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# Modeling Sex-Specific Changes in Myocardial Fibrosis

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## ABSTRACT

Heart disease the leading cause of death for both men and women in the United States. Cardiac fibrosis, or accumulation of extracellular matrix proteins in the heart, can occur after a heart attack and increase the risk for further complications. Current treatments for heart disease do not include extracellular matrix regulators, partly due to the complicated signaling network responsible for the production of these proteins. By using a computational model of the signaling network in cardia fibroblasts, the relationship between particular molecules and downstream extracellular matrix production can be examined.

Biological sex is an important factor for cardiac health and for drug development. In this study, progesterone signaling was added to a previously published signaling network model for cardiac fibroblasts. Progesterone was found to decrease the overall production of extracellular matrix proteins. Additional trials examined the effect of each of three estrogen receptors in the model, finding that estrogen receptor beta largely activates proteases, decreasing overall extracellular matrix production. Finally, a sex-specific drug screen was performed on six drugs that interact with molecules downstream of progesterone, finding that the addition of progesterone to the model slightly changed the predicted effect of the drugs on the extracellular matrix.

## BACKGROUND

The leading cause of death in the United States is heart disease, connected to one out of every five deaths in 2021[1]. Heart disease is a blanket term that can be used to describe a number of heart conditions, notably coronary artery disease, which is characterized by deposits of cholesterol and other substances in the artery, causing atherosclerosis and partially or totally blocking the flow of flood[2]. If left untreated, this process can cause a heart attack, or myocardial infarction, in which part of the heart does not receive enough blood and cells begin to die.

After a heart attack, the infarct region is vulnerable to further complications as it heals. Cardiac fibrosis, the production and accumulation of extracellular matrix (ECM) proteins, such as collagen, is necessary for initial survival and recovery from a myocardial infarction[3]. These proteins are responsible for forming the scar tissue that provides structural support to the infarct region during the healing process[3]. However, if cardiac fibrosis is uncontrolled, either by overproducing ECM proteins or by depositing proteins in regions of the heart that do not need scar tissue, it can increase the risk of arrhythmia by disrupting the electrical stimuli produced by nodes in the heart[4].

The current regimes for treatment for heart failure focus on reducing blood pressure or slowing the heart rate of patients, which can be accomplished with angiotensin-converting enzyme inhibitors and beta-blockers[5]. This strategy aims to reduce the stress on the heart and slow the progression of heart failure, but it does not address the biochemical changes that occur in the heart after a myocardial infarction, such as cardiac fibrosis. No current FDA-approved treatments for heart failure direct target cardiac fibrosis[5]. Current research in this area focuses on creating a drug that is capable of inhibiting cardiac fibrosis or reversing the process, even though

controlled ECM protein deposits can be beneficial and support the healing infarct region. The relationship between environmental conditions and various cell signaling molecules and eventual downstream ECM production is complex, which makes it difficult to design *in vitro* studies that target specific pathways in the signaling network; this lack of knowledge about individual pathways hinders understanding about cardiac fibrosis and the development of an effective treatment regime that can promote cardiac fibrosis only in the areas where it is needed. Until pathways with the potential to control cardiac fibrosis have been identified, treatments will not be able to selectively promote or inhibit cardiac fibrosis.

In cases like this, computational models that can conduct *in silico* simulations of the complex molecular pathways can be a useful tool to help researchers identify potential new drug combinations that can be applied in focused *in vitro* and clinical studies. At the moment, these models have a somewhat limited scope and are primarily used to narrow down potential drug candidates for clinical studies based on a variety of risk factors. As researchers learn more about the biochemical processes and mechanisms that are manipulated by drugs and computational power improves, this field will grow both more precise and more accurate, and therefore become better predictors of how a candidate will perform in a pharmaceutical study. However, in addition to a stronger understanding of molecular pathways and computing power, the accuracy and predictive power of computational models also relies on data that is representative of the diverse population the model is intended to help. Data that is robust across different demographics and conditions must be used to create the models so that they can be accurate for a wider range of patients. Even for a simulation of just one cell, like a cardiac fibroblast, the concentrations of various hormones and signaling molecules, past or current health conditions, age, sex, and other different factors could have a significant difference in the way the cell will respond to new inputs. In order to justify using computational models to choose treatment regimen for patients, the models must be valid across a broad range of conditions and cellular environments.

Historically, experimental studies and clinical trials have primarily used data from males. Until the 1990s, women of childbearing age were banned from participating in clinical trials, even if they were not pregnant or planning to become pregnant, meaning that they were allowed to take drugs that had not been tested on their demographic[6]. It can be dangerous to overgeneralize results– just because a drug is effective for some people does not mean it will be effective for everyone. For drugs that target very specific pathways, the effectiveness of the treatment would be extremely reduced if that pathway was not very active in the first place. Although the ban against female participation in clinical studies was lifted in the early 1990s, the National Institutes of Health did not require both male and female animals in clinical trials until 2016. This has resulted in a backlog of many FDA-approved drugs that were not tested on female participants and have twice the adverse response rate in women as they do in men[7].

Biological sex is particularly important when considering the cardiovascular health of a patient. Men are 3 to 4 times more likely to have heart disease, and women are twice as likely to have an adverse drug response to treatment for heart disease[8]. Pre-menopausal women are less likely to suffer from myocardial infarctions than men of the same age, a difference primarily attributed to the cardioprotective role of the ovarian hormone estrogen, particularly estradiol, the most prevalent estrogen hormone during the reproductive years, excluding pregnancy[8]. Men also produce the hormone estradiol, through a pathway in the testes that converts testosterone to estradiol, but in much lower quantities than pre-menopausal women, more similar to the level produced by women after menopause. Previous research has suggested that many adverse drug

responses occur in a sex-specific manner, further intensifying the need to understand what pathways are involved and how they could be manipulated to improve recovery outcomes after heart failure for both men and women[5].

Other steroid hormones related to biological sex include progesterone and testosterone, but estrogen has been the focus of more research related to sex-differences in cardiovascular health, despite being overshadowed by progesterone in terms of serum concentration, particularly during the female reproductive years and during pregnancy[9]. With respect to cardiovascular health, many of the other signaling molecules responsible for the promotion of ECM proteins, such as interleukin-6, exist in the blood stream at much lower concentrations than both progesterone and estrogen, with a difference up to 3 orders of magnitude[10]. Given that the cellular environment for female cardiac fibroblasts is relatively flooded with estrogen and progesterone, both of these hormones must be considered when creating a computational model of the molecular pathways for these cells; otherwise, the model will not be an accurate reflection of the conditions to which the cell is exposed.

In this study, a previously published signaling network model of cardiac fibroblasts, which includes a pathway for estradiol, will be modified to include a pathway for progesterone as well. This model will then be used to determine the effect that progesterone has on ECM production at a variety of concentrations intended to represent pre-menopausal, post-menopausal, pregnant, and male conditions. The model will also be used to determine the effect of cycling progesterone and estrogen together, modeling a menstrual cycle, to see if there is a difference in ECM production at different points in the menstrual cycle, and if modeling the two hormones together has different outcomes than varying them separately.

