coupled	ET1fb	endothelin 1 feedback	0	1	1
g-coupled	ETAR	endothelin 1 receptor A	0	1	0.1
g-coupled	DAG	diacyl-glycerol	0	1	0.1
g-coupled	PKC	protein kinase C	0	1	0.1
tension	TRPC	transient receptor potential ca	0	1	0.1
g-coupled	NE	norepinephrine	0	1	1
g-coupled	NEin	norepinephrine input	0	1	1
g-coupled	BAR	beta adrenergic receptor 1 or	0	1	0.1
g-coupled	AC	adenylate cyclase	0	1	0.1
g-coupled	cAMP	cyclic adenosine monophosp	0	1	0.1
g-coupled	PKA	protein kinase A	0	1	0.1
g-coupled	CREB	cAMP response-element bind	0	1	0.1
g-coupled	CBP	CREB - binding protein	0	1	0.1
growth factor	TGFB	transforming growth factor b	0	1	1
growth factor	TGFBin	transforming growth factor b	0	1	1
growth factor	TGFBfb	transforming growth factor b	0	1	1
growth factor	TGFB1R	TGFB receptor	0	1	0.1
growth factor	smad3	small mothers against decape	0	1	0.1
growth factor	smad7		0	1	10
growth factor	latentTGFB	TGFB1 with latent protein co	0	1	10
growth factor	BAMBI	BMP and activin bound inhib	0	1	0.1
growth factor	PDGF	platelet derived growth factor	0	1	1
growth factor	PDGFin	platelet derived growth factor	0	1	1
growth factor	PDGFR	platelet derived growth factor	0	1	0.1
g-coupled	NP	natriuretic peptide	0	1	1
g-coupled	NPin	natriuretic peptide input	0	1	1
g-coupled	NPRA	natriuretic peptide receptor	0	1	0.1
g-coupled	cGMP	cyclic guanosine monophosp	0	1	0.1
g-coupled	PKG	protein kinase G	0	1	0.1
tension	tension	membrane or adhesion tensio	0	1	1

tension	tensionin	tension input	0	1	1
tension	tensionfb	tension feedback	0	1	1
tension	B1int	beta 1 integrin	0	1	0.1
tension	Rho	a Rho-dependent GTPase	0	1	0.1
tension	ROCK	rho associated protein kinase	0	1	0.1
tension	Ca	calcium	0	1	0.1
tension	calcineurin	calcineurin	0	1	0.1
tension	NFAT	nuclear factor of activated T-	0	1	0.1
cytokine	IL6	interleukin-6	0	1	1
cytokine	IL6in	interleukin-6 input	0	1	1
cytokine	IL6fb	interleukin-6 feedback	0	1	1
cytokine	gp130	IL-6 receptor complexed to g	0	1	0.1
cytokine	STAT	signal transducers and activat	0	1	0.1
cytokine	IL1	interleukin-1 alpha and beta	0	1	1
cytokine	IL1in	interleukin-1 input	0	1	1
cytokine	IL1RI	IL1 receptor type I	0	1	0.1
cytokine	TNFa	tissue necrosis factor alpha	0	1	1
cytokine	TNFain	tissue necrosis factor alpha in	0	1	1
cytokine	TNFaR	TNF alpha receptor	0	1	0.1
cytokine	NFKB	nuclear factor kappa-light-ch	0	1	0.1
cytokine	PI3K	phosphoinositide 3-kinase	0	1	0.1
cytokine	Akt	protein kinase B	0	1	0.1
MAPK	p38	a MAP kinase	0	1	0.1
MAPK	TRAF	tnf receptor associated factor	0	1	0.1
MAPK	ASK1	apoptosis signal related kinas	0	1	0.1
MAPK	MKK3	mitogen activated protein kin	0	1	0.1
MAPK	PP1	protein phosphatase 1	0	1	0.1
MAPK	JNK	a MAP kinase	0	1	0.1
MAPK	abl	abl tyrosine kinase	0	1	0.1
MAPK	Rac1	a Rho-dependent GTPase	0	1	0.1
MAPK	MEKK1	a MAP3K associated with p3	0	1	0.1
MAPK	MKK4	a MAP2K associated with p3	0	1	0.1
MAPK	ERK	a MAP kinase	0	1	0.1
MAPK	Ras	representing the family of GT	0	1	0.1
MAPK	Raf	family of raf protein serine/th	0	1	0.1
MAPK	MEK1	a MAP2K mainly specific to	0	1	0.1
adhesion	FAK	focal adhesion kinase	0	1	0.1
g-coupled	epac	exchange protein activated by	0	1	0.1
adhesion	Factin	polymerized actin	0	1	1
adhesion	FA	stabilization of focal adhesio	0	1	1
growth	cmyc	myc transcription factor	0	1	0.1
growth	CTGF	connective tissue growth fact	0	1	0.1

growth	proliferation	proliferation	0	1	10
adhesion	SRF	serum response factor	0	1	0.1
ECM	EDAFN	extra domain A of fibronectin	0	1	10
adhesion	aSMA	alpha-smooth muscle actin	0	1	10
MAPK	AP1	activator protein 1	0	1	0.1
ECM	TIMP1	tissue inhibitor of metallopro	0	1	10
ECM	TIMP2	tissue inhibitor of metallopro	0	1	10
ECM	PAI1	plasminogen activator inhibit	0	1	10
ECM	proMMP14	inactive MMP14	0	1	10
ECM	proMMP1	inactive MMP1	0	1	10
ECM	proMMP2	inactive MMP2	0	1	10
ECM	proMMP9	inactive MMP9	0	1	10
ECM	fibronectin	fibronectin	0	1	10
ECM	periostin	periostin	0	1	10
ECM	proCI	procollagen I	0	1	10
ECM	proCIII	procollagen III	0	1	10
tension	B3int	beta 3 integrin	0	1	0.1
adhesion	Src	proto-oncogene tyrosine-prot	0	1	0.1
MAPK	Grb2	growth factor receptor-bound	0	1	0.1
adhesion	p130Cas	breast cancer anti-estrogen re	0	1	0.1
tension	YAP	yes-associated protein 1	0	1	0.1
adhesion	MRTF	myocardin-related transcripti	0	1	0.1
adhesion	Gactin	monomeric actin	0	1	1
ECM	TNC	tenascin-c	0	1	10
growth	mTORC1	mammalian target of rapamy	0	1	0.1
growth	mTORC2	mammalian target of rapamy	0	1	0.1
growth	p70S6K	p70-S6 kinase 1	0	1	0.1
growth	EBP1	eukaryotic translation initiati	0	1	0.1
tension	syndecan4	syndecan 4	0	1	0.1
ECM	proMMP3	inactive MMP3	0	1	10
ECM	proMMP8	inactive MMP8	0	1	10
ECM	proMMP12	inactive MMP12	0	1	10
ECM	thrombospon	thrombospondin 4	0	1	10
ECM	osteopontin	osteopontin	0	1	10
tension	contractility	intracellular tension	0	1	1
tension	RhoGEF	a Rho guanine nucleotide exc	0	1	0.1
tension	RhoGDI	a Rho GDP-dissociation inhi	0	1	0.1
adhesion	talin	talin 1	0	1	0.1
adhesion	vinculin	vinculin	0	1	0.1
adhesion	paxillin	paxillin	0	1	0.1
adhesion	MLC	myosin regulatory light chain	0	1	0.1
g-coupled	AT2R	angiotensin II receptor type 2	0	1	0.1

estrogen	E2	17-beta estradiol (estrogen 2)	0	1	1
estrogen	E2in	17-beta estradiol (estrogen 2)	0	1	1
estrogen	ERX	estrogen receptor alpha	0	1	0.1
estrogen	ERB	estrogen receptor beta	0	1	0.1
estrogen	GPR30	G protein-protein coupled est	0	1	0.1
estrogen	CyclinB1	cyclin beta 1	0	1	0.1
estrogen	CDK1	cyclin-dependant-kinase 1	0	1	0.1
ECM	LOX	lysyl oxidase	0	1	10

Reaction Information

module	ID	Rule	Weight	n	EC50	
input	i1	=> AngIIin	0.1	1.25	0.6	
input	i2	=> TGFBin	0.1	1.25	0.6	
input	i3	=> tensionin	0.1	1.25	0.6	
input	i4	=> IL6in	0.1	1.25	0.6	
input	i5	=> IL1in	0.1	1.25	0.6	
input	i6	=> TNFain	0.1	1.25	0.6	
input	i7	=> NEin	0.1	1.25	0.6	
input	i8	=> PDGFin	0.1	1.25	0.6	
input	i9	=> ET1in	0.1	1.25	0.6	
input	i10	=> NPin	0.1	1.25	0.6	
input	i11	=> E2in	0.1	1.25	0.6	
input	i12	AngIIin => AngII	1	1.01	0.5	
input	i13	TGFBin => TGFB	1	1.01	0.5	
input	i14	tensionin => tension	1	1.01	0.5	
input	i15	IL6in => IL6	1	1.01	0.5	
input	i16	IL1in => IL1	1	1.01	0.5	
input	i17	TNFain => TNFa	1	1.01	0.5	
input	i18	NEin => NE	1	1.01	0.5	
input	i19	PDGFin => PDGF	1	1.01	0.5	
input	i20	ET1in => ET1	1	1.01	0.5	
input	i21	NPin => NP	1	1.01	0.5	
input	i22	E2in => E2	1	1.01	0.5	
middle	r1	AngII & !ERB => AT1R	1	1.25	0.6	
middle	r2	AT1R => NOX	1	1.25	0.6	
middle	r3	NOX => ROS	1	1.25	0.6	
middle	r4	IL6 => gp130	1	1.25	0.6	
middle	r5	ROS => p38	1	1.25	0.6	
middle	r6	ROS => JNK	1	1.25	0.6	
middle	r7	IL1RI & !ERX => NFKB	1	1.25	0.6	
middle	r8	gp130 => STAT	1	1.25	0.6	
middle	r9	TNFaR => PI3K	1	1.25	0.6	
middle	r10	TGFB1R & !PKG & !smad7 & !ERB => smad3	1	1.25	0.6	
middle	r11	ERK & !ERX => NFKB	1	1.25	0.6	
middle	r12	p38 &!ERX => NFKB	1	1.25	0.6	
middle	r13	ETAR => ROS	1	1.25	0.6	
middle	r14	ERK => AP1	1	1.25	0.6	
middle	r15	cAMP => PKA	1	1.25	0.6	
middle	r16	!smad3 => CBP	1	1.25	0.6	
middle	r17	!CREB => CBP	1	1.25	0.6	
middle	r18	TGFB1R => ACE	1	1.25	0.6	
middle	r19	TGFB & !BAMBI => TGFB1R	1	1.25	0.6	
middle	r20	PKA => CREB	1	1.25	0.6	

middle	r21	NE => BAR	1	1.25	0.6
middle	r22	ET1 => ETAR	1	1.25	0.6
middle	r23	IL1 => IL1RI	1	1.25	0.6
middle	r24	PDGF => PDGFR	1	1.25	0.6
middle	r25	BAR => AC	1	1.25	0.6
middle	r26	BAR & AT1R => AC	1	1.25	0.6
middle	r27	AC => cAMP	1	1.25	0.6
middle	r28	FAK =>MEKK1	1	1.25	0.6
middle	r29	cAMP => epac	1	1.25	0.6
middle	r30	Rho => ROCK	1	1.25	0.6
middle	r31	TNFa => TNFaR	1	1.25	0.6
middle	r32	NP => NPRA	1	1.25	0.6
middle	r33	NPRA => cGMP	1	1.25	0.6
middle	r34	cGMP => PKG	1	1.25	0.6
middle	r35	Ras => Raf	1	1.25	0.6
middle	r36	Raf & !ERK => MEK1	1	1.25	0.6
middle	r37	MEK1 & !PP1=> ERK	1	1.25	0.6
middle	r38	p38 => PP1	1	1.25	0.6
middle	r39	MKK3 => p38	1	1.25	0.6
middle	r40	TGFB1R => TRAF	1	1.25	0.6
middle	r41	Rac1 => MEKK1	1	1.25	0.6
middle	r42	MEKK1 => MKK4	1	1.25	0.6
middle	r43	MKK4 & !NFKB => JNK	1	1.25	0.6
middle	r44	PDGFR => abl	1	1.25	0.6
middle	r45	abl => Rac1	1	1.25	0.6
middle	r46	JNK => cmyc	1	1.25	0.6
middle	r47	TNFaR => TRAF	1	1.25	0.6
middle	r48	TRAF => ASK1	1	1.25	0.6
middle	r49	ASK1 => MKK3	1	1.25	0.6
middle	r50	ASK1 => MKK4	1	1.25	0.6
middle	r51	IL1RI => ASK1	1	1.25	0.6
middle	r52	Ras => p38	1	1.25	0.6
middle	r53	TGFB1R => PI3K	1	1.25	0.6
middle	r54	PDGFR => PI3K	1	1.25	0.6
middle	r55	FAK => PI3K	1	1.25	0.6
middle	r56	TGFB1R => NOX	1	1.25	0.6
middle	r57	Akt & !ERX => NFKB	1	1.25	0.6
middle	r58	JNK => AP1	1	1.25	0.6
middle	r59	IL1RI & TGFB => BAMBI	1	1.25	0.6
middle	r60	STAT => smad7	1	1.25	0.6
middle	r61	Rho & !Rac1 => p38	1	1.25	0.6
middle	r62	MKK4 & !Rho => JNK	1	1.25	0.6
middle	r63	TGFB1R => Rho	1	1.25	0.6
middle	r64	AT1R => Ras	1	1.25	0.6

