Cardiac Cell-to-Cell Communication Using Exosomes and Cytokines Regulated By Spectrin-Based Pathways

Thesis

Presented in Partial Fulfillment of the Requirements for the Honors Distinction in Research in the field of Biomedical Engineering in the Undergraduate School of The Ohio State University

By

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The Ohio State University

2021

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Abstract

Increased levels of cardiac fibrosis are characteristic remodeling responses to stress-induced conditions in cardiovascular diseases associated with cardiac dysfunction and arrhythmias. The stress-induced transition of resident/quiescent cardiac fibroblasts (CF) to an activated myofibroblast state displays increased proliferation, contractility, and deposition of extracellular matrix, all crucial steps in the fibrotic pathway. While the precise mechanism associated with this process remains unclear, our group has identified a novel βIV-spectrin/STAT3 complex in the heart crucial for the regulation of normal gene transcription and maintenance of the quiescent CF phenotype. Further, the complex plays a key role in regulating the cardiac-cell response to acute and chronic stress, however the upstream extracellular stress signals that lead to stress-induced βIV-spectrin dysfunction and successive CF activation remain unknown.

The following study explores the upstream extracellular stress signals associated with CF activation, using βIV-spectrin deficient mice. Specifically, primary mouse CFs from WT (control) and βIV-spectrin deficient (qv^{4J}) species were isolated and cultured. Extracellular stress signals (i.e. exosomes and cytokines/chemokines) were examined. Finally, in-vitro assays (e.g. proliferation and collagen gel contractility) were performed to evaluate CF activity in response to the extracellular stress signals. We reported that WT CFs treated with qv^{4J} conditioned media displayed higher rates of proliferation and contraction than CFs treated with control media. We also identified several cytokine/chemokine candidates that are involved in the CF activation pathway. The knowledge gained from this study will provide new insight into understanding the overall fibroblast biology, mechanisms of cardiac cell cross-talk, and for developing therapeutic strategies to target cardiac fibrosis.

Acknowledgements

This work was supported by National Institutes of Health (NIH), American Heart Association (AHA), and Ohio State University Office of Undergraduate Research. The facilities and resources used were supplied by the Hund Lab for Excitable Cell Engineering, the Ohio State Wexner Medical Center, and The Ohio State Department of Biomedical Engineering.

I would be extremely remiss not to acknowledge the people who have supported me in the process of this thesis, in doing it all during a pandemic, and throughout my experience at Ohio State. I am incredibly grateful for the advisement from Dr. Hund, Dr. Childers, Dr. Nehal Patel, and Dr. Drew Nassal, who gave me the opportunity pursue my own research, encouraged me and celebrated my research achievements, inspired me to pursue research as a career, and overall showed me how to be a better scientist. To add, each member of the Hund Lab has been there for me as well, whether they gave me grad school advice, mentored me, allowed me to mentor them, or were there to bring life and conversation to a boring experiment. The experience wouldn't have been bearable without you.

To my friends, who I am so thankful for putting up with me through all of this. You're my companions and my inspirations; whether it's bonding with you in and out of class, pulling late nights to finish homework assignments, or looking up to you as role models, you've helped me stay grounded through this thesis, made my life infinitely more exciting, and inspire me daily to strive in both academics and my personal life.

And finally, to my family, who has been there with me every step of the way. You exposed me to STEM and inspired me to be a scientist from before I can remember—even if I didn't realize it at the time. You're the support that I can always turn to, even if I too often take for granted. You drive me to continue to be a better person, and to be satisfied with where I am.