In addition, this model will be used to simulate sex-specific drug screening with consideration of both estrogen and progesterone signaling. The previously published signaling network model had been used to screen potential drugs for differences in response between men and women, but this model only included estrogen, not progesterone. Once the model is modified to include a pathway for progesterone, the drugs that targeted molecules related to the progesterone pathway will be re-screened to determine whether the concentration of progesterone affects the response of each treatment regimen.

## MATERIALS AND METHODS

### Integration of progesterone into the cardiac fibroblast signaling network

A previously published signaling network model of cardiac fibroblasts was modified and expanded to include the steroid hormone progesterone[5]. The original model was based on data obtained from a manual literature search of over 300 research papers on signaling pathways in cardiac fibroblasts and cardiomyocytes[5]. The original model included 132 nodes, which represent proteins, integrins, cellular receptors, and transcription factors) and 203 edges (reactions)[5]. This model included 11 biochemical inputs, which included: transforming growth factor beta (TGFβ), angiotensin II (AngII), endothelin 1 (ET 1), mechanical tension, (PDGF), norepinephrine (NE), tissue necrosis factor alpha (TNFα), interleukin-1 (IL1), interleukin-6 (IL6), natriuretic peptide (NP), and estradiol (E2)[5]. These inputs were connected to many molecular pathways, which eventually culminated in 22 cellular outputs, which included: alpha-smooth muscle actin (α-SMA), procallagen I & III (proCI and proCIII), several pro-matrix metalloproteinases, (proMMP1, proMMP 2, proMMP3, proMMP8, proMMP9, proMMP12, proMMP14) tissue inhibitors of metalloproteinase (TIMP1, TIMP2), lysyl oxidase (LOX), periostin, fibronectin, latent transforming growth factor beta (latentTGFβ), thrombospondin4,

osteopontin, connective tissue growth factor (CTGF), plasminogen activator inhibitor 1 (PAI1), tenascin-c (TNC), extra domain A of fibronectin (EDAFN), and proliferation[5]. The majority of the studies used to build this model were specific to cardiac fibroblasts[11]. Other cell types referenced in studies used to build this model included other cardiac cells, such as cardiomyocytes, or fibroblast cells from other parts of the body, such as pulmonary fibroblasts[11]. Almost all of the papers used to update the model were performed using neonatal rat cardiac fibroblasts, with a combination of male and female cells, and the results were not separated based on the sex of the cells[5].

In order to add progesterone to the model, signaling pathways for progesterone in cardiac fibroblasts first had to be identified. A manual literature search for signaling pathways involving estrogen returned a study performed using rat cardiomyocytes that described the metabolism of estrogen and progesterone[12]. The progesterone receptor was found to inhibit tissue necrosis factor alpha and interleukin-1, which were two of the inputs in the original signaling network model[12]. Two new nodes were created and added to the original model: one node for progesterone (PG) and one node for the progesterone receptor (PGR). Three reaction edges were also added: one for progesterone activating the progesterone receptor, one for the progesterone receptor inhibiting TNFα, and one for the progesterone receptor inhibiting IL-1 (Figure 1). The rest of the model was left unchanged because those reaction pathways were previously established and validated.

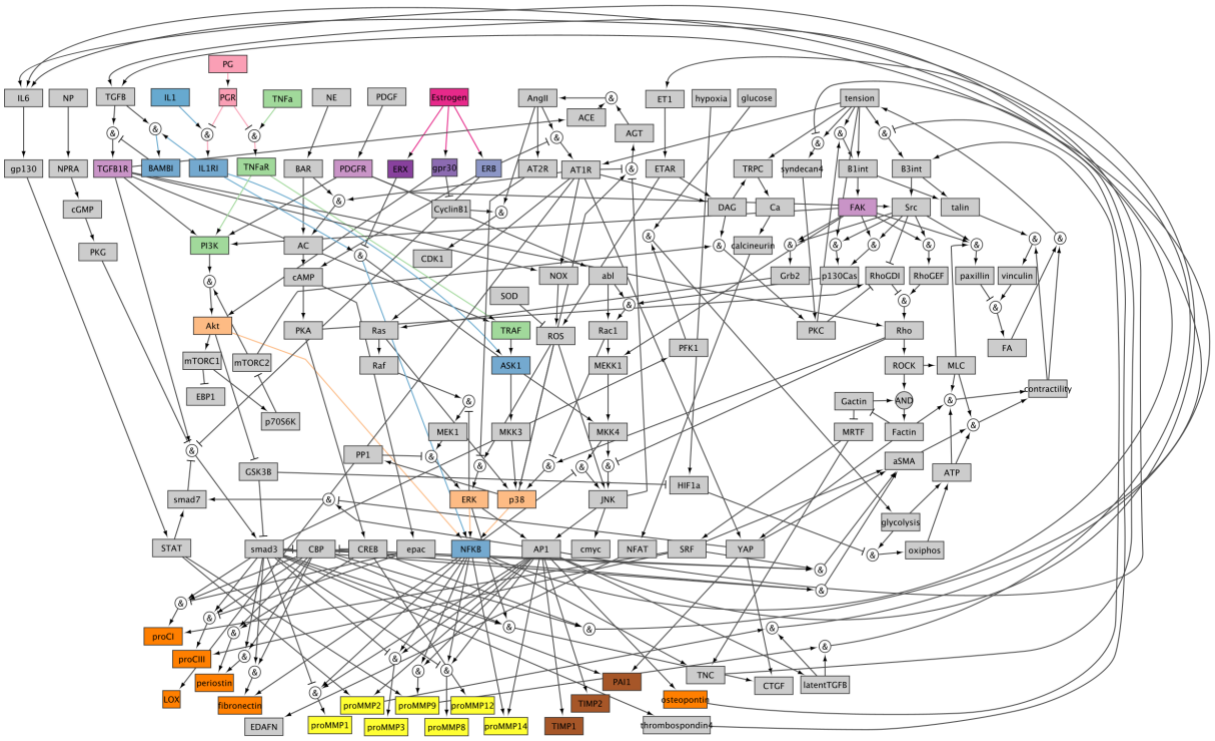


Figure 1. Cardiac fibroblast signaling network model integrated with progesterone (PG, highlighted in pink) signaling (including its interactions with interleukin-1 and tissue necrosis factor alpha, highlighted in blue). Logical NOT (-|, indicating an inhibition reaction), AND (AND in a circle, indicating that at least two species are required for a reaction), and OR (occurs when two or more arrows point to the same species, indicates only one species is required for a reaction) gates were used to define signaling interactions.

The signaling network model used in this experiment uses logic-based ordinary differential equations, modeled as a system of Hill equations, to capture the activity level of each node. Logical NOT, AND, and OR gates were used to govern the 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 $f_{or}(x_1,x_2) = f(x_1)+f(x_2) - f(x_1)f(x_2)$. The differential equations that populate the model were constructed using the open-source software Netflux for MATLAB, which is easily accessible and can be downloaded from github.

