Role of Dopamine Receptor 3 in Cardiac Fibrosis

By

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Abstract

Dopamine receptors are known to exist primarily in the central nervous system and have been extensively studied there. However, other organ systems in the periphery, such as the kidney, have been shown to express these receptors as well. Studies on dopamine receptors in the cardiovascular system are both lacking and contradictory. Dopamine receptor agonists are prescribed to patients with neurological diseases and some medications have been removed from the market due to fibrosis and heart valve regurgitation. We hypothesized that there is an intrinsic cardiac dopaminergic system and changes to that system can lead to left ventricular (LV) remodeling and particularly, cardiac fibrosis. The first aim focused on determining the relative gene expression of all five dopamine receptors in both wild type (WT) mouse heart tissue and LV cardiac fibroblasts. Then, the expression and relationship between excitatory dopamine receptor 1 (D1R) and inhibitory dopamine receptor 3 (D3R) was examined through a dopamine receptor 3 global knock out (D3KO) mouse model. Both receptors were confirmed to express in WT cardiac fibroblast cells and heart tissues through a comprehensive analysis via immunofluorescence, RT-qPCR, and western blot. We found that in the D3KO fibroblasts, D1R expression is significantly

increased around 8-fold compared to WT. In addition, regardless of pharmacological treatment with agonists and antagonists, the increase in D1R expression was seen in the D3KO fibroblasts. The second aim focused on the relative expression of known profibrotic markers in order to determine if dysfunctional D3R can lead to a profibrotic phenotype. Relative gene expression of type I collagen decreased, while relative gene expression of type III collagen increased in the D3KO heart tissue samples. Both matrix metalloproteinases (MMP's), MMP2 and MMP9 were significantly decreased in the D3KO LV cardiac fibroblasts. Finally, known profibrotic marker, transforming growth factor β1 (TGF-β1), and the angiotensin II receptor type I were examined. In the D3KO LV cardiac fibroblasts, angiotensin II receptor type I was significantly increased many folds. TGF-β1 was also increased in D3KO fibroblasts and myocardial tissue. Thus, we determined that there is an intrinsic cardiac dopaminergic system in WT mice, and that the loss of function of D3R in the cardiac system contributes to a profibrotic phenotype. This data provides positive proof for the existence of an intracardiac dopaminergic system as well as highlighting the possibility that dysfunctional D3R can contribute to cardiac fibrosis.

Role of Dopamine Receptor 3 in Cardiac Fibrosis

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Dedication

I lovingly dedicate this thesis to my late father, for without his encouragement, support, and general love of science, I would never have started this journey.

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To start with, I have to acknowledge my mentor Dr. Katwa. He took me into his lab when I was down and out, met me at my skill level, worked with and taught me everything I needed to know to get to the master's level, and all while supporting and encouraging me. He has a true love of teaching that can be so lacking these days and whatever he does, he does from his heart to make others happy with no expectation for anything in return. I just want you to know Dr. Katwa, you made me smile and you gave me a chance that no one else would. I wouldn't be here without you and I will be forever grateful.

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Chapter 1

Introduction

1.1 Dopamine and Dopamine Receptors

Dopamine is a catecholamine found throughout the body that is responsible for inducing a pleasure response in the brains of mammals. Likewise, dopamine controls many bodily functions such as movement, cognition, and overall behavior (1). The localization and function of dopamine and its receptors have been examined and reported meticulously within the brain and central nervous system (CNS) (2). Dopamine receptors are classified as G-protein coupled receptors (2, 3), which are divided into two classes, D1-like and D2-like receptors, based on interactions with the enzyme adenylyl cyclase. The D1-like receptors consist of D1 and D5, while D2, D3, and D4 comprise the D2-like receptors (2-4). D1-like receptors serve to stimulate and increase adenylyl cyclase activity and act through the G_s protein subunit, whereas D2-like receptors inhibit adenylyl cyclase and act through the G_i protein subunit (2, 5-7). Dopamine receptors are located within many tissues in the CNS and peripheral nervous system (PNS), such as the brain, spinal cord, kidney, heart, gastrointestinal tract, and the eyes (2, 3, 8). While there is an abundance of reports regarding dopamine receptor expression in the CNS, information is lacking on the expression, function, regulation, and role of dopamine receptors within the PNS (2). However, the idea of dopamine having a key role in the PNS is not entirely new, as this was first proposed in 1960 (9). In the sixty years since, additional data regarding dopamine and its receptors in the PNS has been uncovered, but considerable research questions remain. These receptors are able to modulate cardiac activity through interactions with the CNS and PNS, the heart and kidneys, as well as smooth muscle cells (10). In addition, it has been postulated that dopamine serves to control sodium excretion in the kidneys, thereby regulating blood pressure (11). Studies can be found in

the literature reporting findings of peripheral and cardiovascular pathologies upon aberrant function of every subtype of the dopamine receptor family (3, 11-15). Despite this, there remains a lack of definitive consensus from studies regarding the dopamine receptor 3 (D3R) subtype in the periphery, specifically in the cardiovascular system. Thus, the role that D3R may have in the cardiovascular system and its potential impact on cardiac fibrosis is of particular interest in this report.

1.2 Possible Functions of Dopamine Receptor 3 in the Cardiovascular System

The roles of D3R in organ systems outside of the CNS remain virtually unidentified. One conceivable function of D3R is the regulation of cellular growth. In neuronal cells, the activation of D3R indirectly caused an upregulation of the phosphorylation of the extracellular signal-regulated kinase (ERK) (16), which has been well established to positively regulate cellular growth and inhibit apoptosis (17). It is possible that in organ systems other than the brain, D3R can regulate the proliferation and inhibition of apoptosis in fibroblasts and other cells. This is supported by a study recently published from our lab wherein the global knock-out of D3R (D3KO) in mice attenuated proliferation of cardiac fibroblast cells compared to the same cell type isolated from wild-type (WT) mice (18). In contrast, stimulation of D3R has been shown to have a significant inhibitory effect on proliferation in vascular smooth muscle cells, mediated predominantly by Protein Kinase C-zeta signaling, but only during concurrent stimulation of dopamine receptor 1 (D1R). Of note though, is that this effect of the dopamine receptors appears to be dependent on adrenergic signaling, as pharmacological stimulation of either D1R or D3R alone did not have any effect on cell proliferation in this cell type (5). This may suggest a link between the regulatory activities of the D1-like family of dopamine receptors, in which heterodimers of D1R and D3R may have more potent regulatory effects than homodimers (5). This notion was also suggested in

another study between D1R and D3R in Wistar-Kyoto (WKY) rats, wherein concurrent pharmacological stimulation of D1R and D3R demonstrated increased relaxation of a preconstricted arterial ring compared to D1R stimulation alone (19).

The function of D3R on pumping volumes and heart rate has been studied briefly. In guinea pigs, a report has indicated that the use of a D3R agonist causes a concentration-dependent reduction in heart rate, with the authors suggesting two potential mechanisms by which this may occur (20). The direct mechanism put forward describes the agonist as stimulating D3R via binding to an allosteric site. However, the 'indirect mechanism' suggested that the agonist has *no* interaction with D3R, since reversal of the effects did not occur until administration of notably high concentrations of D3R antagonist. The authors indicated that the mechanistic aspects of D3R on the regulation of heart rate were beyond the scope of their investigation and so they are left unconfirmed (20). Apart from this, research has been performed to assess cardiac function in WT mice. It was shown that for both sexes of WT mice, D1R and D5R expression increased, while D2R and D3R expression decreased when comparing 12-month-old mice to 6-month-old mice, suggesting an age-dependent shift in receptor expression (21). In conclusion, while evidence exists to support the notion of functional D3R in heart tissue, there is still a lack of data and furthermore, a lack of consensus among the literature.

Through the actions of the peripheral dopaminergic system, dopamine receptors serve as key players that regulate sodium balance and blood pressure (22, 23). Hypertension and renal failure are risk factors for cardiovascular issues in the later stages of life and evidence suggests D3R may regulate these processes. For example, analysis of homozygous and heterozygous D3R mutant mice revealed that both mutants had higher systolic and diastolic blood pressures than their WT counterparts (24). Although blood pressure reduced to the same degree in WT, heterozygous,

and homozygous D3R mice upon administration of losartan, WT mice reverted to their baseline blood pressures 10 minutes faster than the heterozygous mice and 20 minutes faster than the homozygous mice. Thus, altered or reduced D3R activation appears to increase systolic blood pressure and cause diastolic hypertension (24). However, counter to these experiments, Staudacher et. al. found no significant difference in blood pressures of both D3KO and WT mice (11). One significant difference between these two investigations which may prompt variation in blood pressure findings was the consciousness of the mice at the time of blood pressure reading. Asico et. al. measured blood pressures in anesthetized mice (24), while Staudacher et. al. measured blood pressure via tail cuff on conscious mice (11), suggesting other variables, such as stress (5), may influence the dependence of D3R on regulating blood pressure. Navigating away from animal models, blood pressure studies have also been conducted on humans. A clinical trial focused on observing blood pressure in patients suffering from Type 1 Diabetes Mellitus and gene polymorphisms in the D3R gene found no association between gene defects and blood pressure (25). Although unclear as to how these polymorphisms may or may not positively or negatively impact the function of D3R, these differing observations suggest a potential species-specific difference in blood pressure modulation by D3R.

It has been hypothesized that knocking down D3R could result in mechanisms of compensation from other dopamine receptors to maintain homeostasis. Wang et. al. revealed that homozygous D3KO mice fed a normal salt diet demonstrated a significant increase in D5R expression within the kidney (26). In homozygous mice, hypertension was exacerbated by treatment with a D1-like receptor antagonist which also affected D5R. Based on this, it was concluded that the increase in D5R helped regulate blood pressure when on a normal salt diet. Important to note is that this upregulation in D5R may be tissue specific, as the expression of this

receptor remained normal in both heart and brain tissues (26). Another study working with renal proximal tubule cells has shown that increased activation of D3R leads to increased expression of D4R (27). While these findings are within the renal system, the same process could occur in other organ systems as well. This also indicates that regulation in the function of D3R is not only essential, but that crosstalk occurs between excitatory and inhibitory dopamine receptor families, which may be crucial for maintaining normal blood pressure.

1.3 Dopamine Receptor 1 and Dopamine Receptor 3 Interactions

In the last few years, the relationship between dopamine receptor 1 (D1R) and D3R, D1-like and D2-like receptors respectively, has been examined by a handful of studies. In general, dopamine receptors can associate to form either homomers or heteromers. This coupling serves to change the effects of the G-protein coupled receptors. It has been observed that both D1-like and D2-like receptors can be expressed on the surface of the same neuron. In the CNS, D1R and D3R have been shown to coimmunoprecipitate and further associate into heterodimer or heterotetramer configurations (28, 29). This same phenomenon has also been reported in rat striatal tissue (30). It has been established that the disruption of D3R resulted in an increase in D1R protein expression in the spinal cord; this implies that D3R and D1R could be interacting (31). This was similarly confirmed by research which indicated that D1R and D3R interact in the cells of the superficial dorsal horn in the spinal cord (32). An investigation conducted on mice suggests that aging appears to increase excitatory D1R expression, but does not affect inhibitory D3R expression in both the striatum and the spinal cord (33). In addition, after long-term treatment with a D3R agonist, the administration of a D1R blocker reversed the effect of the D3R agonist and caused a significant increase in thermal pain withdrawal latency in mice (34). The coimmunoprecipitation of D1R and D3R was exhibited in the renal proximal tubule cells of WKY rats, as well as a diminished

coimmunoprecipitation of the two receptors in spontaneously hypertensive rats (19). Fenoldopam (a D1R agonist) increased D3R proteins in human coronary vascular smooth muscle cells via D1-like receptors (19). When renal proximal tubule cells were observed, a D3R agonist increased D1R expression in direct relation to treatment duration and concentration. It was indicated that within the rat kidney, D3R plays a role in the regulation of D1R, and it was determined that the stimulation of the D1R by the D3R agonist was selective. It was also reported that the D3R antagonist by itself had no effect on D1R, but it did block the D3R agonist's effects as expected. However, the mechanism for how the modulation of D3R increases D1R protein expression is absent (35). According to these observations, excitatory D1R and inhibitory D3R influence each other's function, but whether this is due to effects from direct interaction between these proteins or whether it is a function of downstream regulation of gene expression of each receptor remains to be determined.

Similar to D3R, the actions of D1R in the heart and vasculature remain unclear (2). In thoracic aorta smooth muscle cells isolated from rats, D1R and D3R were observed to coimmunoprecipitate, with administration of a D1-agonist, fenoldopam, resulted in greater interaction between these proteins over time (36). D1-like receptors have been reported to be expressed in smooth muscle tissue (2, 3, 37), and the unique interactions between D1R and D3R may promote pleiotropy within downstream dopamine receptor signaling, as demonstrated with additive inhibitory effect on norepinephrine-mediated proliferation in the artery with stimulation of both excitatory D1R and inhibitory D3R (5). Within human and mouse models, cardiac D1R expression is increased during heart failure (12). It was established in lung fibroblasts that highly effective D1R agonists were able to block the expression of α-smooth muscle actin and the authors confirmed through microscopy that the agonist was able to reduce the contractile forces generated

by the fibroblasts (38). An important inquiry can be presented based on these analyses: are these two receptors controlling functions via crosstalk? If so, then how? If D1R and D3R are working in conjunction within the heart, this information could lead to the elucidation of mechanisms which may directly contribute to cardiac fibrosis and heart failure. This clarification would allow for possible therapeutic targets to halt or reverse the profibrotic actions of these receptors if there are any, including the prospect that therapies might need to target only one of these receptors. rather than focusing on both, to produce a beneficial effect.

1.4 Extracellular Matrix Collagens

An essential concern of this project is the possible connection between D3R and cardiac fibrosis. In order to understand how fibrosis occurs, however, we must examine the extracellular matrix (ECM). Most cells that make up organ systems and tissues produce and maintain an ECM. In terms of tissue organization, the ECM is responsible for differentiation and, most importantly, the structure on which the cells coordinate and form the tissues which further form into fully functioning organs (39, 40). On a cellular scale, most of the components of the ECM are secreted by cells but the ECM is not cellular itself (39). The ECM is comprised of two components, the basement membrane and the interstitial matrix (41, 42). There are a vast number of proteoglycans and fibrous proteins which comprise a majority of the interstitial matrix of the ECM (39, 41, 42). Of particular importance to this report are the collagen proteins, which are the most common proteins in the ECM (39) and matrix metalloproteinases (MMP's) which are enzymes whose function is to degrade parts of the ECM to maintain homeostasis (40, 43).

MMP's are endopeptidases which are classified into six groups. They contain zinc and are calcium dependent. Commonly, MMP's are synthesized within the cell, then cleaved and excreted into the ECM (40). There are over thirty different MMP's that have been classified and many of

those have been found in cardiac tissue, particularly in the left ventricle (LV) (44). MMP's can degrade ECM substrates varying from only one per MMP to many (41, 45). This project will focus on MMP's which are both present in the LV and whose main functions are contributing to collagen degradation and subsequently, possible accumulation. Collagen type I fragments can be degraded by two gelatinases, MMP2 and MMP9 (41). Both of these MMP's have been identified within the LV of hearts including after myocardial infarction, which can lead to an increase in collagen and thus, an increase in tissue fibrosis and scar formation (44). An increase in MMP2 activity has been postulated to result in cardiac dysfunction in different models of spontaneously hypertensive rats (43). Elevated levels of MMP2 have been found within the plasma of patients with acute myocardial infarction (43). Cardiac fibroblasts in particular are influenced by MMP9 activity (45). Both MMP2 and MMP9 have been implicated as markers of cardiac fibrosis (46). Due to all of these observations, MMP2 and MMP9 will be focused on in the rest of this report.

Collagen is a major structural component of the ECM as it can self-assemble into fibrils and other types of structures (47, 48). There are more than 25 different types of collagens that have been identified, with collagen type I and collagen type III prevailing among these (49). In particular collagen I and collagen III are denoted as fibrillar collagens which assemble into fibrils (49, 50). It has also been postulated that there is an important balance in the ratio between collagen I and III in tissues. If this balance is adjusted, adverse side effects can occur (41). A decrease in the ratio could lead to tissues displaying increased and detrimental flexibility and elasticity, while an increase in this ratio could lead to rigidity and possibly fibrosis (41). In addition, both collagen types have been stained in myocardial tissue as two of the predominant collagens there as well (51). Due to these factors, examining collagen I and collagen III is essential in determining possible routes to fibrosis.

1.5 Dopamine Receptor 3 and Cardiac Fibrosis

Cardiac fibrosis, in general, is the accumulation of excessive amounts of collagen in the ECM. This can be caused by a number of different factors and possible dysfunctions in the ECM systems for homeostasis, as well as a number of cardiovascular affecting diseases such as hypertension, diabetes, and obesity (52). In this report, I am interested in exploring the relationship between D3R and cardiac fibrosis. Global knockout of D3R in mice results in an excessive accumulation of collagen within the heart interstitial, thereby suggesting a correlation between fibrosis and dysfunctional D3R (53). Furthermore, my lab has recently indicated the existence of D3R expression on the surface of mouse cardiac fibroblasts isolated from the LV (18). Still, whether these findings are translatable to the human heart is more obscure, as a 2016 review indicates that rat and human D3R expression is exclusive only to brain tissue, restricted specifically within the limbic region (54). This is further corroborated by a study performed by Cavallotti et. al., as D3R was the only dopamine receptor subtype not identified in human heart tissue sections via immunohistochemistry, even among the epicardium, myocardium, and endocardium (3). At the time of writing, this investigation by Cavallotti et. al. is the only one to report on dopamine receptor expression in the human heart. In contrast, protein analysis from a separate study suggests that within mouse hearts, all dopamine receptors except for D4R are present (21). Considering the dichotomy among these studies regarding D3R expression within cardiac tissue, and that each utilized a different model system, there appears to be species-specific distribution of dopamine receptors within cardiac tissue. Still, due to the apparent lack of comprehensive evidence in the literature, there remains no clear consensus in the field regarding D3R's role in the cardiac system. There are still many inconclusive answers to the question of D3R expression in the mammalian cardiac system. Regardless, gaining a clear consensus of these questions will not only expand the

range of therapeutic modalities for which to treat cardiovascular diseases, but it will also enhance our understanding of the adverse cardiovascular risk effects that may accompany pharmacological targeting of dopamine receptors (55-58).