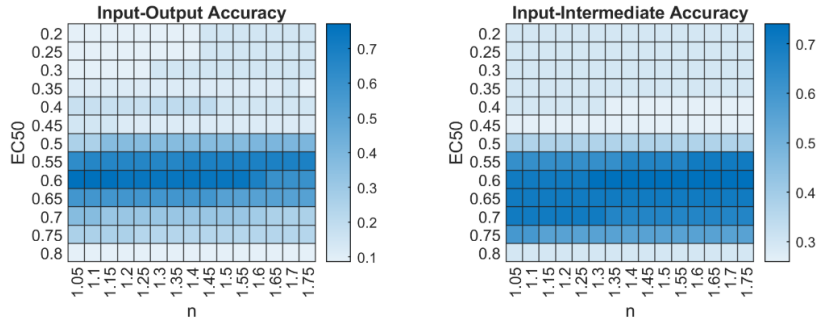
middle	r65	ETAR => DAG	1	1.25	0.6
middle	r66	AT1R => DAG	1	1.25	0.6
middle	r67	DAG => TRPC	1	1.25	0.6
middle	r68	TRPC => Ca	1	1.25	0.6
middle	r69	Ca => calcineurin	1	1.25	0.6
middle	r70	calcineurin => NFAT	1	1.25	0.6
middle	r71	PDGFR => Src	1	1.25	0.6
middle	r72	Akt => mTORC1	1	1.25	0.6
middle	r73	mTORC1 => p70S6K	1	1.25	0.6
middle	r74	!mTORC1 => EBP1	1	1.25	0.6
middle	r75	Akt => smad3	1	1.25	0.6
middle	r76	!p70S6K => mTORC2	1	1.25	0.6
middle	r77	mTORC2 & PI3K => Akt	1	1.25	0.6
middle	r78	mTORC2 & DAG => PKC	1	1.25	0.6
middle	r79	AP1 & !YAP => smad7	1	1.25	0.6
middle	r80	AT1R => YAP	1	1.25	0.6
middle	r81	AngII => AT2R	1	1.25	0.6
middle	r82	ROS & !AT2R => ERK	1	1.25	0.6
middle	r83	tension => B1int	1	1.25	0.6
middle	r84	PKC & tension => B1int	1	1.25	0.6
middle	r85	tension & Src => p130Cas	1	1.25	0.6
middle	r86	tension => TRPC	1	1.25	0.6
middle	r87	tension => AT1R	1	1.25	0.6
middle	r88	B3int => Src	1	1.25	0.6
middle	r89	B1int => FAK	1	1.25	0.6
middle	r90	FAK & Src => Grb2	1	1.25	0.6
middle	r91	Grb2 => Ras	1	1.25	0.6
middle	r92	FAK & Src => RhoGEF	1	1.25	0.6
middle	r93	!Src => RhoGDI	1	1.25	0.6
middle	r94	FAK & Src => p130Cas	1	1.25	0.6
middle	r95	p130Cas & abl => Rac1	1	1.25	0.6
middle	r96	Factin => YAP	1	1.25	0.6
middle	r97	PKA => RhoGDI	1	1.25	0.6
middle	r98	RhoGEF & !RhoGDI & !PKG => Rho	1	1.25	0.6
middle	r99	syndecan4 => PKC	1	1.25	0.6
middle	r100	!PKC => RhoGDI	1	1.25	0.6
middle	r101	NFAT & !Gactin => MRTF	1	1.25	0.6
middle	r102	ROCK & Gactin => Factin	1	1.25	0.6
middle	r103	!Factin => Gactin	1	1.25	0.6
middle	r104	MRTF => SRF	1	1.25	0.6
middle	r105	FAK & Src & MLC => paxillin	1	1.25	0.6
middle	r106	B1int => talin	1	1.25	0.6
middle	r107	B3int => talin	1	1.25	0.6
middle	r108	vinculin & CDK1 & !paxillin => FA	1	1.25	0.6
middle	r109	ROCK => MLC	1	1.25	0.6

middle	r110	E2=> ERX	1	1.25	0.6
middle	r111	E2=> ERB	1	1.25	0.6
middle	r112	E2=> GPR30	1	1.25	0.6
middle	r113	ERB=> cAMP	1	1.25	0.6
middle	r114	ERB=> PKA	1	1.25	0.6
middle	r115	ERX=> Akt	1	1.25	0.6
middle	r116	!GPR30=> CyclinB1	1	1.25	0.6
middle	r117	CyclinB1 & AngII => CDK1	1	1.25	0.6
middle	r118	AT1R & !CDK1=>TGFB	0	1.25	0.6
middle	r119	GPR30=>Akt	1	1.25	0.6
output	r120	CDK1=> proliferation	1	1.25	0.6
output	r121	AP1 => proliferation	1	1.25	0.6
output	r122	CREB => proliferation	1	1.25	0.6
output	r123	CTGF => proliferation	1	1.25	0.6
output	r124	PKC => proliferation	1	1.25	0.6
output	r125	!EBP1 & p70S6K => proliferation	1	1.25	0.6
output	r126	cmyc => proliferation	1	1.25	0.6
output	r127	NFKB & AP1 & !smad3 => proMMP1	1	1.25	0.6
output	r128	AP1 => proMMP2	1	1.25	0.6
output	r129	STAT => proMMP2	1	1.25	0.6
output	r130	STAT => proMMP9	1	1.25	0.6
output	r131	AP1 & NFKB => proMMP9	1	1.25	0.6
output	r132	AP1 => proMMP14	1	1.25	0.6
output	r133	NFKB => proMMP14	1	1.25	0.6
output	r134	NFKB & AP1 & !smad3 => proMMP8	1	1.25	0.6
output	r135	NFKB & AP1 & !smad3 => proMMP3	1	1.25	0.6
output	r136	CREB => proMMP12	1	1.25	0.6
output	r137	AP1 => TIMP1	1	1.25	0.6
output	r138	AP1 => TIMP2	1	1.25	0.6
output	r139	smad3 => PAI1	1	1.25	0.6
output	r140	YAP => PAI1	1	1.25	0.6
output	r141	SRF => proCI	1	1.25	0.6
output	r142	SRF => proCIII	1	1.25	0.6
output	r143	smad3 & CBP & !epac=> proCI	1	1.25	0.6
output	r144	smad3 & CBP & !epac=> proCIII	1	1.25	0.6
output	r145	smad3 & CBP => fibronectin	1	1.25	0.6
output	r146	NFKB => fibronectin	1	1.25	0.6
output	r147	NFAT => EDAFN	1	1.25	0.6
output	r148	smad3 & CBP => periostin	1	1.25	0.6
output	r149	CREB & CBP => periostin	1	1.25	0.6
output	r150	NFKB => TNC	1	1.25	0.6
output	r151	MRTF => TNC	1	1.25	0.6
output	r152	smad3 => thrombospondin4	1	1.25	0.6
output	r153	AP1 => osteopontin	1	1.25	0.6

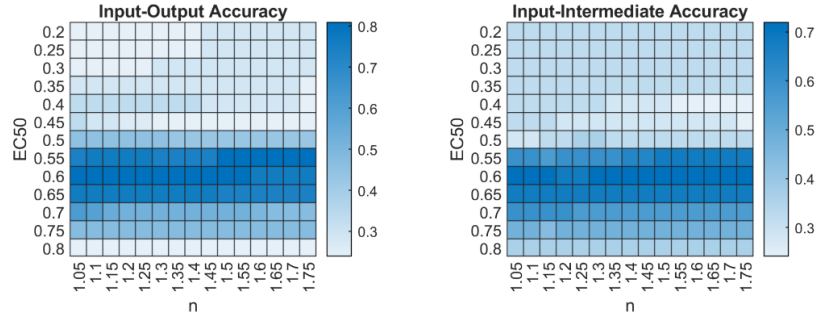
output	r154	smad3 & CBP & ERK => CTGF	1	1.25	0.6
output	r155	YAP => CTGF	1	1.25	0.6
output	r156	Akt => LOX	1	1.25	0.6
output	r157	YAP => aSMA	1	1.25	0.6
output	r158	smad3 & CBP => aSMA	1	1.25	0.6
output	r159	SRF=> aSMA	1	1.25	0.6
fback	r160	AP1 => latentTGFB	1	1.25	0.6
fback	r161	proMMP9 & latentTGFB => TGFBfb	1	1.25	0.6
fback	r162	proMMP2 & latentTGFB => TGFBfb	1	1.25	0.6
fback	r163	tension & latentTGFB => TGFBfb	1	1.25	0.6
fback	r164	!AT1R & !JNK & p38 => AGT	1	1.25	0.6
fback	r165	ACE & AGT => AngIIfb	1	1.25	0.6
fback	r166	CREB + CBP => IL6fb	1	1.25	0.6
fback	r167	NFKB => IL6fb	1	1.25	0.6
fback	r168	AP1 => IL6fb	1	1.25	0.6
fback	r169	AP1 => ET1fb	1	1.25	0.6
fback	r170	Factin & MLC => contractility	1	1.25	0.6
fback	r171	aSMA & MLC => contractility	1	1.25	0.6
fback	r172	!thrombospondin4 & tension => B3int	1	1.25	0.6
fback	r173	osteopontin => B3int	1	1.25	0.6
fback	r174	!TNC & tension => syndecan4	1	1.25	0.6
fback	r175	talin & contractility => vinculin	1	1.25	0.6
fback	r176	contractility & FA => tensionfb	1	1.25	0.6
fback	r177	TGFBfb => TGFB	0.25	1.01	0.5
fback	r178	AngIIfb => AngII	0.25	1.01	0.5
fback	r179	IL6fb => IL6	0.25	1.01	0.5
fback	r180	ET1fb => ET1	0.25	1.01	0.5
fback	r181	tensionfb => tension	0.25	1.01	0.5