The default parameter settings included reaction weights as normalized activity levels between 0 and 1, $y_{int}=0$, $y_{max}=1$, and time constant ($\tau$) = 0.1, 1, or 10 for signaling reactions, transcription reactions, and translation reactions, respectively. The weights for the intermediate reactions were held constant at w=1 throughout all simulations, but the input weights were modified to simulate different cellular conditions. The Hill coefficient (n) for the new signaling model was set at 1.05, and the half-maximum effective concentration (EC50) for each reaction was set to 0.65. These decisions were based on the suggested default parameters for this particular cardiac fibroblast signaling model.

Evaluating the effect of progesterone on extracellular matrix production

In order to initially determine the effect of progesterone on extracellular matrix protein production, the weigh of the reaction representing the progesterone input was varied. This variance was intended to mimic the physiological differences in expression and activity levels of progesterone in male and female cells. Trials were simulated with the weight of the progesterone input node set to 0, 0.1, 0.25, 0.5, and 1.0, with all other inputs set to a weight of 0.1. Then, the same variation in progesterone was modeled with all other inputs set to a weight of 0.5. This was done to determine whether the effects on ECM protein production by differences in progesterone weight would still be significant if other signaling molecules were weighted more heavily than progesterone, i.e. if the effects of progesterone would be easily washed out if other inputs were heightened.

Determining the impact of progesterone on sex-specific drug screens

In a prior study, an approach to use a cardiac fibroblast network model to simulate the effects of known drugs on extracellular matrix production was developed by Zeigler et al[11]. In this work, 121 drugs from DrugBank were identified as including interactions with nodes present in the model, which represented 36 unique interactions. These unique interactions include both agonists, which are molecules that activate receptors or enzymes and antagonists, which are molecules that inhibit activation. Some of the interactions are competitive, meaning that the molecules bind to the activation site of the receptor or enzyme, while others are non-competitive, meaning that the molecules bind to a site other than the active site, leaving the active site available for substrate binding, but still inhibiting or activating the receptor or enzyme.

This framework was used to perform a sex-specific drug screen using the previous model, which only included estrogen. Once progesterone was added, the same framework was used to analyze the drug-target interactions that would be affected by the additional signaling pathways incorporated into the model. Out of the 36 original drug-targets interactions that were analyzed, 6 included interactions with molecules downstream of progesterone that did not have upstream molecules unrelated to progesterone. Molecules further downstream of progesterone that had

other upstream molecules capable of controlling activation were not considered in this initial screening because these nodes were not as directly connected to progesterone.

In this study, only the following 6 interactions will be reviewed: a NKFB and TNFα noncompetitive antagonist, an IL-1 competitive antagonist, an IL-1 noncompetitive antagonist, an IL-1 receptor competitive antagonist, a TNFα noncompetitive antagonist, and a TNFα competitive agonist. Since progesterone is known to inhibit both IL-1 and TNFα, and, the question of whether the presence of progesterone will affect the outcome of these drug-target responses should be addressed.

Four experimental conditions were considered in this screening: male levels of estrogen, male levels of estrogen and progesterone, female levels of estrogen, and female levels of estrogen and progesterone. The female levels of estrogen and progesterone in these simulations were set to represent the average levels of females during the reproductive years[9]. Previous study with this signaling network model found that the differences in drug-target responses between male conditions and post-menopausal female conditions were almost nonexistent, so only male and female (reproductive years) conditions were used in this screening[5]. For the male/post-menopausal female condition, estrogen and progesterone were both weighted at 0.1. For the female (reproductive years) condition, progesterone was weighted at 1 and estrogen was weighted at 0.5.

For each drug-target interaction considered, the administration of a static application of the drug was simulated (w=0.85) with profibrotic stimuli (all inputs except estrogen and progesterone set to w=0.4). In order to compare the potential changes to cardiac fibroblast activity, a Matrix Content Score was determined following the framework established in a previous study[5]. The Matrix Content Score was calculated as the sum of the average matrix activity, the average protease activity, and the average inhibitor activity: $MCS = Activity_{matrix} - Activity_{protease} + Activity_{inhib}$, where $Activity_{matrix}$ = (proCI + proCIII + fibronectin + periostin + osteopontin + LOXL1)/6, $Activity_{protease}$ = (proMMPs 1, 2, 3, 8, 9, 12, 14)/7, and $Activity_{inhib}$ = (TIMP1 + TIMP2 + PAI1)/3.

The Matrix Content Score for a control case for each condition was also calculated, in which all inputs other than progesterone and estrogen were set to w=0.4, and progesterone and estrogen were set to the designated levels for each condition. The difference between the Matrix Content Score for the control case and the Matrix Content Score for each drug was calculated, enabling a comparison in the difference in matrix output caused by each drug for each condition.

Examining the potency of each estrogen receptor

The signaling network model includes three separate receptors for estrogen: estrogen receptor alpha (ERX), estrogen receptor beta (ERB), and g-protein coupled receptor 30 (gpr30). Estrogen receptor alpha and estrogen receptor beta are nuclear receptors[13], while g-protein coupled receptor 30 is a transmembrane receptor[14].

In order to simulate a knockout of each receptor, the $y_{max}$ for two receptors was turned from 1 to 0.1, while the receptor of interest for that trial retained its original $y_{max}$ of 1. For these trials, the input weights for all molecules other than estrogen were held at 0.5 to simulate inflammatory conditions, but the weight for estrogen was held at 1 to ensure that its impact on extracellular matrix production could be analyzed.

**RESULTS**

Integration of progesterone into the cardiac fibroblast signaling network

To validate that progesterone was successfully integrated into the model, the activity levels for downstream molecules were monitored as progesterone weight was increased. The molecules that were monitored and their relationships to progesterone are highlighted in Figure 2. All other input weights were held at w=0.1 for one trial and at w=0.5 for a second trial. The results of these simulations can be found below in Figures 3 and 4.

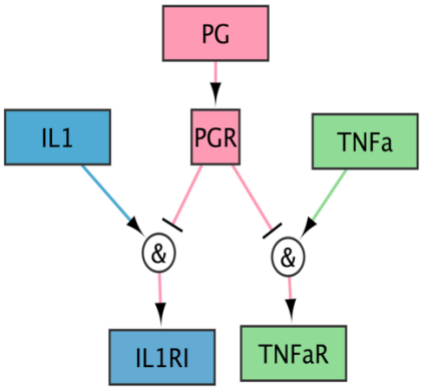


Figure 2. Molecules downstream of progesterone, chosen to validate the integration of progesterone to the signaling network model.