Table of Contents

List of Figures

Introduction

Heart failure (HF) is a leading cause of death in the United States.[1] Between 2013 and 2016, 6.2 million people older than 18 years are affected in the country alone[1], and half of the victims of the disease die within five years of diagnosis.[2] HF is a broad, complex, multifactorial syndrome characterized by maladaptive structural remodeling and compromised heart function. Cardiac fibrosis is a common finding in HF, among a variety of other cardiac disease states, and is heavily associated with maladaptive modeling, including the development of mechanical and electrical cardiac dysfunction.[3] Cardiac fibroblasts (CFs) are essential for maintaining homeostasis of the extracellular matrix (ECM), but are a major contributor to cardiac fibrosis during heart failure. Specifically, upon activation (in response to injury or stress), quiescent CFs assume an activate phenotype (by transdifferentiating into myofibroblasts) characterized by enhanced proliferation, contractility and excess deposition of collagen and ECM proteins resulting in an overall increase in stiffness and cardiac dysfunction.[4]

The actin-associated cytoskeletal protein, β_{IV}-spectrin, is traditionally viewed as an important player in maintaining the cellular architecture and plasma membrane stability as well as coordinating ion channel complexes to regulate excitability.[5,6] Interestingly, recent studies have shown β_{IV}-spectrin to also serve as an important signaling player through its association with the Signal Transducer and Activator of Transcription 3 (STAT3), a stress-activated transcription regulator. Specifically, the β_{IV}-spectrin/STAT3 complex has been identified to regulate gene transcription, fibrosis, and cardiac function in cardiomyocytes and CFs[7,8] (Figure 1). In the context of fibrosis, previous work has reported that knock-out disruptions of the β_{IV}-spectrin/STAT3 complex in cardiomyocytes caused the activation and hypertrophy of nearby quiescent CFs, and a similar disruption of the complex in CFs lead to cardiomyocyte hypertrophy.[8] While β_{IV}-

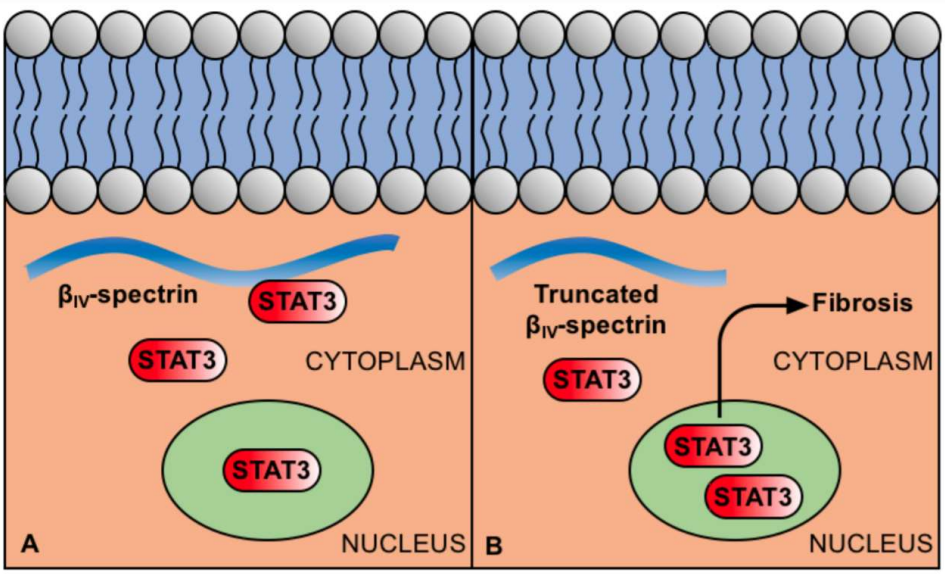

Figure 1. STAT3 and β_{IV}-spectrin associate in cardiac fibroblasts. (**A**) β_{IV}-spectrin targets and retains STAT3 through direct protein-protein interactions. (**B**) The loss of functional β_{IV}-spectrin results in nuclear accumulation of STAT3, changes in gene expression, increased fibrosis and decreased cardiac function.[6]

spectrin/STAT3 disruptions in CFs and cardiomyocytes have been shown to initiate these fibrotic responses,[8] the role of these disruptions in CF activation and the specific long-range, cell-to-cell signaling mechanisms initiating CF activation remains unclear (Figure 2).