It has been hypothesized that improperly functioning or missing D3R can contribute to fibrosis of the heart. For example, a recent study has proposed that activation of D3R signaling may protect against morphine-induced cardiac fibrosis (7). Healthy C57BL/6J mice were subject to chronic administration of morphine (2mg/kg daily for 7 days) with or without seven days of withdrawal, and the effect of time-dependent pramipexole (D3R agonist) administration on cardiac remodeling was determined. Interestingly, regardless of the timing of D3R agonist treatment (i.e., during or after administration of morphine), cardiac hypertrophy was significantly abrogated. Although the only time point able to prevent aberrant collagen deposition compared to naïve heart tissue was pramipexole administration during the withdrawal period (7). Thus, it was concluded that D3R could serve a cardioprotective role as an adjuvant therapy with morphine administration (7). Fibrosis has also been shown to increase with age. An investigation was conducted on WT and D3KO mice of both sexes at different age points, with an observed age-dependent increase in heart fibrosis in both sexes of WT mice. In particular, more intense accumulation of collagen was present in the D3KO mouse heart tissue, suggesting a role for dopamine and D3R in signaling and fibrosis in the left ventricle (21). Additionally, Johnson et. al. measured blood pressures and heart rates of WT and D3KO mice at 2 months, 1 year, and 2 years of age. In the WT mice, blood pressure increased and heart rate decreased during aging. Interestingly, however, blood pressures of 2-month-old D3KO mice were comparable to 2-year-old WT mice. Along with increased blood pressure, there was also comparable cardiac fibrosis between 2-month-old D3KO mice and 2-year-old WT mice (53). Although evidence from isolated mouse cardiac fibroblasts and D3KO mice

support the notion of D3R's direct influence on the homeostasis of the extracellular matrix, it cannot be dismissed that a contributory *indirect* effect of dysfunctional D3R on cardiac fibrosis may be from this receptor's influence on the renin-angiotensin-aldosterone system (24, 59). Still, while this area of research, specifically regarding the mechanisms by which D3R leads to fibrosis, remains largely unexplored, these few studies indicate that D3R has the capacity to influence the pathogenesis of cardiac fibrosis.

1.6 Formation of Hypothesis

Exploring the possibility of D3R contributing to, or directly inducing cardiac fibrosis was fascinating and exciting to me. The university already had a colony of D3KO mice, and further exploring the work done by Johnson et. al. seemed like a good place to start. When I began delving into research on organ systems in the PNS, I noted that there was not much data on dopamine and its receptors in the cardiovascular system. Although dopamine has been prescribed to patients with heart failure and the effects of various concentrations of dopamine on these patients' cardiovascular systems have been investigated, the results are still varied with no conclusive evidence indicating overall beneficial nor detrimental effects (60). While D3R has been found in several arteries and cardiac tissues, the expression and functional significance of D3R in the heart is not well defined. Thus, I created a schematic depicting different major routes to cardiac fibrosis (**Figure 1**) to better visualize possible routes by which cardiac fibrosis may be influenced by the dopaminergic system. The use of global D3KO mice as a tool to elucidate the mechanisms by which D3R can contribute to cardiac fibrosis was my starting point as I had access to the hearts from those mice. Thus, this schematic has a starting point involving dysfunctional D3R as well as D3KO mice. In addition, the interactions between D1R and D3R are of interest when attempting to place D3R in the context of cardiac fibrosis, and so that aspect has been included as well.

Figure 1

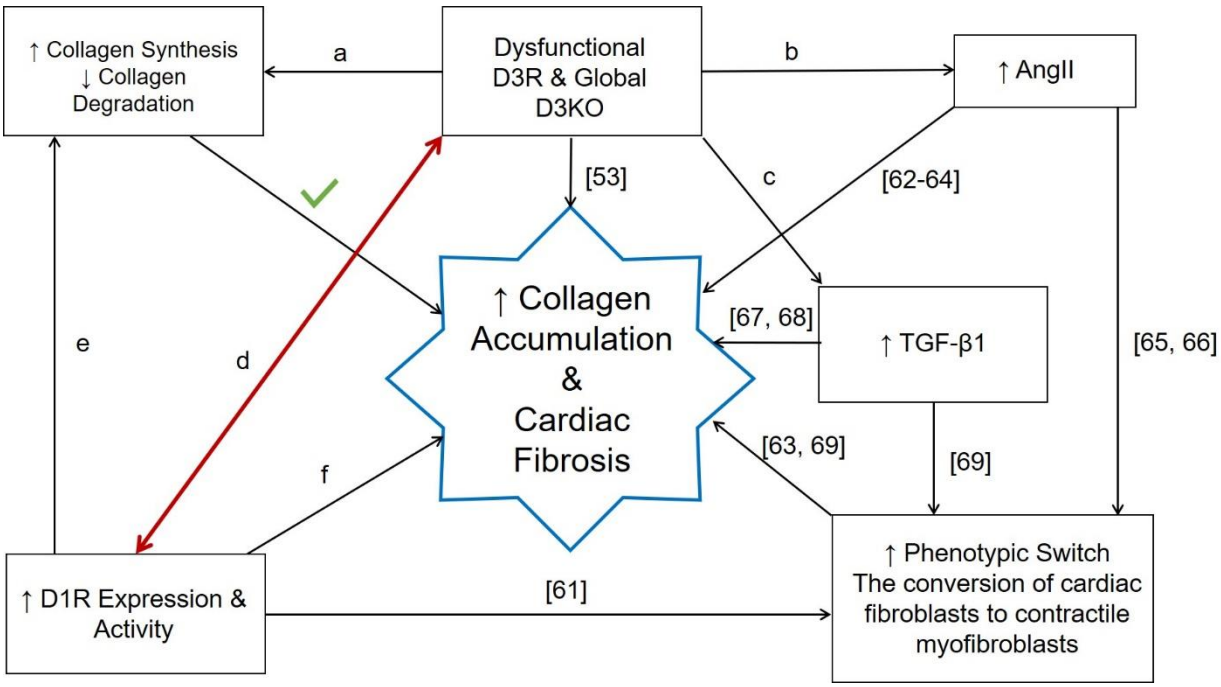


Figure 1: **Possible Pathways Associated with Dysfunctional D3R and D1R Leading to Collagen Accumulation and Fibrosis in the Heart.** Each arrow denotes a pathway and the direction of the pathway which could lead to the increase in collagen expression and accumulation starting with dysfunctional D3R. Of particular interest is pathway d, denoted in red, which is the relationship between D1R expression and activity and a decrease in D3R function. Letters are indicative of pathways which remain unexplored, while other pathways with contributing evidence have been provided with their respective references.

The creation of these pathways involved attempting to answer numerous questions using previously conducted research where such research existed. The first research question we asked was: Does dysfunctional D3R lead to an increase in collagen synthesis, a decrease in collagen degradation, or both? (**Fig. 1, pathway a**) This route could be one way wherein impaired D3R activity indirectly leads to fibrosis, as it is already well established that dysfunctional collagen regulation can result in fibrosis (denoted by the checkmark). From there, as shown in **Figure 1**, the pathways break off into more unknowns involving D1R (**Fig. 1, pathways d, e, f**). This observation led us to another important research question: Do D3R and D1R have a relationship with each other, either direct or indirect, and if they do, how does that lead to fibrosis? If there is a relationship between the two, then D3R may be indirectly leading to fibrosis, and D1R is directly contributing. The opposite of this idea may also be true, where D1R has an indirect contribution to collagen accumulation, while D3R is directly affecting fibrosis of the heart. If either of these theories are proven to be correct, then a shift in thinking will be required in order to move forward. The final pathway connecting D1R expression to fibrosis is indirect and correlates D1R expression to the phenotypic switch of cardiac fibroblasts to myofibroblasts. It has been observed in human lung fibroblasts that the administration of D1-like and D2-like receptor antagonists both caused an increase in the switch from fibroblasts to myofibroblasts, indicating that both receptor types together may play a part in the switch (61). While this experiment was performed in human lung cells and tissues, it is not unheard of to consider that this model could be applied to the cardiac system as well. For the second indirect pathway (**pathway b**) we asked: Does dysfunctional D3R lead to an increase in Angiotensin II (AngII)? If D3KO studies show an increase in AngII, then that would be another indirect pathway where dysfunctional D3R could augment the buildup of collagen. AngII has one direct mechanism and one indirect mechanism through which it

contributes to fibrosis of the heart. Directly, AngII can contribute to collagen accumulation, with this phenomenon having been observed for decades (62-64). Despite this, it has also been observed that AngII can contribute to fibrosis indirectly via promoting the phenotypic switch of cardiac fibroblasts to myofibroblasts (65, 66). Circling back to the original question denoted by **pathway b**, does dysfunctional D3R increase AngII? If the loss of the function of D3R leads to an increase in AngII, then it is very likely that this increase is playing a role in the fibrosis of the hearts of D3KO mice. We posed a different question regarding a third indirect pathway (**pathway c**): Does a loss of function of the D3R lead to an increase in the expression of Transforming Growth Factor β1 (TGF-β1) within the cardiac fibroblasts? Comparable with AngII, an increase in TGF-β1 has been shown to lead to fibrosis via both direct (67, 68) and indirect mechanisms concerning the conversion of cardiac fibroblasts to myofibroblasts (69). TGF-β1 helps to control homeostasis within the ECM by regulating MMP's (45) and thus could lead to fibrosis if the regulation becomes imbalanced. There is an importance in the conversion of fibroblasts to myofibroblasts as contractile myofibroblasts are the main producers of extracellular matrix collagens that contribute to fibrosis of the heart (70). Thereby, this conversion directly influences cardiac fibrosis (63, 69).

Finally, one of the most important research questions for this project is: Does dysfunctional D3R directly contribute to cardiac fibrosis? For this pathway, there is only one study that would indicate this to be true, which looked at fibrosis with respect to age in mice. Cardiac fibrosis was observed in 2-month-old D3KO mice at a similar level to that of 2-year-old WT mice (53). Administration of D3R agonists and antagonists demonstrated regulatory effects on blood pressure in mice. Studies have shown that use of a D3R agonist results in decreased blood pressure (37, 71) and that use of an antagonist increases blood pressure (26). In addition, the administration of these agonists has provided some information necessary to form links between D3R and other dopamine

receptor subtypes such as D1R (35), D4R (27), and D5R (26). However, the mechanisms through which D3R is coupled to these other receptors remain unclear. The field of dopamine receptor research in the PNS is vast and contains numerous unanswered and unconfirmed research questions. Although the production and use of drugs targeting dopamine receptors in the CNS has prevailed for a long time for the treatment of psychological disorders, reports on the effects of these drugs and receptor agonists and antagonists in other organ systems are largely absent from the field.

1.7 Hypothesis

Based on the paper by Johnson et. al. (53), and the previous research performed in my lab relating D3R and attenuation of proliferation and migration as well as an increase in the collagen I to III ratio (18), I formulated the following hypothesis: There is an intrinsic cardiac dopaminergic system and changes to this system can lead to left ventricular remodeling and particularly, development of cardiac fibrosis.

Specific Aim 1

Determine whether dopamine receptors are expressed in myocardial tissue and in cardiac fibroblasts, then assess if a relationship exists between excitatory and inhibitory receptors.

Specific Aim 2

Determine whether a dysfunctional D3R promotes fibrosis by examining the relative expression of profibrotic markers, such as collagen I and collagen III, MMP2 and MMP9, and TGF-β1 and AngII.

Chapter 2

Materials and Methods

2.1 Animals

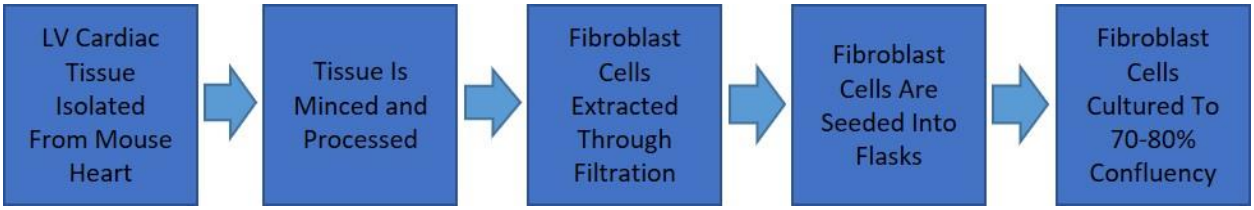
All animal experimental procedures were approved by the Institutional Animal Care and Use Committee at East Carolina University and followed National Institute for Health guidelines outlining animal care and use in a laboratory setting (The Guide-NRC 2011;8th edition). Three to six-month-old, male and female (WT) mice (C57BL/6J, n=15), and dopamine D3 receptor global knockout mice (D3KO; strain B6.129S4-Drd3^{tm1dac}/J, stock # 2958; n=15) were used for experiments. The D3KO mice were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained as a breeding colony at ECU.

2.2 Cardiac Fibroblast Cell Isolation

The left ventricles were removed from whole hearts of WT and D3KO mice for cell culture. Tissues were washed with ice cold 1x PBS (Invitrogen; AM9624) to remove any remaining blood, minced, and then digested with collagenase II (Worthington Biochemical Corp.; 46A034) containing DNaseI (Worthington Biochemical Corp.; LS002139) at 37℃ for 15 minutes. Then, the tissue underwent two rounds of centrifugation (300 xg for 7 minutes each) with removal of supernatant each time to discard any debris remaining from the collagenase digestion. Finally, cell pellets were suspended in 1x DMEM F-12 media (Gibco; 11320-033) supplemented with 10% fetal bovine serum (FBS) (Gibco; 10438-026) and incubated at 37℃ + 5% CO_2 in T25 cell culture flasks until fibroblasts were firmly attached. After the cells were attached, the flasks were washed in warmed 1x DPBS (Gibco; 14190-144) to remove debris and placed back into the incubator and allowed to grow to 70% confluency before subculture. **Experimental Diagram 1** shows the

general timeline for cell isolation and culture. The primary cultures were identified and confirmed as cardiac fibroblasts by spindle shaped morphological appearance and vimentin staining.

Experimental Diagram 1



Experimental Diagram 1: **Left Ventricular Cardiac Fibroblast Cell Isolation.** This diagram depicts the process which left ventricular (LV) cardiac fibroblast cells undergo in order to be utilized for *in vitro* experiments. The process starts with the excising of the murine heart from the animal and subsequently isolating the left ventricle. The LV tissue is minced and digested with collagenase II and washed with media. The cells are filtered to remove large debris and the cells are seeded into T25 flasks. These flasks are grown to sub-confluency (70-80% confluent) and then the cells are further seeded into a larger T75 flask and grown again to sub-confluency before use in experimentation.

2.3 Cell Culturing and Treatments

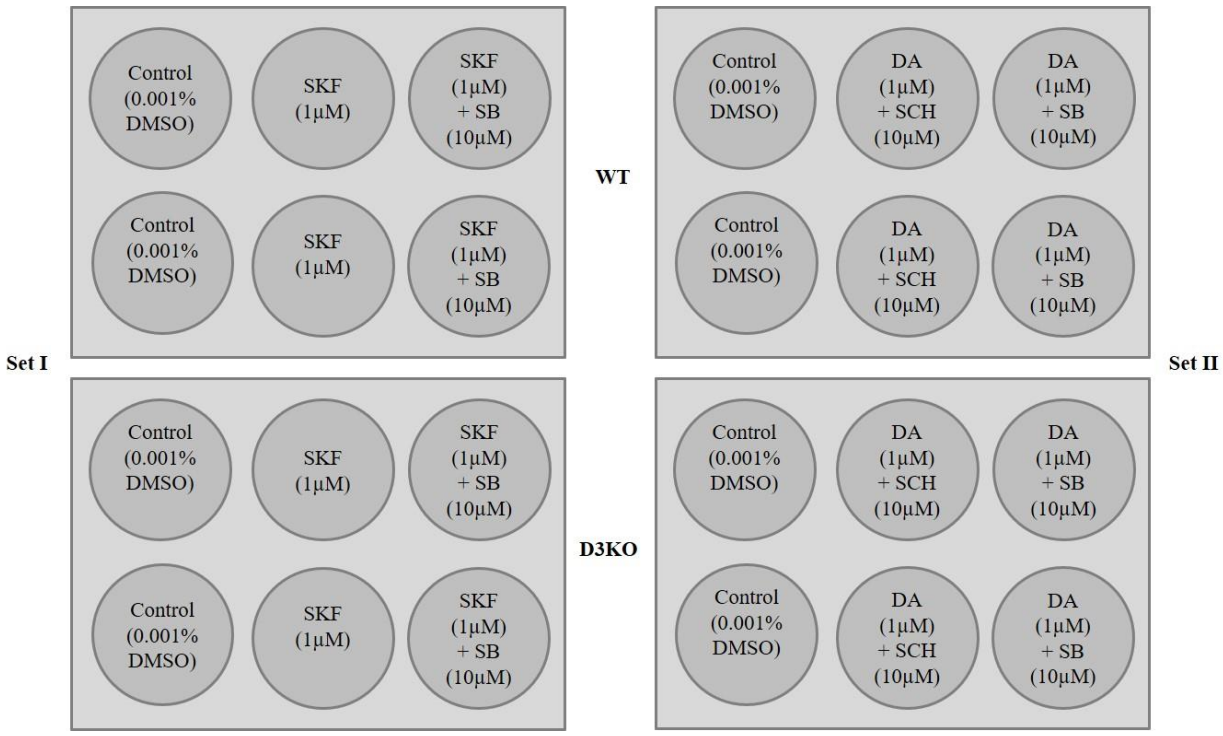
Cell passages 4-8 were used throughout experimentation. Treatments were designed using both agonists and antagonists of D1R and D3R. The first two sets of experiments used D1R agonist SKF 38393 hydrobromide (SKF) (Tocris; 0922, CAS: 20012-10-6), D3R antagonist SB 277011A dihydrochloride (SB) (Tocris; 4207, CAS: 1226917-67-4), D1R antagonist SCH 39166 hydrobromide (SCH) (Tocris; 2299, CAS: 1227675-51-5), and dopamine hydrochloride (DA) (Tocris; 3548, CAS: 62-31-7) with sodium metabisulfite (Sigma-Aldrich; 255556, CAS: 7681-57-4) added as an antioxidant. All reagents were solubilized in dimethyl sulfoxide (DMSO) (Fisher Scientific; BP231-1), high mM stock concentrations of each reagent were made and subsequently diluted, as these reagents were all water insoluble. Thus 0.001% DMSO was added to the control media in order to keep everything constant. **Table 1** shows the composition of these treatments followed by **Experimental Diagram 2**, depicting 6-well plate treatment organization. For each 6-well plate, there were four replicates.