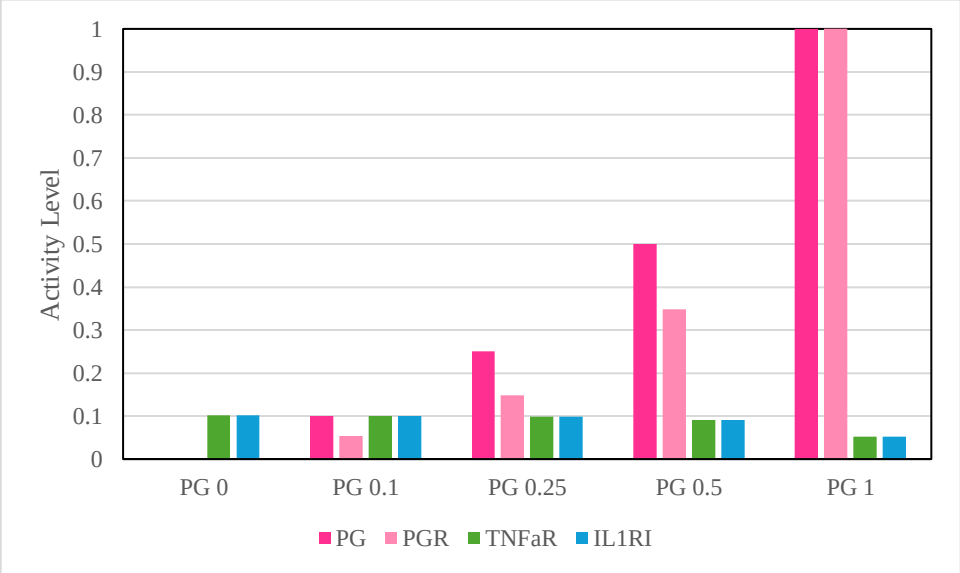


Figure 3. Select Activity Downstream of Progesterone, with Other Inputs w=0.1

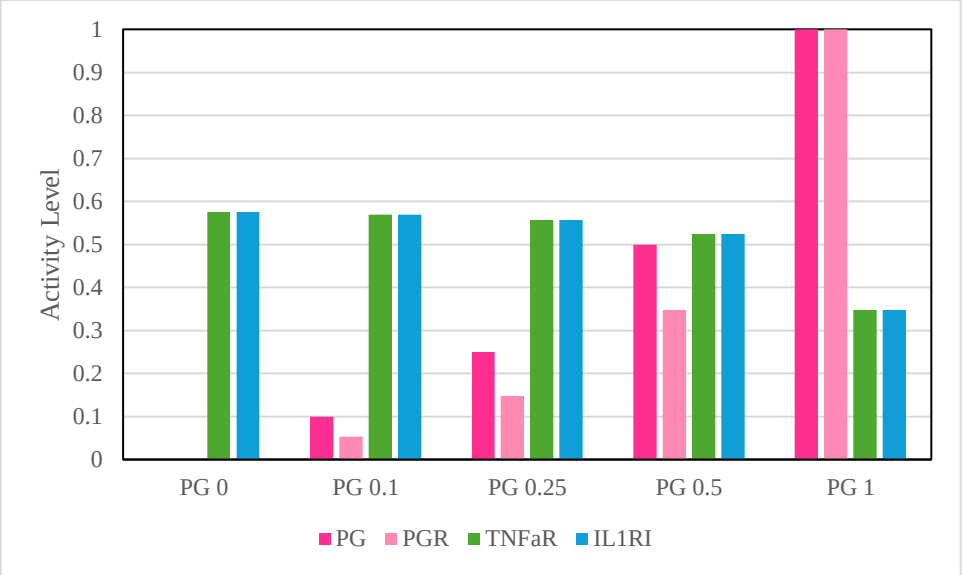

Figure 4. Select Activity Downstream of Progesterone, with Other Inputs w=0.5

The decrease in TNFaR and IL1RI activity levels show that the new progesterone pathway was incorporated correctly, as progesterone inhibits TNFa and IL-1. When other inputs were set at w=0.1, TNFaR and IL1R1 activity decreased by 49% as the progesterone weight was increased from 0 to 1 (Figure 3), but when other inputs were set at w=0.5, TNFaR and IL1R1 activity decreased by 40% (Figure 4). This shows that the impact of progesterone is dampened as other input weights are increased, meaning that progesterone has more impact on healthy cells than cells in a state of inflammation.

Evaluating the effect of progesterone on extracellular matrix production

To determine the effect of progesterone on molecules further downstream, activity levels of tumor necrosis factor receptor (TRAF) and phosphoinositide 3 kinase (P13K), which are both downstream of TNFa, and nuclear factor, apoptosis signal-regulating kinase, and BAMBI, which are all downstream of IL1. A heat map of the activity levels of these molecules can be found below, both with other inputs set at weight 0.1 (Table 1) and with other inputs set at weight 0.5 (Table 2). The overall activity levels are higher for the inflamed cell condition, so the changes in expression rather than the absolute levels are analyzed.

Table 1. Activity Downstream of Progesterone, with Other Inputs w=0.1

| Name | **PG 0** | **PG 0.1** | **PG 0.25** | **PG 0.5** | **PG 1** |
|---|---|---|---|---|---|
| PG | 0 | 0.1 | 0.25 | 0.5 | 1 |
| PGR | 0 | 0.053 | 0.1482 | 0.3482 | 0.9999 |
| TNFain | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| TNFa | 0.0989 | 0.0989 | 0.0989 | 0.0989 | 0.0989 |
| TNFaR | 0.102 | 0.1007 | 0.0979 | 0.0911 | 0.0524 |
| TRAF | 0.0791 | 0.0783 | 0.0768 | 0.0729 | 0.052 |
| P13K | 0.115 | 0.1143 | 0.1128 | 0.1091 | 0.089 |
| IL1in | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| IL1 | 0.0989 | 0.0989 | 0.0989 | 0.0989 | 0.0989 |
| IL1RI | 0.102 | 0.1007 | 0.0979 | 0.0911 | 0.0524 |
| ASK1 | 0.093 | 0.0918 | 0.0895 | 0.0837 | 0.0518 |
| BAMBI | 0.0028 | 0.0028 | 0.0027 | 0.0025 | 0.0014 |
| NFKB | 0.153 | 0.1519 | 0.1498 | 0.1445 | 0.1156 |
| MCS | 0.03464 | 0.03482 | 0.03443 | 0.03384 | 0.03064 |

Table 2. Activity Downstream of Progesterone, with Other Inputs x=0.5

| Name | **PG 0** | **PG 0.1** | **PG 0.25** | **PG 0.5** | **PG 1** |
|---|---|---|---|---|---|
| PG | 0 | 0.1 | 0.25 | 0.5 | 1 |
| PGR | 0 | 0.053 | 0.1482 | 0.3482 | 0.9999 |
| TNFain | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| TNFa | 0.4997 | 0.4997 | 0.4997 | 0.4997 | 0.4997 |
| TNFaR | 0.5749 | 0.5688 | 0.5563 | 0.5249 | 0.348 |
| TRAF | 0.5392 | 0.5345 | 0.525 | 0.5018 | 0.3872 |
| P13K | 0.6959 | 0.6929 | 0.6866 | 0.6714 | 0.5962 |
| IL1in | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| IL1 | 0.4997 | 0.4997 | 0.4997 | 0.4997 | 0.4997 |
| IL1RI | 0.5749 | 0.5688 | 0.5563 | 0.5249 | 0.348 |
| ASK1 | 0.6436 | 0.6372 | 0.6241 | 0.5913 | 0.4155 |
| BAMBI | 0.1507 | 0.1485 | 0.1439 | 0.1329 | 0.0782 |
| NFKB | 0.7854 | 0.7825 | 0.7764 | 0.7615 | 0.6859 |
| MCS | 0.27168333 | 0.27725238 | 0.2755619 | 0.27805952 | 0.25454762 |

With other inputs set at weight 0.1 (Figure 5), increasing progesterone begins to impact downstream molecules once progesterone weight is 5 times greater than that of other inputs. A

greater decrease is seen for TRAF than for P13K, likely because P13K is activated by several other molecules and is less dependent on progesterone signaling. When other input weights are set at 0.5 (Figure 6), a similar trend is seen: once progesterone weight overcomes other input weights, TRAF and P13K activities begin to drop, with a greater decrease present for TRAF than P13K.

