**A NOVEL APPROACH TO DIFFERENTIATING CARDIAC FIBROBLASTS
FROM INDUCED PLURIPOTENT STEM CELLS**

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ABSTRACT

Heart disease remains the leading cause of death in the United State, and such disease is caused by electrical, chemical, or biomechanical imbalances in the heart. The two most abundant cells comprising the heart that are responsible for these functions are cardiomyocytes and cardiac fibroblasts. In normal cardiac tissue, these fibroblasts are found surrounding the myocytes and bridging gaps between myocyte layers in such a way that each cardiomyocyte is closely related to one or more neighboring fibroblasts. Unsurprisingly, defects in cardiac fibroblast function have been implicated in a variety of heart disease conditions including fibrosis, aberrant electrical signaling, and hypertrophy. However, it is difficult to study cardiac fibroblasts due to limited availability of primary human cells and challenges to propagate them long-term in vitro. The aim of this study was to develop a new protocol for the differentiation of human induced-pluripotent stem cells (iPSCs) to cardiac fibroblasts that is more efficient and reproducible than current methods. The proposed method requires 10 days (compared to the established Zhang *et. al*. protocol which takes 20 days) and uses far less expensive reagents. The new proposed protocol resulted in successful differentiation of iPSCs to cardiac fibroblasts and was verified with flow cytometry using iPSC and cardiac fibroblast markers. Having an effective protocol for the differentiation of cardiac fibroblasts allows for the future in vitro study of cardiac

fibroblast activation to myofibroblasts that is implicated in wide variety of heart disease. Understanding the interactions of cardiac fibroblasts with cardiomyocytes provides insights into the causes of cardiac dysfunction as well as a possible direction for the treatment of patients.

INTRODUCTION

As of 2017, heart disease remains the leading cause of death in the United States [8] with 1 in 4 deaths being due to heart disease [9]. Such disease is caused by electrical, chemical, or biomechanical imbalances of the heart. The cells comprising the heart that are responsible for these functions include cardiomyocytes, cardiac fibroblasts, endothelial cells, and vascular smooth muscle cells [1], [10]. Of these cell types, cardiomyocytes and fibroblasts are the most abundant with cardiomyocytes making up 75% of normal myocardial tissue volume and 30-40% of cell numbers [11]. The remaining 60-70% of heart cells are predominantly cardiac fibroblasts. In normal cardiac tissue, these fibroblasts are found surrounding the myocytes and bridging gaps between myocyte layers in such a way that each cardiomyocyte is closely related to one or more neighboring fibroblasts [1]. Defects in cardiac fibroblast function have been implicated in a variety of heart disease conditions including fibrosis [12], [13], aberrant electrical signaling [1]–[7], and hypertrophy [14], [15].

The cellular interactions between cardiomyocytes and cardiac fibroblasts play an important role in both cardiac development [16] and cardiovascular disease. Recent studies have found that cardiac fibroblasts and cardiomyocytes communicate through cell-cell interactions [17], [18] as well as multiple non-physical communication pathways involving cytokine production [19]. Cultured cardiac fibroblasts have also been shown to alter the contractile activity [20] and electrophysiological conditions [21] of the cardiomyocytes. Cardiac fibroblasts become activated under different heart stresses and respond by proliferating, secreting cytokines, and eventually differentiating into myofibroblasts that can assist with the increased stress load. While this acute response of fibroblasts reduces the cardiac stress on cardiomyocytes, chronic activation of cardiac fibroblasts has been implicated as an important step in the development of heart failure [22]–[26]. To develop effective treatments for heart disease, it is essential to further understand the pathways

by which cardiac fibroblasts interact with cardiomyocytes and the role that these interactions play in the development in heart disease.

However, it is difficult to study cardiac fibroblasts due to limited availability of primary human cells and challenges to propagate them long-term in vitro. Animal models have been suggested, but the translational value of animal models is limited in its ability to recapitulate phenotypic hallmarks of disease in humans. For this reason, human induced pluripotent stem cell (iPSC) technology [27] is increasingly utilized for the study of cardiac fibroblasts and the role they play in heart function and disease [28]. Protocols to date that detail the differentiation of iPSCs into cardiac fibroblasts are all extremely expensive and time-consuming. Most involve differentiating iPSCs first into epicardial cells followed by further differentiation to cardiac fibroblasts [29]–[32]. The Zhang *et. al.* protocol [33] allows direct differentiation from iPSCs to cardiac fibroblasts but still takes twenty days to reach full differentiation and results in a cell population that is, on average, 75% cardiac fibroblasts but can range anywhere between 50-100% cardiac fibroblasts. Therefore, there is a need to develop a more effective, efficient, and reproducible differentiation protocol for in the vitro study of cardiac fibroblasts.

The aim of this study was to develop a new protocol for the differentiation of iPSCs to cardiac fibroblasts that is more efficient and reproducible than current methods. The proposed method requires 10 days compared to the Zhang *et. al.* protocol which takes 20 days and uses far less expensive reagents. The successful differentiation of iPSCs to cardiac fibroblasts was determined using several verified cardiac fibroblast markers including DDR2 [1], [34], [35], VIM [33], and PDGFR-$\alpha$ [36], [37]. Having an effective protocol for the differentiation of cardiac fibroblasts allows for the future in vitro study of cardiac fibroblast activation to myofibroblasts that is implicated in wide variety of heart disease [5], [12], [13], [28], [38]. Understanding the

interactions of cardiac fibroblasts with cardiomyocytes provides insights into the causes of cardiac dysfunction as well as a possible direction for the treatment of patients.

BACKGROUND

The heart consists of a diverse cellular community, consisting of cardiac fibroblasts, cardiomyocytes, immune cells, endothelial cells, and more. Early studies surrounding heart research have focused on the functional cell type of the heart, the cardiomyocyte, which is responsible for the contractility of the heart. More recently, research has begun to focus on the other cells found in the heart and their interaction with cardiomyocytes. Of particular interest is the function of cardiac fibroblasts and the role that they play in cardiac remodeling of pathological conditions including hypertension, hypertrophy, and fibrosis.

Cardiac fibroblasts derive from the mesenchyme and serve a structural function in the heart by producing collagen and fibronectin, two major components of the extracellular matrix (ECM) [1], [39]. Viewed morphologically, fibroblasts are flat, spindle-like cells with many processes that emanate from the cell body. While they lack a basement membrane, they have a prominent Golgi apparatus and endoplasmic reticulum. During neonatal development, the 3-dimensional collagen network of the heart is laid down, and cardiac fibroblasts can be found within this network of connective tissue. While this is forming, cardiomyocytes become attached to the collagen through proteins called integrins [40]. Cardiac fibroblasts then surround the groups of myocytes. The cardiac fibroblasts form a network of cells through interconnected cellular processes, allowing them to contract the collagen and exert a mechanical force on the cardiomyocytes they surround. This organization allows the cardiac fibroblasts to maintain the structural integrity of the heart through cell-cell interactions, cell-ECM interactions, proliferation, ECM degradation, and ECM synthesis [1]–[3], [41]. This organization also lets fibroblasts be responsive to a variety of stimuli and dynamically maintain proper form and function of the heart [4]–[7].

