ROLE OF TCF21 IN PERINATAL CARDIAC FIBROBLASTS PROLIFERATION AND GENE EXPRESSION

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ABSTRACT

Cardiac fibroblasts play a dominant role in heart disease, but our understanding of the signals that control these cells during heart development is inadequate. Tcf21 is a coronary artery disease associated gene whose function in the heart is not well understood. Previously using Tcf21 null animals, we have demonstrated that this basic helix loop helix transcription factor is essential for the formation of epicardial-derived cardiac fibroblasts. Lack of Tcf21 resulted in a failure of cardiac fibroblast progenitor migration from the epicardium. Recently, we have found that Tcf21 continues to be expressed in mature cardiac fibroblasts suggesting a continued role for this transcription factor. To evaluate the function of Tcf21 after embryonic development, we generated animals which lacked Tcf21 in the fibroblast lineage. When Tcf21 is removed at birth, we find a 30 percent reduction in the number of fibroblasts. Using EdU, we determined that loss of Tcf21 leads to reduced fibroblast proliferation in the perinatal period. To identify transcriptional targets of Tcf21, we have compared fibroblast transcriptomes in control and Tcf21 deficient fibroblasts. We have found an increase in lipid genes in the Tcf21 deficient fibroblasts. Taken together our results demonstrate that Tcf21 continues to play important roles in fibroblast biology beyond the initial formation of cardiac fibroblasts from the epicardium.

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LIST OF ABBREVIATIONS

CF: Cardiac fibroblast

ECM: Extracellular matrix

MMP: Matrix metalloproteases

EPDC: Epicardially derived cells

PDGFRα: Platelet derived growth factor alpha

TCF21: Transcription factor 21

DDR2: Discoidin receptor 2

CAD: Coronary artery disease

TF: Transcription factor

bHLH: Basic helix loop helix

Chapter 1: Introduction

Understanding cardiac fibroblasts functions and their roles has been an ongoing endeavor in cardiovascular research. Cardiac fibroblasts have previously been characterized by their spindle shapes and mesenchymal origins (Kanekar et al., 1998). Now they are understood to synthesize, secrete, and maintain components of the extra cellular matrix (ECM) within the heart (Krenning et al., 2010)(Pinto et al., 2016). Most importantly they produce collagens which make up the backbone of the ECM. During development cardiac fibroblasts lay down the ECM to support the cells and allow for signaling to occur within the heart. In contrast, after heart injury they rearrange it which compensates for the loss of cardiomyocytes or stress that has been placed on the heart (Sano et al., 2000).

While there have been many studies that point out the importance of cardiac fibroblasts after injury, there are many questions that remain regarding the normal development of function of these cells.

1.1 Cardiac Development

During development dynamic changes in cell types and function occur in the heart. Gene transcription and regulation is tightly controlled to allow the proper differentiation of progenitor cells into mature myocytes and non-myocytes. Although the cardiomyocytes make up around 70% of the myocardial space, they constitute only 30% of the cell numbers in the heart. Non myocyte cells, endothelial cells, leukocytes, pericytes, and cardiac fibroblasts constitute the remaining cell types with endothelial cells constituting

60% (Pinto et al., 2016). Among these cells, cardiac fibroblasts can be thought of as the building blocks that support the structure of the heart.

Cardiac fibroblasts were initially found to mature and migrate from only the pro-epicardial organ to the sub epicardium during development (Dettman et al., 1998). While most fibroblasts undergo epithelial to mesenchymal transition and migrate into their respective areas of the heart, they do not all arise from the same origin. 80% of fibroblasts, the epicardial fibroblasts, eventually reside within the myocardium and line epicardium of the heart, derive from the pro-epicardial organ (Ali et al., 2014). Another 16% of fibroblasts, the endocardial fibroblasts, migrate into the septum and valves and arise from the endocardium (Ali et al., 2014), with the remaining fibroblasts coming from the neural crest (Moore-Morris et al., 2014)(Moore-Morris et al., 2016).

1.2 Transcription factor 21

Tcf21 is a basic helix loop helix (bHLH) transcription factor (TF) that is involved in the embryogenesis of kidneys, testis, lungs, spleen, and the heart. It has been established as one of the factors that determines and guides differentiation of mesenchymal cells in these organs (Lu et al., 1998)(Braitsch et al., 2012)(A. Acharya et al., 2012)(Tandon et al., 2013). This Class B TF is required to dimerize to promote transcription. The typical binding partner for Tcf21 is a Class A TF, known as an E protein, that upon dimerization binds to DNA at a canonical binding sequence (Lu et al., 1998). This sequence is a known E box sequence (CAGCTG) that is common amongst bHLH transcription factors (Lu et al., 1998).

1.2.1 Tcf21 in Cancer

Tcf21 is not only important in organogenesis but is also characterized as a tumor suppressor in many cancers such as kidney, colorectal, lung, breast, and gastric cancer (Yang et al., 2015)(Dai et al., 2017)(Gooskens et al., 2015)(Chen et al., 2018). Its reduced levels in colorectal and breast cancer cells have been shown to lead to poor prognosis in patients (Dai et al., 2017, p. 21)(Wang et al., 2015). In in vitro and in vivo studies, cancerous cells tended to downregulate Tcf21, sometimes by hypermethylation (Gooskens et al., 2015) but the reduced expression was associated with larger tumor size and metastasis. In contrast, overexpression of Tcf21 in colorectal cancer cells and breast cancer cells provided protection by inducing apoptosis and inhibiting cell proliferation (Wang et al., 2015).

1.2.2 Tcf21 in Cardiac Fibroblasts

During embryonic development TF's play an important role in instructing patterns of gene expression. Tcf21 has been shown to be expressed in early mesodermal cells in the heart (Braitsch et al., 2012). Global deletion of Tcf21 results in significant developmental defects leading to perinatal lethality (A. Acharya et al., 2012)(Bhandari et al., 2011). Tcf21 is first seen expressed in early mesenchymal cells in the heart at embryonic day 8.5 (A. Acharya et al., 2012). Tcf21 expression in epicardially derived cells allows EMT and migration of differentiated fibroblasts into the interstitium of the heart. Tcf21 determines the distinction between if a cell becomes a cardiac fibroblast or a coronary vascular smooth muscle cell (A. Acharya et al., 2012).

Cardiac fibroblasts have been an elusive cell to study in its early stages. The hunt to find a reliable marker has proven to be a difficult task. We now know that previously used markers such as Vimentin, Fsp-1 and Thy-1, were shown to also mark lymphocytes and endothelial cells. More specific markers to cardiac fibroblasts like PDGFRα, DDR2, Periostin, and Tcf21 are being used today (Vicente-Steijn et al., 2015)(Ivey & Tallquist, 2016)(Snider et al., 2009).