Appendix C

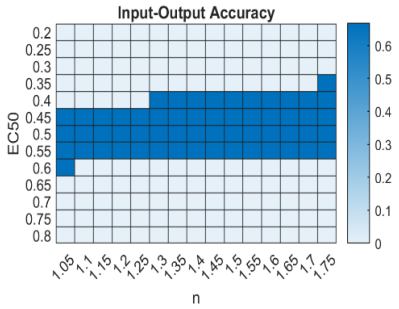
A) Pooled



B) Male



C) Female



Appendix D

Drug name	Binding	Action	Target
Bosutinib	Non-Competitive	Antagonist	abl
Dasatinib	Non-Competitive	Antagonist	abl
Nilotinib	Non-Competitive	Antagonist	abl
Ponatinib	Non-Competitive	Antagonist	abl
Benazepril	Competitive	Antagonist	ACE
Captopril	Competitive	Antagonist	ACE
Cilazapril	Competitive	Antagonist	ACE
Enalapril	Competitive	Antagonist	ACE
Fosinopril	Competitive	Antagonist	ACE
Lisinopril	Competitive	Antagonist	ACE
Moexipril	Competitive	Antagonist	ACE
Perindopril	Competitive	Antagonist	ACE
Quinapril	Competitive	Antagonist	ACE
Ramipril	Competitive	Antagonist	ACE
Rescinnamine	Competitive	Antagonist	ACE
Spirapril	Competitive	Antagonist	ACE
Trandolapril	Competitive	Antagonist	ACE
Arsenic trioxide	Competitive	Agonist	AP1; ERK
Azilsartan medoxomil	Competitive	Antagonist	AT1R
Candesartan	Competitive	Antagonist	AT1R
Eprosartan	Competitive	Antagonist	AT1R
Forasartan	Competitive	Antagonist	AT1R
Irbesartan	Competitive	Antagonist	AT1R
Olmesartan	Competitive	Antagonist	AT1R
Tasosartan	Competitive	Antagonist	AT1R
Telmisartan	Competitive	Antagonist	AT1R
Valsartan	Competitive	Antagonist	AT1R
Losartan	Non-Competitive	Antagonist	AT1R
Saprisartan	Non-Competitive	Antagonist	AT1R
A.T. Globulin	Non-Competitive	Antagonist	B1int
Arbutamine	Competitive	Agonist	BAR
Arformoterol	Competitive	Agonist	BAR
Bambuterol	Competitive	Agonist	BAR
Clenbuterol	Competitive	Agonist	BAR

Dipivefrin	Competitive	Agonist	BAR
Dobutamine	Competitive	Agonist	BAR
Droxidopa	Competitive	Agonist	BAR
Ephedra	Competitive	Agonist	BAR
Epinephrine	Competitive	Agonist	BAR
Fenoterol	Competitive	Agonist	BAR
Formoterol	Competitive	Agonist	BAR
Indacaterol	Competitive	Agonist	BAR
Isoetarine	Competitive	Agonist	BAR
Isoprenaline	Competitive	Agonist	BAR
Norepinephrine	Competitive	Agonist	BAR
Olodaterol	Competitive	Agonist	BAR
Orciprenaline	Competitive	Agonist	BAR
Pirbuterol	Competitive	Agonist	BAR
Procaterol	Competitive	Agonist	BAR
Ritodrine	Competitive	Agonist	BAR
Salbutamol	Competitive	Agonist	BAR
Salmeterol	Competitive	Agonist	BAR
Terbutaline	Competitive	Agonist	BAR
Vilanterol	Competitive	Agonist	BAR
Acebutolol	Competitive	Antagonist	BAR
Alprenolol	Competitive	Antagonist	BAR
Atenolol	Competitive	Antagonist	BAR
Betaxolol	Competitive	Antagonist	BAR
Bevantolol	Competitive	Antagonist	BAR
Bisoprolol	Competitive	Antagonist	BAR
Bopindolol	Competitive	Antagonist	BAR
Bupranolol	Competitive	Antagonist	BAR
Carteolol	Competitive	Antagonist	BAR
Carvedilol	Competitive	Antagonist	BAR
Celiprolol	Competitive	Antagonist	BAR
Esmolol	Competitive	Antagonist	BAR
Labetalol	Competitive	Antagonist	BAR
Levobunolol	Competitive	Antagonist	BAR
Metipranolol	Competitive	Antagonist	BAR
Metoprolol	Competitive	Antagonist	BAR
Nadolol	Competitive	Antagonist	BAR

Nebivolol	Competitive	Antagonist	BAR
Oxprenolol	Competitive	Antagonist	BAR
Penbutolol	Competitive	Antagonist	BAR
Pindolol	Competitive	Antagonist	BAR
Practolol	Competitive	Antagonist	BAR
Propranolol	Competitive	Antagonist	BAR
Sotalol	Competitive	Antagonist	BAR
Timolol	Competitive	Antagonist	BAR
Amiodarone	Non-Competitive	Antagonist	BAR
Amyl Nitrite	Non-Competitive	Agonist	cGMP
Erythrityl Tetranitrate	Non-Competitive	Agonist	cGMP
Isosorbide Dinitrate	Non-Competitive	Agonist	cGMP
Nitroglycerin	Non-Competitive	Agonist	cGMP
Nitroprusside	Non-Competitive	Agonist	cGMP
Ocriplasmin	Non-Competitive	Antagonist	EDAFN
Ambrisentan	Competitive	Antagonist	ETAR
Bosentan	Competitive	Antagonist	ETAR
Maitentan	Competitive	Antagonist	ETAR
Sitaxentan	Competitive	Antagonist	ETAR
Tocilizumab	Competitive	Antagonist	gp130
Canakinumab	Competitive	Antagonist	IL1
Gallium nitrate	Non-Competitive	Antagonist	IL1
Anakinra	Competitive	Antagonist	IL1R1
Siltuximab	Competitive	Antagonist	IL6
Cobimetinib	Competitive	Antagonist	MEK1
Trametinib	Non-Competitive	Antagonist	MEK1
Marimastat	Competitive	Antagonist	MMP1; MMP2; MMP9; MMP14
Glucosamine	Non-Competitive	Antagonist	MMP9
Triflusal	Non-Competitive	Antagonist	NFKB
Thalidomide	Non-Competitive	Antagonist	NFKB; TNFa
Sacubitril	Non-Competitive	Agonist	NP
Entresto	Competitive	Both	NPRA (Agonist); AT1R (Antagonist)

Urokinase	Non-Competitive	Antagonist	PAI1
Becaplermin	Competitive	Antagonist	PDGFR
Pazopanib	Non-Competitive	Antagonist	PDGFR
Sunitinib	Non-Competitive	Antagonist	PDGFR
Sorafenib	Non-Competitive	Antagonist	PDGFR; Raf
Regorafenib	Non-Competitive	Antagonist	PDGFR; Raf; abl
Dabrafenib	Non-Competitive	Antagonist	Raf
Fasudil	Competitive	Antagonist	ROCK
Ripasudil	Competitive	Antagonist	ROCK
Galunisertib	Non-Competitive	Antagonist	TGFB1R
Lucanix	Competitive	Antagonist	TGFBmRNA
Trabedersen	Competitive	Antagonist	TGFBmRNA
Adalimumab	Non-Competitive	Antagonist	TNF
Certolizumab pegol	Non-Competitive	Antagonist	TNF
Infliximab	Non-Competitive	Antagonist	TNF
Pomalidomide	Non-Competitive	Antagonist	TNF
Etanercept	Competitive	Antagonist	TNFa
Golimumab	Competitive	Antagonist	TNFa

Appendix E

Module 1: Tumor Growth Simulation & Limitations
Platform: NetLogo
Following this lesson students should be able to: **1) Describe basic cancer cell metabolism and migration** **2) Utilize NetLogo's built-in sample models** **3) Develop and test predictions of therapeutic targets using a tumor model** **4) Evaluate a model's limitations**
Purpose: This module employees a built-in NetLogo tumor model. Biologically, students will observe the progression and treatment of a disease from a cellular level. Computationally, students will gain experience in using a model to test predictions as well as identifying model limitations.
Biological Terms: **1) Tumor: an abnormal mass of cells** **2) Cancer: the uncontrolled continues replication of cells** **3) Apoptosis: programmed cell death** **4) Metastasis: the spread of cancer cells to other parts of the body** **5) Remission: the disappearance of the symptoms and signs of cancer**
Computational Terms: **1) Computational Model: a mathematical model used to study the behavior of a complex system** **2) Systems Biology: the modeling of complex biological systems** **3) Limitations: refers to simplifications or missing details in a model that make it unable to capture the full natural phenomena** **4) Stem Cell: a term in the model referring to original cancer cells (blue dots). Can replicate into more stem cells and transitory cells. They also can metastasize** **5) Transitory Cell: a term in the model referring to cells derived from an original cancer stem cell (red dots). They can divide into more cancer transitory cells but will slow (white dots) and undergo apoptosis (black dots) after a few replications**

Time Estimation:

1) **In-Class Activity: 30 minutes**
2) **Model Tutorial: 30 minutes**
3) **Model Testing and Advancement: 15 minutes**

Total: ~1 hour 15 minutes

Part One: In-Class Activity

Materials: UNO or standard card decks

Rules:

1) **Split students into groups of ~4-8**
2) **Have only half the number of students play at first and the others observe**
3) **Have them play UNO as normal**
4) **After two minutes, have students give green cards (split evenly) to spectating students who draw an additional 5 cards and join the game (models migration and formation of secondary tumor)**
5) **Keep playing UNO as normal**
6) **After two more minutes, have students discard any card that is an even number (models a treatment of killing transitory cells)**
7) **Have them keep playing as normal**
8) **After two more minutes, have all students draw a card. If a student draws a yellow card then they no longer have to draw cards if they don't have a move for the remainder of the game (models a treatment of killing stem cells)**
9) **Have them keep playing for an additional five-ten minutes or until the game is over**
10) **Discuss observations in small groups or as a class**

Modification for Individual Activity: A single student can deal out hands for all "participants" with cards face-up, then proceed through steps above while playing all hands.

Modification for standard card deck: The game should be easily modified for a standard deck of cards by changing the rules to use suits instead of colors and assigning typical UNO rules like "draw 4" or "skip" to face cards.

Suggested Discussion Questions:

1) **Did the students who benefited from the "treatments" run out of cards faster?**
2) **How is UNO a good model of cancer metabolism and migration?**
3) **How is UNO NOT a good model of cancer metabolism and migration (i.e. what were the limitations)?**

Part Two: Model Tutorial

1) **Open NetLogo**
2) **Under File choose Model Library**
3) **Choose Tumor under Biology>Evolution**
4) **Click Setup button**
5) **Click Go button and watch as the model very quickly expands into a large original tumor and a smaller metastasized tumor. Also note that the graph almost immediately levels off. To stop the model, hit Go again**
6) **Adjust the speed bar so that it is slower (very far left)**
7) **Click the Setup button again to reset the model and run it again by clicking Go**
    a. **What type of relationship does the graph appear to have now?**
8) **Play around with the interventions (Kill Transitory Cells, Kill Moving Stem Cell, and Kill Original Stem Cell).**
    a. **What happens when you Kill Original Stem Cell <u>immediately</u> (before first replication)?**
    b. **What happens when you Kill Original Stem Cell <u>before</u> the tumor has metastasized?**
    c. **What happens when you Kill Original Stem Cell <u>after</u> the tumor has metastasized?**
    d. **What happens when you Kill Transitory Cell <u>immediately</u> (before first replication)?**

e. **What happens when you Kill Transitory Cell before the tumor has metastasized?**

f. **What happens when you Kill Transitory Cell after the tumor has metastasized?**

g. **What happens when you Kill Moving Stem Cell immediately (before first replication)?**

h. **What happens when you Kill Moving Stem Cell before the tumor has metastasized?**

i. **What happens when you Kill Moving Stem Cell after the tumor has metastasized?**

j. **All three interventions achieved remission if applied before the first replication, why is this likely not a feasible treatment option?**

k. **If you could only apply one intervention, which do you think offers the best chance of remission? Does this depend on whether the tumor has metastasized already?**

Part Three: Model Testing and Advancement:

1) **Using your analyses from step 8, predict the best combined treatment (i.e. (1) Kill Transitory Cells and Original Stem Cell, (2) Kill Transitory Cell and Moving Stem Cell, or (3) Kill Moving Stem Cell and Original Stem Cell) for a tumor that has <u>already metastasized</u>.**
    a. **Prediction:**

2) **Test your prediction with the model.**
    a. **Did your treatment achieve remission? Explain.**

3) **Because this model is extremely basic, it has many limitations which make it unlikely to ever to be used to decide an actual treatment plan.**
    a. **In your opinion, what are the model's three biggest limitations?**

    b. **Choose one of these limitations and describe what you think should be added to the model to address this limitation.**

Module 2: Virus Prevention Simulation and Model Inputs

Platform: NetLogo

Following this lesson students should be able to:

1) **Describe the factors that can impact a virus's spread throughout a population**
2) **Modify a given Netlogo model by adding and deleting buttons from the main interface**
3) **Research the literature to supply model with input parameters from published experimental data**
4) **Determine what additional rules and/or variables should be added to a model to address its limitations**

Purpose: This module modifies the built-in NetLogo Virus code to introduce additional parameters that model an intervention to slow the spread of a virus within a population. Biologically, students will examine population dynamics and viral spread. Computationally, students will research and test the effect of input parameters on a model.