Cardiac fibroblasts are also involved in supporting normal cardiac function by maintaining cell-cell communication with cardiomyocytes, endothelial cells, and cardiac fibroblasts. Cardiac

fibroblasts are both sources and targets of different stimuli that help to coordinate chemical, mechanical, and electrical signals between the various cellular and acellular components of the heart [42]. These cell-cell connections influence the electrophysiology of the heart, angiogenesis, and cytokine secretion. Cardiac fibroblasts and cardiomyocytes communicate through cell-cell interactions. Cardiomyocytes that are co-cultured with cardiac fibroblasts show overexpression of IL-6 and TGF-α compared to cardiomyocytes that are cultured alone [18], [19]. Through gap junction connexins (Cx40 Cx43, Cx45), cardiac fibroblasts can directly regulate electrical conduction in cardiomyocytes [1], [10], [13], [43], [44]. Cardiac fibroblasts also communicate with cardiomyocytes through multiple non-physical communication pathways involving cytokine production. These pathways allow in vivo cardiac fibroblasts to regulate the contractile activity [20] and electrophysiological conditions of cardiomyocytes [21].

Fibroblasts also play a critical role in the process of wound healing by migrating to the damaged tissue site, differentiating to active fibroblasts, and promoting tissue repair. Under certain conditions of stress or cellular damage, resting or quiescent fibroblasts can acquire active, synthetic, contractile properties and express several smooth muscle cell markers not usually typical of fibroblasts [45]. This activation from fibroblast to myofibroblast is promoted by several signaling pathways including transforming growth factor (TGF)-β, various cytokines, ECM signaling molecules, and other growth factors [46]–[48]. Myofibroblasts express contractile proteins, become more mobile, can contract collagen fibers, and are thought to be important for wound closure and structural integrity of healing scars [49]. The activation of myofibroblasts plays a key role in reparative fibrosis following myocardial infarction [50] as well as hypertrophic fibrotic scars in various injuries [42]. Additionally, apoptosis of myofibroblasts is an important step of the progression from granulomatous tissue to a mature scar [2]. However, myofibroblasts

that persist for too long without reversing back to fibroblasts or undergoing apoptosis have been linked destructive tissue remodeling [51], [52].

Cardiac fibroblasts serve as a major component in the structure of the heart and play an important role in normal cardiac function. To better understand the heart and diseases that affect heart function, an understanding of cardiac fibroblasts is necessary and can lead to more optimal patient outcomes and effective disease treatments. Because of this, it is important to have an in vitro model with which to study the different properties of cardiac fibroblasts. By better characterizing cardiac fibroblasts in vitro, we can study their direct interactions with cardiomyocytes. Unfortunately, current differentiation protocols for cardiac fibroblasts are time-consuming and not consistently effective. This project explores an alternative protocol which may result in quicker, more reliable differentiation of cardiac fibroblasts.

METHODS

The development of a new protocol for the differentiation of iPSCs to cardiac fibroblasts was conducted in two stages. The first stage involved the culture of iPSCs followed by their differentiation into cardiac fibroblasts using the new protocol. The second stage involved characterization of the cardiac fibroblasts generated by the new protocol as compared to the Zhang *et. al*. protocol [33].

The induced pluripotent stem cells (iPSCs) used in the development of the new protocol were derived from human white blood cells (WBCs). These WBCs were converted into iPSCs by using an adenovirus to express the four Yamanaka factors: Oct4, Sox2, Klf4, and cMyc [53]. The iPSCs were cultured on vitronectin-coated (ThermoFisher Scientific, Waltham, MA) 6-well culture plates in mTeSR Plus medium (Stem Cell Technologies, Vancouver, Canada) and then incubated at 37° C in 5% $CO_2$ and 95% air. Cells were passaged when they reached between 80-100% confluency, approximately every 3-4 days. During passaging, cells were disassociated from the well using TrypLE Express Enzyme (ThermoFisher, Waltham, MA) and pipetted into a 15-mL test tube. They were then centrifuged down to collect cells at the bottom of the tube, the TrypLE solution was removed. The cells were then resuspended in 1 mL of mTeSR Plus median with 1:100 dilution of Rock pathway inhibitor (Stem Cell Technologies, Vancouver, Canada). The suspended cells were then dispersed onto new vitronectin-coated wells on a 6-well plate at a split ratio of 1:5.

The iPSCs were then differentiated into cardiac fibroblasts using the new proposed protocol. The protocol was started at day 0 when the iPSC well had reached 100% confluency. On day 0, the media on the cells was changed to RPMI growth medium (-insulin) (MilliporeSigma, Burlington, MA) with B27 supplement and Activin A (MilliporeSigma, Burlington, MA). On day 1, the RPMI+B27 (- insulin) media was changed, and bone morphogenetic protein 4 (BMP4) (MilliporeSigma, Burlington, MA) and basic fibroblast growth factor (bFGF) (MilliporeSigma,

Burlington, MA) were added to the medium. The cells were kept in this medium for 48 hours without media change. On day 3, the media was changed to DMEM medium (high glucose) (MilliporeSigma, Burlington, MA) with 10% FBS (MilliporeSigma, Burlington, MA) added. This media was changed on the cells every 72 hours until day 10.

A visual representation of this new proposed protocol can be seen in Figure 1 as compared with the Zhang *et. al.* protocol [33]. The Zhang *et. al.* protocol starts on day 0 when the medium was changed to RPMI+B27 (-insulin) and supplemented with CHIR99021. CHIR inhibits glycogen synthase kinase 3 (GSK3) to inhibit the Wnt pathway [54], [55] for differentiation into the cardiac lineage. Cells were treated in this medium for 24 hours (day 1). After day 1, the medium was changed to RPMI+B27 (-insulin), and cells were cultured in this medium for 24 h (day 2). On day 3, the medium was changed to CFBM medium supplemented with bFGF, a fibroblast growth factor that promotes the proliferation of fibroblasts [56]. CFBM media is composed of DMEM (high glucose) with added HLL supplement (human serum albumin, linoleic acid, lecithin), ascorbic acid, GlutaMAX, hydrocortisone hemisuccinate, and insulin. Cells were fed with CFBM+bFGF every other day until day 20. The new proposed protocol starts on day 0 when the medium was changed to RPMI+B27 (-insulin) and supplemented with Activin A. Activin A promotoes cardiac fibroblast proliferation and differentiation via ALK4, activin receptor-like kinase, and the ERK1/2 and p38-MAPK pathways [57]. Cells were treated in this medium for 24 hours (day 1). After day 1, the medium was changed to RPMI+B27 (-insulin) and supplemented with BMP4 and bFGF. BMP4 induces cardiac differentiation of iPSCs [58], and bFGF promotes the proliferation of fibroblasts [56]. Cells were cultured in this medium for 48 hours. On day 3, the medium was changed to DMEM (high glucose) with 10% FBS (fetal bovine serum). Cells were fed with DMEM+FBS every three days until day 10.