The cell-to-cell cross-talk between CFs and cardiomyocytes has begun to be explored by examining the cells' secreted paracrine stress signaling factors (e.g. exosomes, cytokines, and chemokines.[9] These factors have not been extensively studied, particularly in stress or HF conditions, however the secretory activity of CFs makes them very influential on the physiology of other cells in the heart. Based on previous work, the exosomes and cytokines/chemokines factors secreted by activated CFs may potentially be picked up by nearby quiescent cells and may play a role in spreading the activated phenotype.[8] As such, paracrine signals may be promising candidates for the recruitment of quiescent CFs into the activated myofibroblast state.[9]

Here, we used genetic mouse models of β_{IV}-spectrin deficiency to better understand the initiation of CF activation and recruitment of downstream quiescent CFs, and to test the hypothesis

that β_{IV}-spectrin deficient (activated) CFs recruit and alter the activity of distal quiescent CFs. We aimed to determine the role of β_{IV}-spectrin/STAT3 complex in regulating extracellular stress factors that communicate from fibroblast-to-fibroblasts associated with cardiac fibrosis, and to better understand the molecular paracrine stress factors regulating cardiac fibrosis in order to develop new therapies for HF patients.

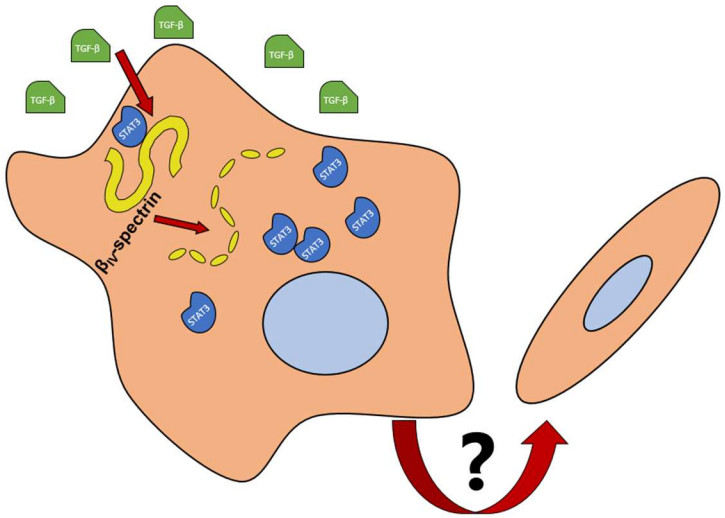

Figure 2. Regulation of fibroblast activation via the βIV-spectrin/STAT3 pathway. Though it is known that the degradation of βIV-spectrin in CFs leads to a transformation into an activated CF, it remains unknown how the activated phenotype can recruit downstream quiescent cardiac fibroblasts.[10]

Materials and Methods

Animals. Adult wild type (WT; control) and truncated β_{IV}-spectrin (qv^{4J}) mice were used. The qv^{4J} mice express a Sptnb4 allele with a premature stop codon proximal to the STAT3 binding region in βIV-spectrin, and were obtained from Jackson Laboratories[12,13] (Figure 3). Animals were cared for in compliance with Ohio State University and NIH guidelines.

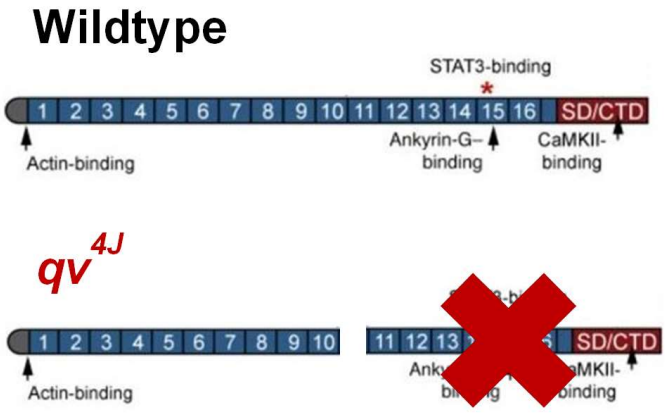

Figure 3. Structure of βIV-spectrin in WT and qv^{4J} models. The βIV-spectrin "deficient" qv^{4J} model contains a pre-mature stop codon prior to STAT-3 ankyrin-binding site.