Table 1

Table 1: **Experimental Media, Sets I and II.** This table depicts the first two sets of medias designed for the cardiac fibroblasts to be treated and collected for RT-qPCR and Western Blot.

Set I: WT and D3KO LV Cardiac Fibroblasts	Set II: WT and D3KO LV Cardiac Fibroblasts
➢ Control: DMEM F12 (Serum Free) + 0.001% DMSO	➢ Control: DMEM F12 (Serum Free) + 0.001% DMSO
➢ DMEM F12 (Serum Free) + 1µM SKF	➢ DMEM F12 (Serum Free) + 1µM DA + 10µM Sodium Metabisulfite + 10µM SCH
➢ DMEM F12 (Serum Free) + 1µM SKF + 10µM SB	➢ DMEM F12 (Serum Free) + 1µM DA + 10µM Sodium Metabisulfite + 10µM SB

Experimental Diagram 2



Experimental Diagram 2: **Shows Treatment Sets I and II Organization in 6-Well Plates.** This is an example of the set up for the first set of experiments, containing D1R agonist SKF and D3R antagonist SB. This example would be used to collect RNA from the control and treated left ventricular fibroblasts. Each 6-well plate was duplicated for an n=4.

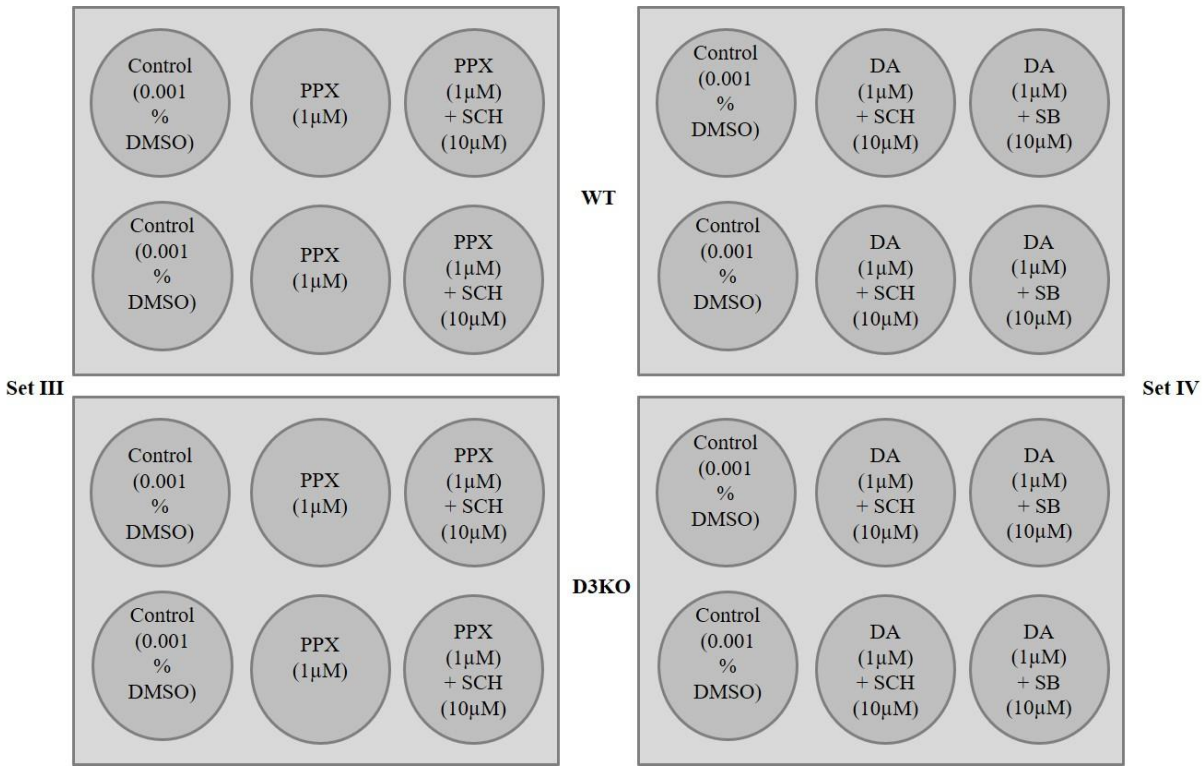
The third set of treatments reversed the agonist and antagonist for D1R and D3R while set IV remained constant. D3R agonist Pramipexole dihydrochloride (PPX) (Tocris; 4174, CAS: 104632-25-9), D1R antagonist SCH, D3R antagonist SB, and DA plus sodium metabisulfite added as an antioxidant for dopamine were all used in these sets. All reagents were solubilized in DMSO (Fisher Scientific; BP231-1) as these reagents were all water insoluble, high mM stock concentrations of each reagent were made and subsequently diluted. Thus, 0.001% DMSO was added to the control media in order to serve as a valid control. **Table 2** shows the composition of these treatments followed by **Experimental Diagram 3**, depicting 6-well plate treatment organization. For each 6-well plate, there were four replicates.

Table 2

Table 2: **Experimental Media, Sets III and IV.** This table depicts the second two sets of medias designed for the cardiac fibroblasts to be treated and collected for RT-qPCR and Western Blot.

Set III: WT and D3KO LV Cardiac Fibroblasts	Set IV: WT and D3KO LV Cardiac Fibroblasts
➢ Control: DMEM F12 (Serum Free) + 0.001% DMSO	➢ Control: DMEM F12 (Serum Free) + 0.001% DMSO
➢ DMEM F12 (Serum Free) + 1µM PPX	➢ DMEM F12 (Serum Free) + 1µM DA + 10µM Sodium Metabisulfite + 10µM SCH
➢ DMEM F12 (Serum Free) + 1µM PPX + 10µM SCH	➢ DMEM F12 (Serum Free) + 1µM DA + 10µM Sodium Metabisulfite + 10µM SB

Experimental Diagram 3



Experimental Diagram 3: **Shows Treatment Sets III and IV Organization in 6-Well Plates.** This is an example of the set up for two more sets of experiments, this time containing D3R agonist, Pramipexole (PPX) and D1R antagonist SCH. This example would be used to collect RNA from the control and treated left ventricular fibroblasts. Each 6-well plate was duplicated for an n=4.

2.4 Immunofluorescence Labeling of D1R and D3R in Heart Tissue and Primary Cardiac Fibroblasts

Hearts were excised from WT animals, rinsed with saline, immediately inserted into OCT compound block molds, and placed on dry ice for ~30 minutes before being stored in a -80ºC freezer. Cryosections of 10 microns thickness were positioned on glass microscope slides and fixed in 4% paraformaldehyde for 15 minutes at room temperature. Similarly, primary cultures of cardiac fibroblasts from the LV of WT animals were seeded (5000 cells/well) and grown on 0.2% gelatin coated glass coverslips in a 24-well plate and then fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. They were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes and blocked in 1% bovine serum albumin (BSA) in 1x PBS containing 0.3% Tween 20 for one hour at room temperature. Both cells and tissues were then incubated with primary antibodies for Vimentin (M0725, lot #027(102), Dako, 1:500 dilution), D1R (NB110-60017AF488, lot #B-3-101620, Novus Biologics, 1:500 dilution), and D3R (bs-1743R-Cy5, lot #AF12125641, Bioss, 1:500 dilution) overnight at 4°C. Both cells and heart tissue were then washed the next day 3 times with 1x PBS containing 0.3% Tween 20. Then they were incubated with secondary antibodies (Donkey anti-Mouse Alexa Fluor Plus 647, 1:1000 dilution or Donkey anti-Mouse Alexa Fluor Plus 555, 1:1000 dilution) for 1 hour at room temperature. Following 3 additional washes with 1x PBS containing 0.3% Tween 20 for 15 minutes, cells and heart tissue were then placed on slides and covered with coverslips using ProLong Diamond antifade reagent with DAPI (ThermoFisher). The images were captured with an Echo Revolve microscope. This protocol has been previously described (18).

2.5 Protein Quantification Assays (DC Assay and BCA Assay)

WT and D3KO mouse whole heart tissue protein concentration was measured via Detergent Compatible (DC) Assay. Whole heart protein extracts were plated into a 96-well plate at 5µL each and each sample was plated in triplicate. A BSA standard curve consisting of 8 protein concentrations was created. Once all standards and samples were plated, 25µL of Reagent A (BioRad; Cat#500-0113) was added to each well. This was quickly followed by 200µL of Reagent B (BioRad; Cat#500-0114) into each well. The 96-well plate was covered and placed on a plate shaker and incubated at room temperature for 15 minutes with gentle shaking. The absorbance was measured at 700nm on the SpectraMAX 190 system and protein was quantified using the SpectraMAX 190 computer program.

WT and D3KO LV cardiac fibroblasts protein concentration was measured via Bicinchoninic Acid (BCA) Assay (Pierce BCA Protein Assay Kit, Cat # 23227). A BSA standard curve consisting of 8 protein concentrations was made. LV cardiac fibroblast cell protein extracts were plated into a 96-well plate at 9µL each, in addition to the 8 standards, all in triplicate. Then, 260µL of working reagent (Combined BCA Reagent A and BCA Reagent B 50:1, from the kit) was added to each well. The plate was covered and shaken at room temperature for 1 minute. After the shaking, the plate was incubated at 37 °C for 30 minutes, then allowed to cool at room temperature for 5 minutes before reading. The absorbance was measured at 562nm on the SpectraMAX 190 system and protein was quantified using the SpectraMAX 190 computer program.

2.6 Western Blot Analysis for Protein in Heart Tissue and Primary Cardiac Fibroblasts Isolated from LV

Hearts were excised from WT and D3KO animals and snap frozen in liquid nitrogen. The whole heart tissue was homogenized in RIPA buffer with 1x protease and phosphatase inhibitors. Protein from the heart tissues and confluent cardiac fibroblasts was isolated using RIPA buffer with 1x protease and phosphatase inhibitors and quantified by DC or BCA assay. Western blots were performed by loading 30 µg of protein into a 4-15% BioRad precast gel (BioRad, Mini-PROTEAN TGX Stain-Free Gels; Cat #: 4568084. & BioRad, Criterion TGX Stain-Free Precast Gels; Cat # 5678084). After electrophoresis, proteins were transferred onto PVDF membranes and immunoblotting was performed for dopamine receptors: D1R (1:1000, #ab216644 Abcam), D3R (1:2000, ab155098 Abcam), Col1a1 (1:1000, Cat # NB600-450 Novus Biologics), Col3a1 (1:2000, Cat # PA5-27828 Invitrogen), and TGF-β1 (20ng/mL, Cat # MA1-21595 Invitrogen). Primary incubation lasted overnight (~12 hours) in 4 °C while membranes were rocked on a plate shaker. Secondaries used were: IRDye 680RD Goat anti-Mouse (1:15,000, Cat # 926-68070 Licor) and IRDye 800CW Goat anti-Rabbit (1:15,000, Cat # 926-32211 Licor). Secondary incubation lasted for 1 hour at room temperature before washing and imaging. Image acquisition was performed using the Licor Odyssey Clx Imaging System, quantification data was collected from Image Studio ver. 5.2, analysis was performed in excel, and the values were graphed on Graphpad Prism.

2.7 Real Time-quantitative PCR

Hearts were excised from WT and D3KO animals and snap frozen in liquid nitrogen. Frozen mouse heart tissue samples were homogenized in TRIzol reagent and confluent cardiac fibroblasts were lysed and suspended in TRIzol reagent (15596-026). RNA was isolated from mouse heart tissue and primary cardiac fibroblasts using TRI/Direct-Zol RNA Miniprep Kit (Zymo

Research; R2072). A cDNA library of all samples was created using Superscript IV reverse transcriptase with ezDNAse enzyme (Invitrogen; 11766050) according to manufacturer's instructions (Pub. No. MAN0015862). A volume of 1µL of cDNA from each sample was added to the respective wells of a 384-well DNase/RNase-free PCR plate as well as the necessary reagents for RT-qPCR. Thus, each well included materials as follows: 0.5µL primer for specified gene, 0.5µL β-actin (housekeeping gene), 4µL TaqMan™ Fast Advanced Master Mix (Applied Biosystems, 4444557), 1µL cDNA, and 4µL DNase/RNase-free ultrapure water (Invitrogen, 10977023) for 10µL total volume in each well. Samples were added in duplicates. TaqMan primers for specified genes were from Applied Biosystems and are listed in **Table 3**. Quantitative real-time-PCR was performed using Applied Biosystems QuantStudio 6 Flex System at manufacturer's recommended settings. C_T values from QuantStudio program were then transformed to relative gene expression values by the comparative C_T method (72, 73).

Table 3

Table 3: **List of RT-qPCR Primers.** This table lists all TaqMan primers (Applied Biosystems) used in RT-qPCR testing for relative gene expression.

➢ *Drd1* (Mm02620146_s1)	➢ *Col1a1* (Mm00801666_g1)
➢ *Drd2* (Mm00438545_m1)	➢ *Col3a1* (Mm00802300_g1)
➢ *Drd3* (Mm00432887_m1)	➢ *MMP2* (Mm00439498_m1)
➢ *Drd4* (Mm00432893_m1)	➢ *MMP9* (Mm00442991_m1)
➢ *Drd5* (Mm04210376_s1)	➢ *Tgfb1* (Mm01178820_m1)
➢ *ACTB* (Mm02619580_g1)	➢ *Agtr1* (Mm01957722_s1)

2.8 Statistical Analysis

Analysis of Variance (ANOVA) was performed to determine if there was statistical significance between 3 sample means. Two-tail, two sample T-tests were performed when only 2 groups were being compared. Differences were considered significant when p-value was less than or equal to 0.05 using GraphPad Prism.

Chapter 3

Results

3.1 Dopamine Receptors are Expressed in Cardiac Tissue and Fibroblasts

In accordance with the first aim, the first key experiment was designed to answer an essential question: Does the cardiac system possess dopamine receptors? We found this to be true. Upon RT-qPCR evaluation of homogenized WT hearts, all five dopamine receptor subtypes were expressed in mouse heart (**Figure 2A**). Using β-actin as a housekeeping gene, the Delta CT (ΔCT) values were calculated to give relative gene expression of each of the receptor subtypes. According to the gene expression data, D2R expression is most abundant, followed by expression of D1R, then D3R, D5R, and D4R (**Figure 2A**). Similarly, we also found that cardiac fibroblasts isolated from the LV of WT mouse hearts also expressed all five receptors (**Figure 2B**). There is less variation among receptor expression in the cardiac fibroblasts, with D1R appearing to be expressed more and the other four receptors sharing similar levels of expression (**Figure 2B**). These results demonstrate the existence of an intracardiac dopaminergic system consisting of all five dopamine receptor subtypes.

Figure 2

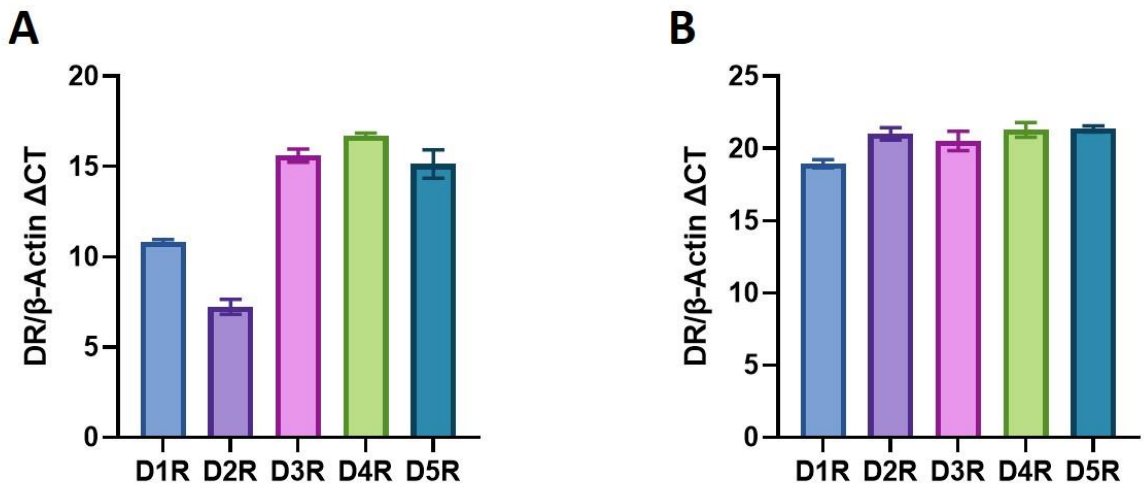


Figure 2: **Dopamine Receptors in Cardiac Tissue and Cardiac Fibroblasts. A)** All five subtypes of dopamine receptors are expressed in mouse cardiac tissue. Values plotted are ΔCT values normalized to β-Actin (housekeeping gene). **B)** All five subtypes of dopamine receptors are expressed in mouse left ventricular cardiac fibroblasts. Values plotted are ΔCT values normalized to β-Actin (housekeeping gene).

3.2 Both Dopamine Receptors 1 and 3 Protein and Gene are expressed in WT Myocardial Tissue and WT Cardiac Fibroblasts

We then focused on excitatory D1R and inhibitory D3R, specifically in the hearts of WT mice. We analyzed protein and gene expression of these two receptors comprehensively through three methods: Immunofluorescence, RT-qPCR, and Western Blot and confirmed the expression of both of these receptors in WT mouse heart tissue. Within the tissue, D1R (**Figure 3A top panel**) and D3R (**Figure 3A bottom panel**) were probed for and imaged using tissue sections from the LV of the heart. The fibroblast specific marker vimentin was used to identify cardiac fibroblasts within the tissue sections. In the third panels of **Figure 3A**, both receptors are shown to be co-localized along with the vimentin stain, indicating expression on the membrane of cardiac fibroblasts. This expression indicates that both D1R and D3R are expressed in the LV cardiac tissue of WT mice. DAPI staining was used to stain the nuclei. Relative levels of D1R and D3R gene expression measured in ΔCT normalized to β-actin are present in **Figure 3B**. The existence of these two receptors was further confirmed by Western Blot. **Figure 3C** shows western blot analyses for D1R (left) and D3R (middle) normalized to the total protein loading control (right). These data give positive proof that excitatory D1R and inhibitory D3R are present within the cardiac system of WT mice at both mRNA and protein levels. The figure was modified from Figure 1 in a previously published article (18).