Biological Terms:

1) **Virus: a microorganism that can only reproduce by infecting living cells**
2) **Infectiousness: the likelihood (probability) that a contagious disease will be transmitted from one person to another**
3) **Transmission: the act of spreading a disease, like a virus, form organism to another**
4) **Immunity: the ability of an organism to fight off an infection. After an organism has survived an initial infection of a virus, they often have immunity from that same virus for a long period of time**

Computational Terms:

1) **Agents: beings in a model that can follow given instructions**
2) **Turtles: agents in NetLogo that can move around in the world**
3) **Patches: agents in NetLogo that make up the 2-D space turtles move around on**
4) **Parameters: a variable included in a model that can be estimated from data**
5) **Buttons: a feature in NetLogo which allows the user to adjust parameters without manipulating the code**

Time Estimation:

1) **In-Class Activity: 30 minutes**
2) **Model Tutorial: 30 minutes**
3) **Model Testing and Advancement: 1 hour**

Total: ~2 hours

Module 2: In-Class Activity

Materials: Dice, meter stick, and note cards or stickers to identify different student groups

Rules:

1) **Split the students up into 4 groups in an area where they can walk around but not so big that they will be too spread out**
2) **Let each group decide input parameters for their group:**
    a. **Define how many people should be in each subgroup to start: Sick-Unmasked ____, Sick-Masked ____, Healthy-Masked ____, Healthy-Unmasked ____**
    b. **Define how close in feet students need to be for it to be considered "close contact" ____**
    c. **Define the infection rate for Mask-Mask contact (i.e. roll a two= 1/6) ____ and Mask-Unmask contact (i.e. roll any even number= 1/2) ____ if one of the individuals is sick**
3) **Have one group perform their simulation at a time while the other groups observe**
4) **For each simulation, have students walk around randomly and stop them after five seconds**
5) **If there is a healthy and sick student within the decided distance have each close contact roll a die to determine if the virus will spread**
6) **Repeat steps 3-4 one more time and tell students who were sick to start with that they are now immune and cannot get sick again**
7) **Repeat steps 3-5, remembering to tell students they are immune after two cycles until all students are either healthy or immune**
8) **Repeat steps 4-7 for the remaining three groups**
9) **Discuss observations in small groups or as a class**

Modification for Individual Activity: A single student can cut out small squares of paper, label one side of each square "healthy" (or a smiling face), and the other side "infected" (or a frowning face, virus picture, etc.). Proceed through the steps above.

Suggested Discussion Questions:

1) **Which input parameter do you think would have had the greatest effect?**
2) **Did each group's simulation take about the same about of time to finish? Why or why not?**
3) **What are some limitations of this model? Would adding/changing any of the input parameters address these limitations?**

Part Two: Model Tutorial
1) Open NetLogo
2) Under File choose Model Library
3) Choose Virus under Biology>Evolution
4) Click Setup button
5) Click Go button and observe
6) To stop the model, hit Go again. This module explains how a virus can spread through a population, but it is not detailed enough to exhibit or test how we could slow or stop the spread of a virus. One proven way to slow the spread of most viruses is to wear masks. This is something Asian countries have been doing for years, but something that did not become popular in the U.S. until the COVID-19 outbreak of 2020
7) Download the Virus_Mask text file. This file has been modified to include variables that allow for the testing of how mask compliance in a population can influence the spread of an infectious disease
8) Copy the text from the file and paste it into the code of the Virus module
9) Navigate back to the main interface, you will likely get an error message that says certain variables have not been defined which is what we will do in the next few steps
10) Right click on the button that says Turtle Shape and delete the box
11) Press the Button drop down box near the top of the screen and choose a Slider to add by clicking on an open area under the sliders already in the interface
12) In the pop-up menu, name the global variable infectiousness-mask and change the units to %. Hint: Make sure to type this and later variable names exactly because it directly refers to the code
13) Repeat step 10 but call this global variable mask-compliance and also change the units to %
14) Repeat step 10 but call this global variable sick-people and also change the units to %. You should now have 7 sliders (number-people, infectiousness, chance-recover, duration, infectiousness-mask, mask-compliance, and sick-people) which allow you to manually adjust the model input parameters
15) Under Tools, choose Turtles Shapes Editors

16) Scroll down to Person and click Duplicate
17) Name the person mask-person
18) Use the shape tools to draw a mask on the person and click Okay when finished
19) Exit out of the Turtles Shapes Editors
20) Click Setup, there should no longer be any errors and many figures should pop up in the black model screen some of whom are healthy (green) and some that are sick (red). The starting amount of people can be adjusted with the slider number-people. Some of the people should also be wearing masks, the percentage of people wearing a mask can be adjusted with the mask-compliance slider

Part Three: Model Testing and Advancement

1) **One important consideration when making a model is to use data from the literature for input parameters to increase its accuracy. Choose a virus and look-up values for infectiousness (likelihood of transmission without mask), infectiousness-mask (likelihood of transmission with mask), chance-recover (survival rate), and duration (average length of infection). Good resources for this include: https://www.cdc.gov/ or https://pubmed.ncbi.nlm.nih.gov/**
   a. **What values did you find?**
      Infectiousness:
      Infectiousness mask:
      Chance recover:
      Duration:
2) **Incorporate these values into the model by using the sliders**
3) **Now, make a hypothesis about the percentage of mask-compliance that would be necessary to eliminate the virus in exactly 6 months (0.5 years) if 25% (sick-people) of the population was already infected. Hint: To make observation easier, you may need to adjust the speed of the model with the speed slider at the top of the interface**
   a. **Hypothesis:**
4) **Test your prediction by running the model until the infection rate reaches 0%.**
   a. **Did you ever reach 0% infection?**
   b. **How long did it take to reach 0% infection?**
   c. **If it did not take exactly 6 months, try adjusting your prediction for mask-compliance and repeat the model test until you get closer to six months. Explain your adjustments and outcomes.**

5) **The main rule governing this model is that if a sick turtle is on the same patch as another turtle it determines if the other turtle is (1) sick (2) immune and (3) wearing a mask. If the turtle is already sick or immune, nothing happens. If the other turtle is not sick or immune already, it will determine if the turtle is wearing a mask and a random number between 0-100 will be generated and if this number is less than the infectiousness rate set by the user for each scenario, the other turtle will get sick.**
   The code for this is:

```
to infect ;; turtle procedure
  ask other turtles-here with [ not sick? and not immune? and not mask? ]
    [ if random-float 100 < infectiousness
      [get-sick]]
   ask other turtles-here with [ not sick? and not immune? and mask]
    [ if random-float 100 < infectiousness-mask
      [get-sick]]
end
```

   A major limitation of this rule is that it does not consider if the sick turtle is wearing a mask; the infection rate is solely decided by if the other non-sick turtle is wearing a mask.
   a. **What other parameters and rules would have to be added to the model to address this limitation? Either describe in words or attempt a coded notation of what should be added.**

6) **This model is extremely basic and has many limitations which make it unlikely to ever to be used to decide an intervention plan**
   a. **In your opinion, besides the one mentioned above what are the model's three biggest limitations? Hint: you might discuss other interventions to slow a virus's spread besides just wearing masks that the current model fails to consider.**

b. Choose one of these limitations and describe what you think should be added to the model to address this limitation.

Module 3: Immune Reaction Simulation Rules

Platform: NetLogo

Following this lesson students should be able to:

1) **Describe the body's immune response due to an allergen**
2) **Define rules for agents of a model given a biological phenomenon**
3) **Code simple rules/commands using NetLogo**
4) **Evaluate how assumptions can increase a model's limitations**

Purpose: This module will guide students through a tutorial to code a NetLogo model from scratch that simulates an allergic reaction. Biologically, students will consider how different cells and molecules interact to elicit an immune response. Computationally, students will define rules and make assumptions to create an agent-based model.

Biological Terms:

1) **Allergen: a substance that when ingested or inhaled can elicit an immune response**
2) **Antibody: a 'Y' shaped protein that recognizes specific antigen such as an allergen**
3) **Immunoglobulin E (IgE): the type of antibody produced by the body after the first encounter with an allergen that will recognize it again**
4) **Mast Cells: are coated with IgEs which will recognize a second encounter with an allergen and cause the mast cell to release granules such as histamines**
5) **Histamine: a compound that can interact with white blood cells and proteins to cause symptoms of an allergic reaction such as itching or sneezing**

Computational Terms:

1) **Agent-based model: a type of modeling governed by agents that have been given a set of rules**
2) **Rules: the instructions given to an agent that defines its decision-making process**
3) **Assumptions: user-determined simplifications that are intended to decrease the complexity of a model**
4) **Command: a term used in programming to indicate a coded instruction for a program or model to carry out**
5) **Breed: a term in NetLogo to define an agent set with distinguished characteristics (i.e. size, shape, color, and rules) from other turtles in the simulation**

Time Estimation:

1) **In-Class Activity: 30 minutes**
2) **Model Tutorial: 1 hour**
3) **Model Testing and Advancement: 1 hour 30 minutes**

Total: 3 hours

Part One: Unplugged Activity

Materials: multicolored beads

Rules:

1) **Split students into groups of ~4-5**
2) **Give each group a bag of multi-colored beads and have them devise their own activity to model an allergic reaction using the beads. Have students define rules for the activity making sure to define what would cause a reaction (e.g., picking a yellow bead), a reaction response (e.g., do ten jumping jacks), and what will end the activity (e.g., everyone picks 5 beads).**
3) **Have students conduct their activities in their groups while the other groups observe**
4) **Discuss as a class**

Suggested Discussion Questions:

4) **What different rules did students come up with?**
5) **Were there certain rules that worked "better" than others to model an allergic reaction?**
6) **Were there any groups that were not able to conduct their activity smoothly because they did have enough rules (or too many)?**

Part Two: Model Tutorial

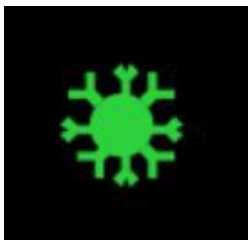
1) **Open NetLogo**
2) **Instead of using one of the built-in codes, you will be making one from scratch today. Click the Code tab to get started**
3) **Define the three breeds of variables that will be part of the model: allergens, mast cells, and histamines using breed [plural_form singular_form]**

```
breed [allergens allergen]
breed [mast-cells mast-cell]
breed [histamines histamine]
```

4) **To set up the simulation, make a command called to setup. Under the command, type 4 actions (1) clear-all, which will clear all variables each time the code is run (2) reset-ticks, which will initialize the time to be 0 (3) set-mast-cells, a command which will initialize the mast-cells on the main interface (4) set-allergens, a command which will initialize the allergens on the main interface. Finish the command by typing end**

```
to setup
 clear-all
 reset-ticks
 set-mast-cells
 set-allergens
end
```