With the use of mouse models Tcf21 has been an instrumental tool in lineage tagging cardiac fibroblasts to help with studying them during development (Maezawa et al., 2012). While expression levels in cardiac fibroblasts during development are maintained, Tcf21 becomes downregulated in differentiating smooth muscle cells (A. Acharya et al., 2012). Expression in cardiac fibroblasts has also been seen in adult hearts at homeostasis (Ivey et al., 2018)(Lu et al., 1998). These differential embryonic expression levels have regarded this transcription factor to be the “fate determining factor” of cardiac fibroblasts. Although Tcf21 expression is imperative for the formation of embryonic cardiac fibroblasts, little is known about the transcription factors role after this early developmental stage.

1.3 Cardiovascular disease and Tcf21

Cardiovascular disease is the leading cause of death in developed countries (Sherry L. Murphy, B.S., Jiaquan Xu, M.D., Kenneth D. Kochanek, M.A., and Elizabeth Arias, Ph.D., 2018). This umbrella term includes not only myocardial infarction but myocardial ischemia, peripheral artery disease, coronary heart disease, and heart failure. After injury to the heart, cardiac fibroblasts become activated, proliferate, and secrete

excess amounts of collagen to compensate for the loss of cardiomyocytes (Porter & Turner, 2009). These activated fibroblasts have been coined myofibroblasts and are typically marked by their expression of periostin and alpha smooth muscle actin (Snider et al., 2009). This fibrotic remodeling forms a scar that helps the heart maintain its structure and function to a limited degree. Ultimately, prolonged and extensive fibrosis leads to heart failure due to either reduced ejection fraction or dilated cardiomyopathies (Porter & Turner, 2009).

Of interest to us is coronary artery disease (CAD). This cardiomyopathy results from buildup of plaque and hardening in the arteries, known as atherosclerosis, leaving narrowed passageway for blood to travel to the myocardium. There is a 55% risk in both men and women in the U.S of developing this disease (Sanchis-Gomar et al., 2016). Intriguingly, the Tcf21 locus has been identified in a Genome Wide Association Study (GWAS) as a CAD associated locus (Sazonova et al., 2015) In a sister study, they found that within the fibrous cap of late atherosclerotic lesions, cells closest to the luminal side expressed Tcf21 (Nurnberg et al., 2015). However, expression of Tcf21 in the fibrous cap was not determined to be detrimental or beneficial in this study. It was later shown that Tcf21 expression within this fibrous cap was protective against the disease progression of CAD (Nagao et al., 2020).

With regards to cardiac fibroblasts, Tcf21 is currently just understood as a marker of resting fibroblasts. Despite its persistent expression, little is known about its function. Elucidating Tcf21 target genes, whether activation or suppression, may help scientists in developing therapies. Tcf21 has the ability to determine the fate of cells during development and there may be potential to access this function after injury in the adult

heart. This may in turn help reduce the progression of cardiomyopathies towards heart failure if targets of Tcf21 are determined.

1.4 Hypothesis and Aims

While it is appreciated that Tcf21 is essential for cardiac fibroblast development, no transcriptional targets or cellular functions have been attributed to Tcf21 within fibroblasts. Therefore, I investigated the role of Tcf21 in perinatal cardiac fibroblasts. Understanding Tcf21 and its target genes will lead to a better understanding of how it regulates cardiac fibroblasts function. The hypothesis that Tcf21 is necessary for proper proliferation of cardiac fibroblasts and regulates cell cycle genes and fibroblast specific genes will be tested with the following aims:

Aim 1: Determine the cellular mechanism that leads to fewer fibroblast numbers in Tcf21 null perinatal cardiac fibroblasts

Deletion of Tcf21 at perinatal (P) day 1 results in fewer CF in the left ventricle (LV). Due to the current understanding, in fetal development, of the importance for Tcf21 in fibroblast differentiation, earlier days during development were taken into account to see when the loss of fibroblasts begins. Interestingly, at P4, no significant reduction is seen between controls and mutants while a 40% decrease begins at P5. This suggests the neonatal deletion of Tcf21 does not result in rapid loss of fibroblasts but shows a slow progression to this reduction

While a gradual reduction in the number of left ventricular CF is seen beginning at P5 there is still a potential for subjective quantifications. In order to remove any preference and investigate that the reduction is a result of Tcf21 loss, a Collagen 1a1 reporter mouse

will be used. The labeling of interstitial and adventitial cardiac fibroblasts, independent of Cre activity, escapes the limitations of the inducible model. No reduction of CF was seen after neonatal deletion of Tcf21 in the Collagen1a1 reporter mice at P4. This suggests the loss of Tcf21, does not affect fibroblast survival. Therefore, in this aim I will measure the colocalization and number of cardiac fibroblasts in this mouse models to determine if Tcf21 has an effect on total left ventricular fibroblast three days and up to one week after Tcf21 deletion.

Previous work has shown the deletion of Tcf21 results in trans-differentiation of CF into coronary vascular smooth muscle cells (Fang et al., 2016). Immunohistochemistry was performed in order to determine if epicardial derived cardiac fibroblasts during perinatal development undergo this change, resulting in the reduction of fibroblasts. αSMA was found only in vascular regions of the heart with no colocalization in the interstitium or adventitia of the heart. This suggests the cardiac fibroblasts do not undergo a trans-differentiation after Tcf21 deletion in early development.

CF undergo similar proliferation rates as cardiomyocytes (Ivey et al., 2018). The peak of proliferation occurs around P4 and begins to taper off at P7 where it subsides almost completely at P10. If CF are not differentiating into smooth muscle cells another mechanism is being deployed. Therefore, in this aim I will use immunohistochemistry to characterize the mechanism by which Tcf21 deletion mediates the observed reduction of cardiac fibroblasts.

To investigate the timeline for when Tc21 deletion results in reduction in the number of CF, I will utilize transgenic mouse lines. TCF21$^{mCrem/fl}$ mice will be crossed with Rosa26$^{tdT/tdT}$ mice to generate conditional knockouts with lineage tagging. Litter mate

controls will be used to compare to. All mice, controls and mutants, are induced with 0.3mg/g of tamoxifen at P1. Hearts will be sectioned and analyzed at P4, P5, and P7 using histology and immunohistochemistry.

Aim 2: Compare gene expression between control and Tcf21 null perinatal cardiac fibroblasts

It is difficult to qualitatively observe how Tcf21 acts to support CF due to their minute number compared to the total number of cells in the heart. For example, in order to see Tcf21 protein loss, in vitro enrichment is required in order to see a band on a Western blot. Additionally, using qPCR of whole heart lysate may show inaccurate CF gene expression, if the normalizing gene is highly expressed within the heart. A more targeted method to observe the genetic handling of Tcf21 is using RiboTag immunoprecipitation. This method allows ribosomes on Tcf21 expressing fibroblasts to be tagged with hemagglutinin. This tag can be pulled down via immunoprecipitation by antibodies conjugated to magnetic beads and the resultant mRNA extracted from the mixture. While this provides a small amount of RNA, it is specific to Tcf21 expressing cardiac fibroblasts and the gene regulation that occurs can be analyzed to elucidate potential binding targets. Therefore, in this aim I will use the results of the RiboTag immunoprecipitation and analyze the gene expression profile via microarray. To identify genes that are being regulated by Tcf21 binding, the RiboTag system will be used. The mutant mice used in this experiment are *TCF21*$^{mCrem/fl}$;*Rosa26*$^{tdT/tdT}$;*Rpl22*$^{HA/+}$.