Figure 1 shows that the new protocol is 10 days shorter and only uses expensive reagents on 2 days as opposed to 11 days. Both features make the new protocol simpler and more cost-effective for the differentiation of iPSCs to cardiac fibroblasts.
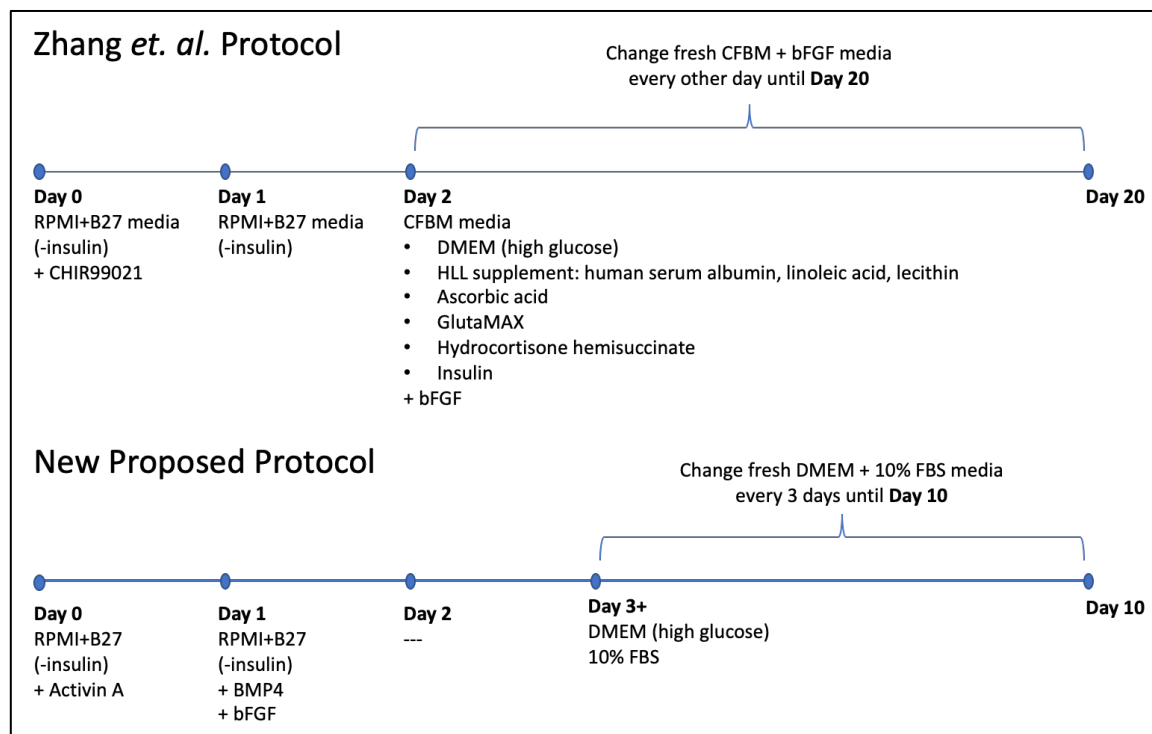


Figure 1. Timeline of Zhang *et. al.* protocol as compared with new proposed protocol to differentiate iPSCs into cardiac fibroblasts. The new protocol is 10 days shorter and requires 2 days of expensive reagents (Tocris, bFGF, Activin A, or BMP4) as opposed to 11 days.

The success of differentiation from iPSCs to cardiac fibroblasts was determined using flow cytometry for a verified iPSC marker and several verified cardiac fibroblast markers. Samples were prepared for flow cytometry by first fixing the cells with 4% paraformaldehyde. They were then disassociated from the well with TrypLE Express Enzyme and were washed twice with DPBS solution (MilliporeSigma, Burlington, MA). The cells were then centrifuged at 1000 g for 3 minutes, and the DPBS solution was removed. 500 μL of 1% Triton X-100 (MilliporeSigma, Burlington, MA) was added, and the cells were left to incubate to 10 minutes to permeabilize their

cell membranes. The cells were then washed twice with DPBS. Next, the fluorescent antibody was added depending on which marker was being evaluated. The cells were left to incubate for 30 minutes with DPBS and the antibodies. Following the incubation, they were washed twice with DPBS and filtered through a 100-micron Nylon filter to remove large clumps of cells.

Flow cytometry was performed using the BD Biosciences FACS Aris™ II. An ultraviolet light source was used for the detection of DAPI signal and identification of cells. A 488 nm laser was used for the detection of GFP signal from conjugated antibodies. Flow events were gated based on forward and side scatter. Only events with a forward scatter in the range of 30,000-250,000 and a side scatter in the range of 1,000-60,000 were counted. Events outside this gate were regarded as cellular debris.

The marker used to identify iPSCs was tumor related antigen TRA-1-60 (podocalyxin). The antibody used was TRA-1-60-AF488 catalog #A25617 (ThermoFisher Scientific, Waltham, MA). TRA-1-60 is a sialylated keratan sulfate proteoglycan that is characteristically expressed by human stem cells [59]. TRA-1-60 is a cell surface antigen in iPSCs that is lost during the differentiation process [60]. The expectation was that TRA-1-60 fluorescence would be strong at day 0 before the differentiation protocol began and subsequently lost throughout the differentiation protocol as the iPSCs differentiated into cardiac fibroblasts. Successful differentiation would include a loss of TRA-1-60 fluorescence.

Several markers were used to characterize the differentiated cardiac fibroblasts including VIM [33], DDR2 [1], [34], [35], and PDGFR-$\alpha$ [36], [37]. Vimentin (VIM) is a type III intermediate filament protein that is expressed in mesenchymal cells [61] and is commonly used to identify cells of fibroblast origin such as cardiac fibroblasts. However, VIM is a marker of immature fibroblasts and, because it is not specific to cardiac fibroblasts, it must be used in conjunction with other cardiac fibroblast markers [62]. Discoidin domain receptor 2 (DDR2) is a

tyrosine kinase receptor expressed on the surface of cells of mesenchymal origin and is a sensitive marker for cardiac fibroblasts. Importantly, DDR2 is not expressed in cardiomyocytes, endothelial cells, or smooth muscle cells [1]. However, DDR2 is only selectively expressed in a small percentage of cardiac fibroblasts, making it a sensitive marker but not a specific marker for cardiac fibroblasts [36], [63], [64]. Platelet-derived growth factor receptor $\alpha$ (PDGFR-$\alpha$) is emerging as a definitive marker of cardiac fibroblasts with a high sensitivity and specificity [36], [64]. PDGFR-$\alpha$ is involved in the formation of cardiac fibroblasts from the epicardium [65]. Together, these three markers allow verification of successful cardiac fibroblast differentiation and characterization of maturity.