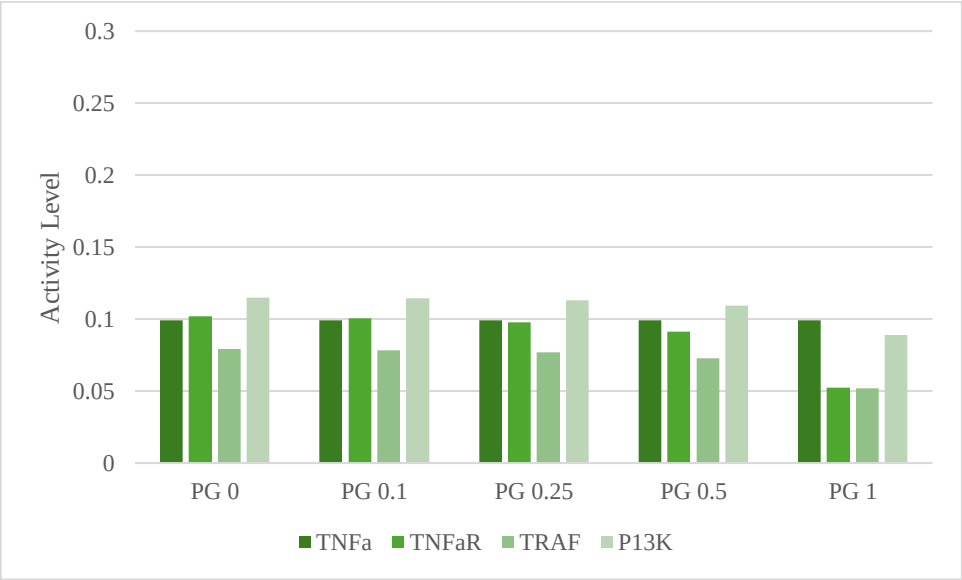


Figure 5. Downstream of TNFa, other inputs w=0.1

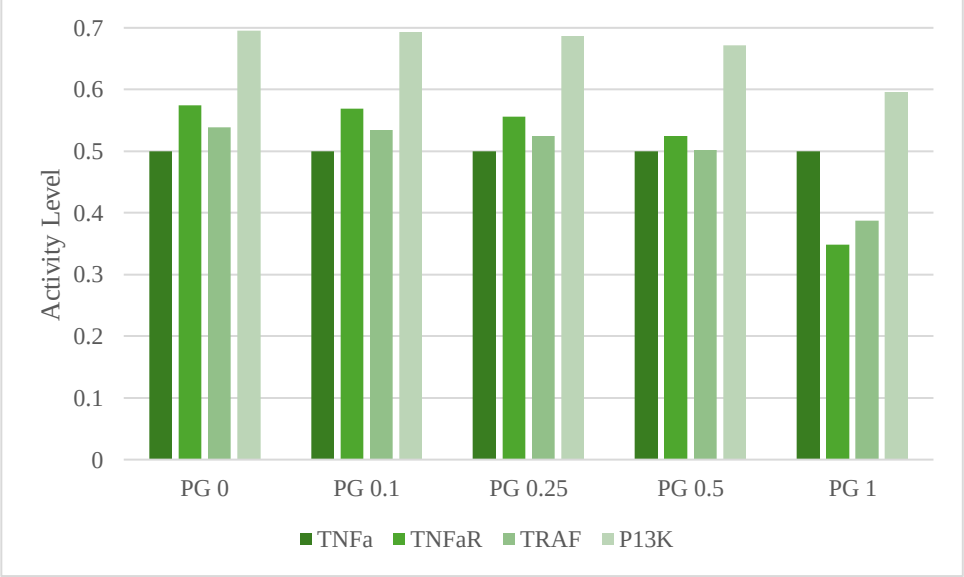


Figure 6. Downstream of TNFa, other inputs w=0.5

With other inputs set at weight 0.1 (Figure 7), increasing progesterone begins to impact molecules downstream of IL1 once progesterone weight is 5 times greater than that of other inputs. Increasing progesterone has a greater impact on ASK1 and BAMBI than NFKB. NFKB is activated by several other molecules and is less susceptible to inhibition by progesterone signaling. When other input weights are set at 0.5 (Figure 8), a similar trend is seen: once progesterone weight overcomes other input weights, ASK1, BAMBI, and NFKB activities decrease, with the greatest change occurring for ASK1 and BAMBI.

