UNIVERSITY OF CALIFORNIA

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Investigation on the Mechanism of Cardiomyocyte Maturation

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Biology

by

Josh Lee

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ABSTRACT OF THE DISSERTATION

Investigation on the Mechanism of Cardiomyocyte Maturation

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Doctor of Philosophy in Molecular Biology

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Professor Yibin Wang, Chair

Cardiovascular disease is the leading cause of death in the world with a dearth of effective therapies. Heart undergoes a complex differentiation and maturation process throughout embryonic and post-natal stages. Intense efforts have been made in the study of cardiomyocyte differentiation, maturation and pathological remodeling. With the aid of stem cells, investigators are able to recapitulate events in early cardiac development; with valuable insight in transcriptional regulatory networks directing early cardiomyocyte differentiation.

However, circumstances that determine myocyte maturation in late stage development, which are especially important for understanding diseases and developing cell based clinical applications, are far less well characterized. Induced Pluripotent Stem cell derived cardiomyocytes (iPSC-CMs) are lineage committed but remain immature and fetal-like in molecular, morphological and functional characteristics. Their application in cell-based therapy for heart failure is limited, in

part due to the lack of sufficient insight to promote their maturation into adult myocytes. Although manly discoveries have been made, the underlining mechanisms of the maturation process is still unknown.

In this study, I discovered that mRNA alternative splicing (AS) is a key event in cardiomyocyte maturation and that manipulating AS patterns via a splicing factor, Rbfox1 is sufficient for improved maturation of cardiomyocytes *in vitro*. Furthermore, using Rbfox1 as a case study on the overall maturation gene expression program, I found that a conserved enhancer sequence upstream of the Rbfox1 TSS may serve as a functional maturation transcription upregulation response element (MATURE). Additional investigation found that MATURE is seemingly regulated by a long non-coding RNA (lncRNA) we termed CARMER (Cardiac Maturation Enhancer RNA). Cardiomyocyte maturation is complex and convoluted, without a defined mechanism. Our findings shed light onto the mechanisms involved in shaping an adult heart, and I hope this knowledge will contribute in understanding how to engineer adolescence

The dissertation of Josh Lee is approved.

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2018

To my parents Yantong and Tony, for their unwavering support and infinite love. You are responsible for the best parts of my being.
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Research Intern 2011
Antibody Group, Allergan, Inc
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PUBLICATIONS

• Jing Z, Gangalum RK, Lee JZ, Bhat SP, (2013). Cell-type-dependent access of HSF1 and HSF4 to aB-crystallin promoter during heat shock. Cell Stress and Chaperones 18: 377-87
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• Lee et al., (2018) In Vitro Maturation of Cardiomyocytes by Rbfox1 (In Submission Process, Cell Stem Cell: Impact Factor 22.4)

CONFERENCE PRESENTATIONS

Poster Presentation

• Lee et a., (2018, May) Maturation of Cardiomyocytes by Rbfox1. In: Cold Spring Harbor Asia: Stem Cell Crossroads
• Lee et al., (2018, March) Maturation of Cardiomyocytes by Rbfox1. In: UCLA DAPM Scientific Symposium
• Lee et al., (2017, July) Maturation of Cardiomyocytes by Rbfox1. In: BCVS Scientific Session.
• Lee et al., Maturation of Cardiomyocytes via Modulation of Alternative RNA Splicing (abstract). In: International Society for Stem Cell Research Symposium. Pluripotency: From Basic Science to Therapeutic Applications; 2016 March 22-24; Kyoto, Japan: CiRA/ISSCR; 2016. Abstract P2-025
• Lee et al., (2016, September). Maturation of Cardiomyocytes via Modulation of Alternative RNA Splicing. In: UCLA Cardiovascular Symposium

Oral Presentation

• Presented at UCLA Musculoskeletal Seminar Series, February, 2018

Chapter I

Introduction – Current State of Stem Cell Derived Cardiomyocytes Technology

Background

Heart disease is the number one cause of morbidity and mortality in the United States. There exists a tremendous need for new and more effective therapies to combat this disease which is on average, is responsible for 800,000 deaths a year in the United States [1]. The lack of regenerative potential in adult human hearts [2] has led researchers to explore the possibilities of *de novo* derivation of cardiomyocytes. With the discovery of human embryonic stem cells (hESCs) and more recently the landmark discovery of human induced pluripotent stem cells (hiPSCs) [3-5], researchers have focused efforts to efficiently and reliably derive cardiomyocytes from stem cell sources [6-10].