Chapter 2: Tcf21 is required for perinatal cardiac fibroblast proliferation and regulation of lipid metabolism genes

2.1 Introduction

Cardiac fibroblasts make up 12% of the non-myocyte cells in the fully developed heart (Pinto et al., 2016) Fibroblasts are spindle shaped, mesenchymal cells that have been routinely characterized by their heterogeneous characteristics, activation markers, and importance during cardiac fetal development as well as after cardiac injury (Baudino et al., 2006)(Souders et al., 2009)(Camelliti et al., 2005). In the heart, studies show that cardiac fibroblasts synthesize and secrete collagen to provide mechanical strength by the formation of the extra cellular matrix in the cardiac interstitium (Kanekar et al., 1998)(Carver et al., 1991). During development, they originate from the proepicardial organ and migrate into the heart. The migration of these epicardial derived cardiac fibroblasts into the myocardium initiates the production of the extra cellular matrix and proper formation of the heart via cell-cell interactions (Souders et al., 2009). Collagen I, III, and VI are the major interstitial collagens (Conrad, 1980)(Camelliti et al., 2005)(Fan et al., 2012). While we have some appreciation for the function of fibroblasts after adult injury, less is understood about the factors that direct cardiac expansion and matrix production in the developing heart.

Tcf21 is a basic helix loop helix (bHLH) transcription factor that determines cardiac fibroblast fate and promotes their differentiation and migration during development (A. Acharya et al., 2012). It is necessary for not only the heart development but lung, kidney, and testes (Tandon et al., 2013). In the heart mice that are null for Tcf21 do not form epicardial derived fibroblasts (A. Acharya et al., 2012). Embryos null for Tcf21 do not develop cardiac fibroblasts, demonstrating an important role for Tcf21 in fibroblast

development, but Tcf21 continues to be expressed in cardiac fibroblasts after this early embryonic phase. Therefore, we hypothesize that Tcf21 continues to play an important role in cardiac fibroblast functions in perinatal development.

With the use of conditional knock out mouse models, we show Tcf21 is necessary for cardiac fibroblast proliferation in the maturing heart. The distinction thatTcf21 expression in differentiating cardiac fibroblasts during embryonic development led others into testing if its absence may result in an increase of smooth muscle cells (Fang et al., 2016). Our Tcf21 deficient hearts were shown to not undergo transdifferentitation as was previously thought, evident by no increase in smooth muscle cells marker.

We also demonstrate, that Tcf21 may suppress lipid metabolism genes within the heart either directly or indirectly. Using microarray to analyze cardiac fibroblast specific gene dysregulation, the most upregulated genes in Tcf21 deficient hearts compared to controls were Uncoupling protein-1 (Ucp-1), Adiponectin (Adipoq), and Perilipin 1 (Plin-1) suggesting Tcf21 plays a role in lipid metabolism in the heart. This paper aims to define the role Tcf21 plays in perinatal development of cardiac fibroblasts.

2.2 Materials and Methods

2.2.1 Mice

Tcf21$^{mCrem/+}$ (A. Acharya et al., 2012), *Collagen1a1-GFP, PDGFR*$\alpha^{GFP/+}$ (Jackson Labs, 007669) and *R26R*^{tdT} *(*Jackson Labs, 007914) , *Rpl22*$^{tm1.1Psam}$ (ribosomal protein L22; Jackson,no. 011029) (Sanz et al., 2009), furthermore to be called *Rpl22*^{HA} (Jackson Labs, 011029) were maintained on a mixed C57Bl/6 background. Tamoxifen induction of *Rpl22*$^{HA/+}$*; Tcf21*$^{mCrem/+}$ enables the isolation of ribosomal RNA specifically from Tcf21 lineage cells. All procedures were approved by the University of Hawaii Institutional

Animal Care and Use Committees and were conducted in accordance with the NIH guidelines for care and use of laboratory animals. *Tcf21$^{mCrem/+}$; R26$^{tdT/tdT}$* mice were backcrossed a minimum of four generations to C57BL/6 and contained the J mutation of the NNT gene. Perinatal pups of both sexes were analyzed.

2.2.2 Tamoxifen induction

Tamoxifen (MP Biomedicals; 0215673891, Adipogen; 10540-29-1, 20 mg/mL stock solution) was dissolved in sunflower seed oil containing 10% ethanol. Tamoxifen was diluted in sunflower seed oil at concentration of 5mg/mL and administered to the mice at postnatal day (P)1 at a dose of 0.3 mg/g body weight by a single intragastric injection.

2.2.3 Histology and Immunostaining

Hearts were prepared for frozen embedding by fixing in 4% paraformaldehyde (PFA) overnight at 4°C. They were then placed in 30% sucrose at RT for 1 and a half hours. Next, 1:1 30% sucrose and Tissue-Tek® O.C.T™ medium was added for 1 hour and a half at RT. Finally, hearts were left in Tissue-Tek® O.C.T™ in plastic molds for two hours before being placed in the -80°C freezer. Sections were made at 10μm each and left to adhere to the slides for 30 minutes at least. To look at lineage labeled cells, slides had 4',6-diamidino-2-phenylindole (DAPI, Roche, 10-236-276-001) added to them at 1:50 dilution in DPBS and were mounted. Slides processed for antibody staining were permeabilized with 0.1% Triton-X100 (Sigma-Aldrich, T8532-50ml)/DPBS, blocked for 1 hour with 1% bovine serum albumin (BSA), 1.5% serum (normal goat or normal donkey serum) in DPBS, incubated with primary antibody overnight at 4°C; anti-αSMA-FITC (Thermo Fischer, 53-9760-82). Secondary antibody used according to primary antibody.

Images were taken at 10X magnification spanning the entire left ventricle of three non-sequential sections. Total raw counts of Tdtomato and Collagen GFP cells were divided by the percent area and added together to give whole ventricle numbers of cells. Percent area of the tissue was measured converting image with the 8-bit tool, adjusting threshold and analyzing particles.

2.2.4 Lipidtox staining

Perinatal day 5 heart sections were permeabilized for 20 minutes with 0.1% Triton-X100 (Sigma-Aldrich, T8532-50ml)/DPBS. They were washed twice with deionized water and incubated for one hour with LipidTOX Green Neutral Lipid Stain (Thermo Fischer, H34475). Sliders were washed again with DPBS, stained with DAPI, and mounted. 40x images were taken and quantification was done using ImageJ. Lipid droplets were counted by converting the image using the 16-bit tool, adjusting threshold, and analyzing particles. The droplets were normalized to percent area of five images of the left ventricle.