Isolation of Cardiac Fibroblasts and Extraction of Conditioned Media. Hearts were isolated from WT and qv^{4J} animals and mouse CFs were isolated from left and right ventricles. Briefly, mouse tissue was minced in 2 mg/mL of collagenase II dissolved in 1× Ham's F-10 buffer. After digestion, the extract was collected and debris was separated with two serial centrifugations at 2000 RPM for 5 min. The supernatant was discarded and pelleted cells were resuspended in DMEM; 1×, 10% FBS, 1% L-glutamine, and 1% Pen/Strep. Cells were allowed to adhere to culture plates for approximately 4 to 5 hours prior to media removal containing non-adhering cells. Fresh media was replenished and cells were grown for 5-7 days at 37°C in 5% CO_2, until confluent. Conditioned cell-culture media from both the WT and qv^{4J} cells was removed and preserved after

24 hrs. The following experiments used this procedure to produce viable WT cells. Three biological (N=3) and three or four technical (n=3, 4) replicates were used, as specified.

Proliferation Assay as a Marker of Altered Gene Expression. A portion of the collected conditioned cell-culture media from either WT (control) or activated qv^{4J} CFs was treated onto WT CFs at 80% confluency. Treated CFs were seeded into 96-well culture-treated plates, as described.[14] Cells were adhered for 24 hours with serum starvation. Proliferation was then assessed by manual cell counting using a hemocytometer at desired time points of 24, 48 and 72 hours after applying conditioned media. Experiments were conducted in technical triplicates to evaluate proliferative response.

Collagen gel formation and macroscopic gel contraction measurements. WT CF were prepared to 80% confluency and plated overnight in 10 cm dishes with WT or qv^{4J} conditioned media. Type I rat-collagen gels (1.5 mg/mL) were prepared by mixing 10x PBS, sterile H_2O, acidic rat tail collagen, and 1M NaOH. Cells were added (100,000 cells/mL) and mixed in 12-well plates before gel solidification.[8] At 24 and 48 hours after gelation, the gels were imaged with an optical macroscope and the diameter of the gel was taken and averaged in two directions using ImageJ software. Isotropic compaction was assumed to measure the volume ratio of gels before and after compaction.[15] Experiments were conducted in technical quadruplets.

Conditioned Media Separation. To examine the exosome and cytokine/chemokine content, samples of conditioned media were split into multiple molecular weight (MW) fractions.[16] The samples were centrifuged at 2,000×g for 10 min at 4 °C to remove cell pellet, dead cells and large extracellular vesicles (EV), and the supernatant was aspirated and retained. Supernatant was then centrifuged at 10,000×g for 30 min at 4 °C to remove cell debris. The supernatant was then ultracentrifuged at 100,000xg for 70 min at 4 °C to pellet exosomes and contaminating proteins,

and the pellet was resuspended in PBS, and ultracentrifuged once more at 100,000xg for 70 min at 4 °C. Supernatant was collected and saved for cytokine measurements. The exosome pellets were resuspended in PBS and saved for exosome measurements.

Cytokine Immunoassay. The profile and content of various cytokines and chemokines were measured in small MW-fractions of conditioned media supernatants from WT and qv^{4J} mouse CFs using a mouse cytokine array panel (Proteome Profiler™ Mouse XL Cytokine Array Kit, Cat. #ARY028, R&D Systems) according to the manufacturer's instructions. The commercially available kit tested for 111 mouse pro-inflammatory, pro-angiogenic, and pro-fibrotic cytokines simultaneously.[11]

Nanosight Measurement of Exosomes. Large EV fractions of media were analyzed to determine the secreted exosome concentration and size. NanoSight NS300 nanoparticle tracking equipment was used obtain particle size distributions and concentrations.[16] Briefly, samples were diluted 1:100 in particle-free PBS and placed in the analyzer. Three representative samples for each system were analyzed by video captures of 60 seconds each to generate averaged concentrations of EV/ml ± SEM and mean ± SEM particle size (nm).