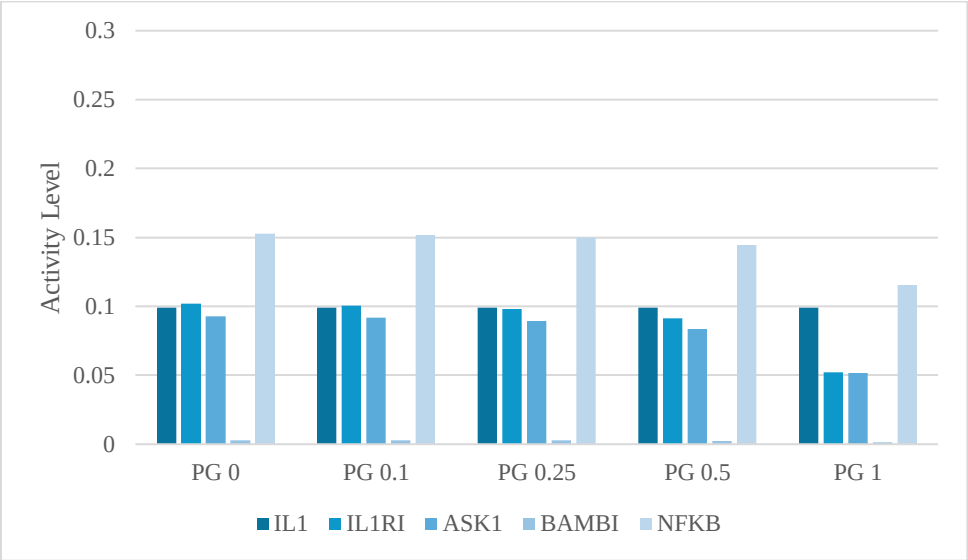


Figure 7. Downstream of IL1, other inputs w=0.1

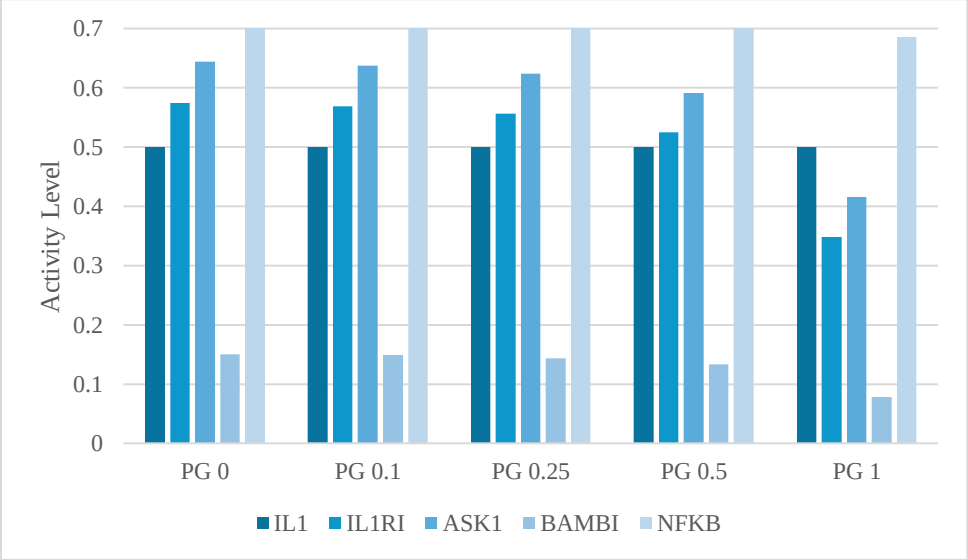


Figure 8. Downstream of IL1, other inputs w=0.5

The effect of progesterone even further downstream can be determined by comparing the Matrix Content Score for different input settings. With other input weights set to 0.1, increasing progesterone causes a decrease in the overall MCS for the cardiac fibroblast model (Figure 9). With other input weights set at 0.5, high progesterone weights still cause a decrease in the overall MCS, but this effect is not seen until progesterone weight is 1 (Figure 10). This trend matches what was observed when examining the activity levels of molecules immediately downstream: when progesterone weight is less than the weight of other inputs, it does not greatly inhibit downstream molecules.

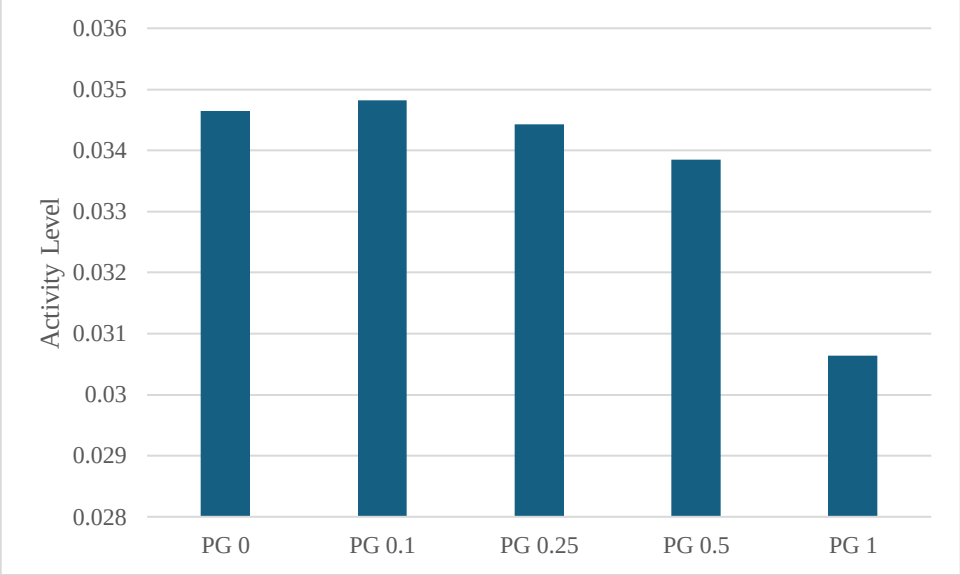


Figure 9. Matrix Content Score, other inputs w=0.1

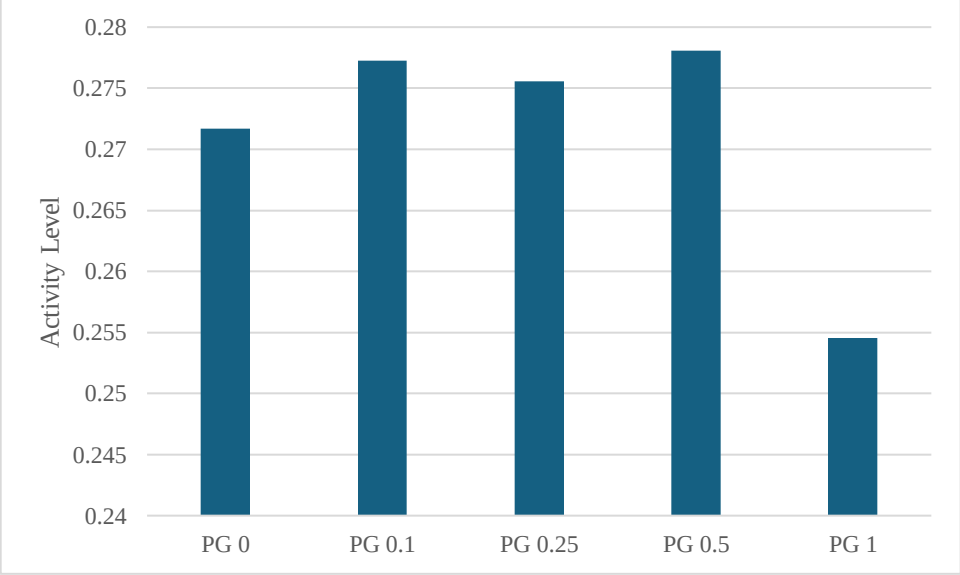


Figure 10. Matrix Content Score, other inputs w=0.5

To further determine the effect of progesterone on the extracellular matrix, individual components of the Matrix Content Score metric were examined. Both with other input weights at 0.1 (Figure 11) and at 0.5 (Figure 12), increasing progesterone weight from 0 to 1 causes a decrease in activity for all components except for matrix metalloprotease 12.