5) **To create mast-cells on the main interface, make a command called to set-mast-cells. Under the command, define the shape of the turtle (variable) by typing set-default-shape turtle "mast cell".**
6) **Because mast cell is not already in the shape catalog for NetLogo, you will need to draw your own. Under Tools click Turtles Shapes Editor and press New. Name the shape mast cell and once you finish drawing click Ok and exit the shapes editor. An example of a shape that resembles a mast cell with IgE antibodies attached:**



7) **Under set-default-shape turtled “mast cell” type create-mast-cells initial-number-mast-cells which will allow you to initialize the number of mast cells manually on the main interface**

8) **To define the position, size, and color of the mast cells in the simulation, under create-mast-cells initial-number-mast-cells type [followed by (1) setxy random-xcor random-ycor which will randomize the distribution of mast cells across the screen. (2) set size 5 which will make the mast cells larger than the other variables. If you would like to try different sizes, try choosing other numbers besides 5. (3) set color green which will make the mast cells green, like with the size you can customize to any color of your choosing. Finish the command by typing] and end**

```
to set-mast-cells
set-default-shape turtles "mast cell"
create-mast-cells initial-number-mast-cells
  [
  setxy random-xcor random-ycor
  set size 5
  set color green
  ]
end
```

9) **To create allergens on the main interface, make a command called to set-allergens. Under the command define the shape of the turtle (variable) by typing set-default-shape turtle “circle”.**

10) **Under set-default-shape turtled “circle” type create-allergens initial-number-allergens which will allow you to initialize the number of allergens manually on the main interface**

11) **To define the position, size, and color of the allergens in the simulation, under create-allergens initial-number-allergens type [followed by (1) setxy random-xcor random-ycor which will randomize the distribution of allergens across the screen. (2) set size .5 which will make the allergens smaller than the mast cells (3) set color one-of-base-colors which will make each of the allergens a random color. Finish the command by typing] and end**

```
to set-allergens
  set-default-shape turtles "circle"
  create-allergens initial-number-allergens
  [
  setxy random-xcor random-ycor
  set size .5
  set color one-of base-colors
  ]
end
```

12) Navigate back to the main interface by clicking Interface. You might receive an error message saying certain variables have not been defined but that is what we will do in the next few steps

13) Under Button click Button, place it near the top of the white part of the main interface, name it setup, and click Ok. This will call back to the to setup command you made earlier

14) Under Button click Slider, place it the white area of the main interface and name it initial-number-mast-cells, and click Ok. This will allow you to define the initial number of mast cells in the simulation

15) Repeat step 14, but name this slider initial-number-allergens which will allow you to define the initial number of allergens in the simulation

16) You should no longer have any error messages. Click the setup button which should populate the black screen with number of mast cells and allergens indicated on the sliders in the colors, sizes, and shapes you defined in the code

17) As it is now, the simulation is stagnant and you will need to code in movement and interactions. Navigate back to the code by clicking Code

18) Make a command called to go. Under to go type ask turtles [move], followed by tick and end. You will need to also define move (step 19) and tick indicates a passage of time in the simulation

```
to go
 ask turtles
  [
    move
  ]
  tick
end
```

19) Make a command called to move. Under the command type (1) rt random 100 which will have the turtles turn right at a random angle, (2) lt random 100 which will have the turtles turn left at a random angle, and (3) fd 1 which will move the turtle forward 1 patch. Finish the command by typing end

```
to move
  rt random 100
  lt random 100
  fd 1
end
```

20) Navigate back to the main interface by clicking Interface. Under Button choose Button and add it near the top of the white part of the main screen, name it go, check the box that says Forever, and click Ok. This will call back to you to go command in the code and will allow the simulation to run continuously until go is clicked again. You should now see the turtles moving around the black screen. They are likely moving very fast, to slow the speed move the speed slider near the top of the screen towards the left

21) As it is now the turtles do not interact in the simulation. In part 3 you will code in an interaction between the mast cells and allergens which will model an allergic reaction

Part Three: Model Testing and Advancement:

1) **To define rules for the agents in the simulation you will need to determine what you want the rules to be. Consider what happens during an allergic reaction to an allergen and a mast cell. Specifically, think about:**
   - **a. How a reaction is initiated (e.g., is direct contact between a mast cell and allergen necessary?).**
   - **b. Are allergens used up during a reaction or can they renter the blood steam?**
   - **c. Are mast cells used up during a reaction?**
   - **d. Can mast cells recognize multiple different types of allergens?**
   - **e. What products are formed due to an allergen and mast cell interaction? [Hint: keep it simple, remember you already defined a third breed of turtle that should suffice as your products]**

2) **Using your considerations above determine at least 3 rules that you will code into the model. For example:**
   a. **A reaction will be initiated if a mast cell comes into contact with an allergen for which it has a specific IgE antibody**
   b.
   c.

3) **Identify any assumptions you may want to make in order to decrease the complexity of your model. For example, in the tutorial the assumption was made that IgE was already present on the mast cells and therefore was not included in the simulation. An additional assumption based on the rule defined above could be that a mast cell will only need to come in contact with one allergen to elicit a response.**
   a. **Explain any additional assumptions:**

4) **Using these rules, determine the code that will need to be added to the model to carry out the rules. Use NetLogo Dictionary (http://ccl.northwestern.edu/netlogo/docs/index2.html) for help on syntax and codes that are available. [Hint: the codes hatch, ask, and die might be helpful]. An example of a code for the first rule would be:**

```
to have-reaction
  if any?  mast-cells-on allergens with [color = alle
```

This code essentially asks if any mast cells come into contact with a certain colored allergen. If true additional commands (what you will write) should follow to simulate the response. It will likely take several tries to get a code that works and is in the correct syntax. To aid in troubleshooting, click Check at the top of the screen which will indicate any errors. Also important, I introduced a new variable allergy so if you used my code, you will need to define what allergy is or simply change it to a color in the code [Hint: try using the buttons in the main interface to allow manual input of a color]. Finally, make sure to add have-reaction under the to go command in order to initiate it when the simulation is started.

5) **Once you have a working code, it is important to test that it works. For example, to test the code provided, you could slow the**

simulation down and decrease the number of allergens and mast cells in order to be able to watch carefully and ensure that a reaction was *only* initiated when a mast cell came into direct contact with an allergen of the color chosen. Come up with at least two additional tests that you could perform to determine if your code works. [Hint: this can include adding a plot button to measure the amount of a certain variable, manipulating variables to see the effect on the simulation, or visually watching for something to happen].

a.

b.

6) **Perform your tests. If your code is not performing how you anticipated try troubleshooting or writing a new code and retry your test.**
   a. **Describe the outcome of your tests and troubleshooting process.**

7) **This model is extremely basic and has many limitations which do not capture the full response of an allergic reaction.**
    a. **In your opinion, what are the model's three biggest limitations? Especially consider any assumptions that were made when answering this question.**

    b. **Choose one of these limitations and describe what you think should be added to the model to address this limitation.**

Module 4: Gene Regulation and Stability Using Boolean Logic

Platform: NetLogo

Following this lesson students should be able to:

1) **Describe the components that make up a lac operon and how it functions**
2) **Apply Boolean logic to biological phenomena**
3) **Identify stability in a biological process and computational system**
4) **Evaluate model outputs to determine emergent phenomena**

Purpose: This module will guide students through a tutorial to code a NetLogo model from scratch that uses Boolean Logic to create a simulation of the lac operon. Biologically, students will be exposed to a common model of gene regulation. Computationally, students will define rules using Boolean logic and analyze model outputs to identify a system's emergent phenomena.

Biological Terms:

1) **Lac operon: the set of genes that regulate the creation of enzymes to break down lactose**
2) **Promotor: the binding site of RNA polymerase**
3) **Repressor: a protein that inhibits the transcription of the lac operon when lactose is not present**
4) **Operator: the binding site of the repressor protein**
5) **Lactose: the backup energy source**
6) **Glucose: the preferred energy source**
7) **Catabolite Activator Protein (CAP): a protein that becomes inactive with high levels of glucose**
8) **RNA polymerase: the protein that transcribes the lac operon**
9) **CAP Site: the binding site of CAP which speeds up the transcription of the lac operon when CAP is inactive**
10) **Transcription: the first step of expressing new proteins from genetic material**

Computational Terms:

1) **Boolean Logic: a problem which reports only a True or False value**
2) **If: used to carry out a function only when a Boolean problem is True**
3) **If Else: used to carry out two functions, one when a Boolean problem is True and one when it is False**

4) **Emergent phenomena: property that arises from the collective behavior of a dynamic system** 5) **Stability: the ability to converge to an equilibrium across a range of inputs or perturbations (unstable systems do not move toward an equilibrium)** 6) **Bistability: two states of equilibrium in a dynamic system**
Time Estimation: **1) In-Class Activity: 30 minutes** **2) Model Tutorial: 1 hour** **3) Model Testing and Advancement: 2 hours 30 minutes** **Total: 4 hours**

Part One: In Class Activity

Materials: none

Rules:

1) **Assign each student (or group of students) a role of the lac operon (promotor, repressor, lactose, glucose, CAP, RNA polymerase, enzymes)**
2) **Have students stand to represent an active state and sit to represent an inactive state**
3) **Have students decide what must happen for them to reach an active state (i.e. if they are the promotor, the repressor will need to be sitting) and what will need to happen to reach an inactive state (i.e. if they are lactose, enzymes will need to be standing)**
4) **Start with an inactive lac operon [Standing CAP and repressor] [Sitting: lactose, glucose, RNA polymerase, enzymes]**
5) **The lactose and glucose students are the inputs. Play a game of Simon Says If and If Else to determine their activation (i.e. If you are wearing a red shirt stand, if else remain seated)**
6) **After each If/If Else command allow the students to reach stability (i.e. no more changing of sitting vs. standing) before introducing another command**
7) **Keep introducing commands to the lactose and glucose inputs for multiple rounds. Make sure to give commands that reach all input possibilities at least once [(1) just glucose standing, (2) just lactose standing, and (3) both standing]**

Suggested Discussion Questions:

1) **How is sitting or standing a good representation of Boolean logic?**
2) **What were the different points of stability reached throughout the activity?**
3) **Were there any components of the lac operon that Boolean logic does not fully cover?**

Part Two: Model Tutorial

1) **Open NetLogo**
2) **Instead of using one of the built-in codes, you will be making one from scratch today. Click the Code tab to get started**
3) **Define the breeds of variables that will be part of the model using breed [plural_form singular_form]. The components of this model should include lactose, glucose, promotor, operator, repressor, lac genes, RNA polymerase, enzymes, cap, and cap site**

```
breed [lactose a_lactose] ;user added with button
breed [glucose a_glucose] ;user added with button
breed [promotor a_promotor] ;part of operon
breed [operator a_operator] ;part of operon
breed [repressor a_repressor] ;part of operon
breed [lac_genes lac_gene] ;part of operon
breed [RNApolymerase a_RNApolymerase] ;part of operon
breed [enzymes enzyme] ;product of lac genes
breed [cap a_cap] ;part of operon
breed [cap_site a_cap_site] ;part of operon
```

4) **Define the global variable transcribe? with globals [transcribe?]. This variable will use Boolean logic to determine if the conditions are met to turn on the lac operon**

```
globals [transcribe?] ;will lac enzymes be created?
```

5) **Define the turtle owned global variable age with turtles-own [age]. This variable will keep track of the age of the turtles**

```
turtles-own [age] ;age of turtle
```

6) **To set up the simulation, make a command called to setup. Under the command, type 4 actions (1) clear-all, which will clear all variables each time the code is run, (2) reset-ticks, which will initialize the time to be 0, (3) make_operon, a command which will create the lac operon, and (4) set transcribe? false, a command that will set the Boolean logic gate as false at beginning of the simulation. Finish the command by typing end**