Statistics. SigmaPlot 14.5 was used for statistical analyses. P-values were determined for single comparisons using Student's t-tests. A *P*-value of <0.05 was considered statistically significant; P>0.05 was not considered significant.

Results

Extracellular Signals from β_{IV}-spectrin Deficiency Alter Cardiac Fibroblast Phenotype. We first sought to understand the general mechanism for communicating the activated fibroblast phenotype. Previous work has shown that knocking-out β_{IV}-spectrin in cardiac myocyte cells

induces a loss of β_{IV}-spectrin in CFs, and vice-versa.[6] This loss of β_{IV}-spectrin induces a change in CF genetic expression and phenotype, notably increasing rate of proliferation and the cell contractility.[8] Based on this work, the exosomes and cytokines/chemokines secreted by activated CFs may potentially be picked up by nearby quiescent cells and may play role in spreading the activated phenotype. Viable, healthy primary CFs from WT and qv^{4J} mice were successfully isolated and cultured for study, and conditioned media from both sets of cells was extracted. The

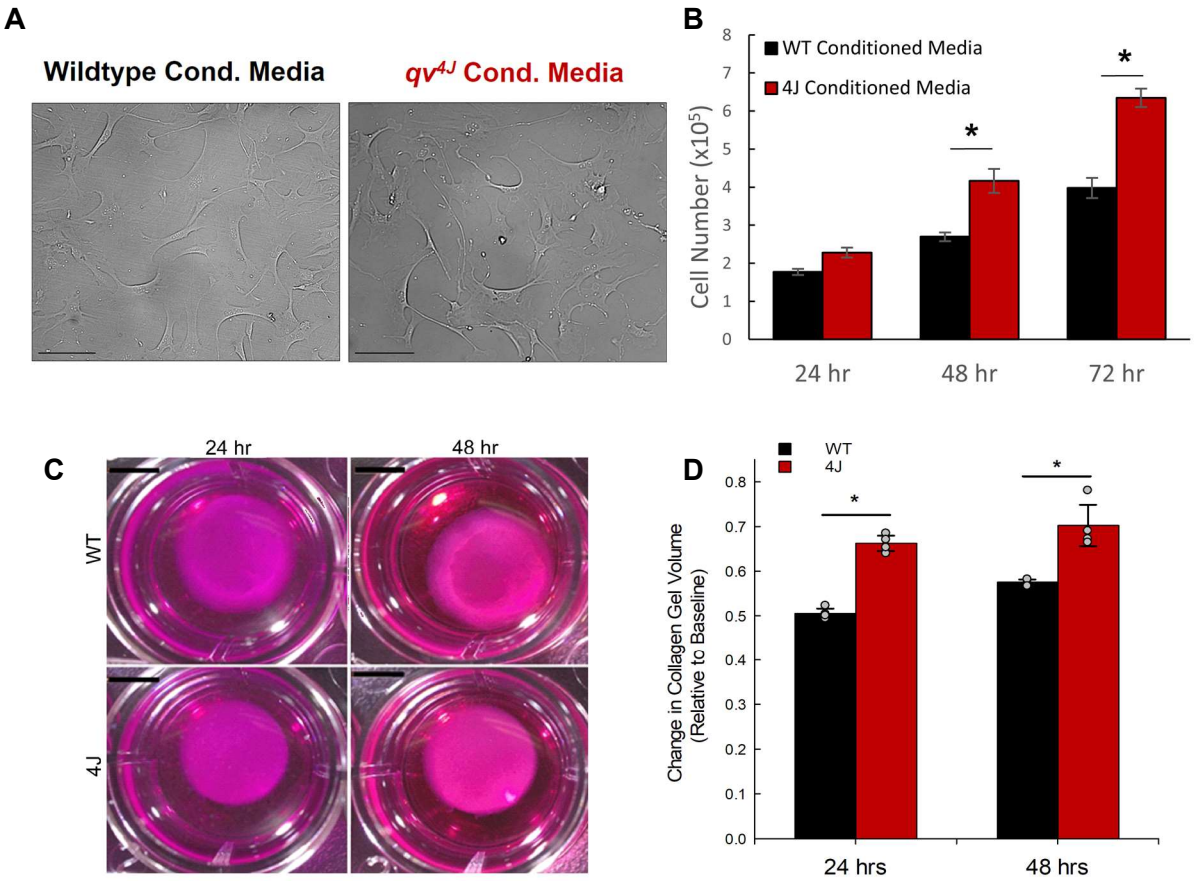

Figure 4. Extracellular signals from βIV-spectrin deficient cells alters activity of quiescent cardiac fibroblasts. (**A**) Representative brightfield images of primary mouse cardiac fibroblasts isolated from ventricles of WT hearts, treated with WT and qv^{4J} conditioned media. qv^{4J} CFs are more contractile and with more defined spindles. 20x magnification. Scale bar = 100µm. **(B)** Proliferation of WT CFs with conditioned media from WT and qv^{4J} CFs evaluated at 24, 48, and 72 hours of treatment. Data presented as mean±SEM; N=3. * denotes significance *P < 0.05 by 2-tailed t test vs. WT conditioned media. (**C**) Representative images of collagen gels seeded with WT CFs treated with WT or qv^{4J} conditioned media over 48-hour time course. Scale bar = 5mm. (**D**) Summary data (mean ± SEM) of change in collagen gel volume over the 48-hour time course in WT and qv^{4J} CFs. n = 4 for both WT and qv^{4J}, where N represents the number of independent preparations; * denotes significance *P < 0.05 by 2-tailed t test.