Figure 3

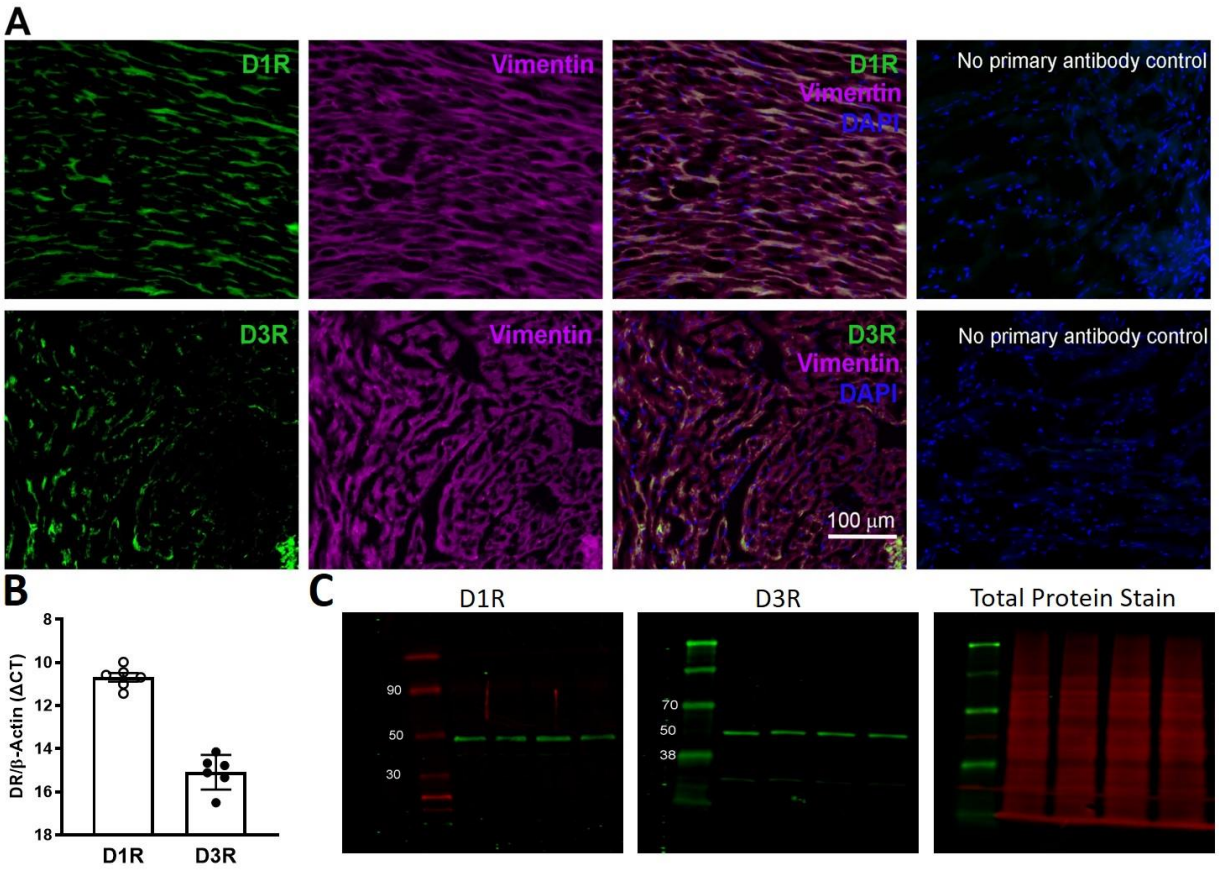


Figure 3: **Both Dopamine Receptors 1 and 3 Protein and Gene are Expressed in WT Myocardial Tissue. A)** Immunofluorescence images representative of D1R (top panel) and D3R (bottom panel); vimentin was used as a marker for cardiac fibroblasts, DAPI stains for the nuclei, and the right images are negative controls. **B)** Gene expression (ΔCT) for D1R and D3R normalized to β-Actin expression. **C)** Immunoblot against D1R and D3R in WT myocardial tissue, the left panel is fluorescent staining of D1R (49 kDa), the middle panel is fluorescent staining of D3R (44 kDa), and the right panel is the total protein stain used for quantification. Modified from Kisling, A. Byrne, S. et. al. Frontiers in Cardiovascular Medicine, October 11, 2021.

We isolated and cultured LV cardiac fibroblasts of WT and D3KO mice, then fixed and stained them for the same receptors, D1R and D3R (**Figure 4A**). Similar to the cardiac tissue, the WT LV cardiac fibroblasts also indicate expression of both D1R and D3R with the fibroblast specific marker vimentin, used to identify the cells as cardiac fibroblasts. DAPI staining was used to stain the nuclei. The D3KO LV cardiac fibroblasts expressed D1R but did not show visual expression of D3R on the membrane, which was expected. This validated the D3KO mouse model as a functional knockout. Relative levels of D1R and D3R gene expression measured in ΔCT normalized to β-actin are present in **Figure 4B**. This gene expression data may indicate that D1R expression is higher than D3R expression in the LV cardiac fibroblasts. **Figure 4C** shows western blot images of LV cardiac fibroblasts which have been probed for both D1R and D3R to demonstrate that both of these receptors are expressed in LV cardiac fibroblasts.

Figure 4

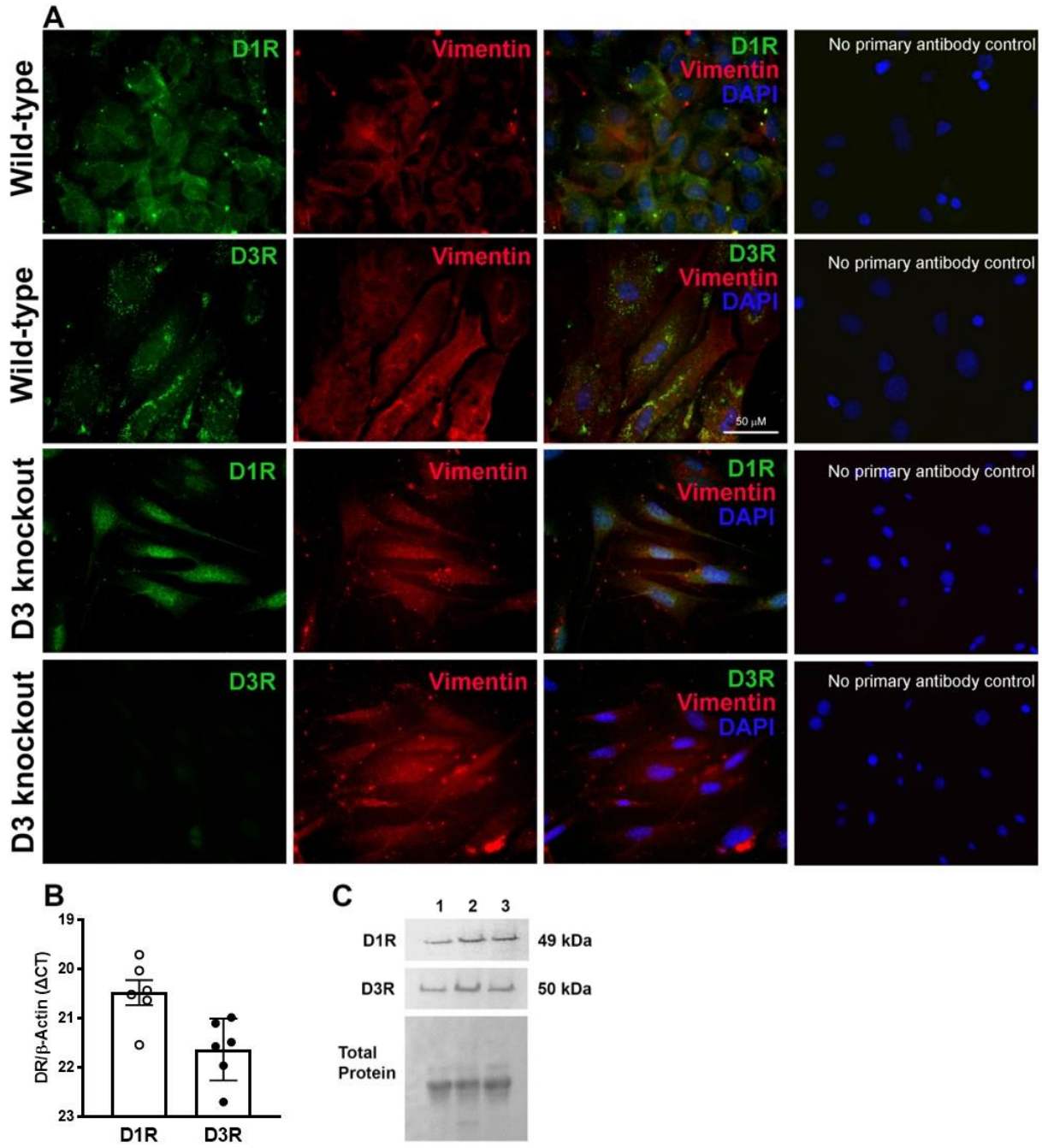


Figure 4: **Both Dopamine Receptors 1 and 3 Protein and Gene are Expressed in Cardiac Fibroblasts. A)** Immunofluorescence images representative of D1R (top panel) and D3R (bottom panel); vimentin was used as a marker for cardiac fibroblasts, DAPI stains for the nuclei, and the right images are negative controls. **B)** Gene expression (ΔCT) for D1R and D3R normalized to β-Actin expression. **C)** Immunoblot against D1R and D3R, the top panel is protein probed for D1R expression, under that is D3R protein expression, followed by a depiction of the total protein stain.
Taken from Kisling, A. Byrne, S. et. al. Frontiers in Cardiovascular Medicine, October 11, 2021.

3.3 Loss of Function of D3R Induces Overexpression of D1R

After the confirmation of D1R expression, the next objective was to compare basal level expression of this receptor in both WT and D3KO tissues and fibroblasts. Whole heart tissue RNA and protein extracts were used to give an overall level of expression in the heart rather than just in the LV tissue. Relative gene expression was measured in both whole heart tissue and LV cardiac fibroblasts and both showed an increased expression of D1R in the D3KO samples when compared to WT. **Figure 5A** is indicative of RT-qPCR data for D1R expression in D3KO cardiac tissue normalized to β-actin expression and converted to fold change compared to the WT cardiac tissue control. There is a significant increase ($p<0.01$) of D1R expression in the D3KO cardiac tissue. Similarly, the relative gene expression measured by RT-qPCR in LV cardiac fibroblasts also shows a significant increase ($p<0.0001$) in D1R expression in the D3KO cells (**Figure 5B**). This is an interesting point that may support the notion that the loss of function of D3R leads to an upregulation of other receptors in order to maintain homeostasis.

Figure 5

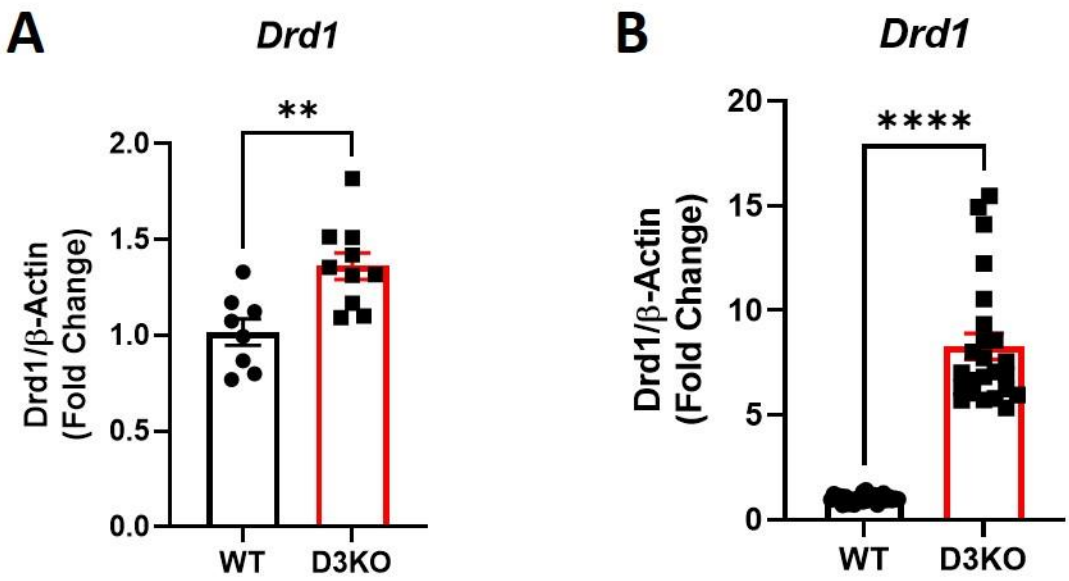


Figure 5: **Loss of Function of D3R Induces Overexpression of D1R. A)** A measure of the relative gene expression of *Drd1* between WT and D3KO whole hearts. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. **$p<0.01$, statistically significant; bars represent standard error of mean. **B)** A measure of the relative gene expression of *Drd1* between WT and D3KO LV cardiac fibroblast cells. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. ****$p<0.0001$, statistically significant; bars represent standard error of mean.

Furthermore, examination of whole heart tissue proteins from both WT and D3KO mice via western blot corroborated this claim. The protein quantification matched the RT-qPCR data shown in **Figure 5**. The blot was stained for D1R protein and normalized to the total protein loading control. The expected molecular weight for the D1R protein is approximately 49kDa. The first well contains a pre-labeled protein ladder standard while the final well contains a 10ul (1ug/ul) of brain protein extract isolated from mouse brain serves as a positive control for dopamine receptors, as the brain contains all five subtypes of dopamine receptors (**Figure 6A**). **Figure 6A** shows the staining for D1R in the green 800 channel with the protein ladder in red. **Figure 6B** is the total protein stain used to normalize the blot and for the quantification in **Figure 6C**. The density normal measurements of this western blot indicate that there is a significant increase in D1R protein in the membranes of the D3KO heart tissue cells ($p<0.05$). The RT-qPCR and western blot data together, point to an increase in D1R receptor expression in the cardiac system in mice with dysfunctional D3R.

Figure 6

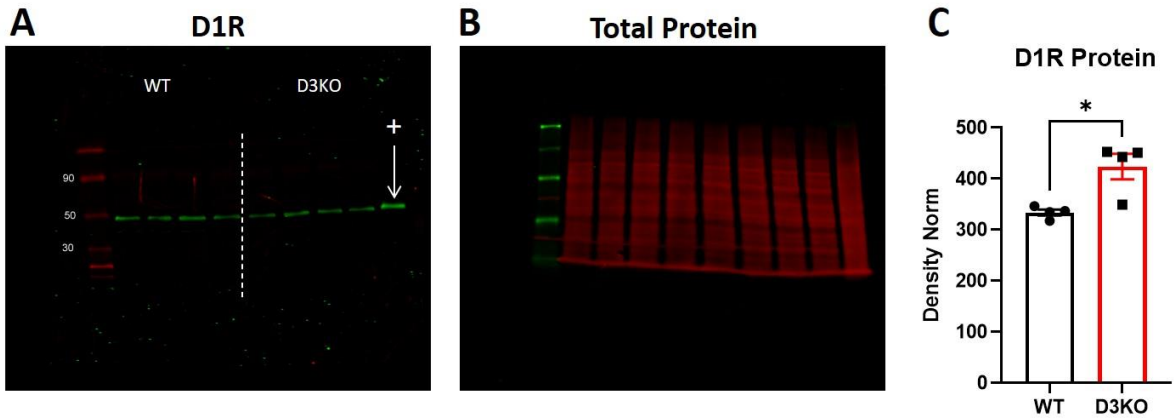


Figure 6: **Absence of Functional D3R Induces Overexpression of D1R Proteins. A)** Protein expression of D1R (49kDa) determined by western blot and measured in samples of whole heart tissues from WT and D3KO mice (n=4 per mouse type). The ladder consists of standardized protein markers and the positive control (plus sign) was from mouse brain. **B)** An image of the total protein stain used to establish relative density normal and to quantify the amount of protein present. **C)** A measure of the relative density normal of the D1R expression between WT and D3KO cardiac tissue. *$p<0.05$, statistically significant; bars represent standard error of mean.

3.4 Loss of Functional D3R Results in Alterations of D3R Gene and Protein Expression compared to Wild Type

In addition to the changes in D1R expression, there were also noticeable alterations in D3R gene expression measurements. Within the D3KO mice, there is still expression of D3R proteins, which are dysfunctional and are left in the cytosol but not inserted in the membrane. Thus, it is important to still ascertain its effects on gene and protein expression. **Figure 7** shows the gene expression exhibited by whole heart tissue collected from WT and D3KO mice (**Figure 7A**) and from cardiac fibroblasts isolated from the LV of WT and D3KO mouse hearts (**Figure 7B**). Gene expression of *Drd3* in both groups shown in **Figure** 7 were normalized to the housekeeping gene β-actin and converted to fold change values. When the gene expression of *Drd3* for whole heart tissue is quantified and converted to fold change, the D3KO heart shows a significant reduction of almost half in gene expression of D3R (**Figure 7A**) ($p<0.05$). Conversely, the LV cardiac fibroblast data shows the opposite, that cardiac fibroblasts from D3KO animals have an almost three-fold increase in *Drd3* expression (**Figure 7B**) ($p<0.01$). This data could indicate that the lack of functional D3R has differing effects between the cellular and tissue levels of organization.