RESULTS

The efficacy of the proposed iPSC-cardiac fibroblast differentiation protocol was assessed using flow cytometry. One iPSC marker, TRA-1-60, was used to verify the initial iPSC cells and assess the differentiation of cardiac fibroblast away from pluripotency. Three cardiac fibroblasts markers were used to determine the success of differentiation and characterize CF maturity: VIM, DDR2, and PDGFR-α. VIM is an immature fibroblast marker with low sensitivity and high specificity for cardiac fibroblasts. DDR2 is a mature CF marker with a high sensitivity and low specificity for cardiac fibroblasts because it is selectively expressed in a small percentage of cardiac fibroblasts. PDGFR-α is a mature CF marker with high sensitivity and high specificity for cardiac fibroblasts. Table 1 compares and contrasts the different markers used for flow cytometry characterization of cardiac fibroblasts and describes each of their sensitivities and specificities.

| | Flow Cytometry Marker | iPSC Sensitivity | iPSC Specificity | CF Sensitivity | CF Specificity | Additional Comments |
|---|---|---|---|---|---|---|
| iPSC marker | TRA-1-60 | **high** | **high** | - | - | |
| CF marker | VIM | - | - | **low** | **high** | immature fibroblasts |
| | DDR2 | - | - | **high** | **low** | only small percentage of CFs |
| | PDGFR-α | - | - | **high** | **high** | emerging as a definitive marker for CFs |

Table 1. Summary of markers used for flow cytometry. TRA-1-60 is an iPSC marker that is both sensitive and specific. VIM, DD2, PDGFR-α are all cardiac fibroblast markers. VIM has low sensitivity and high specificity for CFs. DDR2 has high sensitivity and low specificity for CFs. PDGFR-α has high sensitivity and high specificity for CFs.

Flow cytometry was performed using the iPSC marker, TRA-1-60. TRA-1-60 is a sialylated keratan sulfate proteoglycan that is characteristically expressed by human stem cells

[59]. TRA-1-60 is a cell surface antigen in iPSCs that is lost during the differentiation process [60]. The expectation was that TRA-1-60 fluorescence would be strong at day 0 before the differentiation protocol began and subsequently lost throughout the differentiation protocol as the iPSCs differentiated into cardiac fibroblasts. Successful differentiation would include a loss of TRA-1-60 fluorescence. Figure 2 shows the results of flow cytometry with TRA-1-60 in iPSCs and cardiac fibroblasts differentiated from iPSCs using the new protocol. Figure 2A shows that iPSCs, on average, exhibited significantly more fluorescence of TRA-1-60 than CFs, and Figure 2B shows that iPSCs exhibited a higher number of cells with TRA-1-60 fluorescence than CFs. As expected for successful differentiation, iPSCs exhibit TRA-1-60 fluorescence, and CFs lose TRA-1-60 fluorescence with differentiation.