Applications of Stem Cell Derived Cardiomyocytes

One of the most enticing goals for stem cell derived cardiomyocytes is to provide a source of donor cardiomyocytes as cell replacement therapy for damaged hearts. The loss of terminally differentiated, non-regenerative cardiomyocytes is irreversible and therapeutic regimes are limited in effectiveness, is not curative and heart transplantation remains as the only option for end stage heart failure. In the case myocardial infarctions, cell therapy would need to replace roughly 1 billion cells [11], underlining the importance of for high-throughput and efficient ways to cardiomyocyte production. It also raises the question of effective engraftment upon translation, which to this day remains a major challenge in the field [12, 13]. Other concerns

such as long-term cell survival electrical maturation, electrical coupling and arrhythmia still need to be addressed. In a recent study being done in non-human primates, hiPSC-CMs transplanted into infarcted macaque hearts have shown to cause arrhythmia (Unpublished data presented by Charles E. Murry at BCVS 2017).

A second application of stem cell derived cardiomyocytes lies in its potential for novel cardiac drug discovery, development and safety testing. The pipeline for drug development is long, arduous and expensive, plagued by the inability to mimic the human heart in a reliable and economical method. Many drug programs have faltered during the transition from animal models to human subjects. The average cost to successfully bring a drug to market is estimated to be $1.5 billion, and the drugs that make it to market, many are later withdrawn chiefly due to side effects associated with detrimental effects to heart function [14]. The use of hiPSC-CMs offers the pharmaceutical industry a powerful tool to screen candidate drugs for potential cardiac symptoms and/or secondary therapeutics against cardiotoxicity before heavy investments into clinical trials. With many such undertakings already reported [15-17], the use of hiPSC-CMs in this process will only become more prevalent in the foreseeable future.

A third application for this technology is the modeling of disease using iPSC-CMs derived from patients of both known and unknown cardiac diseases. The promise of using patient's own fibroblast generated hiPSC-CMs to conduct electrophysiological and molecular studies can open new ways to uncover the mechanisms of disease [18-20]. In addition, the use of these cells in novel drug discovery and efficacy testing is potentially a revolutionary step in personalized medicine.

The excitement around stem cell derived cardiomyocytes has yielded tremendous progress in all three areas of application. However, many roadblocks still exist. Chiefly among them is the fact

that stem cell derived cardiomyocytes are not able to mature and resembles fetal cardiomyocytes. This phenomenon of being permanently "stuck" in development limits the capacity and effectiveness of stem cell derived cardiomyocytes in therapeutic as well as *in vitro* modeling/screenings.

Characteristics of Stem Cell Derived Cardiomyocytes

Despite the excitement around stem cell derived cardiomyocytes and the potential it holds, the cells remain immature and resembles fetal cardiomyocytes [21]. This inability to recapitulate postnatal maturation seen in adolescence has impaired the advancement of this field. In this section, I will attempt to dissect and highlight the differences between human pluripotent stem cell derived cardiomyocytes (hPSC-CMs) and adult human cardiomyocytes.

Morphology

The morphological difference of hPSC-CMs and adult ventricular cardiomyocytes are stark (Fig. 1). The growth of post-natal heart is predominantly contributed by physiological hypertrophy and up to a 30-40 fold increase in cardiomyocyte size [22]. It is important to note that this dramatic increase in cell size is distinct from pathological hypertrophy observed in disease [23]. The dimensions for hPSC-CMs are oblong and usually 30 μm x 10 μm [21], significantly smaller than the cylindrical adult cardiomyocytes (150 μm x 10 μm) [24]. In addition, most mature cardiomyocytes are bi-nucleated, whereas hPSC-CMs remain mono-nuclear. Also, hPSC-CMs lack a robust and organized sarcomere and the network of t-tubules distinctly featured in adult ventricular cardiomyocytes is absent in hPSC-CMs. As a result, excitation-contraction coupling in these cells is slower, and calcium cycling is weak due to the fact that calcium enters the cells

through the sarcolemma instead of being released from the sarcoplasmic reticulum (SR).