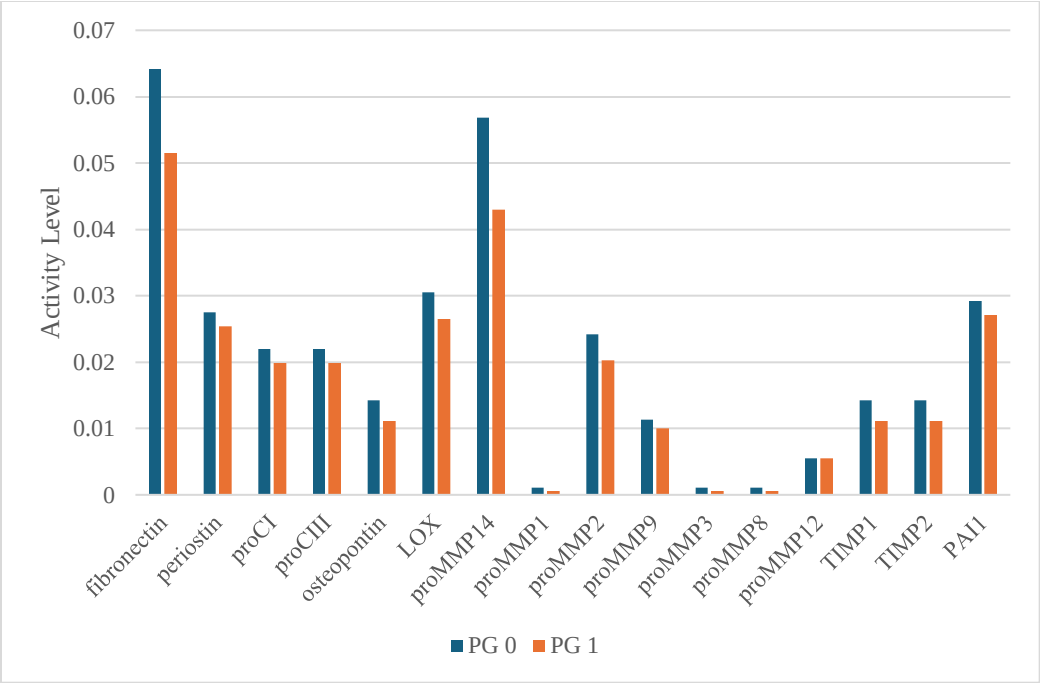


Figure 11. Individual Components of Matrix Content Score, other inputs w=0.1

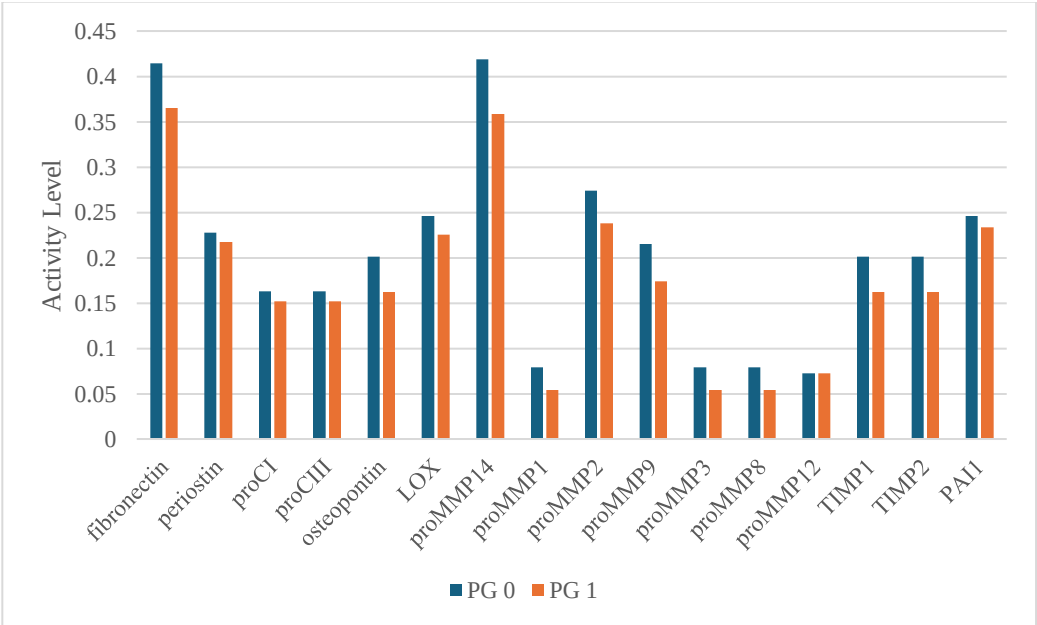


Figure 12. Individual Components of Matrix Content Score, other inputs w=0.5

To better understand the effect of progesterone, the relative decreases in activity in response to progesterone of these molecules was calculated and graphed below in Figure 13.

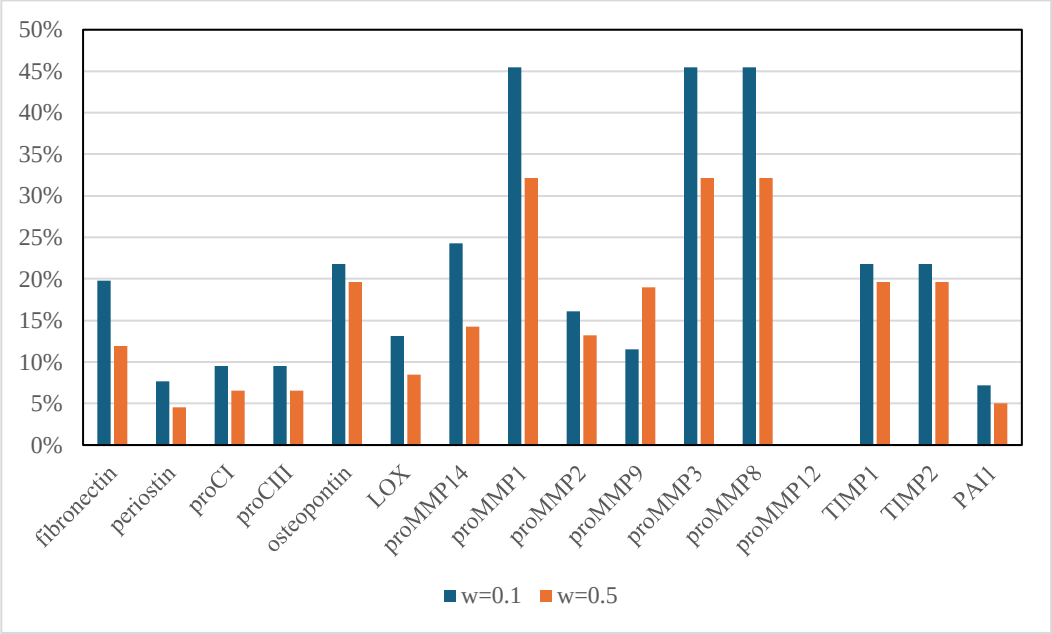


Figure 13. Percent Relative Decrease in MCS Components

Progesterone caused the greatest relative decrease for matrix metalloproteases 1, 3, and 8 in both the healthy and inflamed cell conditions, and matrix metalloprotease 12 was not affected by progesterone in either condition. This explains how progesterone decreased the Matrix Content Score: by decreasing the production of matrix proteins and protease inhibitors but leaving a protease fully active, the overall effect on the extracellular matrix decreased growth.

<u>Determining the impact of progesterone on sex-specific drug screens</u>

Each drug-target interaction was modeled separately, and the Matrix Content Score for each simulation was calculated. The differences between the MCS for the drug trials and the MCS for each condition, without any drug interactions, can be found below in Table 3. These values represent the effect on the extracellular matrix caused by the addition of drug-target interactions.

Table 3. Matrix Content

| | | | | |
|---|---|---|---|---|
| NKFB + TNFa antagonist | -0.01581 | -0.01087 | -0.01811 | -0.01107 |
| IL1competitive antagonist | 0.01266 | 0.01598 | 0.01071 | 0.01596 |
| IL1 noncompetitive antagonist | 0.01266 | 0.01598 | 0.01071 | 0.01596 |
| IL1R competitive antagonist | 0.03962 | 0.02676 | 0.03398 | 0.02647 |
| TNFa noncompetitive antagonist | -0.00035 | -0.00206 | -0.00204 | -0.00220 |
| TNFA competitive agonist | 0.12184 | 0.13476 | 0.12340 | 0.13491 |
| | F, no PG | M, no PG | F, PG | M, PG |

For all drugs in this study, there were differences between the female and conditions. Although for many drugs these differences were very small, even a slight difference could impact the outcome of a treatment regimen. Slight differences were also noted when progesterone signaling was incorporated, with a greater effect seen for the female conditions as progesterone signaling is more prevalent for females then for males.