Flow cytometry was also performed using the fibroblast marker, Vimentin. Vimentin (VIM) is a type III intermediate filament protein that is expressed in mesenchymal cells [61] and is commonly used to identify cells of fibroblast origin such as cardiac fibroblasts. However, because Vimentin is a marker of immature fibroblasts, it has a low sensitivity but a high specific for cardiac fibroblasts as discussed in Table 1. The expectation was that Vimentin fluorescence would be low at day 0 before the differentiation protocol began and subsequently increase in strength throughout the differentiation protocol as the iPSCs differentiated into cardiac fibroblasts. Successful differentiation would include a gain of Vimentin fluorescence. Figure 3 shows the results of flow cytometry with Vimentin in iPSCs and cardiac fibroblasts differentiated from
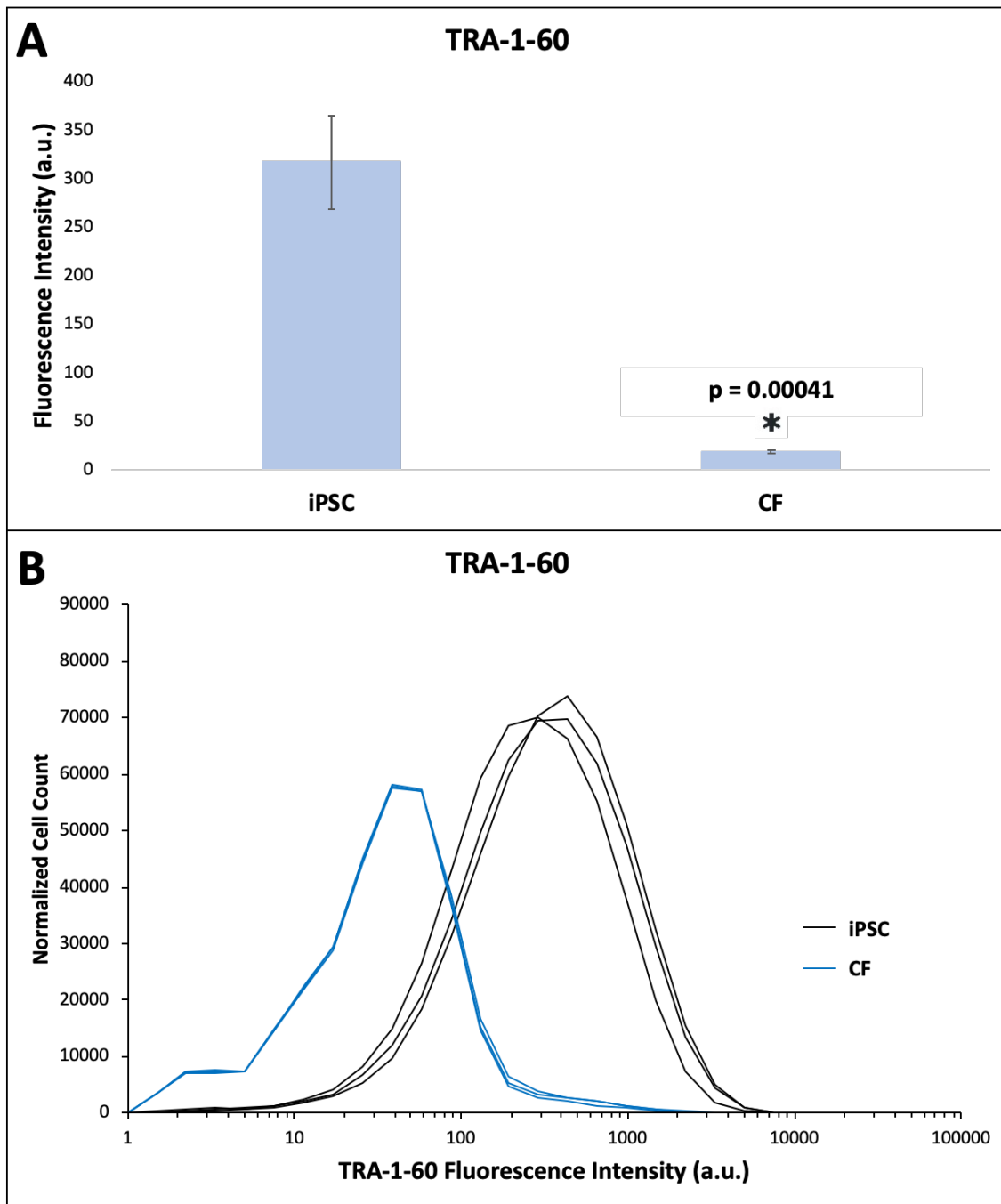
Figure 2. TRA-1-60, an iPSC marker, fluorescence in iPSCs as compared to cardiac fibroblasts (CF) differentiated with the new protocol. IPSCs exhibit significantly higher TRA-1-60 fluorescence than CFs. (A) Average fluorescence intensity of TRA-1-60 is significantly higher in iPSCs than in CFs. Significance was calculated using a student's T-test and shown with * where $p < 0.05$. (B) A histogram showing the number of cells at various levels of TRA-1-60 fluorescence demonstrates that iPSCs consistently exhibit higher fluorescence of TRA-1-60 than CFs. Separate lines represent different cell preparations, and 3 cell preparations each were analyzed for iPSCs and CFs.
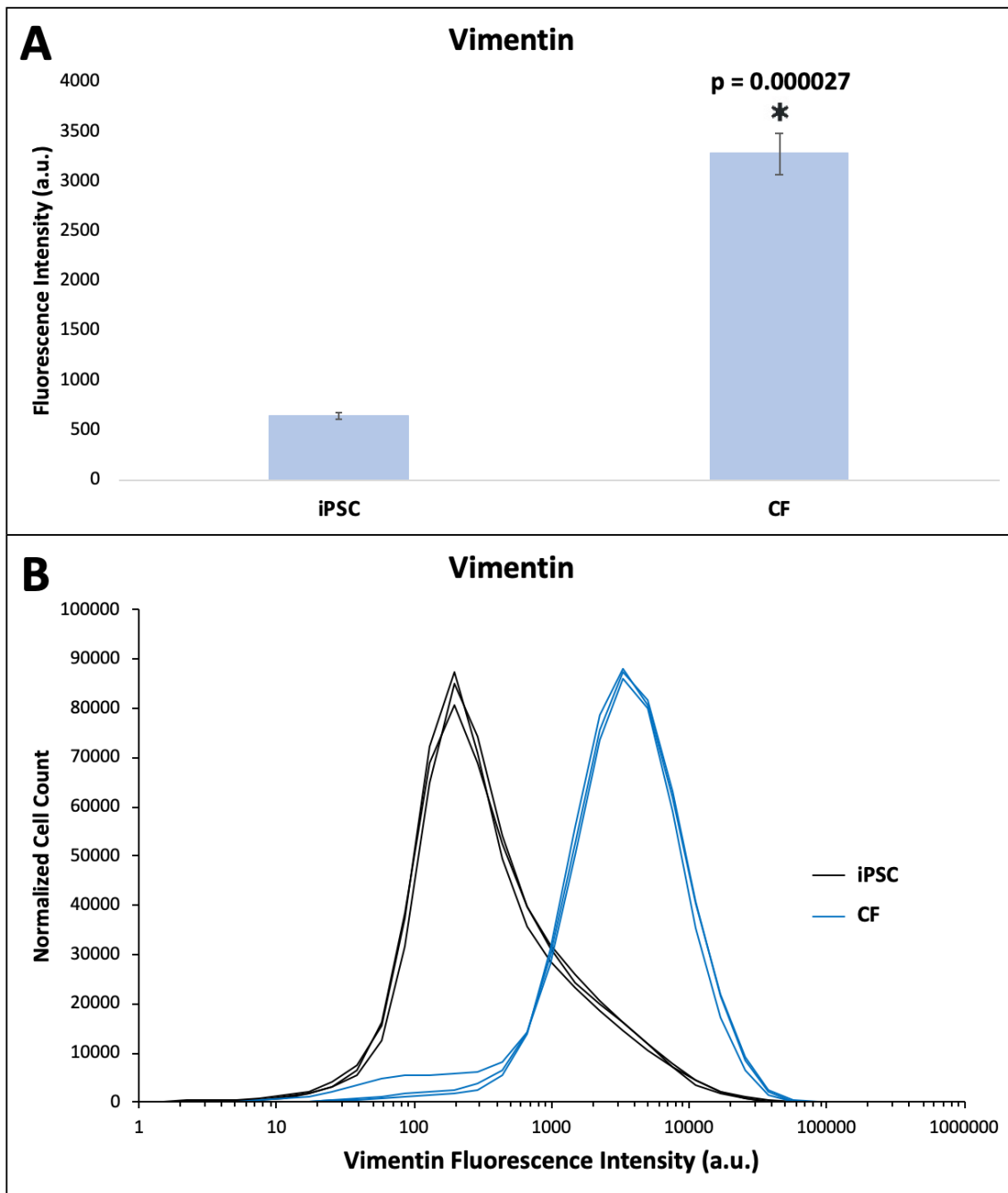
Figure 3. Vimentin (VIM), an immature fibroblast marker with low sensitivity and high specificity for cardiac fibroblasts (CF), fluorescence in iPSCs as compared to cardiac fibroblasts differentiated with the new protocol. CFs exhibit significantly higher VIM fluorescence than iPSCs. (A) Average fluorescence intensity of VIM is significantly higher in CFs than in iPSCs. Significance was calculated using a student's T-test and shown with * where $p < 0.05$. (B) A histogram showing the number of cells at various levels of VIM fluorescence demonstrates that CFs consistently exhibit higher fluorescence of VIM than CFs. Separate lines represent different cell preparations.
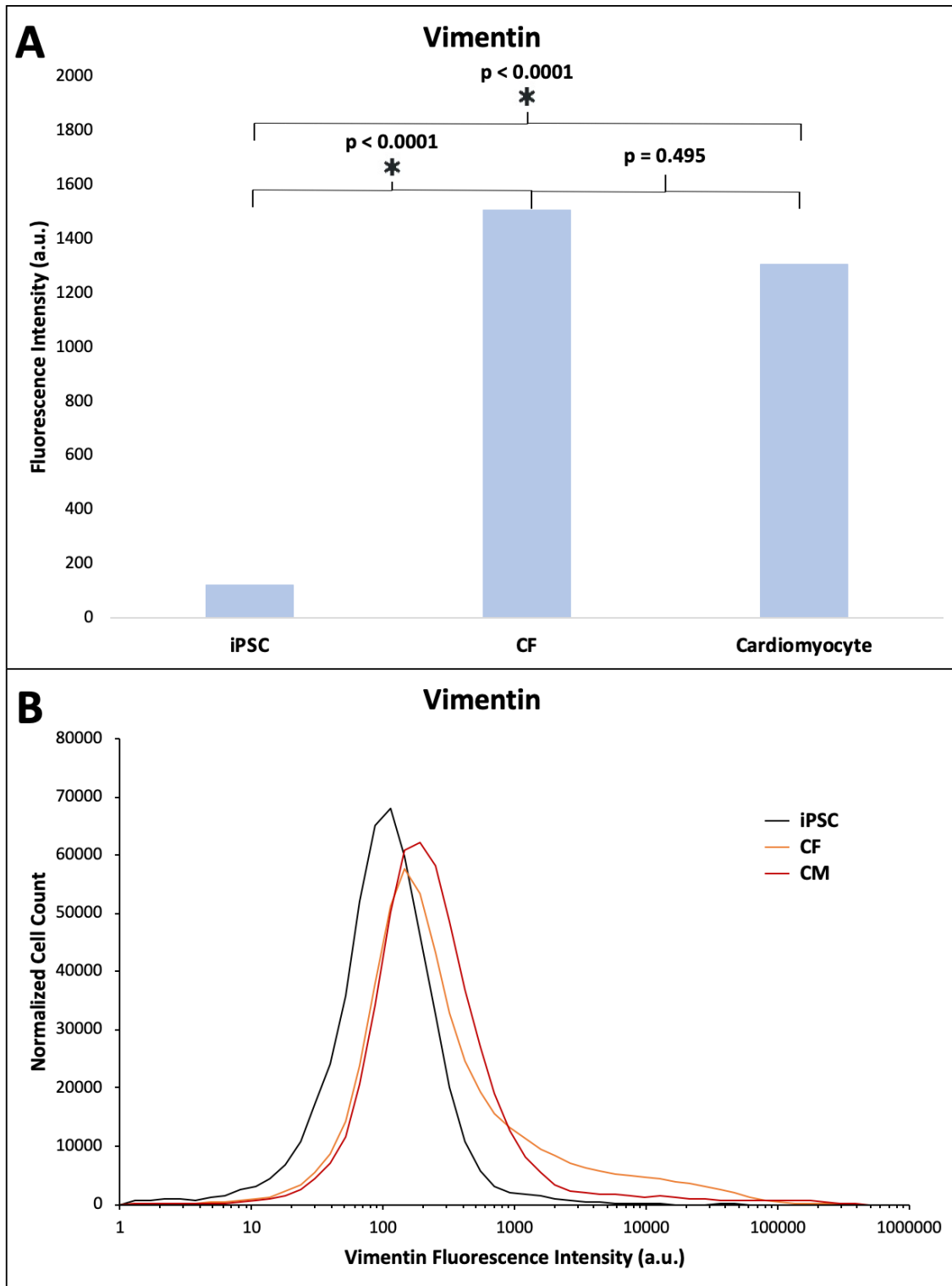
Figure 4. Vimentin (VIM) fluorescence in iPSCs compared to cardiac fibroblasts (CF) and iPSC-derived cardiomyocytes (CM). (A) Average fluorescence intensity of VIM is significantly higher in CFs and CMs than in iPSCs. Significance was calculated using a student's T-test and shown with * where $p < 0.05$. (B) A histogram showing the number of cells at levels of VIM fluorescence.