observed proliferation and contraction would confirm if messengers present in the qv^{4J} media affected the WT cells similarly to the fibrotic pathway seen *in vivo.* Conditioned media experiments were run to determine whether extracellular paracrine signals were secreted by active CFs and activating downstream quiescent CFs. Fibroblast proliferation was assessed to test for functional consequences during interactions with signals from β_{IV}-spectrin deficient CFs. A significant increase in proliferation (measured as cell number) was observed in the qv^{4J}-conditioned CFs at the 48- and 72-hour time points (Figure 4B). The 24-hour time point also displayed a noticeable increase in qv^{4J}-conditioned CFs over the WT-conditioned CFs. Furthermore, qv^{4J}-conditioned CFs showed a significant increase in contractility relative to WT, assessed by the compaction measured from the collagen gel volume (Figure 4D). When viewed together, these data show that treating otherwise quiescent WT CFs with conditioned media containing extracellular factors from activated qv^{4J} CFs alters the phenotype of the WT CFs, transforming them into a more active cell type.

Conditioned Media Separation and Characterizing Exosome, Cytokine, and Chemokine Content. The final goal was to characterize the exosomes, cytokines and chemokines secreted from the activated CFs. To examine the exosome and cytokine/chemokine content, the qv^{4J} conditioned media was be separated by mass; the higher and lower molecular weight fractions were analyzed for the expression of certain exosomes and cytokines, respectively. The cytokine and chemokine immunoassays yielded several pro-inflammatory cytokine-coding genes that were significantly upregulated in qv^{4J} conditioned media when compared to WT media. Most strikingly was MMP-3, at nearly 5 times more than any other gene. Periostin/OSF-2, CCL17/TARC, Coagulation Factor III/Tissue Factor were also heavily upregulated (Figure 5A).