2.2.5 Edu labeling and staining

EdU (Lumiprobe, 50-114-3043) was dissolved to 5mg/ml in DPBS and administered via intraperitoneal injection at 0.3mg/ml one hour before isolation. Hearts were prepared as stated above. EdU Click-iT® Plus reaction cocktail (1X Click-iT® reaction buffer, Copper protectant, Alexa Fluor® picolyl azide 488, Reaction buffer additive) was added onto sections for 30 minutes followed by addition of 4',6-diamidino-2-phenylindole (DAPI, Roche, 10-236-276-001) before being mounted with 66% glycerol.

2.2.6 Immunoprecipitation of Polyribosomes

Immunoprecipitation and purification of polysome-bound mRNAs was performed from snap frozen hearts isolated from *Tcf21*$^{mCrem/+}$*; Rpl22*$^{HA/+}$ mice. Heart tissue was

homogenized in (10% wt/vol) of ice-cold polysome buffer (50 mM Tris-HCl pH 7.5, 100mM KCl, 12 mM $MgCl_2$, 1 mM DTT, 1% IPEGAL, 200 U/ml RNasin® Plus RNase Inhibitor (Promega, PAN2615), 1 mg/ml heparin, and 0.1 mg/ml cycloheximide) and homogenates were centrifuged at 10,000 g for 10 min to create a post mitochondrial supernatant. 1% of supernatant was reserved as input before immunoprecipitation. The remaining supernatant immunopurified using anti-HA-tag mAb (MBL, M180-11) at 4 °C for 4 hrs. Beads were washed with high salt buffer (50 mM Tris-HCl pH 7.5, 300 mM KCl, 12 mM $MgCl_2$, 1 mM DTT, 1% IPEGAL) and RNA extracted using Quick-RNA™ MicroPrep kit (Zymo Research, R1050) according to manufacturer's instructions.

2.2.7 qPCR

Heart tissues were homogenized and lysed, respectively in IBI Isolate (IBI Scientific, IB47600). Total RNA was prepared according to IBI Scientific RNA extraction protocol. RNA quality and concentration were determined by NanoDrop ND-1000 (Thermo Fisher Scientific). For first strand cDNA synthesis from total heart RNA, M-MLV Reverse Transcriptase and buffer (Sigma, M1302-40KU) and random hexamer (Thermo Fisher Scientific, S0142) were used. RNA from input and IP samples was reverse transcribed using qScript™ cDNA Synthesis Kits (QuantaBio, 101414-098). RT-qPCR reactions were performed PowerUp™SYBR™ Master Mix (Applied Biosystems™, A25776) on the LightCycler® 480 instrument (Roche). Genes were normalized to Gapdh expression. Primer sequences available upon request.

2.2.8 Western Blot

Protein lysates were prepared from flash frozen hearts. They were homogenized using a 2mL dounce and 500μl of ice cold RIPA buffer (10% SDS, 1M Tris, pH 7.6, 20%

NP-40, 5M NaCl, 10% sodium deoxycholate, milliQ water). The homogenates were spun at 12,000 rpm for 20 minutes at 4°C. The supernatant was removed and the concentration was tested using the BCA kit (label). 50μg of lysate was loaded into a 1.5mm, 15% SDS-PAGE gel. The gel was run for 2 hours at 100V, transferred onto a nitrocellulose membrane (for Tcf21) or PVDF membrane for 30 minutes at 100V, 250mA. Nitrocellulose membrane was left to fully dry for 2 hours. The membrane was then blocked for 1 hour on a rocker at RT and incubated in primary antibody overnight on a rocker at 4°C; αSMA (Sigma A2547). Corresponding Li-Cor IRDye secondary antibody was incubated for 1 hour on rocker at RT. An Odyssey CLx imaging system was used for detection and images were analyzed using Image Studio 5.2.5 software (Li-Cor Biosciences, Lincoln, NE).

2.2.9 Microarray

mRNA was prepared as stated under Immunoprecipitation of Polyribosomes. RNA concentration was analyzed using a bioanalyzer in the University of Hawaii Cancer Center. Microarray analysis was done using the Clariom S Pico Mouse Chip protocol (Thermofischer, 902932).

2.2.10 Statistical Analysis

All statistical calculations were performed using Prism 8 (Graph Pad). Data was analyzed using two-sided t-tests or one-way ANOVA as noted in the figure legends. Statistical variability is expressed as mean ± SD.

2.3 Results

2.3.1 Neonatal Deletion of Tcf21 in Cardiac Fibroblasts

Cardiac fibroblasts aid in the proper development of the heart by laying down and maintaining the ECM. The epithelial mesenchymal transition and migration of cardiac fibroblasts into the interstitium of the heart has been shown to be dependent on Tcf21, a transcription factor (Maezawa et al., 2012)(Asha Acharya et al., 2011). Given the importance of Tcf21 in the embryonic migration and differentiation of cardiac fibroblasts, we wanted to investigate if Tcf21 has a similar role in cardiac fibroblasts during the perinatal development stage. However, Tcf21 null mice die at birth due to pulmonary hypoplasia (A. Acharya et al., 2012). To examine the role of Tcf21 in cardiac fibroblasts after birth, we conditionally deleted Tcf21 using a tamoxifen inducible *Tcf21*^{mCrem} mouse line and one floxed allele of Tcf21. We refer to these mice as Tcf21 FKO. We also used a tdTomato Cre reporter to follow Cre recombinase activity. During perinatal development, it has been shown that cardiac fibroblasts reach their peak proliferation at P4 (Ivey et al., 2018) Therefore, we initially decided to delete Tcf21 at perinatal day (P)1 by tamoxifen administration and examine the hearts at P4 (Figure 1.1. A). However, we observed similar levels of fibroblasts at this time point. Next we looked at P5 (Figure 1.1. B) and at P7 (Figure 1.1. C) to see if Tcf21 loss caused an abrupt loss of cardiac fibroblasts or a gradual reduction. We observed a 40% decrease in the number of cardiac fibroblasts in the left ventricle at P5 (Figure 1.1. E) and a 30% reduction at P7 (Figure 1.1. F). These results indicate neonatal deletion of Tcf21 results in a gradual reduction in the number of left ventricular cardiac fibroblasts.

To verify the efficiency of Tcf21 deletion using our model, we used RT-PCR to measure mRNA transcript levels of Tcf21. P4 hearts induced at P1 showed a range of

deletion from 50% to 90%. P7 hearts induced at P1 showed a range of deletion from 30% to 50%. We found another way to determine the efficiency of deletion by examining epicardial labeling of the heart. Using this method, we were able to check labeling in hearts more rapidly. Because we observed a range of Tcf21 deletion, we chose to only examine hearts that showed 50% induction or higher.

To determine fibroblast loss independent of Cre activity, we used a collagen1a1-GFP reporter mouse (Yata, 2003)(Carver et al., 1991) which expresses GFP under the control of the *Col1a1* promoter. After P1 deletion and observations at P4 (Figure 1.2. A-B) P7, no reduction was seen in collagen expressing cardiac fibroblasts (Figure 1.2. C-D). These data demonstrate that Tcf21 does not affect fibroblast survival, but allows us to study fibroblasts that lack Tcf21.