iPSCs

using the new protocol. Figure 3A shows that CFs, on average, exhibited significantly more fluorescence of Vimentin than iPSCs, and Figure 3B shows that CFs exhibited a higher number of cells with Vimentin fluorescence than iPSCs. Figure 4A shows that both CFs and iPSC-derived cardiomyocytes (CMs), on average, exhibited significantly more fluorescence of Vimentin than iPSCs, and Figure 4B shows that CFs and CMs exhibited a higher number of cells with Vimentin fluorescence than iPSCs. As expected for successful differentiation, iPSCs do not exhibit Vimentin fluorescence, and CFs gain Vimentin fluorescence with differentiation.

Flow cytometry was also performed using the fibroblast marker, DDR2. Discoidin domain receptor 2 (DDR2) is a tyrosine kinase receptor expressed on the surface of cells of mesenchymal origin and is a specific marker for cardiac fibroblasts. The expectation was that DDR2 fluorescence would be low at day 0 before the differentiation protocol began and subsequently increase in strength throughout the differentiation protocol as the iPSCs differentiated into cardiac fibroblasts. Successful differentiation would include a gain of DDR2 fluorescence. Figure 5 shows the results of flow cytometry with DDR2 in iPSCs and cardiac fibroblasts differentiated from iPSCs using the new protocol. Figure 5A shows that CFs do not exhibit significantly more fluorescence of DDR2 than iPSCs, and Figure 5B shows that CFs do not exhibit a higher number of cells with DDR2 fluorescence than iPSCs. While these were not the results expected for successful differentiation, DDR2 is only expressed in a small subset of CFs, and therefore, lack of DDR2 does not rule out the possibility of successful CF differentiation.

Flow cytometry was also performed using the fibroblast marker, PDGFR-α. Platelet-derived growth factor receptor α (PDGFR-α) is emerging as a definitive marker
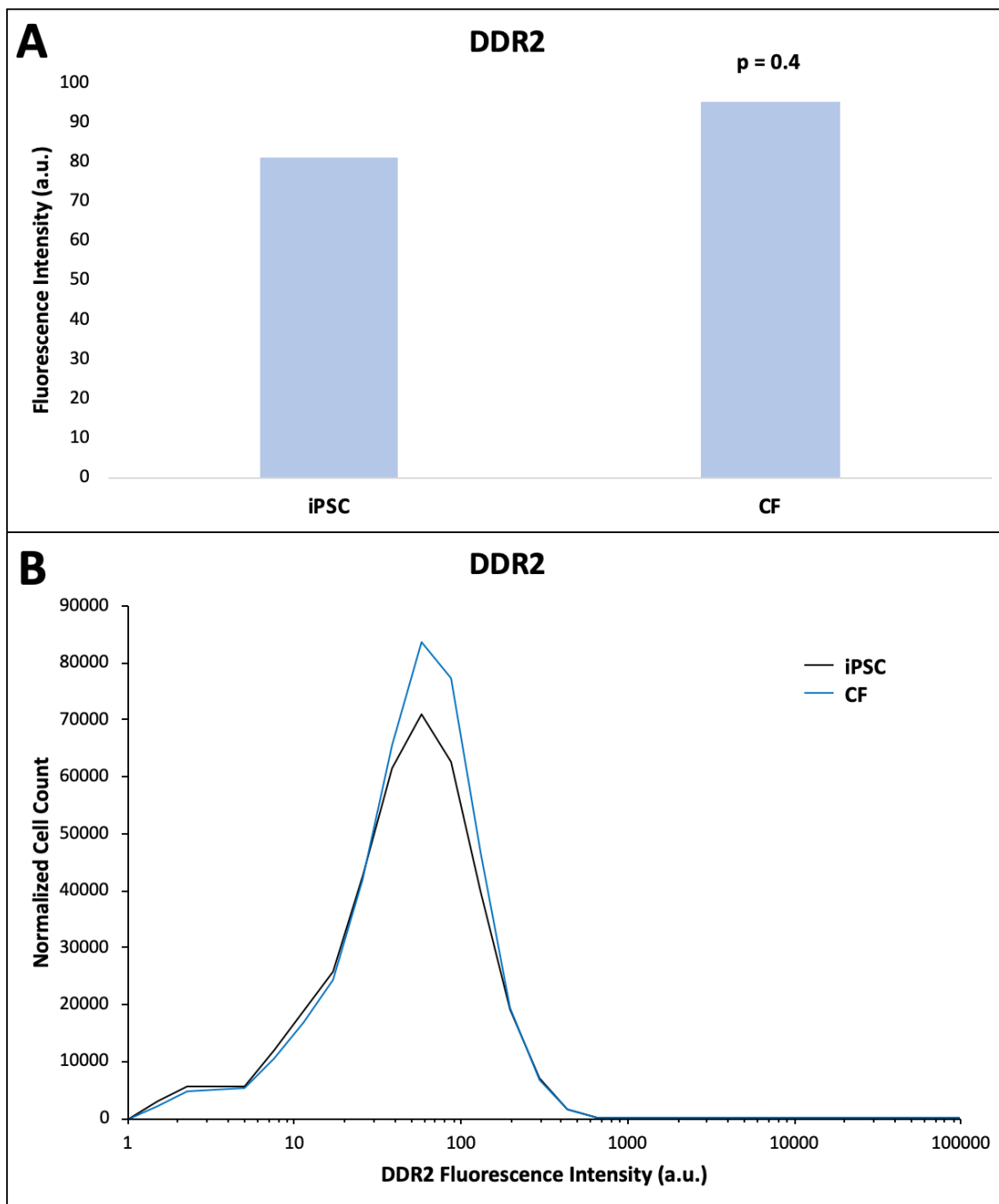
Figure 5. DDR2, a marker with high sensitivity and low specificity for cardiac fibroblasts (CF), fluorescence in iPSCs as compared to cardiac fibroblasts differentiated with the new protocol. CFs and iPSCs exhibit no significant difference in DDR2 fluorescence. (A) Average fluorescence intensity of DDR2 is not significantly different in CFs and iPSCs. Significance was calculated using a student's T-test and shown with * where $p < 0.05$. (B) A histogram showing the number of cells at various levels of DDR2 fluorescence demonstrates no significant difference in the number of cells with DDR2 fluorescence between CF and iPSC cell populations. Separate lines represent different cell preparations. 1 cell preparation each was analyzed for iPSCs and CFs.