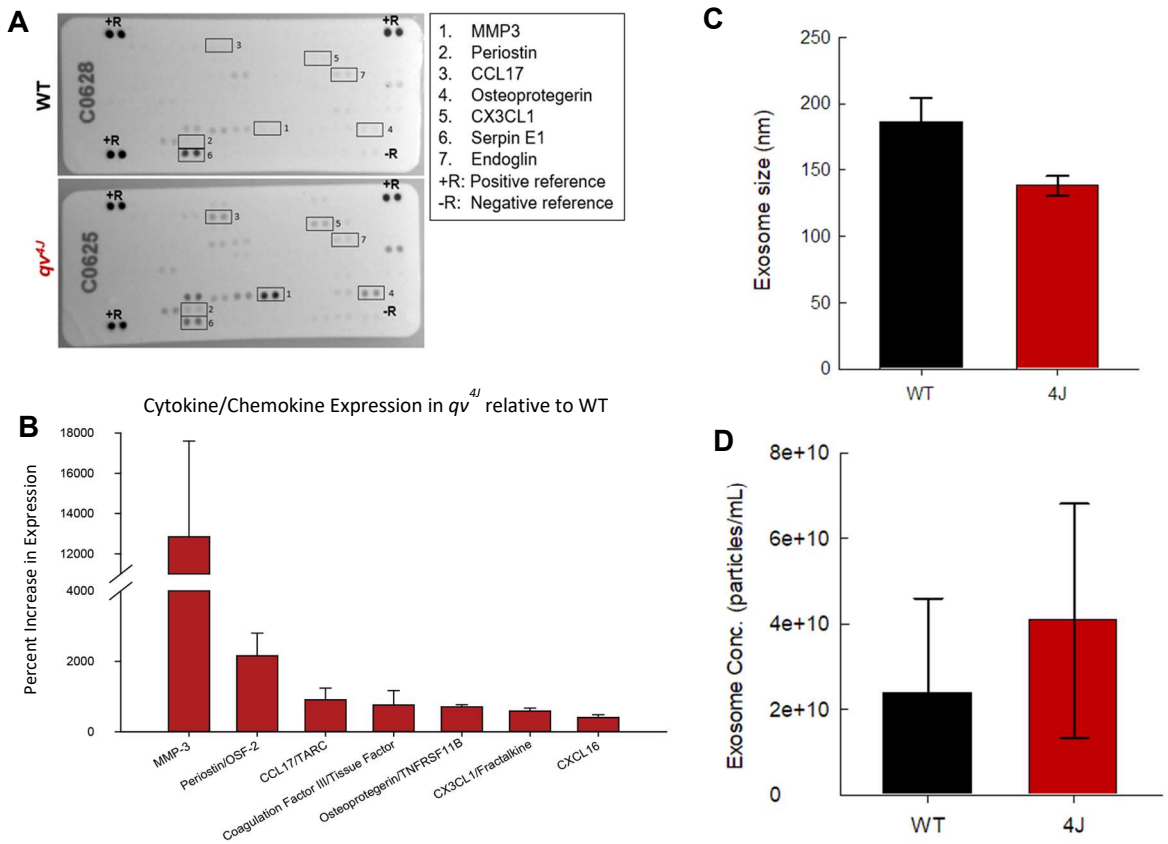

Figure 5. Characterization of qv^{4J} conditioned media exosome and cytokine components. **(A)** Representative image of cytokine immunoassay: intensity is measured from dots. **(B)** Relative expression of significantly upregulated cytokine-transcribing genes in qv^{4J} CF conditioned media. qv^{4J} expression values were normalized to the expression of corresponding genes in WT. **(C)** Exosome size and **(D)** exosome concentration of both WT and qv^{4J} conditioned media higher MW fractions. Data presented as mean ± SEM; N=3. * denotes significance, *P < 0.05 by 2-tailed t-test. P > 0.05, so significance could not be determined.

The NanoSight nanoparticle tracking uses light scattering and Brownian motion to obtain particle size distributions and concentrations.[16] Interestingly, the exosome nanoparticle tracking analysis yielded that the exosomes in qv^{4J} conditioned media were generally smaller than WT media, but not significantly (Figure 5C). Similarly, the analysis determined that the qv^{4J} media generally had a higher concentration of exosomes, but once again not significantly (Figure 5D).