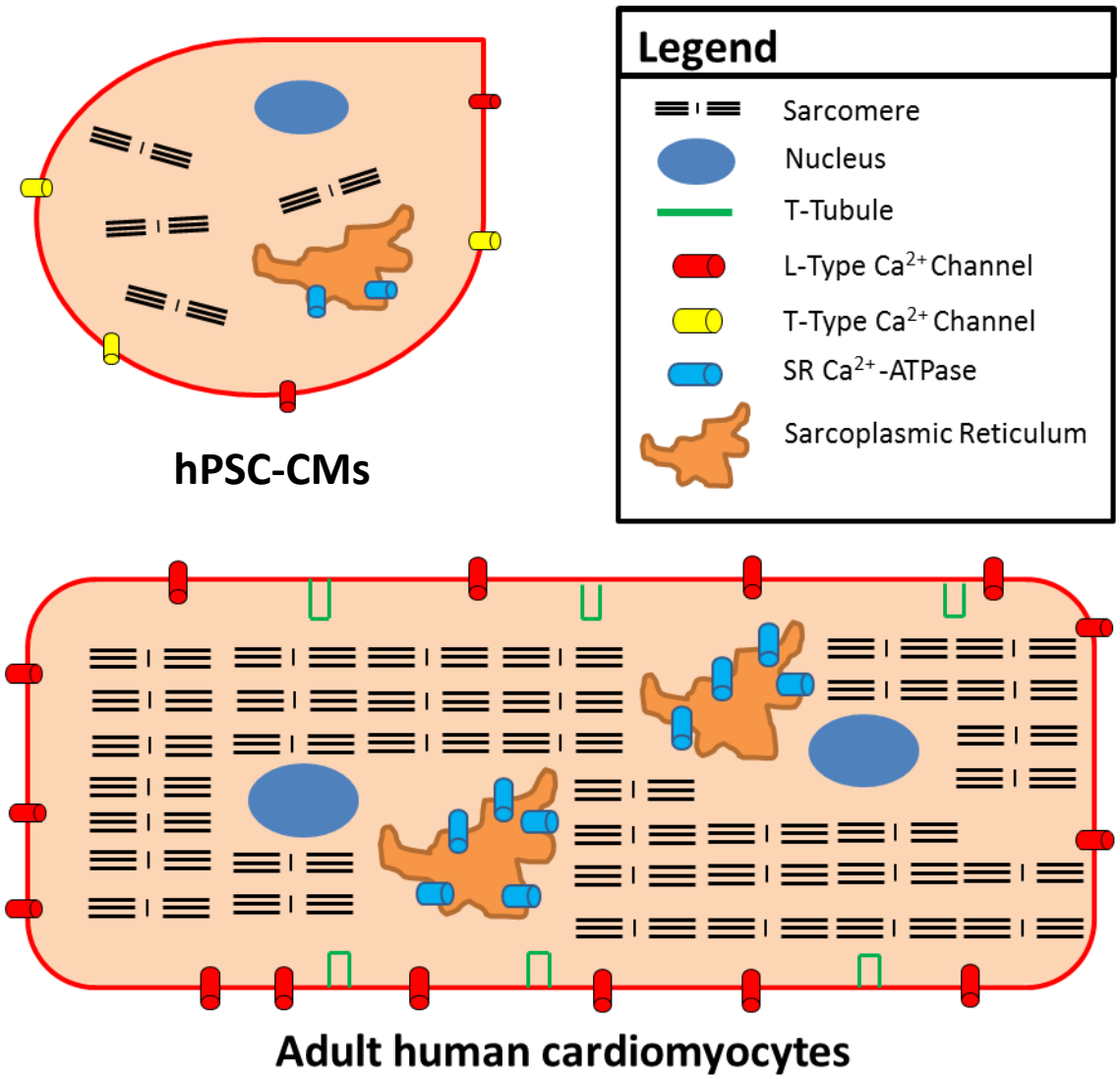

Figure 1. An illustration of the morphological differences of hPSC-CMs and adult ventricular cardiomyocytes. hPSC-CMs lacks the size (not to scale) and the cylindrical shape of adult cardiomyocytes, is mono-nuclear rather than bi- or multinuclear, lacks a rand organized sarcomere, network t-tubules and key channel proteins are lowly expressed. Therefore, the physiological and functional properties of hPSC-CMs resembles that of a fetal cardiomyocyte