Examining the potency of each estrogen receptor

The Matrix Content Score for each knockout trial was calculated, showing that estrogen receptor alpha increases matrix production the most, and estrogen receptor beta decreases matrix production the least (Figure 14).

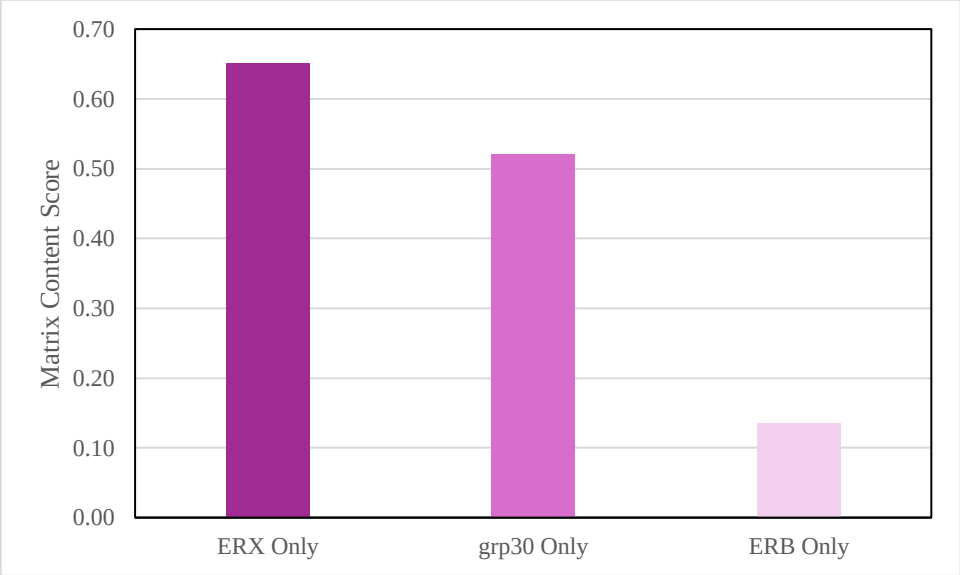


Figure 14. Matrix Content Score for E2 Receptor Knockdown

The matrix index, protease index, and inhibitor index values for each knockout trial are reported below in Figure 15. While estrogen receptor alpha and g-protein coupled receptor 30 primarily activate matrix production and protease inhibitors, estrogen receptor beta primarily activates proteases.

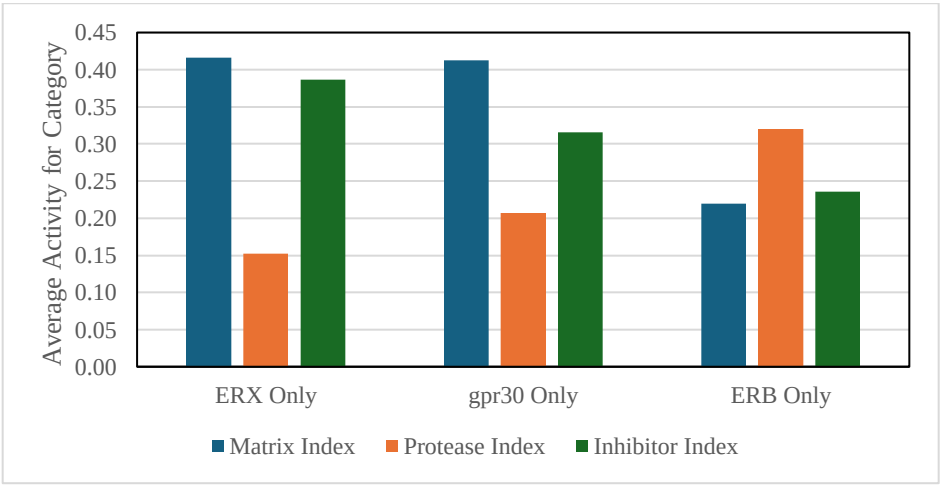


Figure 15. Matrix, Protease, and Inhibitor Indices for E2 Receptor Knockdown

## DISCUSSION

The development of drugs that are capable of selectively impacting cardiac fibrosis has been a slow process due to the complicated nature of the signaling mechanisms in cardiac fibroblasts. These signaling networks can be influenced by a variety of factors, including biochemical interactions, mechanical forces, and biological sex. In this study, a previously published sex-specific signaling network model for cardiac fibroblasts was further developed by integrating progesterone signaling. This model was then used to understand the relationship between progesterone and extracellular matrix production. Additionally, the impact of each of three estrogen receptors on extracellular matrix production was analyzed, and a sex-specific drug screen determined the effect of progesterone and estrogen signaling on predicted drug outcomes on extracellular matrix production.

Progesterone was shown to decrease extracellular matrix production by inhibiting matrix production and protease inhibitors while maintaining the activity of matrix metalloprotease 12. Estrogen receptor beta was found to have a smaller promotional effect on extracellular matrix production than estrogen receptor alpha and g-protein coupled receptor 30. Estrogen receptor beta promotes protease activity at greater levels than the other two estrogen receptors in this model, decreasing overall extracellular matrix production.

The sex-specific drug screen confirmed previously published differences between drug outcomes for male and female cells. These differences were maintained when progesterone signaling was added. A greater difference was seen between male and female conditions than presence and absence of progesterone conditions, suggesting that estrogen signaling plays a greater affect in regulating extracellular matrix production than progesterone.

The original cardiac fibroblast signaling network model was previously validated against 47 independent research papers that directly measured intermediate and outputs nodes in the model[5]. The model was found to be 81.8% accurate in predicting experimental activity levels of outputs as certain inputs were changed and of intermediates as certain inputs were changed[5]. Then, additional papers were added to the bank of data in order to validate the response of the model to estrogen. The female-specific data that was incorporated to the validation set measured either the direct output secretion or an intermediate signaling response to a single input stimulus in fibroblasts. The completed validation set included 185 perturbation experiments, the results of which were based on cells that were 39% male, 36% pooled male and female, 17% unreported, and 8% female[5]. The model was found to be 77% accurate in predicting model changes once estrogen was added[5]. The model was specifically found to be 59% accurate in predicting the outcomes of estrogen treatment[5]. This lower accuracy is likely due to the smaller number of papers that reported female-specific data for cardiac fibroblasts.

Since progesterone has not been the center of many specific research studies focused on cardiac fibroblast signaling, no validation was performed for the changes implemented in this study. However, since the majority of the interactions in the signaling network model that involve progesterone were previously validated either with general studies or with sex-specific studies, further validation was not deemed necessary for this study. Before this model is used in a setting other than the academic world, further investigation should determine its accuracy in predicting outcomes for conditions that include varying levels of progesterone.

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