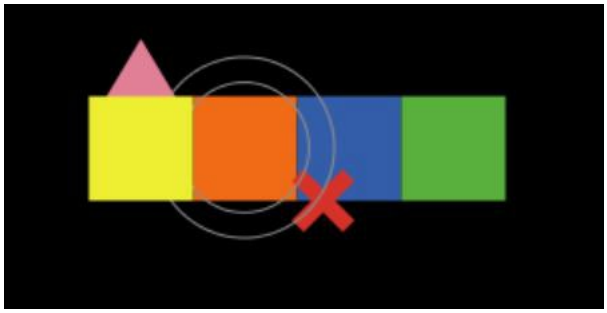
```
to setup ;initialize main interface
  clear-all
  reset-ticks
  make_operon
  set transcribe? false
end
```

7) **To create the lac operon, make a command called to make_operon. Under the command, create the 7 turtles that comprise the lac operon: operator, promotor, repressor, cap site, cap, lac genes, and RNA polymerase using the format create-breed 1 [set color ____ set shape "____" setxy ____ ____ set size____]. You can customize with your own preferences in the ____s and finish the command with end**

```
to make_operon ;create operon
  create-RNApolymerase 1
  [
    set color grey
    set shape "circle 3"
    setxy  -4 0
    set size 10
  ]
  create-cap_site 1
  [
    set color yellow
    set shape "square"
    setxy -8 0
    set size 5
  ]
  create-promotor 1
  [
    set color orange
    set shape "square"
    setxy -4 0
    set size 5
  ]
  create-operator 1
  [
    set color blue
    set shape "square"
    setxy 0 0
    set size 5
  ]
```

```
create-lac_genes 1
[
  set color green
  set shape "square"
  setxy 4 0
  set size 5
]
  create-repressor 1
[
  set color red
  set shape "x"
  setxy -1 -2
  set size 3
]
    create-cap 1
[
  set color pink
  set shape "triangle"
  setxy -8 3
  set size 3
]
end
```

8) **Navigate to the main interface by clicking Interface and check to see if your lac operon was built correctly. Under Button click Button, place it near the top of the white part of the main interface, name it setup, and click Ok. Click the setup button and your lac operon should appear on the screen. If you used the above commands, it will give you the lac operon pictured bellow. [Hint: circle 3 for the RNA polymerase is not a default shape in the NetLogo Turtle Shapes Library and will need to be created]**



9) **To add glucose to the model, make a command called to add-glucose. Under the command type create-glucose 10 [set color cyan set shape “circle” set size 1 setxy random-xcor random y-cor] end.**

```
to add-glucose ;will run when gucose button pressed
    create-glucose 10
  [
    set color cyan
    set shape "circle"
    set size 1
    setxy random-xcor random-ycor
  ]
end
```

10) Navigate to the main interface by clicking Interface. Under Button click Button, place it near the top of the white part of the main interface, name it add-glucose, and click Ok. When this button is clicked it will add 10 glucose molecules to the simulation

11) Repeat steps 9 & 10 to add lactose to the model in the code and with the button. Instead of the color cyan, use magenta

```
to add-lactose ;will run when lactose button pressed
   create-lactose 10
  [
    set color magenta
    set shape "circle"
    set size 1
    setxy random-xcor random-ycor
  ]
end
```

12) While on the main interface, also add a plot to the screen. Under Button click Plot and add it to the bottom of the white part of the main screen. Right click on the plot and choose Edit. Name the plot Gene Regulation, the x axis label Time, and the y axis label Amount

13) Under pen update commands change plot count turtles to plot count glucose and change to default color from black to cyan by clicking on the black box, choosing cyan, and clicking Ok.

14) Click add pen and repeat step 13 by naming the pen command, plot count lactose and making the color magenta

15) Click add pen and repeat step 13 by naming the pen command, plot count enzymes and making the color green. Click Ok to exit the plot

16) Navigate back to Code. Make a command called to go under the command type tick and end

17) **Navigate to the main interface by clicking Interface. Under Button click Button, place it near the top of the white part of the main interface, name it go, and click Ok. When this button is clicked it will start the simulation. More commands will need to be added under to go and the model to simulate the lac operon which you will do in part three**

Module 4: Gene Regulation and Stability Using Boolean Logic

1) **To define rules for the agents in the simulation you will need to determine what you want the rules to be. Consider how the lac operon works (https://www.khanacademy.org/science/ap-biology/gene-expression-and-regulation/regulation-of-gene-expression-and-cell-specialization/a/the-lac-operon). Specifically think about:**
    a. **When lactose is present what happens to the repressor?**

    b. **How does the removal of the repressor effect transcription?**

    c. **When glucose is present what happens to CAP?**

    d. **How does the removal of CAP affect transcription?**

    e. **What is produced as a result of transcription?**

    f. **What parts of the lac operon including its inputs and products move?**

2) **Using your considerations from above define the outcomes (rules) for the Boolean Logic problems below. The first two have been completed as examples**
    d. **If lactose > 0**

True: move repressor and start transcription
False: nothing

e. **If else lactose >= (greater or equal to) glucose**
True: fast transcription
False: slow transcription

f. **If glucose > 0 [Hint: what happens to CAP?]**

True:

False

g. **If enzyme > lactose**

True:

False:

h. **If enzyme on lactose [Hint: what is the purpose of the enzymes produced by the lac operon?]**

True:

False:

3) **Identify any assumptions you may want to make in order to decrease the complexity of your model.**

a.

4) **Using these rules and assumptions, determine the code that will need to be added to the model to carry out the rules. Use NetLogo Dictionary (http://ccl.northwestern.edu/netlogo/docs/index2.html) for help on syntax and codes that are available. [Hint: the codes if and ifelse should be very helpful and comprise most of the added components. Also count, ask, die, any? and move should be helpful]**

5) **Once you have a working code, it is important to test that it works. Come up with at least 5 tests (one per rule) to determine if your simulation is working properly.**

a.

b.

c.

d.

e.

6) **Perform your tests. If your code is not performing how you anticipated try troubleshooting or writing a new code and retry your test.**
   b. Describe the outcome of your tests and the troubleshooting process.

7) **Analyzing how a simulation performs when varying input parameters (i.e. changing the amount of lactose or glucose added to the simulation) can uncover emergent phenomena of the model. An emergence of the lac operon biologically and in this simulation is it bistability. Using the Gene Regulation plot from the simulation explain how we can make this conclusion.**
   a.

8) **This model is extremely basic and has many limitations which do not capture the full workings of a lac operon.**
   a. **In your opinion, what are the model's three biggest limitations? Especially consider any assumptions that were made when answering this question.**

b. **Choose one of these limitations and describe what you think should be added to the model to address this limitation.**

Module 5: The Menstrual Cycle Using Boolean Logic

Platform: NetLogo

Following this lesson students should be able to:

1) **Describe the hormones that contribute to the menstrual cycle and how they function**
2) **Apply Boolean logic to biological phenomena**
3) **Identify stability in a biological process and computational system**
4) **Evaluate model outputs to determine emergent phenomena**

Purpose: This module will guide students through a tutorial to code a NetLogo model from scratch that uses Boolean Logic to create a simulation of the menstrual cycle. Biologically, students will be exposed to the hormones involved in the menstrual cycle. Computationally, students will define rules using Boolean logic and analyze model outputs to identify a system's emergent phenomena.

Biological Terms:

1) **Follicular Phase: the period of the menstrual cycle from the first day of the period until ovulation. Starts with the release of FSH and continues as estrogen slowly builds up**
2) **Luteal Phase: the period of the menstrual cycle from the start of ovulation until the period starts. Progesterone peaks and then drops**
3) **Follicle Stimulating Hormone (FSH): released from the pituitary gland and stimulates the maturation of an egg in the ovaries**
4) **Progesterone: released by the corpus luteum after ovulation. If fertilization of an egg does not occur, the corpus luteum dies and levels drop**
5) **Estrogen: released by the ovaries. Levels rise and fall twice within the menstrual cycle**
6) **Luteinizing Hormone (LH): released from the pituitary gland due to high estrogen levels**
7) **Ovulation: the release of an egg. Triggered by a spike in LH**

Computational Terms:

1) **Boolean Logic: a problem which reports only a True or False value**
2) **If: used to carry out a function only when a Boolean problem is True**
3) **If Else: used to carry out two functions, one when a Boolean problem is True and one when it is False**

4) **Emergent phenomena: property that arises from the collective behavior of a dynamic system** 5) **Stability: the ability to converge to an equilibrium across a range of inputs or perturbations (unstable systems do not move toward an equilibrium)**
Time Estimation: **1) In-Class Activity: 30 minutes** **2) Model Tutorial: 1 hour** **3) Model Testing and Advancement: 2 hours 30 minutes** **Total: 4 hours**

Part One: In Class Activity

Materials: none

Rules:

1) **Assign each student a component/hormone in the menstrual cycle (5 FSH, 5 LH, 5 progesterone, 5 estrogens, 1 egg (ovulation))**
2) **Have students stand to represent an active state and sit to represent an inactive state**
3) **Have students decide what must happen for each component to reach an active state (i.e. all LH stand up if 5 estrogen and 3 FSH are standing) and what will need to happen to reach an inactive state (i.e. all LH sit if egg is standing)**
4) **Start with everyone sitting**
5) **Play a game of Simon Says If and If Else to determine their activation (i.e. If you are wearing a red shirt stand, if else remain seated)**
6) **After each If/If Else command allow the students to determine if who is standing would be part of the menstrual cycle (i.e. if it follows one of the rules outlined in step 3). If so have them cycle**
7) **Keep introducing commands until they have cycled at least three times**

Suggested Discussion Questions:

1) **How is sitting or standing a good representation of Boolean logic?**
2) **What components of the menstrual cycle does Boolean logic does not fully cover?**

Part Two: Model Tutorial

1) **Open NetLogo**
2) **Instead of using one of the built-in codes, you will be making one from scratch today. Click the Code tab to get started**
3) **Define the breeds of variables that will be part of the model using breed [plural_form singular_form]. The components of this model should include estrogen, progesterone, LH, FSH, ovaries, fallopian tubes, uterus, and eggs**

```
breed [estrogen a_estrogen] ;menstral cycle hormone
breed [progesterone a_progesterone] ;menstral cycle hormone
breed [LH a_LH] ;menstral cycle hormone
breed [FSH a_FSH] ;menstral cycle hormone
breed [ovaries ovary] ;part of reproductive system
breed [fallopian-tubes fallopian-tube] ;part of reproductive system
breed[uteruses uterus] ;part of reproductive system
breed [eggs egg] ;created as part of menstral cycle
```

4) **Define the global variable ovulate? with globals [transcribe?]. This variable will use Boolean logic to determine if the conditions are met to turn on the lac operon**

```
globals [ovulate?] ;will luteal phase be started?
```

5) **Define the turtle owned global variable age with turtles-own [age]. This variable will keep track of the age of the turtles**

```
turtles-own [age] ;age of turtles
```

6) **To set up the simulation, make a command called to setup. Under the command, type 4 actions (1) clear-all, which will clear all variables each time the code is run, (2) reset-ticks, which will initialize the time to be 0, (3) make_reproductive-system, a command which will create the lac operon, and (4) set ovulate? false, a command that will set the Boolean logic gate as false at beginning of the simulation. Finish the command by typing end**

```
to setup ;initialize main interface
  clear-all
  reset-ticks
  make_reproductive-system
  set ovulate? FALSE
end
```

7) **To create the lac operon, make a command called to make_reproductive-system. Under the command, create the 7**

turtles that comprise the beginning of the model: uterus 1, uterus 2, fallopian tube 1, fallopian tube 2, ovaries x2, and FSH using the format create-breed 1 [set color ____ set shape "____" setxy ____ ____ set size____]. You can customize with your own preferences in the ____s and finish the command with end

```
to make_reproductive-system ;create reproductive system and FSH
  create-uteruses 1 [
    set shape "uterus"
    setxy  0 0
    set size 15 ]
  create-uteruses 2 [
    set shape "square"
    set color magenta
    setxy  0 -8
    set size 7.5]
  create-fallopian-tubes 1 [
    set shape "fallopian-tube"
    setxy  -8 3
    set size 10]
  create-fallopian-tubes 2 [
    set shape "fallopian-tube-2"
    setxy  8 3
    set size 10]
    set-default-shape turtles "ovaries"
  create-ovaries 1 [
    setxy  9 0
    set size 8]
  create-ovaries 1 [
    setxy  -9 0
    set size 8]
  create-FSH 30 [
    set color cyan
    set shape "circle"
    set size .5
    setxy random-xcor random-ycor]
end
```