Proliferative capacity

Early stage hPSC-CMs have proliferative potential [25], but gradually loses the ability as it matures in culture, decreasing from a 24-48 hour doubling time to very low levels (10% of cells were $BrdU^+$ at 4 weeks in culture) [25], similar to changes seen in fetal cardiac development [26]. In addition, human cardiomyocytes proliferate for the first few months after birth and begins to exhibit bi-or multinucleation, the percentage of cardiomyocytes with this phenomenon has been debated, varying from a range of 25% to 60% [27]. Furthermore, mature human cardiomyocytes have been observed to have polyploidy, the result of DNA replication without nuclear division or cytokinesis, the levels of ploidy can reach 8N in healthy hearts.

Gene Expression

The differences between hPSC-CMs and adult ventricular cardiomyocytes is stark. However, the most of what we and others have observed are not drivers of transcriptional regulation, rather they are downstream consequences of the overall gene expression program. Among them are structural and sarcomere genes, ion transporters and channels, calcium handling and sarcoplasmic reticulum associated proteins [28-30]. Compiled in Table 1 are highlighted genes differentially expressed in hPSC-CMs and adult cardiomyocytes.

	Genes Upregulated in Adult Heart Compared to hPSC-CMs
Structural	MYL2, TNNI3, ACTN2, MYH7, MYL3, TNNC1, TNNT2, MYH11, SORBS1, MYOM2, MYOM1, TCAP, DES
Ion Transport	KCNA4, KCNA5, KCNAB1, KCNAB2, KCND2, KCND3, KCNE4, KCNG1, KCNH2, KCNH7, KCNIP2, KCNJ2, KCNJ3, KCNJ5, KCNJ8, KCNK1, KCNQ1, KCNV1, SCN1A, SCN1B, SCN2B, SCN3A, SCN4B, SCN5A, HCN1, HCN4, CACNA1C, CACNA1D, CACNA1H, CACNA1G, CACNA2D1, CACNB2 SLC8A1, TRPC3, TRPC4, TRPC6, CFTR
Calcium Handling (SR)	ATP2A2, PLN, CASQ2, RYR2, RYR3, TRDN, ITPR1, ITPR3, ASPH, S100A1, HRC, S100A1

Table 1. Selected major cardiac genes enriched in adult human heart vs. hPSC-CMs.

Electrophysiological Properties: Action Potential

Due to the insufficient expression of ion transport and calcium handling genes illustrated in Table 1, the electrophysiological properties of hPSC-CMs differs significantly from adult cardiomyocytes. In the best cases, late stage hPSC-CMs have an observed maximum diastolic potential of approximately -60 to -75 mV [31], compared to a consistently observed -85 mV in adult ventricular cardiomyocytes [32]. Along with MDP, hPSC-CMs have a much slower maximum rate of depolarization (dv/dt_{max} or V_{max}), ranging from 10-40 V/second [31, 33] compared to 300 V/second in healthy adult hearts. [34]

Electrophysiological Properties: Calcium Handling

Expression of calcium channels necessary for contractility, such as NCX and HCN is lower in hPSC-CMs when compared to adult cardiomyocytes [29]. Furthermore, calcium induced calcium release (CICR) from the sarcoplasmic reticulum is extremely weak in hPSC-CMs, with calcium transients that are smaller and slower, relying mostly on diffusion of ions through the cell membrane [35-38]. This passive and abnormal diffusion of calcium reduces synchrony in contraction and results in a significantly weaker force generation [39].

It is important to note that when paced, adult cardiomyocytes show a positive force-frequency relationship. As pacing rates increased, a greater calcium transient and force of contraction are seen [40], a product of large amounts of intracellular calcium stores, expression and localization of key transporter proteins and electrical coordination across the cell. In contrast, hPSC-CMs have poorly ion transporter expression and coordination, low levels of intracellular calcium storage and exhibit a negative force-frequency relationship [41]. As pace frequency increases, the cellular machinery to cycle calcium stalls, resulting in a slower calcium transient and weaker force of contraction.