cardiac fibroblasts with a high sensitivity and specificity [36], [64]. PDGFR-$\alpha$ is involved in the formation of cardiac fibroblasts from the epicardium [65]. The expectation was that PDGFR-$\alpha$ fluorescence would be low at day 0 before the differentiation protocol began and subsequently increase in strength throughout the differentiation protocol as the iPSCs differentiated into cardiac fibroblasts. Successful differentiation would include a gain of PDGFR-$\alpha$ fluorescence. Figure 6 shows the results of flow cytometry with PDGFR-$\alpha$ in iPSCs and cardiac fibroblasts differentiated from iPSCs using the new protocol. Figure 6A shows that both CFs and iPSC-derived cardiomyocytes (CMs), on average, exhibited significantly more fluorescence of PDGFR-$\alpha$ than iPSCs and that CMs exhibited significantly more fluorescence than CFs. Figure 6B shows that CFs and CMs exhibited a higher number of cells with PDGFR-$\alpha$ fluorescence than iPSCs. As expected for successful differentiation, iPSCs do not exhibit Vimentin fluorescence, and CFs gain Vimentin fluorescence with differentiation.
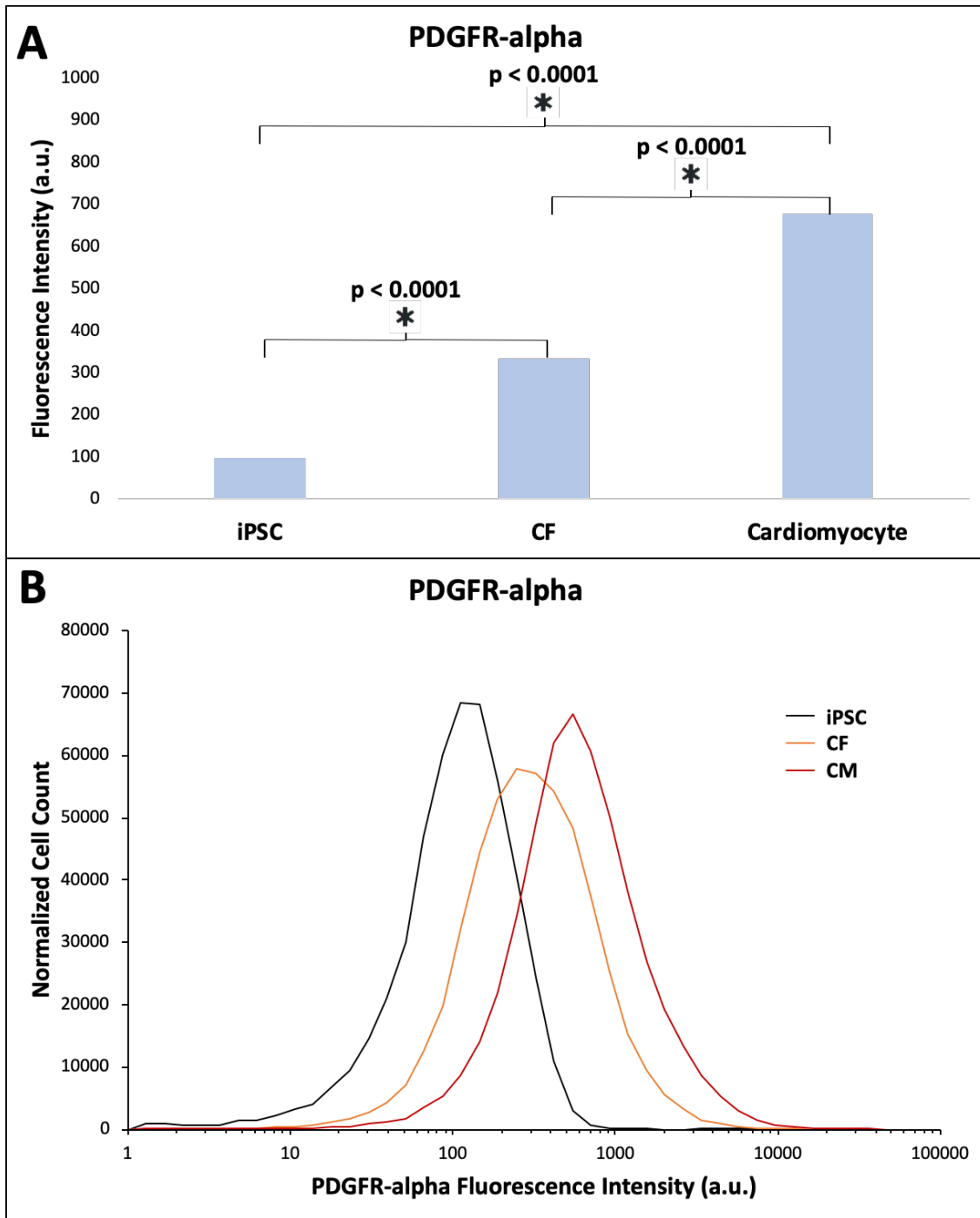
Figure 6. PDGFR-α, a highly sensitive and highly specific marker for cardiac fibroblasts (CF), fluorescence in iPSCs compared to iPSC-derived cardiomyocytes (CM) compared to CFs differentiated with the new protocol. (A) Average fluorescence intensity of PDGFR-α. Notably, PDGFR-α fluorescence is significantly higher in CFs than iPSCs. Significance was calculated using a student's T-test and shown with * where $p < 0.05$. (B) A histogram showing the number of cells at various levels of PDGFR-α fluorescence demonstrates that iPSCs exhibit less fluorescence of PDGFR-α than CFs which exhibit less fluorescence than CMs. 1 cell preparation each was analyzed for iPSCs, CFs, and CMs.

DISCUSSION

Motivated by the in-depth study of cardiac fibroblasts in vitro and their interaction with cardiomyocytes, this project was designed to establish a cost-effective, efficient, and effective protocol for the differentiation iPSCs to cardiac fibroblasts. The new protocol, as seen in Figure 1, used far less expensive reagents than the protocol established by Zhang *et. al*. [33], and it also cut the differentiation time in half, from 20 days to 10 days. The success of differentiation from iPSCs to cardiac fibroblasts was determined using flow cytometry for a verified iPSC marker (TRA-1-60) and several verified cardiac fibroblast markers (Vimentin, DDR2, and PDGFR-α. A summary of the markers along with relevant results can be seen in Figure 7.