Figure 7

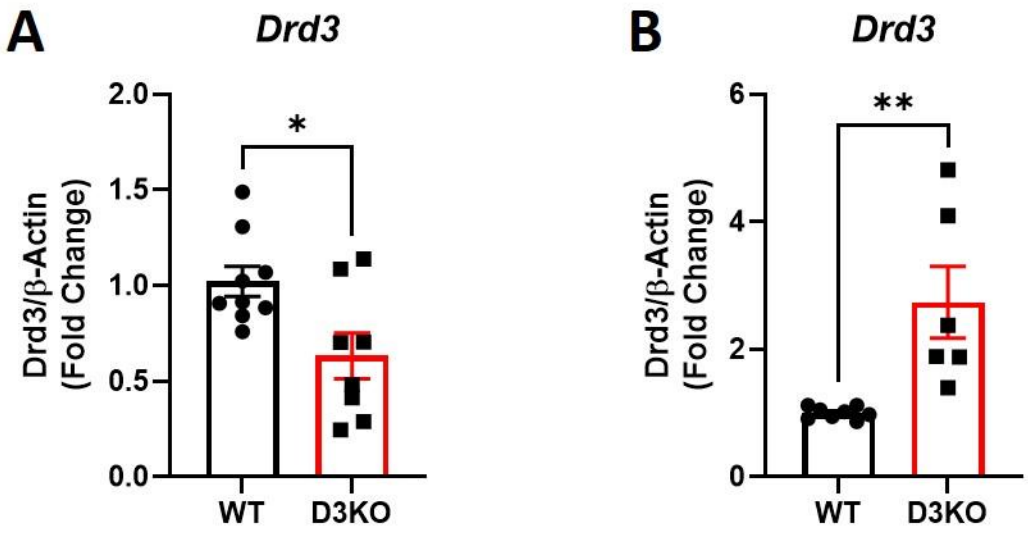


Figure 7: **Dysfunctional D3R Results in Alterations of D3R Gene Expression. A)** A measure of the relative gene expression of *Drd3* between WT and D3KO whole hearts. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. *$p<0.05$, statistically significant; bars represent standard error of mean. **B)** A measure of the relative gene expression of *Drd3* between WT and D3KO LV cardiac fibroblast cells. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. **$p<0.01$, statistically significant; bars represent standard error of mean.

Protein quantification of whole hearts from both WT and D3KO mice via western blot corroborated the RT-qPCR data from **Figure 7**. The mice are a global D3R knock out, however, the cells still express D3R, though the protein is misfolded and thus cannot be inserted into the membrane to be utilized by the cells. Due to this, however, the protein still exists at approximately 44kDa and can still be measured via western blot. The blot was stained for D3R protein and normalized to the total protein loading control. The first well contains a pre-labeled protein ladder standard while the final well contains a positive protein control isolated from mouse brain, as the brain contains all five subtypes of dopamine receptors (**Figure 8A**). **Figure 8A** shows the staining for D3R in the green 800 channel. **Figure 8B** is the total protein stain used to normalize the blot and for the quantification in **Figure 8C**. The density normal measurements of this western blot indicate that there is a significant decrease in D3R protein in the D3KO heart tissue ($p<0.05$). The RT-qPCR and western blot data together, point to a reduction in D3R receptor expression in the cardiac system in mice with dysfunctional D3R.

Figure 8

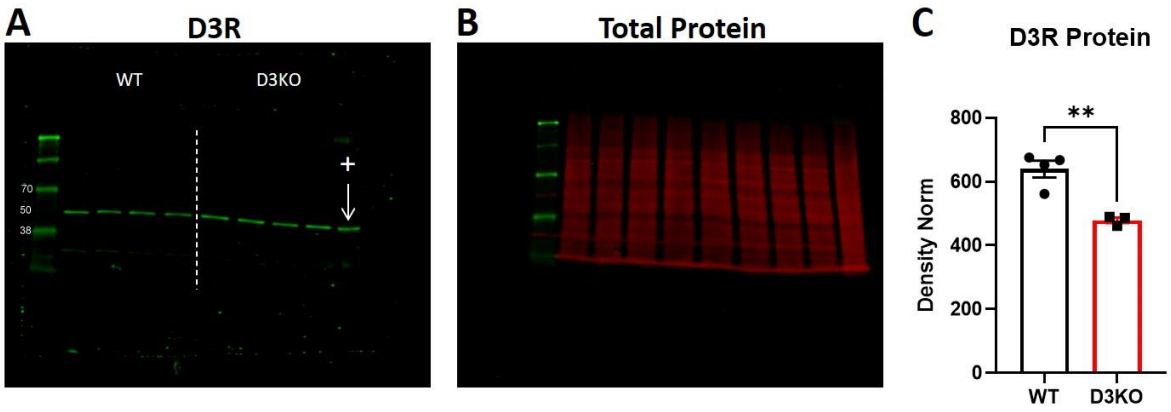


Figure 8: **Loss of Function of D3R Results in Alterations of D3R Protein Expression. A)** Protein expression of D3R (44kDa) determined by western blot and measured in samples of whole heart tissues from WT and D3KO mice (n=4 per mouse type). The ladder consists of standardized protein markers and the positive control (plus sign) was from mouse brain. **B)** An image of the total protein stain used to establish relative density normal and to quantify the amount of protein present. **C)** A measure of the relative density normal of the D1R expression between WT and D3KO cardiac tissue. **p<0.01, statistically significant; bars represent standard error of mean.

3.5 Validation of Agonists, Antagonists, and D3KO

To understand the interdependence of D1R and D3R, we decided to incorporate a pharmacological approach using D1R and D3R agonists and antagonists. To validate the experimental design, we used D1R and D3R specific agonists and antagonists, but initially, we started with some simple experiments. The D1R agonist SKF and antagonist SCH are validated in **Figure 9**. The WT LV cardiac fibroblasts showed increased gene expression of D1R when treated with the D1R agonist (**Figure 9A**) and normal expression when treated with both DA (**Figure 9C**) and the D1R antagonist SCH (**Figure 9B**). This would indicate that the D1R agonist SKF, does serve to increase D1R gene expression and that the D1R antagonist SCH neither increases nor decreases D1R expression and that the natural ligand dopamine has no significant impact in WT cells.

Figure 9

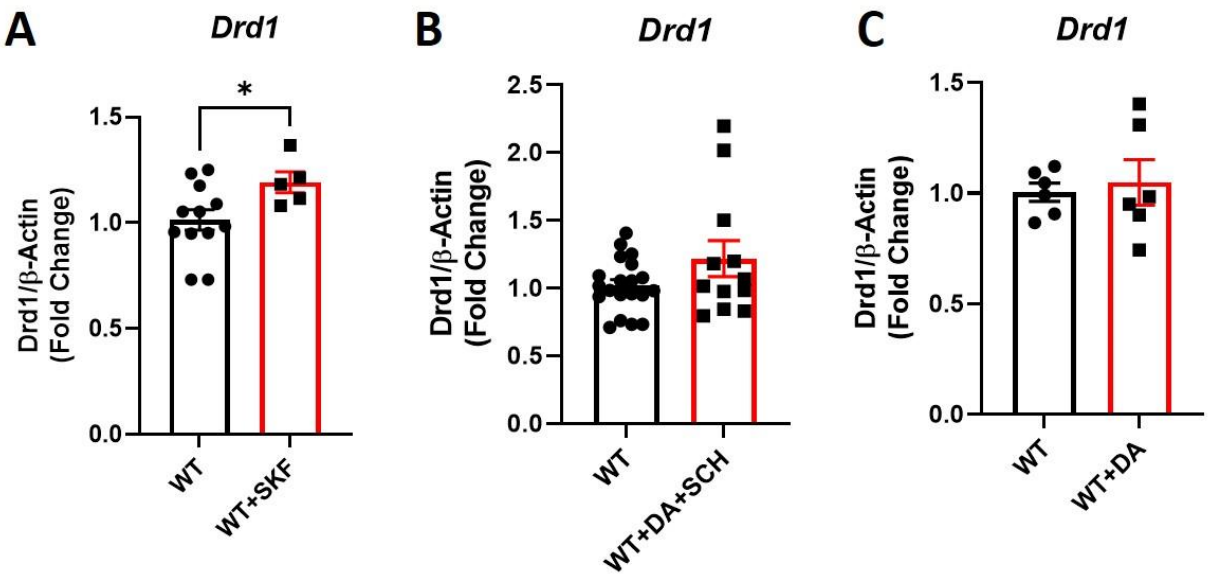


Figure 9: **Validation of D1R Agonist SKF and D1R Antagonists SCH. A)** A measure of the relative gene expression of *Drd1* between WT LV cardiac fibroblasts control cells and cells treated with D1R agonist SKF. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. *$p<0.05$, statistically significant; bars represent standard error of mean. **B)** A measure of the relative gene expression of *Drd1* between WT LV cardiac fibroblast control cells and fibroblasts treated with dopamine and the D1R antagonist SCH. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change, bars represent standard error of mean. **C)** A measure of the relative gene expression of *Drd1* between WT LV cardiac fibroblast controls and fibroblast cells treated with the natural receptor ligand dopamine. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change, bars represent standard error of mean.

In addition, the D3R antagonist SB and the D3KO animals were validated. **Figure 10** shows the relative gene expression for *Drd1* using cardiac fibroblasts isolated from the LV of WT and D3KO animals as well as intervention experiments with various pharmacological treatments. **Figure 10A** serves as a baseline of *Drd1* expression in control fibroblasts. While D3R antagonist SB caused an increase in D1R expression in WT cardiac fibroblasts (**Figure 10B**) ($p<0.001$), however, this same treatment showed no significant difference in the D3KO cells (**Figure 10C**). This was expected and demonstrated that the D3KO cells indeed possess dysfunctional D3R and thus are not influenced by D3R agonists or antagonists.

Figure 10

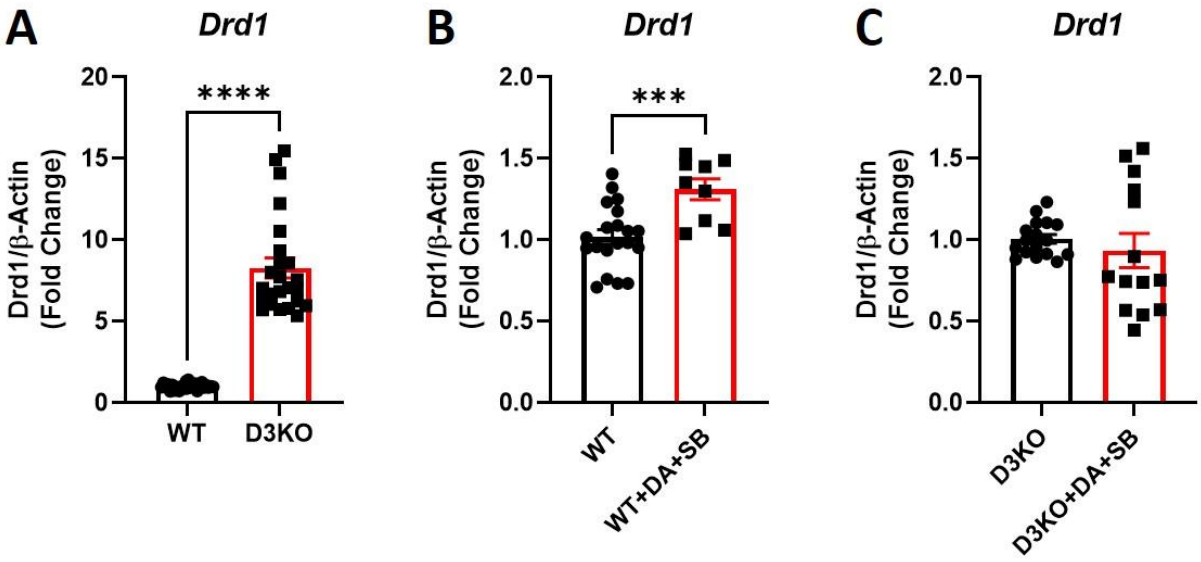


Figure 10: **Validation of D3R Antagonist SB and the Global D3KO. A)** Relative gene expression of *Drd1* in LV cardiac fibroblasts from WT and D3KO animals. This serves as a control for the figure. ****p<0.0001, bars represent standard error of mean. **B)** Relative gene expression of *Drd1* in WT LV cardiac fibroblasts, using both control cells and cells treated with the natural receptor ligand dopamine and D3R antagonist SB. ***p<0.001, bars represent standard error of mean. **C)** Relative gene expression of *Drd1* in D3KO LV cardiac fibroblasts, using both control cells and cells treated with the natural receptor ligand dopamine and D3R antagonist SB, bars represent standard error of mean.

3.6 Regardless of Treatment, D3KO Mice Continuously Overexpress D1R

The last question that was essential to answer was the ability of pharmacological agents to influence the expression of the D1R protein in the D3KO fibroblasts. Ultimately, regardless of treatment, the D3KO LV cardiac fibroblasts consistently exhibited increased expression of D1R when compared to the WT counterpart.

3.7 Loss of D3R Function Leads to a Decrease in Collagen Type I Expression in Myocardial Tissue and LV Cardiac Fibroblasts

The secretion of type I and type III collagens is essential for the homeostasis of the ECM in the heart. Fibroblasts in particular are responsible for these secretions. Gene expression measurements were taken using the comparative ΔCT method normalized to β-actin and converted to fold change. Relative gene expression was measured in both whole heart tissue and LV cardiac fibroblasts and both showed a reduction in expression of *Col1a1* in the D3KO samples when compared to WT (**Figure 11**). **Figure 11A** shows the relative gene expression in WT and D3KO cardiac tissue. When compared to WT heart tissue, the D3KO hearts seem to express slightly less collagen type I ($p<0.05$). In **Figure 11B**, the measurement of the relative expression in LV cardiac fibroblasts, this decrease is significantly more pronounced ($p<0.0001$) and appears to be almost three-fourths lower than the WT control fibroblast expression. This could mean that the alteration of dopamine receptor expression is influencing collagen type I production.

Figure 11

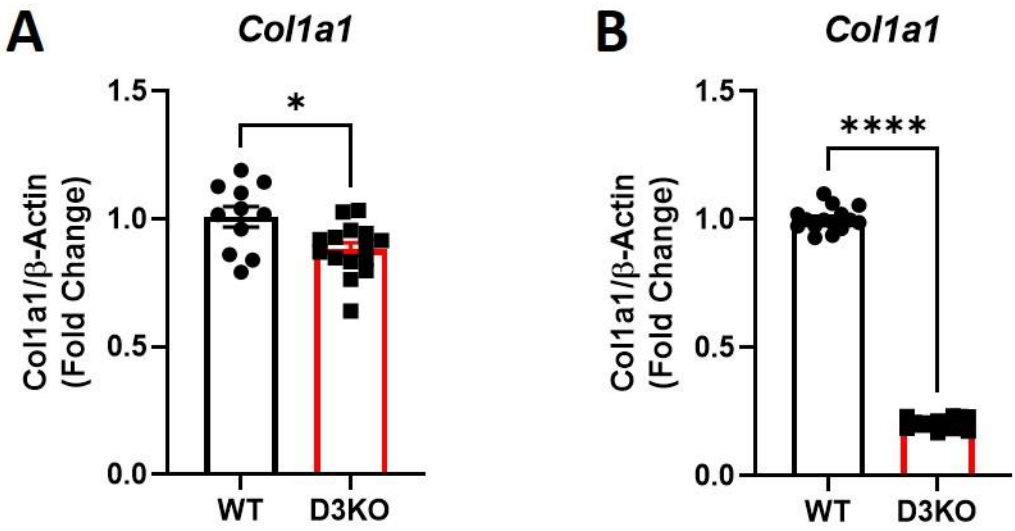


Figure 11: **Loss of D3R Function Leads to a Decrease in Collagen Type I Expression in Myocardial Tissue and Cardiac Fibroblasts. A)** A measure of the relative gene expression of *Col1a1* between WT and D3KO whole hearts. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. *p<0.05, statistically significant; bars represent standard error of mean. **B)** A measure of the relative gene expression of *Col1a1* between WT and D3KO LV cardiac fibroblast cells. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. ****p<0.0001, statistically significant; bars represent standard error of mean.

3.8 Use of Pharmacological Agents cause WT to Mimic D3KO

Upon exploring the effect of pharmacological agents on collagen type I expression in the WT and D3KO cardiac fibroblasts, an interesting observation was made. The WT cells, when treated with the D3R antagonist SB, exhibit a reduced expression of *Col1a1*. The relative gene expression for WT cells treated with D1R agonist SKF (**Figure 12A**) and with both SKF and D3R antagonist SB (**Figure 12B**) are shown in **Figure 12**. This specific set of treatments shows no significant change when treated solely with SKF (**Figure 12A**), however, the inclusion of SB results in a significant decrease in *Col1a1* in the WT cells (**Figure 12B**) ($p<0.05$). This treatment serves as a pseudo-D3KO treatment, causing the WT cells to mimic the D3KO control cell phenotype. In addition, the converse is also true, stimulation of the WT cells with D3R agonist PPX resulted in a decrease in *Col1a1* expression (**Figure 12C**) ($p<0.01$). At the same time, stimulation with D1R antagonist SCH produces an increase in *Col1a1* expression (**Figure 12D**) ($p<0.001$). This may suggest that D1R is playing an essential role in the up or downregulation of collagen type I expression.