8) **Navigate to the main interface by clicking Interface and check to see if your reproductive system was built correctly. Under Button click Button, place it near the top of the white part of the main interface, name it setup, and click Ok. Click the setup button and your lac operon should appear on the screen. If you used the above commands, it will give you the reproductive system and FSH pictured below. [Hint: uterus, fallopian tube 1 and 2, and ovaries**

are not a default shape in the NetLogo Turtle Shapes Library and will need to be created]

9) **Navigate back to the Code and create a command called to follicular-phase. Initiate Boolean logic with if not ovulate? This will run only if ovulate? is set to FALSE. Create command if count LH= 0 and if any? FSH on ovaries ask n-of 1 FSH on ovaries to hatch-eggs and hatch-estrogen and ask n-of 3 FSH [die]. When LH=0 while ovulate? is FALSE, when FSH is on ovaries, eggs will develop and estrogen will be created. Create command ask eggs and if age= 100 [set size .5] and if age=500 [set size .75]. This will have eggs get larger as they mature**

```
to follicular-phase
if not ovulate? [
    if count LH = 0 [
      if any? FSH-on ovaries [
        ask n-of 1 FSH-on ovaries [
          hatch-eggs 1 [
            set color white
            set shape "circle"
            set size .25
            set age 0]
          hatch-estrogen 2 [
            set color yellow
            set shape "circle"
            set size .5
            setxy random-xcor random-ycor]]
        ask n-of 3 FSH [die]]
      ask eggs [
        if age = 100 [set size .5]
        if age = 500 [set size .75]]]
```

10) **Within to follicular-phase if not ovulate?, create code if count estrogen = 20 and hatch-LH 100, hatch-FSH 30, and hatch-**

estrogen 40. This will have LH, FSH, and estrogen spike when ovulate? is FALSE and estrogen = 20

```
if count estrogen = 20 [
  hatch-LH 100 [
    set color orange
    set shape "circle"
    set size .5
    setxy random-xcor random-ycor]
  hatch-FSH 30 [
    set color cyan
    set shape "circle"
    set size .5
    setxy random-xcor random-ycor]
  hatch-estrogen 40 [
    set color yellow
    set shape "circle"
    set size .5
    setxy random-xcor random-ycor]]
```

11) Within to follicular-phase if not ovulate?, create code if count estrogen = 60 and ask up-to-n-of 9 eggs [die] and set ovulate? TRUE. This will have all but 1 egg die and set ovulate? TRUE when estrogen= 60

```
    if count estrogen = 60 [
      ask up-to-n-of 9 eggs [die]
      set ovulate? true]]
end
```

12) Create command to go and tick, ask turtles [follicular-phase, move, age-up, if ovulate? [luteal-phase]]

```
to go ;will run simultion
 tick
 ask turtles [
 follicular-phase
 move
 age-up
 if ovulate? [luteal-phase]]
end
```

13) Create command to age-up [ask eggs [set age age + 1]. This will have eggs age up

```
to age-up
  ask eggs [
  set age age + 1]
end
```

14) Create command to move ask FSH, estrogen, LH, and progesterone to randomly move

```
to move
  ask FSH [
  rt random 100
  lt random 100
  fd 1]
 ask estrogen [
  rt random 100
  lt random 100
  fd 1]
  ask LH [
  rt random 100
  lt random 100
  fd 1]
  ask progesterone [
  rt random 100
  lt random 100
  fd 1]
end
```

15) **Navigate to the main interface by clicking Interface. Under Button click Button, place it near the top of the white part of the main interface, name it go, and click Ok. When this button is clicked it will start the simulation**

16) **While on the main interface, also add a plot to the screen. Under Button click Plot and add it to the bottom of the white part of the main screen. Right click on the plot and choose Edit. Name the plot Hormone Levels, the x axis label Time, and the y axis label Amount**

17) **Under pen update commands change plot count turtles to plot count FSH and change to default color from black to cyan by clicking on the black box, choosing cyan, and clicking Ok.**

18) **Click add pen and repeat step 17 by naming the pen command, plot count estrogen and making the color yellow**

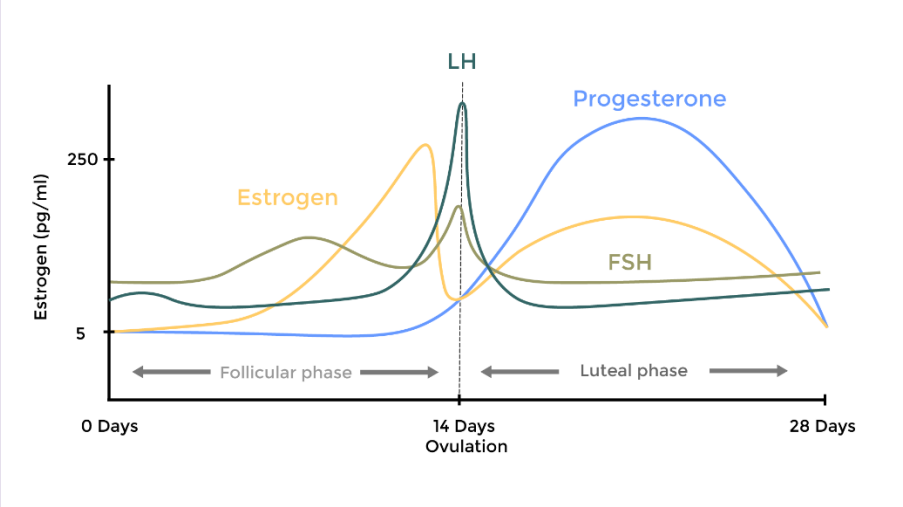
19) **Click add pen and repeat step 17 by naming the pen command, plot count LH and making the color orange. Click Ok to exit the plot**

20) **Click add pen and repeat step 17 by naming the pen command, plot count progesterone and making the color red. Click Ok to exit the plot**

21) **This tutorial has helped create half the menstrual cycle, you will need to create the luteal phase and a way for the model to continue cycling. [Hint: setting the time step slower will help]**

Part Three: Model Advancement and Testing

1) **To define rules for the luteal phase the simulation you will need to determine what you want the rules to be. Consider this graph of the hormone levels in the menstrual cycle:**



a. **When does progesterone start to rise?**

b. **What happens to LH and FSH during the luteal phase?**

c. **What happens to estrogen during the luteal phase?**

d. **What happens to the egg during the luteal phase, how does it move?**

e. **What must occur for the simulation to cycle?**

2) **Using your considerations from above define at least five outcomes (rules) using Boolean Logic. The end goal should be to have your graph look very similar to the one provided and for the egg to ovulate. Anything else you do to make the model more accurate is up to you! [Hint: look through provided code of follicular phase for assistance]**

a.

b.

c.

d.

e.

3) **Identify any assumptions you may want to make in order to decrease the complexity of your model.**

a.

4) **Using these rules and assumptions, determine the code that will need to be added to the model to carry out the rules. Use NetLogo Dictionary (http://ccl.northwestern.edu/netlogo/docs/index2.html) for help on syntax and codes that are available. [Hint: the codes if and ifelse should be very helpful and comprise most of the added components. Also count, ask, die, any? and move should be helpful. Look at the provided code for the follicular phase for assistance]**

5) **Once you have a working code, it is important to test that it is accurate. Come up with at least 5 tests (one per rule) to determine if your simulation is working properly.**

a.

b.

c.

d.

e.

6) **Perform your tests. If your code is not performing how you anticipated try troubleshooting or writing a new code and retry your test.**

c. **Describe the outcome of your tests and the troubleshooting process.**

7) **This model is extremely basic and has many limitations which do not capture the full workings of a lac operon.**
   a. **In your opinion, what are the model's three biggest limitations? Especially consider any assumptions that were made when answering this question.**

   b. **Choose one of these limitations and describe what you think should be added to the model to address this limitation.**

Appendix F

Please complete this survey, which will be used to evaluate the computational biology learning modules. Completion is voluntary and will not affect your standing with Clemson or the Emerging Scholars Program. Any open-ended comments will be completely de-identified. All survey respondent information will remain confidential.

1. Name

* 2. Gender Identity

- ◯ Male
- ◯ Female
- ◯ Prefer not to say
- ◯ Other (please specify)

3. Race

4. How would you rate your computational experience prior to this course?

- ◯ Extensive Experience
- ◯ Moderate Experience
- ◯ Minimal Experience
- ◯ No Experience

5. Which module did you complete for our fourth module?

- ◯ Lac Operon
- ◯ Menstrual Cycle

Menstrual Cycle

6. Please rate your pre-class and current (post-class) levels of understanding of the following computational biology elements. 1= No Understanding, 2= Minimal Understanding, 3= Confused Understanding, 4= Good Understanding, 5= Excellent Understanding

	Pre	Post
1) Describe basic cancer cell metabolism and migration		
2) Utilize NetLogo's built-in sample models		
3) Develop and test predictions of therapeutic targets using a tumor model		
4) Evaluate a model's limitations		
5) Describe the factors that can impact a virus's spread throughout a population		
6) Modify a given Netlogo model by adding and deleting buttons from the main interface		
7) Research the literature to supply model with input parameters from published experimental data		
8) Determine what additional rules and/or variables should be added to a model to address its limitations		
9) Describe the body's immune response due to		

an allergen		
10) Define rules for agents of a model given a biological phenomenon		
11) Code simple rules/commands using NetLogo		
12) Evaluate how assumptions can increase a model's limitations		
13) Describe the hormones that contribute to the menstrual cycle and how they function		
14) Apply Boolean logic to biological phenomena		
15) Identify stability in a biological process and computational system		
16) Evaluate model outputs to determine emergent phenomena		

7. Please rank the modules from most (1) to least (5) favorite.

☰ ☐	Module 1 Tumor Growth Simulation & Limitations
☰ ☐	Module 2 Virus Prevention Simulation and Model Inputs
☰ ☐	Module 3 Immune Reaction Simulation Rules
☰ ☐	Module 4: Modeling the Menstrual Cycle Using Boolean Logic

8. Did having a female focused project influence your confidence in being able to contribute to the team on the fourth module? Why or why not?

Lac Operon

9. Please rate your pre-class and current (post-class) levels of understanding of the following computational biology elements. 1= No Understanding, 2= Minimal Understanding, 3= Confused Understanding, 4= Good Understanding, 5= Excellent Understanding

	Pre	Post
1) Describe basic cancer cell metabolism and migration		
2) Utilize NetLogo's built-in sample models		
3) Develop and test predictions of therapeutic targets using a tumor model		
4) Evaluate a model's limitations		
5) Describe the factors that can impact a virus's spread throughout a population		
6) Modify a given Netlogo model by adding and deleting buttons from the main interface		
7) Research the literature to supply model with input parameters from published experimental data		

8) Determine what additional rules and/or variables should be added to a model to address its limitations		
9) Describe the body's immune response due to an allergen		
10) Define rules for agents of a model given a biological phenomenon		
11) Code simple rules/commands using NetLogo		
12) Evaluate how assumptions can increase a model's limitations		
13) Describe the components that make up a lac operon and how it functions 14) Apply Boolean logic to biological phenomena		
15) Identify stability in a biological process and computational system		
16) Evaluate model outputs to determine emergent phenomena		

10. Please rank the modules from most (1) to least (5) favorite.

- ☐ Module 1 Tumor Growth Simulation & Limitations
- ☐ Module 2 Virus Prevention Simulation and Model Inputs
- ☐ Module 3 Immune Reaction Simulation Rules
- ☐ Module 4 Gene Regulation and Stability Using Boolean Logic

11. Please rate your confidence from (1= No Confidence to 4=Extremely Confident) in the ability to

	1= Not Confident	2= Minimally Confident	3= Moderately Confident	4= Extremely Confident
1) Explain computational terms to your peers	○	○	○	○
2) Explain biological terms to your peers	○	○	○	○
3) Code a simple biological process using Netlogo individually	○	○	○	○
4) Code a simple biological process using Netlogo as part of a team	○	○	○	○
5) Break down complex problems into smaller, simpler problems.	○	○	○	○
6) Make connections between similar problems and experience.	○	○	○	○
7) Identify important information while ignoring unrelated or irrelevant details	○	○	○	○
8) Design an algorithm to solve a problem	○	○	○	○

12. Overall please rate the value this class had on your understanding of computational biology.

13. Did you feel that having the hands-on activities before doing the coded tutorial help you understand the biological and computational concepts better? Why or why not?