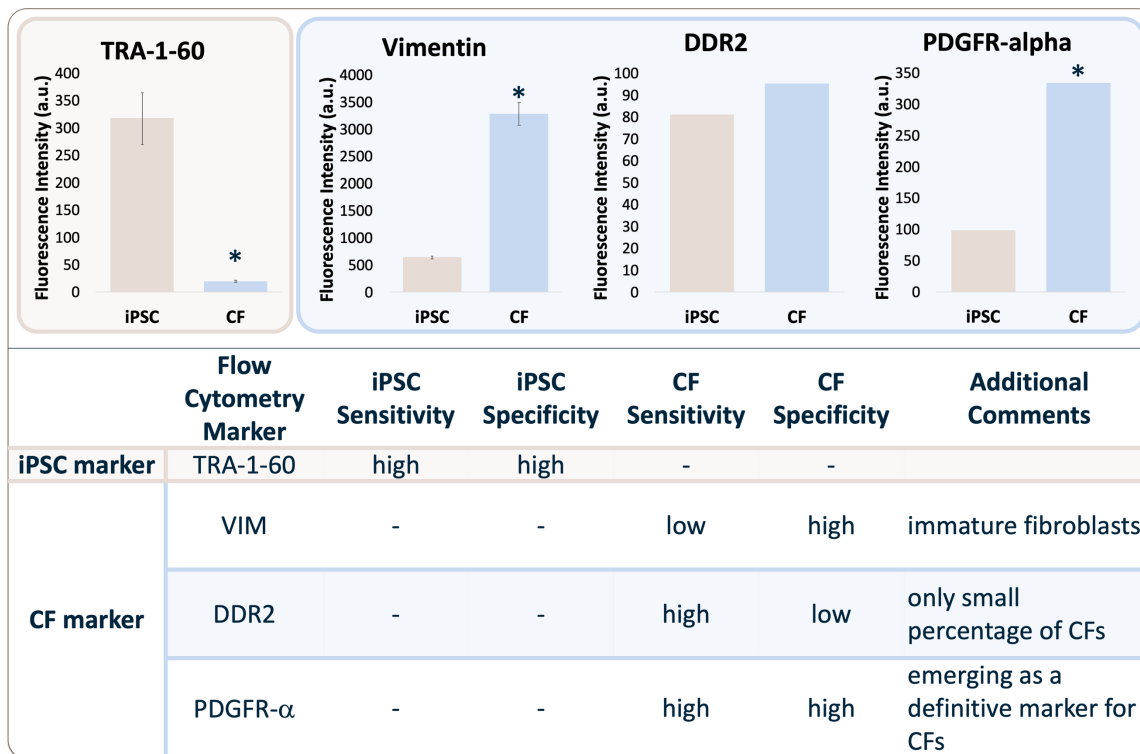| | Flow Cytometry Marker | iPSC Sensitivity | iPSC Specificity | CF Sensitivity | CF Specificity | Additional Comments |
|---|---|---|---|---|---|---|
| iPSC marker | TRA-1-60 | high | high | - | - | |
| CF marker | VIM | - | - | low | high | immature fibroblasts |
| | DDR2 | - | - | high | low | only small percentage of CFs |
| | PDGFR-α | - | - | high | high | emerging as a definitive marker for CFs |

Figure 7. Summary of flow cytometry markers, and summary of fluorescence data from flow cytometry for all markers.

Figure 7 summarizes the flow cytometry results of cardiac fibroblasts differentiated from iPSCs using the new protocol proposed in this project. TRA-1-60 is an iPSC marker with high

sensitivity and high specificity. Figure 7 shows that the differentiated CFs exhibited significantly less fluorescence of TRA-1-60 than iPSCs, indicating success of the protocol in differentiating iPSCs away from their pluripotent state. Vimentin is an immature fibroblast marker with a low sensitivity and high specificity for cardiac fibroblasts. Figure 7 shows that the differentiated CFs exhibited significantly more fluorescence of Vimentin than iPSCs, indicating success of the protocol in differentiating iPSCs toward cells within the fibroblast lineage. DDR2 is a cardiac fibroblast marker with high sensitivity and low specificity for cardiac fibroblasts. CFs exhibited no significant difference in fluorescence of DDR2 compared to iPSCs. While lack of DDR2 expression was not an expected result for successful differentiation, DDR2 is a CF marker with low specificity because it is only expressed in a small percentage of CFs. Therefore, lack of DDR2 expression may indicate unsuccessful differentiation into cardiac fibroblasts, it can also indicate that the new protocol led to differentiation of a DDR2- population of cardiac fibroblasts. Evidence from other markers would be needed to make this distinction. PDGFR-α is a cardiac fibroblast marker with a high sensitivity and high specificity for cardiac fibroblasts. Importantly, PDGFR-α is emerging as a definitive marker for CFs [36], [64]. Figure 7 shows that the differentiated CFs exhibited significantly more fluorescence of PDGFR-α than iPSC. This is the most definitive preliminary result that the new protocol was successful in differentiation iPSCs to cardiac fibroblasts.

A limitation of the study to date is that relatively few samples have been prepared for characterization. More samples must be prepared and characterized with flow cytometry to prove that the new protocol reproducible and effectively produces cardiac fibroblasts. Another limitation is the use of only 4 markers for characterization of the differentiated cardiac fibroblasts. More markers are needed to verify the presence of cardiac fibroblasts as well as to characterize the subpopulations of cardiac fibroblasts produced by the new protocol. Lastly, the new protocol needs

to undergo a direct head-to-head comparison with the Zhang *et. al.* [33] protocol. While we compared our results to those outlined in their paper, further analysis would have iPSCs cells from the same batch split into two populations and differentiated with both protocols. Subsequent analysis with flow cytometry would help characterize the differences in cardiac fibroblast populations generated by both protocols.

Despite these limitations, the project is successful in establishing a new protocol for the differentiation of in vitro iPSCs to cardiac fibroblasts that is more cost-effective and quicker. The new protocol generates cardiac fibroblasts that are VIM-positive, PDGFR-$\alpha$-positive, TRA-1-60-negative, and DDR2-negative. Future work should be aimed at further characterization of the cardiac fibroblasts generated by the new protocol as well as direct comparison to established protocols of differentiation.

Having a new, effective protocol for differentiation will aid in the in vitro study of cardiac fibroblasts and the role they play in cardiac pathology. Defects in cardiac fibroblast function have been implicated in a variety of heart disease conditions including fibrosis [12], [13], aberrant electrical signaling [1]–[7], and hypertrophy [14], [15]. Considering the fact that cardiomyocytes and cardiac fibroblasts are the two most abundant cells in the body [11], co-culture of these cells can further the study of how cardiomyocyte are closely related in structure and in function to one or more neighboring fibroblasts [1]. Such in vitro cell culture models will help to elucidate the mechanism behind how various heart diseases develop as well as further discovery of novel treatment methods aimed at correcting synergistic cardiomyocyte and cardiac fibroblast function.

ACKNOWLEGMENT

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