Figure 12

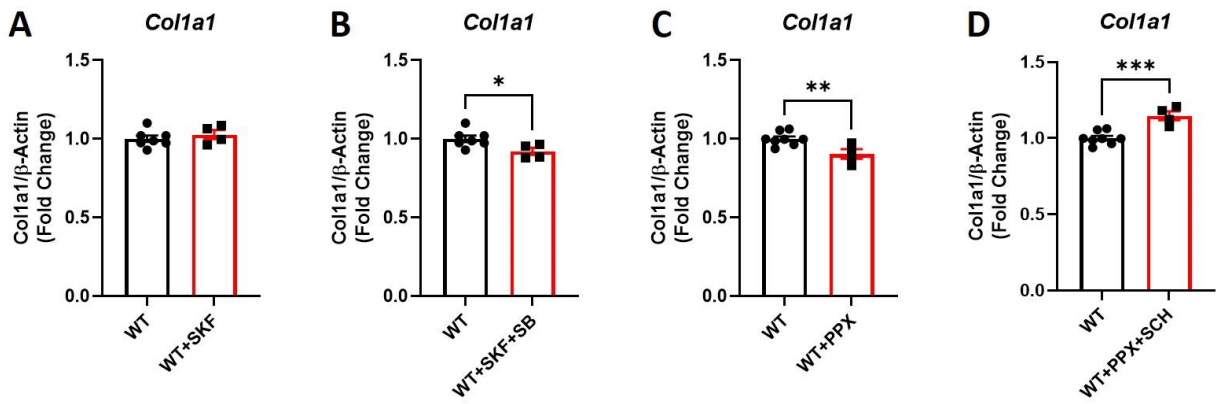


Figure 12: **Use of Pharmacological Agents Cause WT to Mimic D3KO. A)** A measure of the relative gene expression of *Col1a1* in WT control and treated cardiac fibroblasts. There is no significant difference with treatment of D1R agonist SKF. Bars represent standard error of mean. **B)** A measure of the relative gene expression of *Col1a1* in WT control and treated cardiac fibroblasts. The addition of D3R antagonist SB causes a reduction in expression. *$p<0.05$, statistically significant; bars represent standard error of mean. **C)** A measure of the relative gene expression of *Col1a1* in WT control and treated cardiac fibroblasts. The use of D3R agonist PPX resulted in a significant decrease in *Col1a1* expression. **$p<0.01$, statistically significant; bars represent standard error of mean. **D)** A measure of the relative gene expression of *Col1a1* in WT control and treated cardiac fibroblasts. The addition of both PPX and D1R antagonist SCH causes a significant increase in *Col1a1* expression. ***$p<0.001$, statistically significant; bars represent standard error of mean.

A similar phenomenon is observed with the treatment of the WT cardiac fibroblast cells with the natural ligand dopamine and the use of the D3R antagonist SB. The inclusion of the D3R antagonist causes a significant reduction in the expression of collagen type I. The relative gene expression for WT cells treated with the natural receptor ligand dopamine (**Figure 13A**) and with both DA and the D3R antagonist SB (**Figure 13B**) are shown in **Figure 13**. The treatment with the natural ligand dopamine causes an increase in *Col1a1* (**Figure 13A**) ($p<0.0001$), while treatment with DA and D3R antagonist SB results in a significant decrease in *Col1a1* (**Figure 13B**) ($p<0.0001$). These data may suggest that the blocking of or otherwise dysfunctional D3R causes a reduction in collagen type I expression and production.

Figure 13

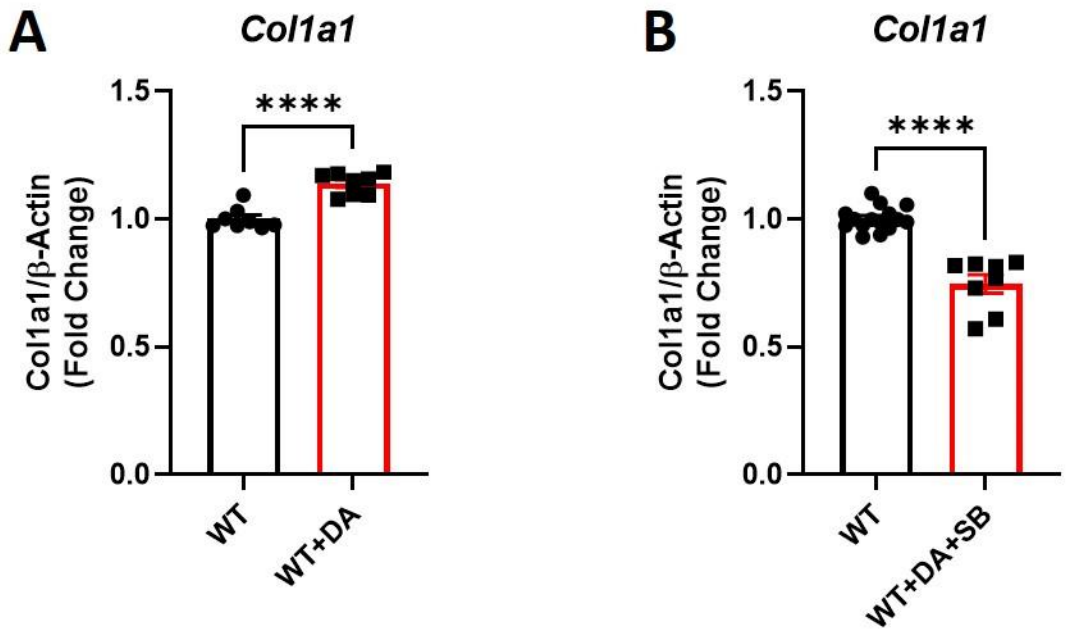


Figure 13: **Use of Pharmacological Agent SB Causes WT to Mimic D3KO Properties. A)** A measure of the relative gene expression of *Col1a1* in WT LV cardiac fibroblasts both with control cells and with treatment. The addition of the receptor ligand dopamine presents an increase in collagen type I expression. ****p<0.0001, statistically significant; bars represent standard error of mean. **B)** A measure of the relative gene expression of *Col1a1* in WT LV cardiac fibroblasts both with control cells and with treatment. The addition of the receptor ligand dopamine and the D3R antagonist SB cause a decrease in *Col1a1* expression. ****p<0.0001, statistically significant; bars represent standard error of mean.

3.9 Dysfunctional D3R Causes an Increase in Collagen Type III Expression that Can Be Augmented with Pharmacological Agents

Just like collagen type I, collagen type III is an essential component of the cardiac fibroblast ECM. Relative gene expression measurements were taken using the comparative ΔCT method normalized to β-actin and converted to fold change. Gene expression was measured in both whole heart tissue and LV cardiac fibroblasts and both showed a significant increase in expression of *Col3a1* in the D3KO derived samples when compared to WT (**Figure 14**). **Figure 14A** shows the relative gene expression in WT and D3KO cardiac tissue. When compared to WT heart tissue, the D3KO hearts appear to express about 1.5 times more collagen type III ($p<0.01$). In **Figure 14B**, the measurement of the relative expression in LV cardiac fibroblasts, this increase is a bit more pronounced at 2 times more *Col3a1* expression ($p<0.01$) than the WT LV cardiac fibroblast control. This would suggest that the loss of function of D3R results in an increase of collagen type III expression.

Figure 14

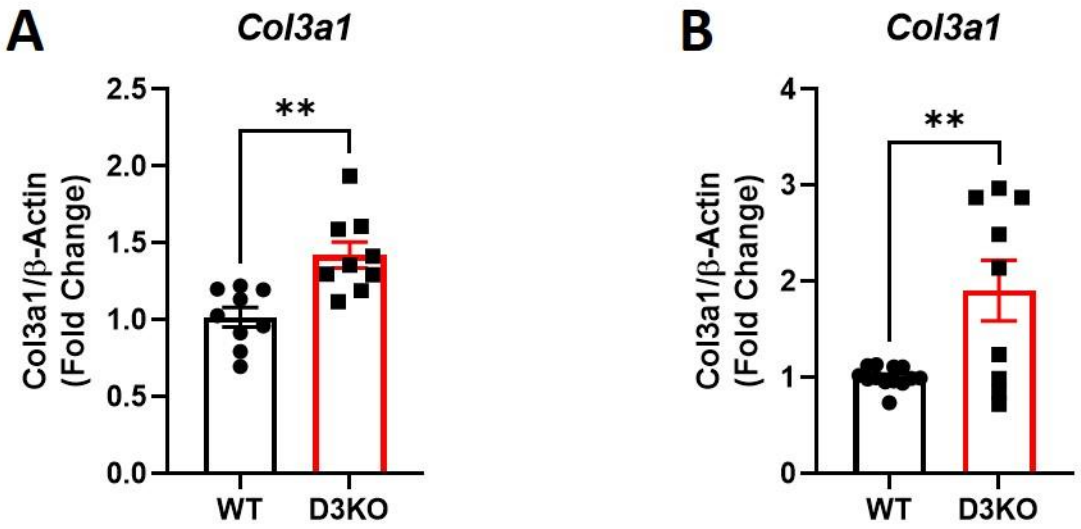


Figure 14: **Dysfunctional D3R Causes an Increase in Collagen Type III Expression in Myocardial Tissue and Cardiac Fibroblasts. A)** Relative gene expression measurements of *Col3a1* in whole heart tissue of WT and D3KO mice. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. **p<0.01, statistically significant; bars represent standard error of mean. **B)** Relative gene expression measurements of *Col3a1* in LV cardiac fibroblasts isolated from WT and D3KO mice. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. **p<0.01, statistically significant; bars represent standard error of mean.

Fascinatingly, in this study it was observed that with respect to the expression of collagen type III, the fibroblasts could be prompted to reverse this overexpression with the tested pharmacological treatments. First, the use of the natural receptor ligand dopamine resulted in a decrease in expression when compared to the D3KO control fibroblast cells (**Figure 15A**) ($p<0.05$). Subsequently, the use of both the receptor ligand dopamine and the D1R antagonist SCH resulted in a decrease of *Col3a1* expression as well (**Figure 15B**) ($p<0.05$), indicating that it is possible that the overexpression of D1R receptors in the D3KO fibroblasts is playing a role in inducing the increase of expression of type III collagen.

Figure 15

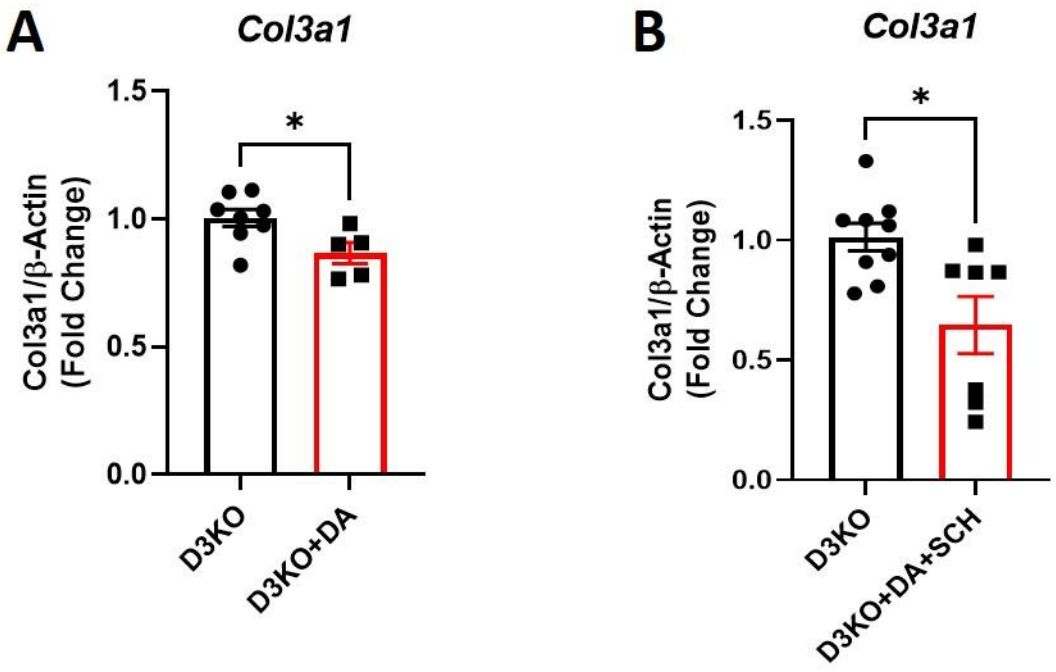


Figure 15: **Collagen Type III Expression Can Be Augmented in D3KO Fibroblasts with Pharmacological Treatments. A)** Relative gene expression measurements of *Col3a1* in cardiac fibroblasts isolated from the LV of D3KO mice using both control fibroblasts and a treatment with the natural ligand dopamine. The treatment resulted in a significant reduction in collagen type III expression in the D3KO LV cardiac fibroblasts. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. *$p<0.05$, statistically significant; bars represent standard error of mean. **B)** Relative gene expression measurements of *Col3a1* in LV cardiac fibroblasts isolated from D3KO mice using both control fibroblasts and a treatment with the natural ligand dopamine along with D1R antagonist SCH. The treatment resulted in a significant reduction in collagen type III expression in the D3KO LV cardiac fibroblasts. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. *$p<0.05$, statistically significant; bars represent standard error of mean.

3.10 Type I Collagen and Type III Collagen Proteins are Expressed in Myocardial Tissue and LV Cardiac Fibroblasts

Protein quantification of whole hearts and LV cardiac fibroblasts from both WT and D3KO mice via western blot did not match the data obtained from RT-qPCR experiments. However, it is possible to deduce from the western blot images that collagen type I and collagen type III both express in these mice. The blots were stained for Col1a1 and Col3a1 proteins and normalized to the total protein loading control. The first well contains a pre-labeled protein ladder standard. Within **Figure 16** there are both western blot images as well as graphs of the density normal which was calculated using the total protein stain to normalize the data. **Figure 16A** shows the staining for Col1a1 in protein isolated from whole heart tissue in the topmost panel. Underneath that panel is the total protein stain which was used to normalize the quantification values. Finally, the graphical representation of the density normals of both WT and D3KO tissue Col1a1 protein expression is under both of the western blots. There was no statistical significance to these data, however they can still be used to indicate expression in both types of heart tissue. **Figure 16B** shows the staining for Col3a1 in protein isolated from whole heart tissue in the topmost panel. Underneath that panel is the total protein stain which was used to normalize the quantification values. Finally, the graphical representation of the density normals of both WT and D3KO tissue Col3a1 protein expression is under both of the western blots. There was no statistical significance to these data, however they can still be used to indicate expression of Col3a1 in both types of heart tissue. **Figure 16C** has the staining for Col3a1 in protein isolated from LV cardiac fibroblasts in the topmost panel. Underneath that panel is the total protein stain which was used to normalize the quantification values. Finally, the graphical representation of the density normals of both WT and D3KO LV cardiac fibroblast Col3a1 protein expression is under both of the western blots. There

was no statistical significance to these data, however they can still be used to indicate expression of Col3a1 in both types of LV cardiac fibroblasts. All of these western blot experiments will need to be repeated again to ensure the consistency of the data.

Figure 16

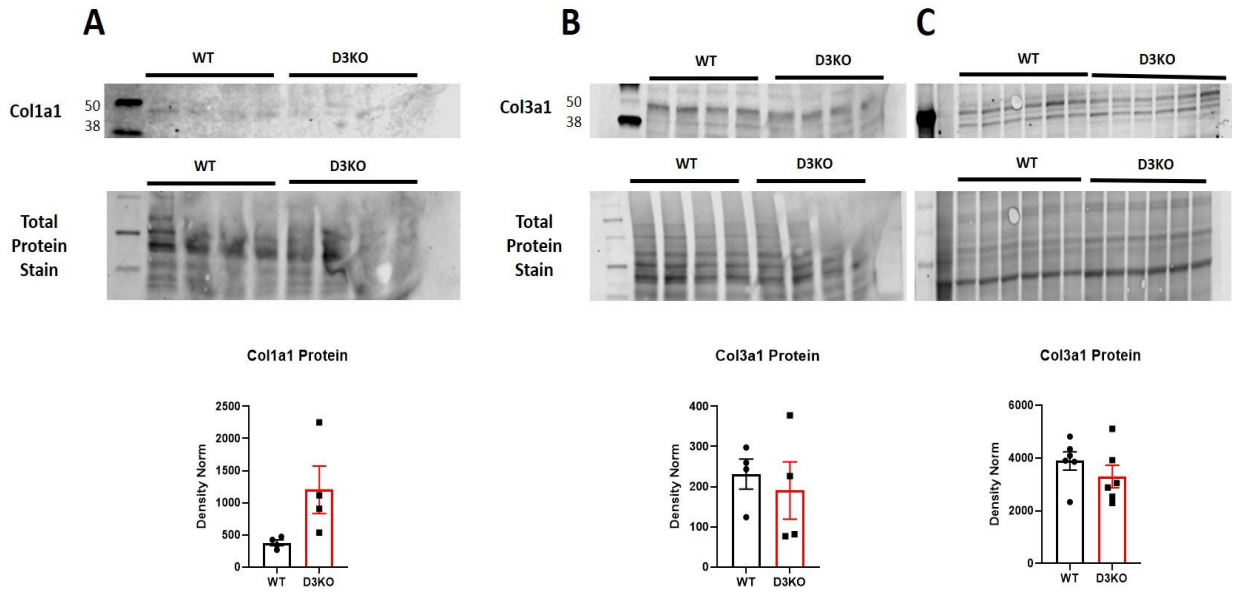


Figure 16: **Type I Collagen and Type III Collagen Proteins are Expressed in Myocardial Tissue and LV Cardiac Fibroblasts. A)** The top panel shows protein expression of Col1a1 fragments determined by western blot and measured in samples of whole heart tissues from WT and D3KO mice (n=4 per mouse type). The middle panel is an image of the total protein stain used to establish relative density normals and to quantify the amount of protein present. The ladders consist of standardized protein markers. The graph at the bottom is a measure of the relative density normals of the Col1a1 expression between WT and D3KO cardiac tissue. Not statistically significant; bars represent standard error of mean. **B)** The top panel shows protein expression of Col3a1 fragments determined by western blot and measured in samples of whole heart tissues from WT and D3KO mice (n=4 per mouse type). The middle panel is an image of the total protein stain used to establish relative density normals and to quantify the amount of protein present. The ladders consist of standardized protein markers. The graph at the bottom is a measure of the relative density normals of the Col3a1 expression between WT and D3KO cardiac tissue. Not statistically significant; bars represent standard error of mean. **C)** The top panel shows protein expression of Col3a1 fragments determined by western blot and measured in samples of LV cardiac fibroblasts from WT and D3KO mice (n=6 per mouse type). The middle panel is an image of the total protein stain used to establish relative density normals and to quantify the amount of protein present. The ladders consist of standardized protein markers. The graph at the bottom is a measure of the relative density normals of the Col3a1 expression between WT and D3KO cardiac fibroblasts. Not statistically significant; bars represent standard error of mean.