The inefficient handling of calcium in hPSC-CMs is at least partially due to the fact that the sarcoplasmic reticulum (SR) proteins in these cells are largely unorganized or have low levels of expression. RYR expression is observed, but only at a small fraction of the adult cardiomyocyte level [42], and the colocalization of RYR with L-type calcium channels, which would allow for effective CICR, is observed with conflicting results and is debated in the field [24, 43, 44]. Much like RYR SERCA, the Ca^{2+} ATPase Pump is also lowly expressed in hPSC-CMs, at comparable levels to fetal cardiomyocytes [44]. Calsequestrin, which binds calcium ions for dense packing and storage in the SR, along with Phosphlamban, an endogenous inhibitor of SERCA are largely missing from studies [36, 44].

Contractile Force

In both immature and mature cardiomyocytes, force generation are used as an important functional readout. Previous findings of a strip of human myocardium measures the contractile force to be 44 ± 11.7 mN/mm^2, and 56.4 ± 4.4 mN/mm^2 for rat myocardium [45]. In studies involving hPSC-CMs the results were inconsistent, but shows immaturity when compared to adult cardiomyocytes. The reported force generation of these cells ranged from 0.08 nM/mm^2 to 0.62 nM/mm^2 in a sheet or construct [46-49]. Different strategies of maturation have been applied to increase contractile force, interestingly, similar methods have yielded vastly different results, ranging from marginal increases of 1.34 nM/mm^2 to more mature contractile force measurements of 23.2 nM/mm^2 [50-54].

Strategies for Maturing hPSC-CMs

The hPSC-CMs have remained immature and fetal-like since their discovery almost two decades ago[6]. The tremendous challenge of realizing the potential of this technology now shifts from derivation to maturation. The difficult task of engineering adolescence is in large part due to the lack of understanding on the drivers of postnatal cardiac maturation. The cardiac differentiation process from pluripotent stem cells is marked by a distinct transcription factor signature (Figure 2). However, as progenitor cells reach terminal differentiation this transcription factor signature disappears (Figure 2). This has limited the deployable strategies used to mature hPSC-CMs *in vitro*, with methods focusing on improving isolated parameters and relying heavily on hypertrophic signaling, mechanical manipulations and engineered niches [55]. This has yielded incremental but marginal improvements in the maturation of hPSC-CMs, with little molecular insight on how the gene program is implemented and controlled. In the following section, I will highlight the different strategies reported to engineer adolescence in hPSC-CMs.