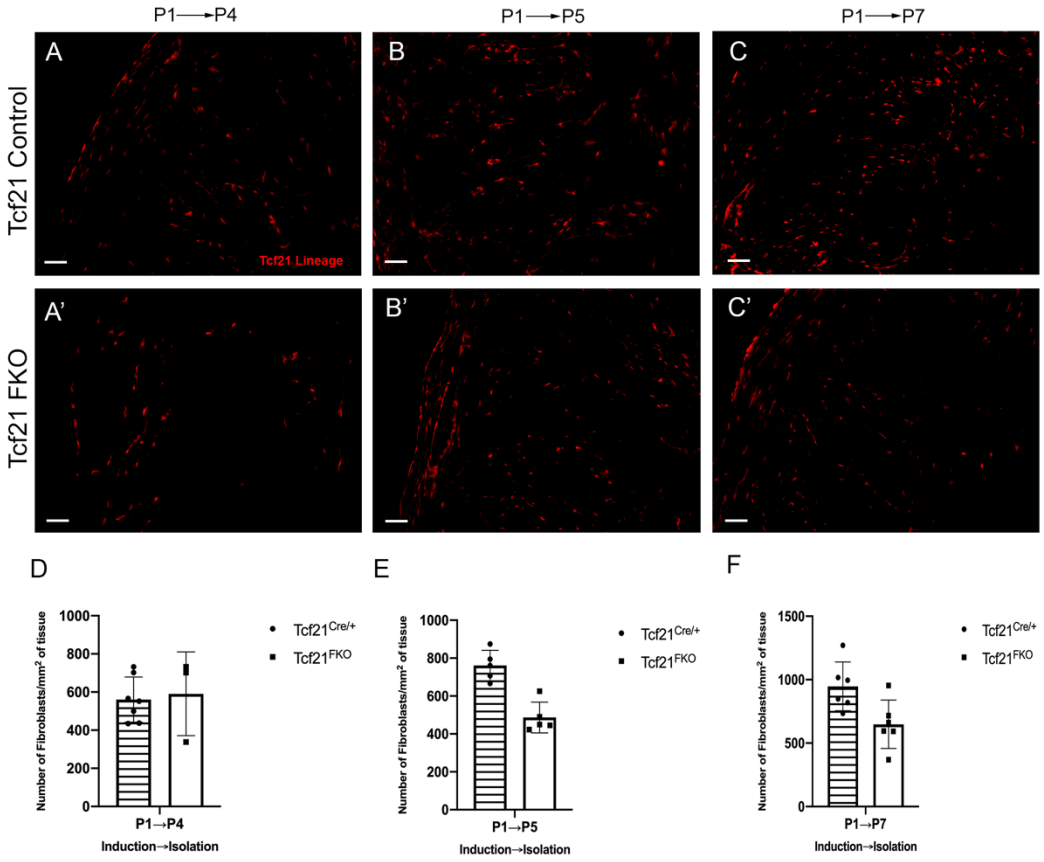

Figure 1. 1 Neonatal deletion of Tcf21 in cardiac fibroblasts

A-C) Representative images of Tcf21 lineage tagged cardiac fibroblasts in *Tcf21*$^{mCrem/+}$*; R26R*$^{tdT/tdT}$ (control) and *Tcf21*$^{mCrem/fl}$*; R26R*$^{tdT/tdT}$ (Tcf21 FKO) hearts at corresponding time points. (**D-F**) Quantifications of Tcf21 lineage tagged cardiac fibroblasts. Dots within bar graphs represent biological replicates. All images are in the left ventricle (LV) of the heart. Results are mean ± SD. Scale bars: 100μm

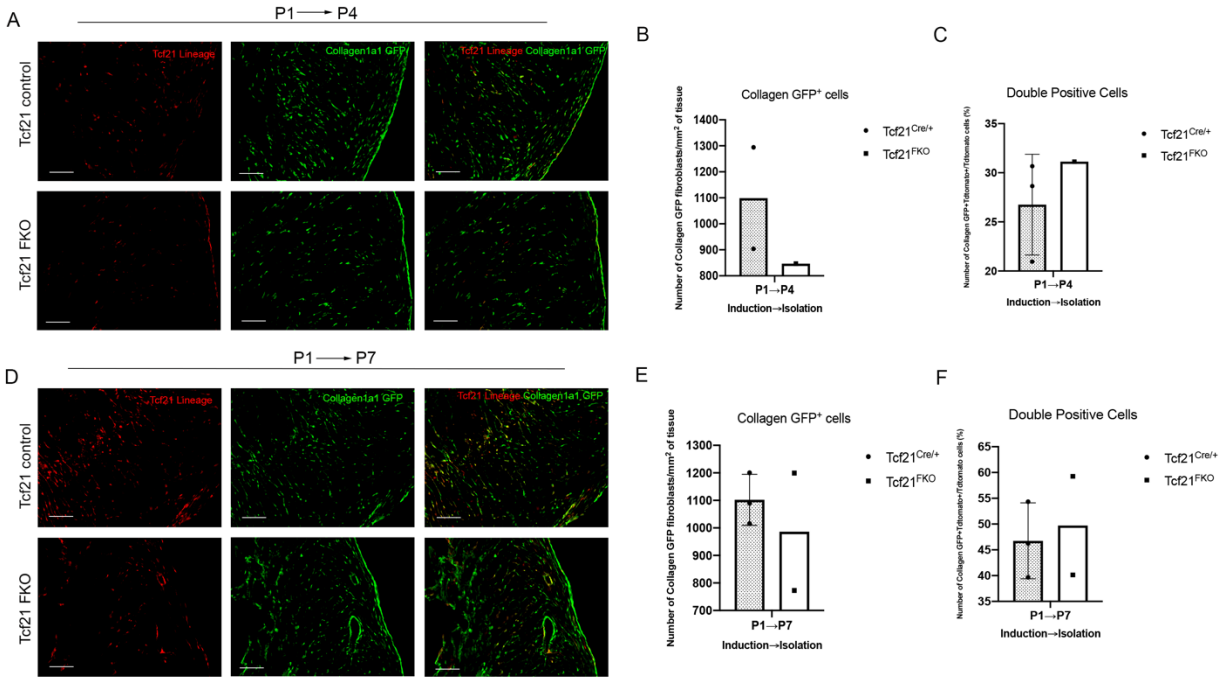

Figure 1. 2 Collagen1a1 reporter labeled cardiac fibroblasts

(**A,D**) Representative images of Tcf21 lineage and Collagen1a1-GFP cells at specified time points. (**B,E**) Quantification of Collagen1a1-GFP^{+}. (**C,F**) Quantifications of Tcf21 lineage^{+} Collagen1a1-GFP^{+}. Dots within bar graphs represent biological replicates. All images are in the left ventricle (LV) of the heart. Results are mean ± SD. Scale bars: 100μm

2.3.2 Smooth Muscle Cell Gene Expression in Tcf21 deleted fibroblasts

In the embryonic heart, Tcf21 expressing cells have the ability to differentiate into fibroblasts or vascular smooth muscle cells. After E12.5, Tcf21 is a factor in determining epicardial cell fate and is solely expressed by fibroblasts(Fang et al., 2016). Therefore, we were curious if loss of Tcf21 would result in an increase of smooth muscle cell markers on cardiac fibroblasts during perinatal development. Staining with alpha smooth muscle actin (αSMA) at P5 and P7 showed that Tcf21 expressing cardiac fibroblasts in the myocardium do not express smooth muscle cell markers after P1 deletion (Figure 1.3. A).