3.11 Loss of D3R Can Alter MMP Expression in Myocardial Tissue and LV Cardiac Fibroblasts

MMP's are an essential part of maintaining the ECM homeostasis through degradation of collagens. To help determine if the MMP's could be affecting the collagen accumulation, MMP2 and MMP9 expression were determined through RT-qPCR. The basal relative gene expression measurements for MMP2 and MMP9 found in both whole heart tissue and LV cardiac fibroblasts from WT and D3KO animals are shown in **Figure 17**. When comparing WT and D3KO whole heart tissue gene expression, there is no significant difference for MMP2 (**Figure 17A**). However, there is a marked reduction in MMP2 expression when comparing the gene expression within the LV cardiac fibroblasts (**Figure 17B**) ($p<0.0001$). This could mean that accumulation of collagen type III could be caused by the lack of MMP2 being expressed by the fibroblasts. With regard to MMP9, the D3KO cardiac tissue exhibited drastically increased expression (**Figure 17C**) ($p<0.0001$) and the D3KO cardiac fibroblasts showed a significant decrease in MMP9 expression (**Figure 17D**) ($p<0.0001$). Similar to MMP2, the lack of MMP9 expression in the LV cardiac fibroblasts could be responsible for a pro-fibrotic phenotype.

Figure 17

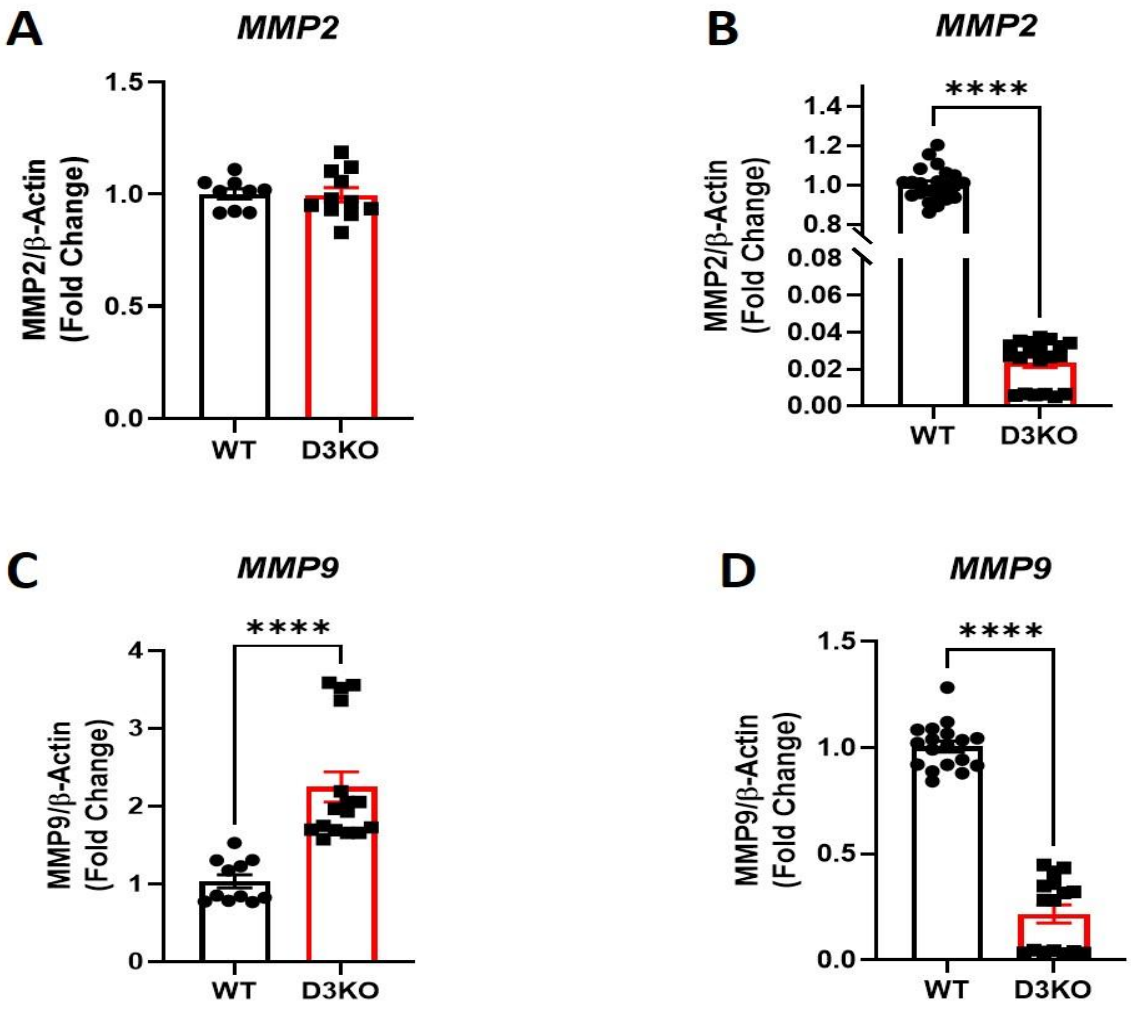


Figure 17: **Loss of D3R Can Alter MMP Expression in Myocardial Tissue and LV Cardiac Fibroblasts. A)** Relative gene expression measurements of *MMP2* in whole heart tissue of WT and D3KO mice. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. No significant difference; bars represent standard error of mean. **B)** Relative gene expression measurements of *MMP2* in LV cardiac fibroblasts isolated from WT and D3KO mice. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. ****$p<0.0001$, statistically significant; bars represent standard error of mean. **C)** Relative gene expression measurements of *MMP9* in whole heart tissue of WT and D3KO mice. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. ****$p<0.0001$, statistically significant; bars represent standard error of mean. **D)** Relative gene expression measurements of *MMP9* in LV cardiac fibroblasts isolated from WT and D3KO mice. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. ****$p<0.0001$, statistically significant; bars represent standard error of mean.

3.12 Absence of Functional D3R Results in an Increase in TGF-β1 Expression in Myocardial Tissue and LV Cardiac Fibroblasts

TGF-β1 serves to regulate MMP functions as well as contributing to cardiac fibrosis through both direct and indirect mechanisms. As such, it was an important aspect of Aim 2 to include in my study. Upon examining relative levels of gene expression in cardiac tissue and LV cardiac fibroblasts, the data points to significantly increased levels of TGF-β1 in the cardiac system of D3KO mice (**Figure 18**). The whole heart tissue was examined first and exhibited an approximate 1.3 times more expression in D3KO compared to WT (**Figure 18A**) ($p<0.001$). The LV cardiac fibroblasts showed slightly more significant expression at approximately 1.5 times more than the WT fibroblasts (**Figure 18B**) ($p<0.0001$). These findings were further confirmed in western blot examination of the TGF-β1 protein expression in whole heart tissues as well. **Figure 18C** shows the western blot with TGF-β1 expression, the total protein stain used to calculate the density normals of both types of tissues, and it also shows the graph of the density normals, which show the increase in TGF-β1 proteins in D3KO tissue compared to WT. These increases in TGF-β1 expression could be responsible for the downregulation of MMP's 2 and 9 that I observed, as well as possibly contributing to an increase in collagen expression or an increase in the ratio of collagen expression.

Figure 18

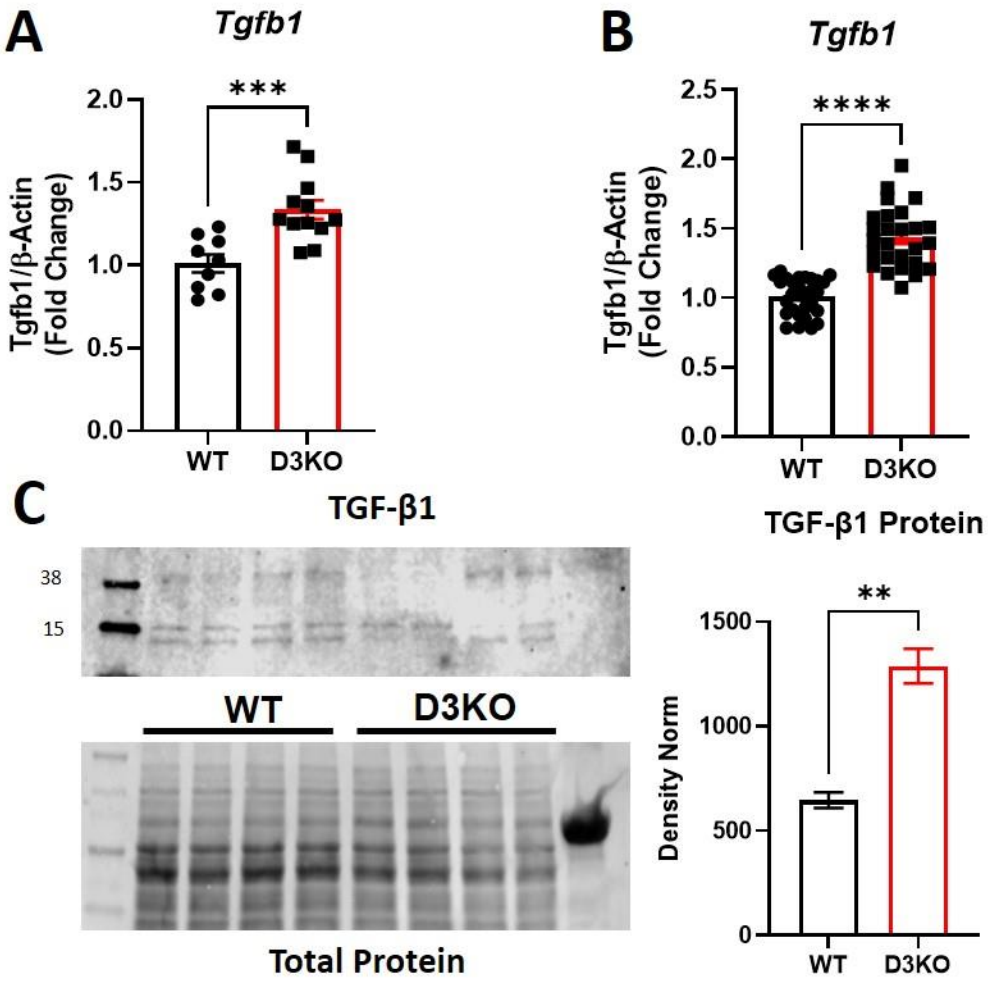


Figure 18: **Absence of Functional D3R Results in an Increase in TGF-β1 Expression in Myocardial Tissue and Cardiac Fibroblasts. A)** A measure of the relative gene expression of *Tgfb1* in WT and D3KO whole heart tissue. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. ***p<0.001, statistically significant; bars represent standard error of mean. **B)** A measure of the relative gene expression of *Tgfb1* in WT and D3KO LV cardiac fibroblast controls. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. ****p<0.0001, statistically significant; bars represent standard error of mean. **C)** The left upper panel is a western blot stained for TGF-β1 in WT and D3KO cardiac tissue. Under that panel is a panel containing the total protein stain which was used in the calculation of the density normals. On the right is the graphical representation of the density normals, indicating that D3KO has an increase in TGF-β1 protein expression when compared to WT. **p<0.01, statistically significant; bars represent standard error of mean.

3.13 Blocking D1R Activity Induces a Reduction in TGF-β1 Expression

Interestingly, a noticeable trend began to emerge after conducting experiments on the LV cardiac fibroblasts using pharmacological agents, particularly D1R antagonist SCH. **Figure 19** displays the most notable significant differences. **Figure 19A** serves as the baseline for the WT fibroblasts which were treated with DA. There was no significant alteration of *Tgfb1* expression when the cells were treated with only the natural ligand dopamine, however, there was a significant decrease of nearly 30% in expression compared to the WT control with the addition of SCH, the D1R antagonist (**Figure 19B**) ($p<0.0001$). In addition, a similar decrease was observed when the WT cells were treated with SCH and the D3R agonist PPX (data not shown), indicating that regardless of D3R activation, SCH induces a decrease in TGF-β1. This significant reduction in expression remained the case in the D3KO LV cardiac fibroblasts as well. **Figure 19C** consists of D3KO LV cardiac fibroblasts, both controls and those treated with D1R antagonist SCH. Once again, the treatment with the antagonist results in a significant decrease in TGF-β1 expression. These data could indicate that regardless of D3R function, that D1R could be playing a role in the regulation of TGF-β1.

Figure 19

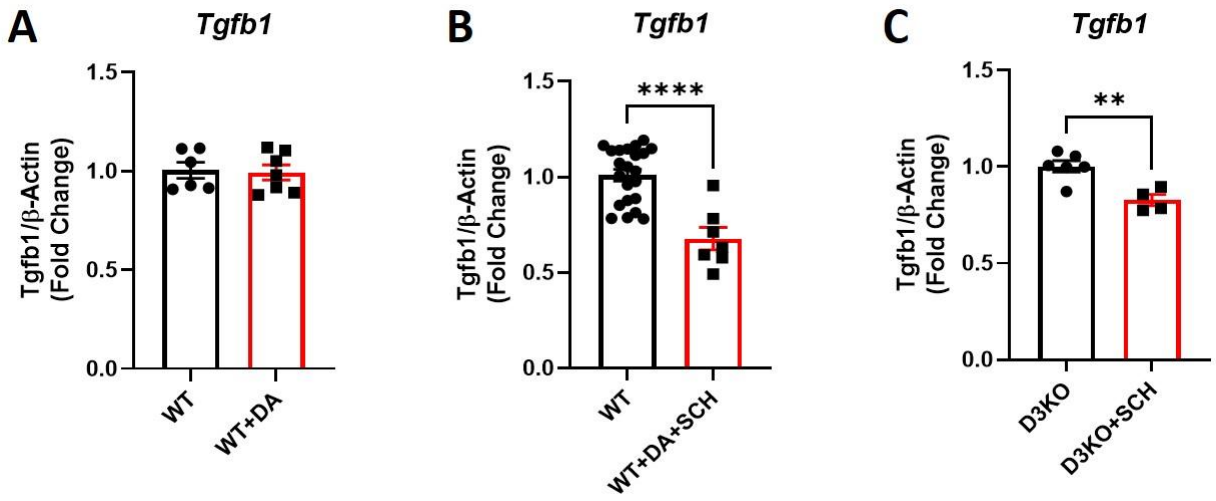


Figure 19: **Blocking D1R Activity Induces a Reduction in TGF-β1 Expression. A)** A measure of the relative gene expression of *Tgfb1* in WT control and treated LV cardiac fibroblasts. The addition of natural receptor ligand dopamine did not induce any significant effect on *Tgfb1* expression in the WT fibroblasts. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. No significance; bars represent standard error of mean. **B)** A measure of the relative gene expression of *Tgfb1* in WT control and treated LV cardiac fibroblasts. The addition of natural receptor ligand dopamine along with D1R antagonist SCH caused a significant decrease of *Tgfb1* expression in the WT fibroblasts. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. ****$p<0.0001$, statistically significant; bars represent standard error of mean. **C)** A measure of the relative gene expression of *Tgfb1* in D3KO control and treated LV cardiac fibroblasts. The addition of the D1R antagonist SCH caused a significant decrease of *Tgfb1* expression in the D3KO fibroblasts. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. **$p<0.01$, statistically significant; bars represent standard error of mean.

3.14 Dysfunctional D3R Produces Alterations in Angiotensin II Receptor Type 1 in Myocardial Tissue and Cardiac Fibroblasts

Angiotensin II also has direct and indirect routes to the induction of cardiac fibrosis. The Angiotensin II Type 1 Receptor (AT1R) is one of the predominant AngII receptors and its expression in the cardiac systems of these animals was important to my Aim 2. **Figure 20** shows the alterations that dysfunctional D3R can cause to the AT1R in both whole cardiac tissue and LV cardiac fibroblasts. Within the whole hearts, expression was reduced overall in the D3KO hearts (**Figure 20A**). Fascinatingly, this was not the case for the LV cardiac fibroblasts which exhibited a drastically marked increase in *Agtr1* gene expression as much as nearly 175-fold more than WT LV cardiac fibroblasts (**Figure 20B**). The huge amount of receptor expression in the D3KO fibroblasts could indicate some sort of imbalance in the homeostasis of the fibroblasts and possibly be the root cause for some of the pro-fibrotic phenotypes exhibited previously in D3KO hearts.

Figure 20

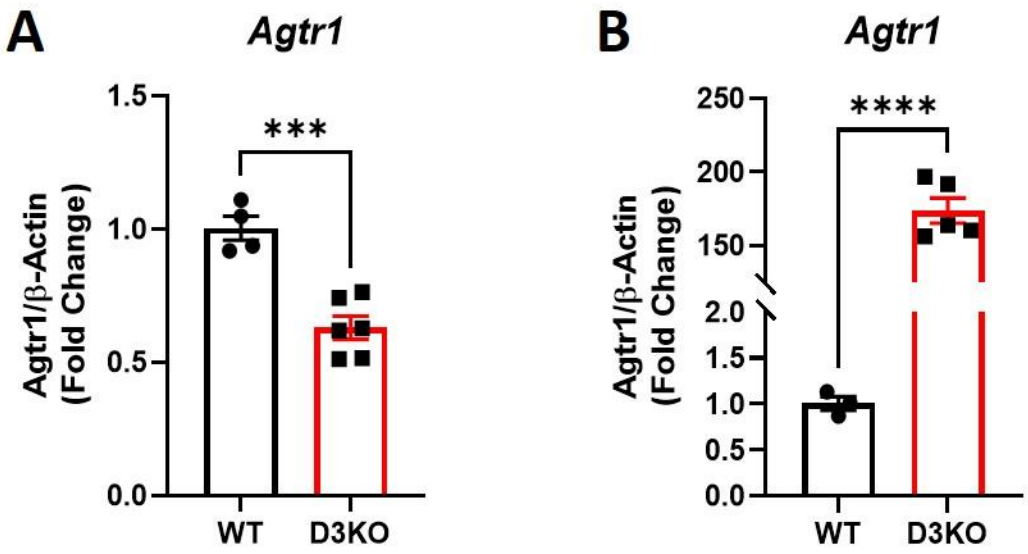


Figure 20: **Dysfunctional D3R Produces Alterations in Angiotensin II Receptor Type 1 in Myocardial Tissue and Cardiac Fibroblasts. A)** A measure of the relative gene expression of *Agtr1* in WT and D3KO whole heart tissue. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. ***p<0.001, statistically significant; bars represent standard error of mean. **B)** A measure of the relative gene expression of *Agtr1* in WT and D3KO LV cardiac fibroblast controls. Gene expression was measured using the comparative delta CT method with gene expression normalized to β-actin and converted to fold change. ****p<0.0001, statistically significant; bars represent standard error of mean.