Discussion

Here we define a role for β_{IV}-spectrin in producing paracrine signaling factors that tune the phenotype of downstream quiescent CFs. Preliminary studies have shown that the loss of β_{IV}-spectrin encourages the accumulation of STAT3 in the CF nucleus, causing transcriptional changes and resulting in a more proliferative, more contractile cell type. In this study, we demonstrated that cells without functional β_{IV}-spectrin will also produce pro-inflammatory extracellular factors, which are then taken up by other nearby quiescent cells. This resulted in the transformation of quiescent cells to have this more proliferative, contractile cell type (Figure 4C,D). Ultimately, these changes at the cellular level result in increased fibrosis, cardiac remodeling, and defective electrical signal propagation at the organ-level. These findings suggest that the β_{IV}-spectrin/STAT-3 complex could be an important target for improving cardiac mechanical and electrical function in several diseases.

The transfer of the activated phenotype between activated and quiescent CFs—and their successive activation—suggested that significant levels of pro-inflammatory exosomes, cytokines, chemokines, or a combination of these paracrine signals were secreted by qv^{4J} CFs compared to WT CFs. While the cytokine and chemokine immunoassay was able to identify potential candidates of CF activation, it was interesting that the exosome analysis did not yield significant differences between the two cell types. (Figure 5A) These upregulated signaling factor genes are potential targets for reducing the activation of CFs, and thereby inhibiting the remodeling effects of cardiac fibrosis. Limiting the activation of STAT-3 in cardiac fibroblasts may decrease cardiac fibrosis, and thereby reduce the negative effects of hypertrophy and cell death in heart failure. Further work could explore methods of interrupting the cross-talk by cytokines, whether through inhibitors of the upregulated cytokines and chemokines or via other therapeutic methods.

Most immediately, further investigation on the exosome component of the conditioned media is warranted. It was initially expected for there to be a difference between the exosomes of the two cell lines, however the study could not conclude a difference (Figure 5B). One limitation of the study is that the nanoparticle tracking only looks at population-wide trends in exosome size and concentration; it could be possible that the concentration of certain individual exosomes types were fact being upregulated, and that other specific exosomes were being downregulated. The lack of significance could also be explained by potentially only a very slight change in the concentration of certain exosomes is needed in the signaling cascade, and in combination with the upregulation of other cytokines, the cell is extremely sensitive to the change in those exosomes. Future work could explore other methods of identifying specific categories of exosomes to see changes on the individual level. Additionally, other signaling pathways could be explored, such as the transfer of lipid signals and how they are involved in the cell cross-talk process.[17]

Although we demonstrated that the presence of certain cytokines and chemokines have a significant effect on the activation of CFs, we cannot rule out the involvement of other signaling pathways. Previous studies have shown the TGF-β/SMAD protein complex signaling pathway is involved in the loss of β_{IV}-spectrin.[18] It would be interesting to study potential links between the pathways for controlling the CF phenotype. Additionally, paracrine signals are not the only signaling pathway; other recent research has shown that mechanical stressors are also involved in the cardiac fibrosis remodeling process.[19] It would be interesting to study how the mechanical effects of the influence the release of signaling factors produced by the cells. Finally, the cross-talk between CFs and cardiac myocytes would be critical for a more systemic understanding of communication. It would be interesting to observe the factors released by myocytes and their effect on CFs, and vice-versa, in a co-culture experiment.

Conclusion

The stress-induced transition of quiescent cardiac fibroblasts to an activated myofibroblast state contributes to cardiac fibrosis, and produces mechanically and electrically deleterious effects cardiac remodeling. The activated phenotype is communicated between cardiac fibroblasts using exosomes, cytokines, and chemokines. Although not each category of paracrine signal was found to be impactful, this work lays the foundation for the importance of these paracrine factors on changes in fibroblast phenotype, and highlights potentials for gene targets as well as studies on other methods of cellular cross-talk. It will be exciting for future studies to further explore the interplay between these different signals and the tissue-level remodeling changes.

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