In both controls and Tcf21 deficient hearts, αSMA staining was visible around vessels with little to no colocalization with the Tcf21 lineage of cells and minimal expression in the interstitium (Figure 1.3. A-B). Moreover, total protein levels of αSMA were similar between control and Tcf21 FKO hearts (Figure 1.3. C).

Because we observed that loss of Tcf21 does not result in the expression of smooth muscle cell protein in fibroblasts, we wanted further investigate if loss of Tcf21 resulted in increase in smooth muscle cell transcript levels. To examine transcript levels specifically in fibroblasts, we used a system to immunoprecipitate out hemagglutinin tagged ribosomes in Tcf21 specific cardiac fibroblasts referred to as Ribotag. RT-PCR in P5 Ribotag immunoprecipitated hearts revealed that transcript levels of αSMA were decreased in Tcf21 deficient cardiac fibroblasts (Figure 1.3. D). These results suggest that Tcf21 deletion does not result in smooth muscle cell markers being upregulated within cardiac fibroblasts.

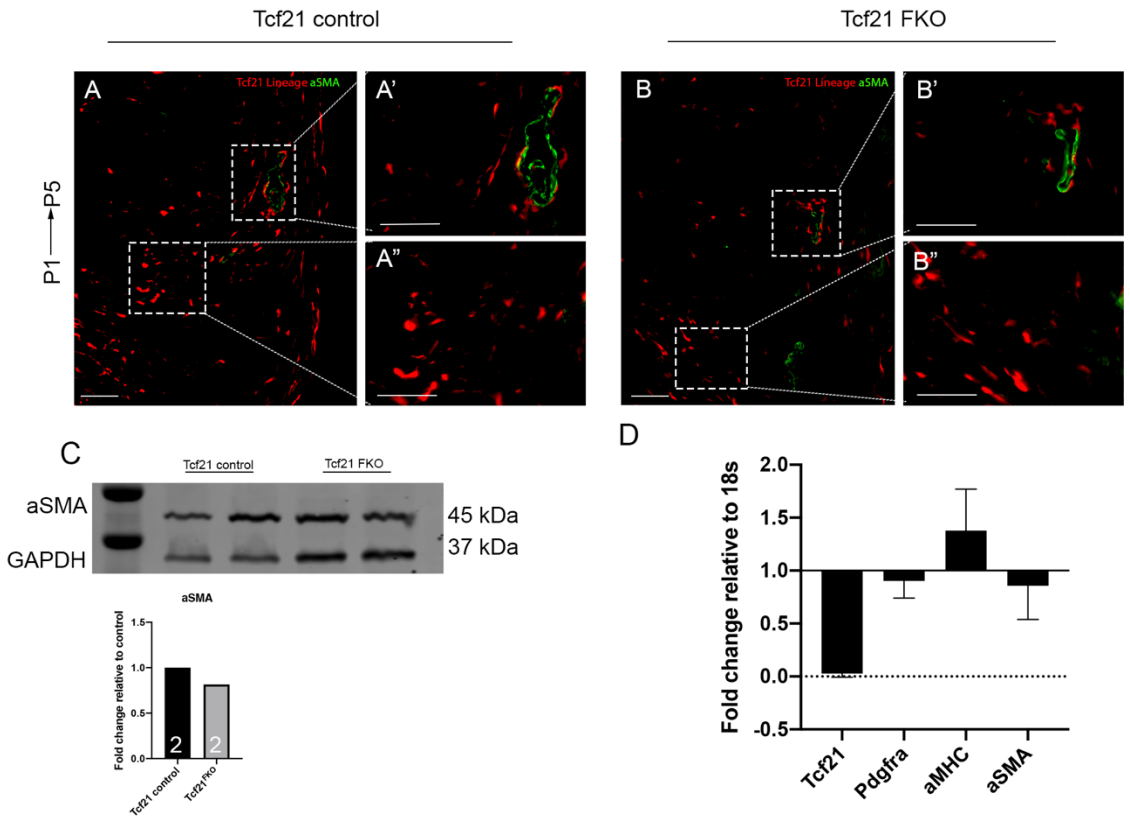

Figure 1. 3 Smooth muscle cell gene expression in Tcf21 deleted fibroblasts

(**A-B**) Representative images of Tcf21 lineage and αSMA staining in the LV of P5 hearts. (**A'-B'**) Cardiac vessel inset and (**A"-B"**) Myocardium inset. (**C**) P5 Whole heart protein lysate was analyzed by Western blot. GAPDH was used as a loading control. (**D**) P5 Ribotag RNA from control and Tcf21 FKO heart analyzed by RT-PCR for selected fibroblast (Tcf21, Pdgfrα), cardiomyocyte (αMHC), and smooth muscle (αSMA) genes n=3. Numbers within bar graphs represent biological replicate n values. Scale bars: (A) 100μm, (A',A" inset) 20μm

2.3.3 Tcf21 Deletion Shows a Reduction in DNA synthesis

During perinatal development, our lab has shown that cardiac fibroblasts reach their peak proliferation around P4 and begin to taper to baseline levels by P10 (Ivey et al., 2018). Because we observed a reduction in Tcf21 lineage cells at P5 within this perinatal

proliferative window, we investigated whether Tcf21 plays a role in the proliferative ability of cardiac fibroblasts at P5. To examine fibroblasts undergoing DNA synthesis, we injected pups with 5-Ethynyl-2´-deoxyuridine (EdU) and isolated the hearts after one hour. We found that P5 control hearts had around a 6% proliferation rate for cardiac fibroblasts (Figure 1.4. A-B). Interestingly, in Tcf21 deficient hearts, the proliferation rate dropped to around 2.5% (Figure 1.4. B). This data suggest Tcf21 play a role in cardiac fibroblast proliferation during perinatal development.