14. After completing this course, do you feel like you are more or less likely to pursue a career in computational biology? Explain.

15. Do you have any suggestions to improve the learning modules?

Appendix G

The Comprehensive Assessment of Team Member Effectiveness (CATME) is copyrighted. For permission to use outside of research or classroom purposes, please contact Matthew W. Ohland at Ohland@purdue.edu or Misty L. Loughry at MLoughry@georgiasouthern.edu.

The same content is measured in multiple ways. We have a Behaviorally Anchored Rating Scale (BARS) version. We also have short and long versions that use Likert scales. The BARS version of the instrument can be administered on-line. See www.CATME.org for instructions.

To cite any of the Likert scale versions, please use the following citation:

Loughry, M. L., Ohland, M. W., & Moore, D. D. (2007). Development of a theory-based assessment of team member effectiveness. *Educational and Psychological Measurement*, 67(3), 505-524.

To cite the BARS version, please cite the paper above and the following:

Ohland, M. W., Loughry, M. L., Woehr, D. J., Bullard, L. G., Felder, R. M., Finelli, C. J., Layton, R. A., Pomeranz, H. R., & Schmucker, D. G. (2012). The comprehensive assessment of team member effectiveness: Development of a behaviorally anchored rating scale for self and peer evaluation. *Academy of Management Learning & Education,* 11(4), 609-630.

For assessment-related uses, please cite the above and the following:

Loughry, M. L., Ohland, M. L., & Woehr, D. J. (2014). Assessing teamwork skills for assurance of learning using CATME Team Tools. *Journal of Marketing Education*, 36(1), 5-19.

If you use the Team-Maker tool, available online at www.CATME.org, please cite the following:

Layton, R. A., Loughry, M. L., Ohland, M. W., & Ricco, G. D. (2010). Design and validation of a web-based system for assigning members to teams using instructor-specified criteria. *Advances in Engineering Education*, 2(1), 1-28.

	Your name					**Peer Evaluation** **Section Number** _____ **Team Number** _____ ← Write the names of the people on your team including your own name. This self and peer evaluation asks about how you and each of your teammates contributed to the team during the time period you are evaluating. For each way of contributing, please read the behaviors that rating. Then confidentially rate yourself and your teammates by placing a mark in the relevant box.
Contributing to the Team's Work						• Does more or higher-quality work than expected. • Makes important contributions that improve the team's work. • Helps to complete the work of teammates who are having difficulty.
						Demonstrates behaviors described in the row just above and just below.
						• Completes a fair share of the team's work with acceptable quality. • Keeps commitments and completes assignments on time. • Fills in for teammates when it is easy or important
						Demonstrates behaviors described in the row just above and just below.
						• Does not do a fair share of the team's work. Delivers sloppy or incomplete work. • Misses deadlines. Is late, unprepared, or absent for team meetings. • Does not assist teammates. Quits if the work becomes difficult.
Interacting with Teammates						• Asks for and shows an interest in teammates' ideas and contributions. • Improves communication among teammates. Provides encouragement or enthusiasm to the team. • Asks teammates for feedback and uses their suggestions to improve.
						Demonstrates behaviors described in the row just above and just below.
						• Listens to teammates and respects their contributions. • Communicates clearly. Shares information with teammates. Participates fully in team activities. • Respects and responds to feedback from teammates.
						Demonstrates behaviors described in the row just above and just below.
						• Interrupts, ignores, bosses, or makes fun of teammates. • Takes actions that affect teammates without their input. Does not share information. • Complains, makes excuses, or does not interact with teammates. Accepts no help or advice.
Keeping the Team on Track						• Watches conditions affecting the team and monitors the team's progress. • Makes sure that teammates are making appropriate progress. • Gives teammates specific, timely, and constructive feedback.
						Demonstrates behaviors described in the row just above and just below.
						• Notices changes that influence the team's success. • Knows what everyone on the team should be doing and notices problems. • Alerts teammates or suggests solutions when the team's success is threatened.
						Demonstrates behaviors described in the row just above and just below.
						• Is unaware of whether the team is meeting its goals. • Does not pay attention to teammates' progress. • Avoids discussing team problems, even when they are obvious.
Expecting Quality						• Motivates the team to do excellent work. • Cares that the team does outstanding work, even if there is no additional reward. • Believes that the team can do excellent work.
						Demonstrates behaviors described in the row just above and just below.
						• Encourages the team to do good work that meets all requirements. • Wants the team to perform well enough to earn all available rewards. • Believes that the team can fully meet its responsibilities.
						Demonstrates behaviors described in the row just above and just below.
						• Satisfied even if the team does not meet assigned standards. • Wants the team to avoid work, even if it hurts the team. • Doubts that the team can meet its requirements.
Having Relevant Knowledge, Skills, and Abilities						• Demonstrates the knowledge, skills, and abilities to do excellent work. • Acquires new knowledge or skills to improve the team's performance. • Able to perform the role of any team member if necessary.
						Demonstrates behaviors described in the row just above and just below.
						• Has sufficient knowledge, skills, and abilities to contribute to the team's work. • Acquires knowledge or skills needed to meet requirements. • Able to perform some of the tasks normally done by other team members.
						Demonstrates behaviors described in the row just above and just below.
						• Missing basic qualifications needed to be a member of the team. • Unable or unwilling to develop knowledge or skills to contribute to the team. • Unable to perform any of the duties of other team members.

	Your name					**CATME Likert -Short Section Number _____ Team Number _____** ← Write the first and last names of the people on your team including your own name. This self and peer evaluation asks about how you and each of your teammates contributed to the team during the time period you are evaluating. Please read each item that describes a way of contributing. Then confidentially rate yourself and your teammates using the following scale: 1 Strongly Disagree — 2 Disagree — 3 Neither Agree Nor Disagree — 4 Agree — 5 Strongly Agree
Contributing to the Team' s Work						Did a fair share of the team's work.
						Fulfilled responsibilities to the team.
						Completed work in a timely manner.
						Came to team meetings prepared.
						Did work that was complete and accurate.
						Made important contributions to the team's final product.
						Kept trying when faced with difficult situations.
						Offered to help teammates when it was appropriate.
Interacting with Teammates						Communicated effectively.
						Facilitated effective communication in the team.
						Exchanged information with teammates in a timely manner.
						Provided encouragement to other team members.
						Expressed enthusiasm about working as a team.
						Heard what teammates had to say about issues that affected the team.
						Got team input on important matters before going ahead.
						Accepted feedback about strengths and weaknesses from teammates.
						Used teammates' feedback to improve performance.
						Let other team members help when it was necessary.
Keeping the Team on Track						Stayed aware of fellow team members' progress
						Assessed whether the team was making progress as expected.
						Stayed aware of external factors that influenced team performance.
						Provided constructive feedback to others on the team.
						Motivated others on the team to do their best.
						Made sure that everyone on the team understood important information.
						Helped the team to plan and organize its work.
Expecting Quality						Expected the team to succeed.
						Believed that the team could produce high-quality work.
						Believed that the team should achieve high standards.
						Cared that the team produced high-quality work.
Having Relevant Knowledge, Skills, and Abilities						Had the skills and expertise to do excellent work.
						Had the skills and abilities that were necessary to do a good job.
						Had enough knowledge of teammates' jobs to be able to fill in if necessary.
						Knew how to do the jobs of other team members.

Comprehensive Assessment of Team Member Effectiveness (CATME)

Past Tense Long Form

CONTRIBUTING TO THE TEAM'S WORK

Did a fair share of the team's work.
Carried at least a fair share of the team's workload.
Did an acceptable portion of the team's work.

Fulfilled responsibilities to the team.
Kept commitments to the team.
Followed through on obligations to the team.

Came to team meetings prepared.
Arrived on time for team meetings.
Prepared for meetings ahead of time.

Completed work in a timely manner.
Did assigned tasks by the time the team needed the work.
Completed team assignments by the due date.

Did work that was complete and accurate.
Used care when completing work for the team.
Carefully completed tasks assigned by the team.

Made important contributions to the team's final product.
Provided insights and ideas that improved the team project.
Made recommendations that improved the team's performance.

Kept trying when faced with difficult situations.
Remained effective under pressure.
Did not give up when faced with a challenge.

Offered to help teammates when it was appropriate.
Helped other team members when they needed assistance.
Helped teammates who were having difficulty.

INTERACTING WITH TEAMMATES

Communicated effectively.
Was specific when communicating information or feelings to teammates.
Expressed ideas clearly.

Facilitated effective communication in the team.
Helped improve communication among team members.
Facilitated the exchange of ideas among team members.

Exchanged information with teammates in a timely manner.
Shared information that affected others on the team.
Kept other team members informed about work-related issues.

Provided encouragement to other team members.
Was supportive of other team members.
Helped build teammates' confidence.

Expressed enthusiasm about working as a team.
Enjoyed working as part of a team.
Enjoyed teamwork.

Heard what teammates had to say about issues that affected the team.
Listened to teammates who had different perspectives.
Paid attention to what teammates had to say.

Got team input on important matters before going ahead.
Gave all team members a chance to participate in team decisions.
Requested a response from teammates before making important decisions.

Accepted feedback about strengths and weaknesses from teammates.
Was receptive to constructive criticism from other team members.
Accepted constructive criticism from other team members.

Used teammates' feedback to improve performance.
Took teammates' feedback seriously.
Acted on teammates' constructive criticism.

Let other team members help when it was necessary.
Asked teammates for help when necessary.
Allowed teammates to assist when help was needed.

KEEPING THE TEAM ON TRACK

Stayed aware of fellow team members' progress.
Monitored teammates to discover effective or ineffective performance.
Noticed whether or not teammates were doing their job correctly.

Assessed whether the team was making progress as expected.
Checked whether the team was on the right track.
Evaluated the team's work to improve team effectiveness.

Stayed aware of external factors that influenced team performance.
Monitored external conditions that influenced team success.
Noticed situations outside the team's control that affected the team's performance.

Provided constructive feedback to others on the team.
Provided feedback to teammates about their strengths and weaknesses.
Let teammates know how they were performing.

Motivated others on the team to do their best.
Encouraged others on the team to focus on meeting the team's objectives.
Motivated teammates to do a good job on their part of the team's work.

Made sure that everyone on the team understood important information.
Checked to make sure that everyone was clear on what needed to be done.
Made sure that everyone on the team knew what they were supposed to do.

Helped the team to plan and organize its work.
Made sure that the team had the necessary supplies, information, and resources.
Helped the team to develop clear objectives.

EXPECTING QUALITY

Expected the team to succeed.
Believed that the team could produce high-quality work.
Was confident that the team could get a lot done when it worked hard.

Cared that the team produced high-quality work.
Believed that the team should achieve high standards.
Wanted the team to excel at its work.

HAVING RELEVANT KNOWLEDGE, SKILLS, and ABILITIES

Had the skills and expertise to do excellent work.
Had the skills and abilities that were necessary to do a good job.
Had the skills necessary to contribute to the team's work.

Had enough knowledge of teammates' jobs to be able to fill in if necessary
Knew how to do the jobs of other team members.
Was able to perform other team members' roles.

Had skills and abilities that other team members lacked.
Had talents that the team needed and other team members did not have.
Had different skills and abilities than teammates had.