Chapter 4

Discussion

The goal of this research project was to investigate whether dopamine receptors D1 and D3 express both in mouse cardiac tissue and primary cultures of cardiac fibroblasts and to explore the potential role of D3R in cardiac fibrosis. We have utilized the D3KO mouse model which had been used in a previous article (53). While the article by Johnson et. al. implies that a dopaminergic system may exist in the cardiac system, as cardiac fibrosis was observed specifically in D3KO animals (53), there was very little concrete evidence of the expression of dopamine receptors in the cardiac system. The primary goal of Aim one was to validate the assumption that the cardiac system possesses an intrinsic dopaminergic system. **Figure 2** depicts the relative gene expression of each of the five dopamine receptor subtypes in both cardiac tissue (**Figure 2A**) and cardiac fibroblasts (**Figure 2B**), showing evidence that all five receptors are intrinsically expressed in the heart. The LV cardiac fibroblasts demonstrate relatively similar levels of expression between receptors, with D1R showing slightly increased expression (**Figure 2B**). However, there is a larger noticeable difference in expression of the receptors in whole hearts. D2R is expressed the most, followed by D1R, with the other three receptors D3R, D4R, and D5R being expressed at around the same amount. This suggests that other cardiac cells besides fibroblasts, express high levels of D2R. This report shows for the first time that both mouse cardiac tissue and cardiac fibroblasts express all five dopamine receptors at the gene level.

In addition, this is the first study where D1R and D3R were shown to be present through a comprehensive analysis consisting of visualization via immunofluorescence, gene expression via RT-qPCR, and protein quantification via western blot. This aspect of my thesis has been recently published in an article in which I am a co-author (18). The shift in focus from all five receptor

subtypes to just two was made due to several reports which indicate that D1R and D3R interact directly with one another (29-32). It is also important to note that we wanted to observe both an excitatory dopamine receptor (D1R) as well as an inhibitory receptor (D3R) in order to address any functional significances which may arise when the two types are interacting. **Figure 3** displays three different techniques to show expression of D1R and D3R proteins in WT cardiac tissue. The first of these is immunofluorescence, which showed the presence of both receptor subtypes in WT cardiac tissue sections (**Figure 3A**). The cardiac fibroblasts were marked by the fibroblast specific stain, vimentin. However, there was staining of D1R and D3R in the cells that were not just fibroblasts as well. This is to be expected, as the heart is a complex tissue comprised of a number of different cell types. It has been shown that D1R and D2R express in rat cardiomyocytes (74), so that could feasibly be the case in mice as well. **Figure 3B** indicates that both D1R and D3R express in cardiac tissue at the gene level. This data suggests that D1R is expressed more than D3R in WT tissue. **Figure 3C** confirms the expression of D1R and D3R proteins in WT mouse cardiac tissue. Overall, **Figure 3** confirms the expression of these two receptors in cardiac tissue in a complete and comprehensive way. Similarly, LV cardiac fibroblasts (**Figure 4**) expressed both D1R and D3R, as visualized by immunofluorescence (top two panels of **Figure 4A**). From these images, it is clear that both receptors express in the membrane of the cardiac fibroblast cells. The bottom panels of **Figure 4A** show both receptors in D3KO LV cardiac fibroblasts. Just like in the WT cells, D1R is expressed. However, there is a difference between the WT and D3KO cells when it comes to the expression of D3R. There is no visual indication of D3R expression on the membranes of the D3KO LV cardiac fibroblast cells. This is due to how the global knock out was generated. This knock out has been created by altering the D3R gene, but not by removing it entirely, which causes the synthesis of D3R proteins which are dysfunctional and therefore cannot

be inserted into the cell membrane (75). Due to this, the D3R is not seen in the images depicting immunofluorescence and that helps to confirm the validity of the D3KO mouse as a tool for these experiments. **Figure 4B** indicates that both D1R and D3R express in LV cardiac fibroblasts at the gene level and **Figure 4C** confirms the expression of D1R and D3R proteins in WT mouse cardiac fibroblasts.

To explore the second half of aim one, WT and D3KO tissues as well as cardiac fibroblasts were used. The RT-qPCR data showed something interesting. The D3KO tissue and LV cardiac fibroblasts both exhibited increased gene expression of D1R (**Figure 5**). The tissue expression of D1R was less than the fibroblast expression at around 1.3-fold increased from WT tissues. It is possible, as with any sort of tissue analysis, that the increase in expression was muted from varying levels of expression in the multiple cell types located in the heart tissue, as whole hearts were homogenized. The LV D3KO cardiac fibroblasts had increased gene expression of D1R at nearly 8-fold more than the WT fibroblasts. This supports evidence from other organ systems that D1R and D3R can interact with each other such as in the brain and spinal cord (31, 33), and in the kidney (19). It may also indicate that the loss of D3R is being compensated for by the increase of D1R expression. The increase in D1R expression in D3KO heart tissue was also confirmed by western blot (**Figure 6**).

We then quantified the expression (gene and protein) of D3R in the WT and D3KO LV cardiac fibroblasts and whole heart tissues. Within the whole heart tissue, D3R expression overall was significantly decreased in D3KO hearts which may be due to the dysfunctional D3R protein present in the tissue (**Figure 7**). Alternately, it may be possible that due to the myriad of other cell types present in the cardiac tissue, the gene expression was ultimately affected and that would be the cause for the discrepancy between D3KO LV cardiac fibroblast cells and D3KO whole heart

tissues. The D3KO LV cardiac fibroblasts showed significantly increased *Drd3* gene expression. This may be due to the fibroblasts specifically overcompensating for the dysfunctional D3R production, by increasing gene expression in an attempt to create functional D3R to be inserted into the membrane. **Figure 8** corroborates the whole heart tissue data obtained from RT-qPCR with western blot expression. Specifically, D3R protein expression is reduced in the D3KO heart tissue compared to WT tissue.

Finally, to conclude the last part of aim one, cardiac fibroblast cells of both genotypes were treated with pharmacological agents specific to both D1R and D3R. The first step of those treatments was to validate the actions of the agonists, antagonists, and the global D3KO model. In **Figure 9** the validation of the D1R agonist SKF and the D1R antagonist SCH can be seen in terms of *Drd1* gene expression. The D1R agonist SKF promoted the expression of D1R in WT cells when compared to a control (**Figure 9A**). While the administration of the natural ligand dopamine did not have any significant effect on the expression of D1R, the inclusion of dopamine and D1R receptor antagonist SCH also had no significant effect on D1R receptor expression (**Figure 9B & 9C**). This shows that the antagonist is working and that the dopamine administration had no significant effect on the expression either way. Moreover, it was also imperative to confirm the D3R antagonist SB as well. This validation is included in **Figure 10**. **Figure 10A** is the basal level of *Drd1* expression found in the LV cardiac fibroblasts and serves as a point of reference. In **Figure 10B**, WT cells are compared with a treatment including dopamine and the D3R antagonist SB. Since the WT LV cardiac fibroblasts exhibit fully functional D3R, the antagonist SB has a receptor with which to bind and the effect of increased *Drd1* expression is seen, though it nowhere near matches the fold change seen in the D3KO fibroblasts. Whereas in **Figure 10C** the same treatment has been used on the D3KO LV cardiac fibroblasts and the D1R gene expression is compared to

the D3KO control. There is no significant change with this treatment. Not only does this validate the antagonist SB, it also shows that SB does not affect D3KO LV cardiac fibroblasts, as it should not, considering that these fibroblasts lack functional D3R in the membrane. From there, a variety of treatment combinations were used as was described in the methods section, however, regardless of pharmacological treatment, the D3KO LV cardiac fibroblasts consistently expressed more D1R than the WT LV cardiac fibroblasts.

Once the expression of the dopamine receptor system in the mouse heart tissues and cardiac fibroblasts was confirmed, we then moved to Aim two. Aim two focused on using knowledge of profibrotic markers in the cardiac system to explore the possibility that a dysfunctional D3R contributes to cardiac fibrosis due to excessive accumulation of collagen in the heart tissue (53). These profibrotic markers were chosen based on their important influence on cardiac fibrosis and adverse remodeling of the heart. As type I and type III collagens are the most common collagens in the heart (51), and a hallmark of cardiac fibrosis is an excessive accumulation or a decreased degradation of these collagen types, they were picked as key genes and proteins to study. MMP's, also known as collagenases, are the enzymes which degrade and break down the collagens once they have been excreted into the extracellular matrix and loss or improperly functioning MMP activity can lead to increased collagen accumulation (41, 44). Finally, Angiotensin II and TGF-β1 are known profibrotic markers and regulate blood pressure and can have a negative impact on heart health. So, these were the final two profibrotic factors we chose to study.

First, we observed basal level gene expression of type I collagen in WT and D3KO whole heart tissues and LV cardiac fibroblasts (**Figure 11**). In both tissue and fibroblasts, the collagen I expression decreased in D3KO samples, but this decrease was more pronounced in the LV cardiac fibroblasts (**Figure 11B**). In addition, the use of pharmacological treatments presented data similar

to D3KO in WT LV cardiac fibroblasts (**Figure 12**). In **Figure 12A** the D1R agonist SKF is shown to have no significant effect on *Col1a1* expression in WT fibroblasts, however, the addition of the D3R antagonist SB (**Figure 12B**), which would mimic the dysfunctional D3R of the D3KO fibroblasts, results in decreased *Col1a1* expression, though not as drastic as what was seen in the basal level expression in the D3KO cells. Then in **Figure 12C & 12D** the use of the D1R antagonist SCH to block the D1R receptors resulted in an increase in the expression of *Col1a1* in WT cells. Further, **Figure 13** shows the effects of the treatment with the natural ligand dopamine and the D3R antagonist SB. Once again, the D3R antagonist caused a significant decrease in the expression of type I collagen (**Figure 13B**), with enough impact to overcome the increase in *Col1a1* expression caused by treatment with dopamine alone (**Figure 13A**). Together, these data indicate that both D1R and D3R may play a role in the regulation of type I collagen expression. The blocking of both receptors caused a significant alteration to the expression of *Col1a1*. At the same time, it was also important to observe the basal level of expression for collagen type III in cardiac tissue and LV cardiac fibroblasts. **Figure 14** shows the basal expression. This expression differed from the *Col1a1* expression as in both tissue and fibroblasts, the *Col3a1* expression increased significantly. Interestingly, the use of dopamine and the blocking of the D1R receptor in the D3KO fibroblasts both caused a significant decrease in collagen type III (**Figure 15**), further providing evidence that the D1R may have a direct or indirect effect on the production of both types of collagens. After the gene expression experiments, it was also essential to determine protein expression at the cellular and tissue levels. While measuring the protein expression of collagen types I and III, I encountered several technical difficulties. When probing for collagens using a western blot, they are notoriously hard to probe for. Collagens can present as full-length molecules, as well as a number of fragments with different molecular weights. In addition, most of the

collagens are secreted into the media; thus, collagen quantification of the cell lysate does not represent the overall levels of collagen protein in the cells. Finally, standardizing the optimal dilution for the antibodies is also time consuming since it varies between sample type. **Figure 16** shows western blot analyses for both collagens I and III in cardiac tissue. **Figure 16A** shows that collagen type I is present in WT and D3KO myocardial tissue; however, there was no significant difference in the protein expression of collagen type I between WT and D3KO whole heart tissue. This could have occurred due to the issues with trying to probe for collagens via western blot, and most certainly, this experiment in particular will need to be repeated. **Figure 16B** shows the blots for collagen type III in both WT and D3KO whole heart tissue. There was no significant difference in expression between the two types of tissues and to confirm these results, further replicates of this experiment will need to be performed. **Figure 16C** displays the western blot for type III collagen performed on LV cardiac fibroblasts from both WT and D3KO mice. Again, no significant difference between the WT and D3KO LV cardiac fibroblasts was seen. Cellular protein expression is much more reduced than tissue protein expression simply by the nature of cells versus tissues. It is harder to quantitate and probe for these proteins in cell lysates. Further experiments will need to be conducted with LV cardiac fibroblasts to confirm the protein expression data found here.

The next set of profibrotic markers investigated were MMP2 and MMP9. The basal level gene expression of both of these gelatinases was examined in WT and D3KO myocardial tissue and LV cardiac fibroblasts (**Figure 17**). In **Figure 17A** the basal level gene expression of MMP2 for whole heart tissue has been converted to fold change and graphed. Between the WT and D3KO tissues, there was no significant difference in MMP2 expression. This could be explained by the other cells in the heart tissue other than fibroblasts, which could be making up for the significant

decrease in expression in the fibroblasts (**Figure 17B**). Furthermore, an interesting dichotomy exists regarding the expression of MMP9 between the cardiac tissue and the cardiac fibroblasts. In the whole heart tissue, there is nearly a 2-fold increase in MMP9 expression in the D3KO cardiac tissue (**Figure 17C**), however, in the D3KO cardiac fibroblasts, the relative gene expression of MMP9 is significantly reduced (**Figure 17D**). This could also be accounted for by the possibility of the other cardiac cells compensating and overexpressing MMP9 to make up for the lack of production from the cardiac fibroblasts. Particularly, leukocytes are one of the main cellular sources of MMP9, it would be interesting to investigate whether D3KO present a higher level of tissue inflammation compared to the WT hearts. In addition, MMP production, or a lack thereof, does affect the collagen degradation as well. If the MMP's are being secreted less in D3KO fibroblasts, this could lead to the increase in collagen type III that has been shown here. Additionally, if there is increased MMP expression in the tissues, there could be too much degradation of collagens and that could be the cause of the reduced collagen type I.

Finally, we focused on the profibrotic marker TGF-β1 and the expression of the angiotensin II type I receptor, which is a binding site for angiotensin II. Here we show that TGF-β1 gene expression is significantly increased in both D3KO whole heart tissue and LV cardiac fibroblasts, and significantly increased TGF-β1 protein expression is present in D3KO cardiac tissue (**Figure 18**). The basal level of gene expression of *Tgfb1* in D3KO myocardial tissue is shown in **Figure 18A**. These values when converted to fold change, reveal a 1.3-fold increase in the gene expression of the D3KO tissue when compared to WT cardiac tissue. A similar increase of approximately 1.5-fold is seen in the basal level gene expression of *Tgfb1* in the D3KO LV cardiac fibroblasts as well (**Figure 18B**). **Figure 18C** corroborated the relative gene expression data and seems to suggest that TGF-β1 expression is increased in D3KO heart tissue and cardiac fibroblasts. Furthermore,

the blocking of D1R caused a decrease in TGF-β1 expression in both WT and D3KO cardiac fibroblasts (**Figure 19**). This data indicates that in both types of fibroblasts, that D1R is playing a role in the TGF-β1 pathway, and this could be leading to the increase in collagen accumulation that have been shown in these experiments and that was proposed in a previous study (53). The last profibrotic aspect that was looked into was the angiotensin II receptor type I. We observed the basal level of relative gene expression in both the WT and D3KO heart tissue and the LV cardiac fibroblasts. In the tissues, there was a significant decrease in *Agtr1* expression in the D3KO hearts (**Figure 20A**). However, there was a fascinatingly high statistically significant increase in the receptor expression in the D3KO LV cardiac fibroblasts (**Figure 20B**). This increase was nearly 175-fold greater than the WT fibroblasts. With such a large amount of *Agtr1* expression, any possible increases in AngII would likely have a very potent effect on these cells, inducing a profibrotic response. It is also possible that this increase came from the phenotypic switch of fibroblasts to the contractile myofibroblast, which is a large producer of collagens in the injured heart.

There are still unanswered questions and unconfirmed data surrounding this study. For the future, the first step is to confirm data reproducibility by repeating some of the experiments, mainly the profibrotic marker western blots. These need to be performed on both WT and D3KO myocardial tissue and cardiac fibroblasts. In addition to probing for type I and type III collagens and TGF-β1, the fibroblasts and tissues should also be probed for the angiotensin II type I receptor. There is also another mouse model that could be utilized in this research. There are commercially available D3KO mice which have organ specific knock outs. Thus, buying and having cardiac specific knock outs could provide further insight into the mechanistic aspects of dysfunctional D3R and its relationship with collagen and TGF-β1 that are currently lacking. The media used in

the cardiac fibroblast treatments was collected and stored. Conducting ELISA analysis on those medias could be more illuminating than the western blots in terms of the expression of the secreted proteins and factors. Collagen, MMP's, TGF-β1, and Angiotensin II are all secreted into the media surrounding the cells. Finally, expanding the research to commercially available human cardiac fibroblasts and seeing the effects of blocking D3R in those cells could help to validate the findings across species.

Chapter 5

Conclusion

This study has uncovered a few novel observations. To start with, all five dopamine receptor subtypes have been confirmed to express at the gene level in murine cardiac tissue and LV cardiac fibroblasts. In addition, the expression of excitatory D1R and inhibitory D3R was confirmed via three comprehensive analyses: Immunofluorescence, RT-qPCR, and Western Blot. From there, it was determined that with dysfunctional D3R, the expression of D1R increases. This result matches data obtained from the CNS in the same mouse model (8). The D3KO LV cardiac fibroblasts were found to consistently overexpress D1R regardless of pharmacological treatment. This study also investigated relative expression of profibrotic markers. In the model of dysfunctional D3R, collagen type I decreased in both tissue and fibroblast gene expression. In the same model, collagen type III gene expression increased. Within the LV cardiac fibroblasts, the expression of both MMP2 and MMP9 decreased significantly, possibly indicating a link to collagen accumulation in the decreased degradative action of these enzymes. The levels of TGF-β1 that were observed were increased in both the D3KO heart tissue and LV cardiac fibroblasts. TGF-β1 is a known profibrotic marker and these increased mRNA and protein levels indicate a profibrotic phenotype. The use of the D1R antagonist SCH to reduce *Tgfb1* expression is interesting and warrants further study as a possible anti-fibrotic therapeutic. Finally, the expression of angiotensin II receptor type I was dramatically increased in D3KO LV cardiac fibroblasts and further experimentation is necessary to confirm these findings. Ultimately, the cardiac system in the mouse does exhibit an intrinsic cardiac dopaminergic system and D3KO fibroblasts exhibit a profibrotic phenotype.

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