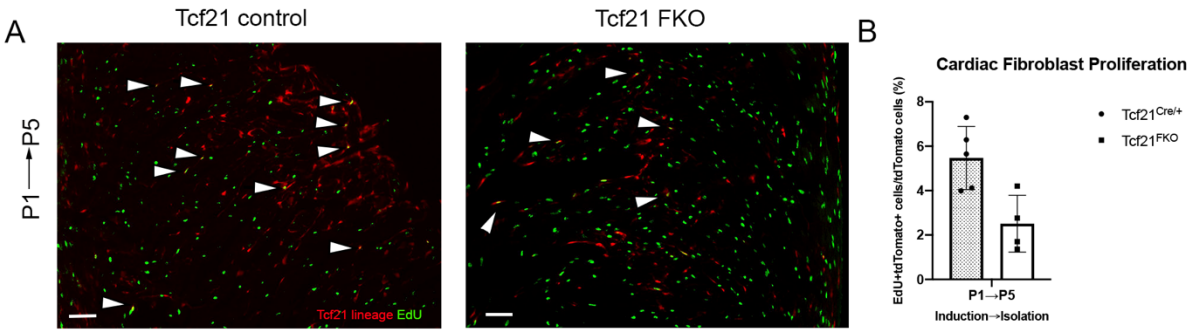

Figure 1. 4 Tcf21 deletion reduced DNA synthesis in cardiac fibroblasts

(**A**) Representative images of EdU in the LV of P5 hearts. White arrowheads indicate EdU⁺ Tcf21 lineage cells. (**B**) Quantification of EdU⁺ Tcf21 lineage cells. Dots within bar graph represent biological replicates. Results are mean ± SD. Scale bars: 100μm

2.3.4 Tcf21 Deletion Results in Upregulation of Lipid Metabolism Genes

In the fully developed heart, cardiac fibroblasts make up around 15% of the non-myocyte cells (Pinto et al., 2016). This means that the ratio of cardiac fibroblast genes and proteins relative to cardiomyocyte or endothelial cell genes and proteins is low. Generally, studying the genetic effects that a transcription factor has on cells relies on RNA or protein extraction of total heart. But, with less than half the cells being cardiac fibroblasts, using

total RNA may result in unclear results when examining fibroblast genes. A way of circumventing this issue is using tissue specific ribosome labeling and immunoprecipitation. Using a hemagglutinin (HA) tagged Rpl22 (Sanz et al., 2009), we can mark ribosomes within our Tcf21 specific cardiac fibroblast population, use magnetic bead pull down to extract out RNA bound to these labeled ribosomes, and isolate the RNA for analysis. This method is a targeted approach to screening for gene regulation within cardiac fibroblasts.

Ribotag isolation and immunoprecipitation was performed on P5 hearts. RNA was screened by Microarray (Figure 1.5. A). Interestingly, we found the most upregulated genes were Uncoupling Protein-1 (Ucp1) followed by Adiponectin (Adipoq) and Perilipin-1 (Plin1) in the Tcf21 FKO hearts relative to controls (Figure 1.5. B). Microarray results were validated by RT-PCR (Figure 1.5. C). These data were particularly striking because lipid metabolism has not been extensively investigated in the perinatal heart especially in relation to cardiac fibroblasts. One study showed that lipid metabolism genes were upregulated in hearts undergoing heart failure (AbdAlla et al., 2011). However, our data suggests that lipid metabolism is regulated partially by fibroblast in the perinatal heart. Whole heart RNA extraction was performed on P5 hearts without Rpl22 labeling to demonstrate the ambiguity of analyzing total RNA which may lead investigators toward inaccurate results when studying cardiac fibroblast gene expression (Figure 1.5. D).

To further investigate lipid metabolism in Tcf21 deficient hearts, P5 hearts were stained with LipidTox which labels neutral lipids. Tcf21 deficient hearts show numerous lipid droplets while controls have none (Figure 1.5. E). However, the lipid droplets do not seem

to be specific to cardiac fibroblasts. These results may imply Tcf21 has some ability to regulate lipid droplet formation in the heart.

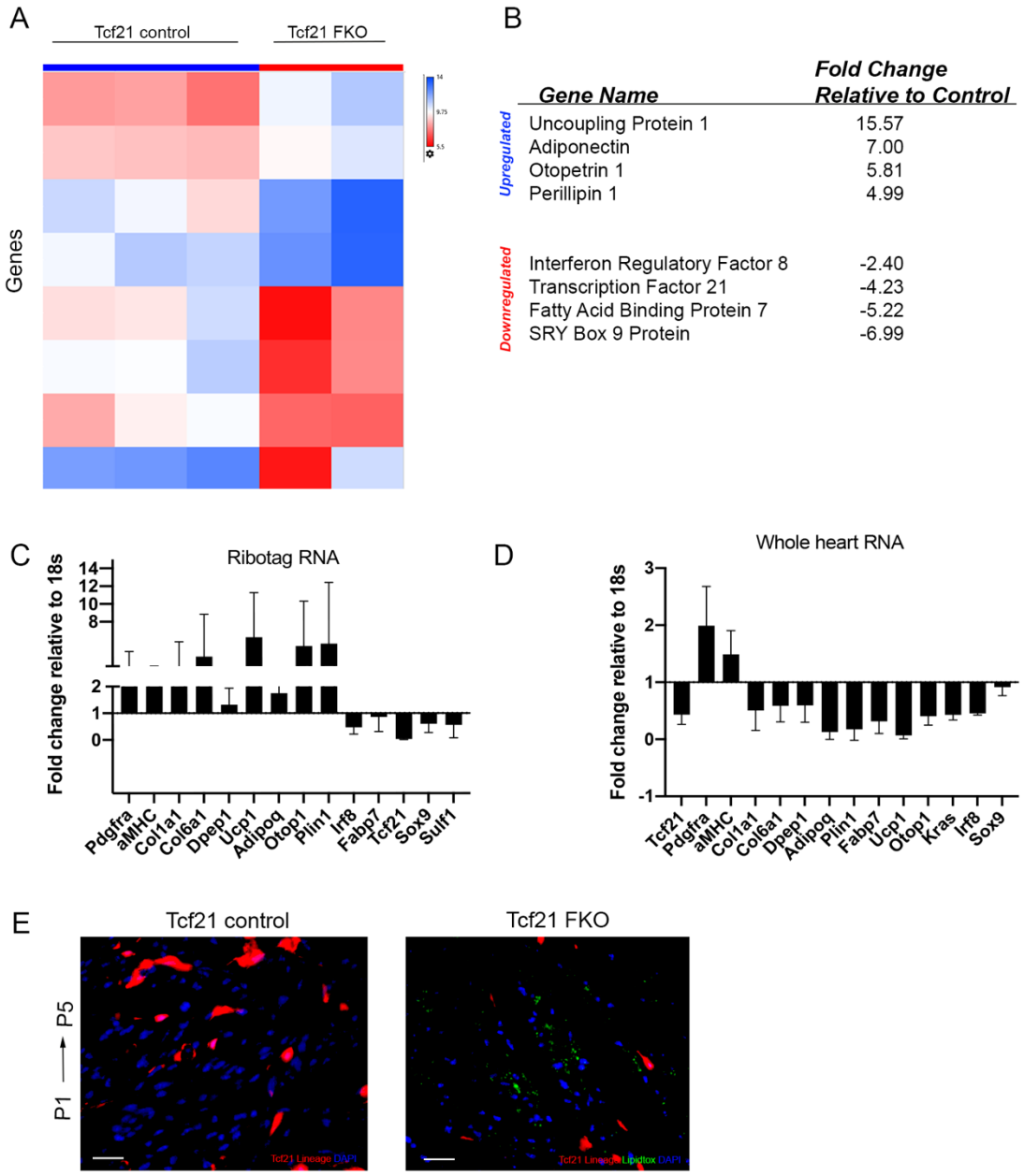

Figure 1. 5 Tcf21 deletion results in upregulation of lipid metabolism genes

(**A**) Heat map of differentially expressed genes from microarray at P5 from control and Tcf21 FKO hearts. Scale is log 2 and indicates signal. (**B**) The 4 most upregulated and

downregulated determined by fold change (> or < 2.0) and p-value (≤0.05). (**C**) P5 Ribotag RNA from control and Tcf21 FKO heart analyzed by RT-PCR to verify transcript levels of genes identified in microarray. n=3 per genotype. αMHC was used as a cardiomyocyte contamination check for Ribotag RNA. (**D**) P5 Whole heart RNA from control and Tcf21 FKO heart analyzed by RT-PCR for fibroblast genes and differentially expressed genes identified in microarray. n=3 per genotype (**E**) Representative images of lipid droplets in P5 hearts. Scale bars: 20μm

2.4 Discussion

2.4.1 Cardiac fibroblast proliferation

Although Tcf21's role in cardiac fibroblast differentiation has been extensively studied during embryonic development, there still remains a lack of knowledge in perinatal development. Our Tcf21 conditional knockout shows that neonatal deletion of Tcf21 results in a gradual reduction of cardiac fibroblasts within the left ventricle. The possibilities for this reduction may be the result of reduced or in proper cell proliferation, increased apoptosis, or transdifferentiation into another cell type. With previous research showing loss of Tcf21 in the embryonic hearts results in no epithelial to mesenchymal migration of cardiac fibroblasts we sought to find out if a similar trend occurs later in development. The 3% reduction in cardiac fibroblast proliferation at P5 implies Tcf21 may play a role in cell cycle regulation in these cells.

There has been some speculation into the downregulation of Tcf21 resulting in a more smooth muscle cell like phenotype (Fang et al., 2016). While this may suggest no increase in coronary smooth muscle cells, more experiments need to be done looking into other markers. Due to a study which found that coronary artery smooth muscle cells upregulated Tcf21, within the fibrous cap, as a protective means of phenotypic modulation during coronary artery disease progression (Nagao et al., 2020) we question Tcf21's role in these two seemingly different cells types. Thus, these data show Tcf21 is still being

expressed in cardiac fibroblasts postnatally and although it does not affect fibroblast survival it is necessary for epicardially derived fibroblast proliferation.

2.4.2 Lipid metabolism in the heart

Although some groups have proposed that Tcf21 is involved in adipose tissue differentiation, no research has shown how Tcf21 may play a role in cardiac lipid metabolism. Our lab has shown that Tcf21 is more restricted in the lung fibroblast population that harbors lipid vesicles (Park et al., 2019). Given the results of the microarray indicating the most upregulated gene in Tcf21 deficient hearts compared to controls being Ucp-1 we began looking more into the possibility of lipid metabolism regulation occurring. Ucp-1 is an uncoupling protein said to only be found in the mitochondria of brown adipose tissue (Fedorenko et al., 2012). Uncoupling proteins inhibit the synthesis of ATP by impeding the coupling of protons with ATP synthetase and turning the energy into heat(Nicholls & Locke, 1984) (Terada, 1990). This protein has a unique ability to allow non shivering thermogenesis. It is said that high expression levels of Ucp-1 are correlated with metabolic inefficiency. One particular example of this is during perinatal development as the heart is switching from glucose to fatty acid oxidation as the main source of energy.

Lipid metabolism involves the synthesis and breakdown of lipids within the cell. The heart regularly obtains lipids through circulation of free fatty acids or by fatty acids bound to lipoproteins (Goldberg et al., 2012). These fatty acids are then used as the main source of energy for the adult heart. Changes to the heart in the form of some cardiac dysfunction can cause a switch from fatty acid oxidation to glycolysis resulting in the

buildup of lipids within the heart typically seen in heart failure (AbdAlla et al., 2011). Currently, there is no research that has shown a connection between cardiac fibroblasts and lipid metabolism in the perinatal heart. At most, it is understood that the perinatal heart is transitioning to fatty acid oxidation as more oxygen becomes more readily available. Loss of Tcf21 showed an accumulation of lipid droplets within the myocardium. Although there was no colocalization with cardiac fibroblasts and lipid droplets this preliminary data may suggest a role for cardiac fibroblasts as lipid vesicles that is regulated by Tcf21. Potentially these fibroblasts may act in a similar manner to autophagosomes in that they clear out free lipids in the heart. While this is speculation more experiments will be run to examine this further.

Chapter 3: Conclusion

3.1 Final Remarks

This work suggests a role for Tcf21 in cardiac fibroblast perinatal development. While Tcf21 has been shown in multiple organs as an important transcriptional regulator during organogenesis it has not be studied extensively during this time period. We have demonstrated a role for Tcf21 in left ventricle cardiac fibroblasts proliferation and showed that although it determines mesenchymal cell fate embryonically, cardiac fibroblasts do not transdifferentiate later in development with the loss of Tcf21.

Our work has begun to elucidate more target genes for Tcf21 in the perinatal heart. Tcf21 is solely expressed in cardiac fibroblasts after their EMT into the interstitial space in the myocardium. We show that Tcf21 may be controlling, whether indirectly or indirectly

lipid metabolism genes as well as known fibroblast genes. One limitation to our work is the lack of apoptosis experiments. This mechanism needs to be examined to see if Tcf21 deletion, resulting in the reduction of fibroblasts, is more drastically due to an increase in apoptosis. Overall, we have begun filling in the gap of knowledge involving Tcf21 during perinatal cardiac development.

3.2 Future Directions

A reduction in cardiac fibroblasts after Tcf21 deletion in perinatal development may have implications in heart injury studies. One way to make this more translatable would include using human cell lines. Utilizing CRISPR Cas9 or adenovirus knockout systems to see if similar results occur. While in vivo studies allow us to investigate the role of cells in their environment, in vitro human cell studies would be the first step in translational medicine with this project.

After myocardial infarction (MI) the activation and proliferation of fibroblasts into the area of cardiomyocyte cell death results in extensive scarring. If the scar could be reduced in size to allow more contractility to occur that would be advantageous in potentially reducing the progression to heart failure. While this may seem far reaching, one future experiment that we would like to try is deleting Tcf21 before MI and measuring the ejection fraction of the heart. This would be coupled with examining the hearts by histology either by immunohistochemistry or trichrome to see the extent of scar formation and fibroblast deposition.

The ultimate goal when studying transcription factors is to find direct binding targets. ChIP seq is a logical next experiment that will be executed in the future. The

experiments in this thesis will help narrow in on key genes if they arise in the results of the ChIP seq data. Overall, Tcf21 is a diverse transcription factor that deserves to be studied in order to have an appreciation of cardiac fibroblast development and the hope of developing therapies that can lessen the risk of heart failure in the future.

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