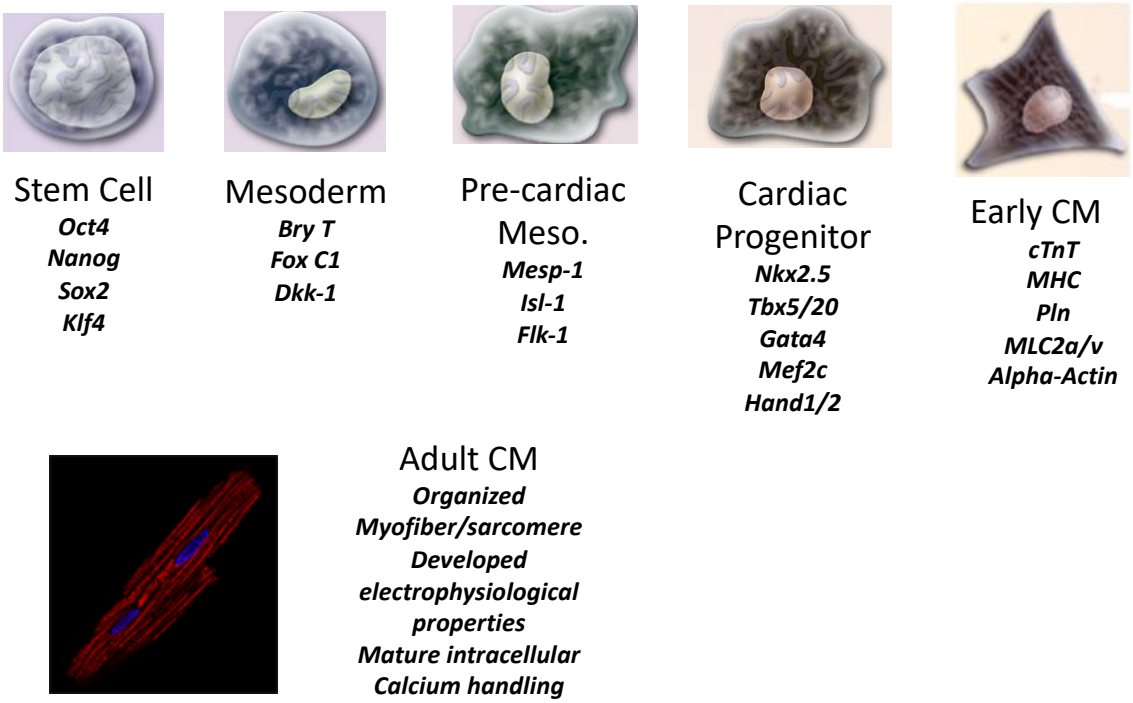

Figure 2. Each stage of differentiation from stem cell to cardiomyocyte is defined by the expression of distinct transcription factors that drives the expression program. However, as cells commit to the cardiac lineage, this distinct signature disappears. In its stead are expressions of genes as consequences of the gene program.

Long Term Culture

Long-term culturing of hPSC-CMs has been reported to induce maturation *in vitro*. Culturing of hPSC-CMs up to one year showed the presence of M-bands containing sarcomeres, a distinctive feature of sarcomeric structural maturation in adult cardiomyocytes [56]. Fetal cardiomyocytes are marked by double positive expression of the structural genes MLCv and MLC2a, around 56% of day 30 hPSC-CMs have these two myosin light chain proteins expressed whereas the one-year prolonged culture cardiomyocytes have only around 36% double positive in its population. In addition, one-year cultured cells also had well-organized myofibrils and a significantly increased size when compared to shorter cultures of 14, 30, 60, 90 and 180 days

[56]. Long-term culturing of hPSC-CMs has also improved functional characteristics, calcium cycling and action potential amplitudes and upstroke velocities has also been observed [57].

Substrate Stiffness

Cardiomyocyte maturation coincides with dynamic environment changes in development. For example, collagen accumulates from embryonic development until several weeks after birth. This, along with other niche changes result in a twofold increase in passive stiffness from neonatal rat hearts to adult rat hearts [58]. It is then not a surprise that reports show hPSC-CMs develop a more mature profile on substrates that are comparable to its native myocardium of 10 kPa [59, 60]. In a related study examining polyacrylamide hydrogels with a range of stiffness from 4.4 to 99.7 kPa, NRVMs and hPSC-CMs were shown to have the greater mechanical force on gels with higher stiffness, and cell area was the greatest on a substrate stiffness of 49.4 kPa [61]. Interestingly, contrary to previous studies, this group observed well defined sarcomeres on all gel stiffnesses.

Cell Patterning

It is well known that adult cardiomyocytes *in vivo* maintain a distinct rod-like shape. The shape of cardiomyocytes have been proven to regulate sarcomere alignment [62], and with a cell length to width ratio of 7:1 (approximate of what occurs naturally) cardiomyocytes exhibit directional anisotropy, scientists have utilized this to develop cell culture systems that mimic the cell alignment of cardiomyocytes in native tissue via substrate patterning as well as topographical nanogrooves. This has yielded culturing systems in NRVMs as well as hPSC-CMs with better

morphology, bipolar localization of connexin 43 and N-cadherin as well as connexin 43 expression [63-65].

Electrical Stimulation

Electrical stimulation is essential for cardiomyocyte function, electrical pulses regulate the rhythm of cardiomyocyte contraction. Subjugating isolated rat cardiomyocytes to electric pacing while in culture improved contractility and sarcomere structure when compared to cardiomyocytes cultured in absence of electrical stimulus[66]. Notably the up-regulation of myosin heavy chain 7 (Myh7) and myosin light chain 2 (Myl2) was observed and potentially contributed to the organization of the sarcomere structure. In hPSC-CMs it was reported that prolonged electrical stimuli yielded cardiomyocytes with longer action potential duration and higher calcium influx and a more matured gene expression profile including the up-regulation of SERCA, Kv4.3, HCN1 and SCN5A [67].

Combinatory Engineering Approaches

Substrate context, patterning and electrical stimuli have all yielded marginal improvements in hPSC-CM maturation *in vitro*. It was clear that manipulating the niche in which cardiomyocytes are cultured in can improve overall maturation. In line with this reasoning are strategies to engineer a more comprehensive environment to culture and mature hPSC-CMs. The proof of concept was elegantly done with NRVMs cast in hydrogels of collagen I plus basement membrane proteins. The cells were subjected to phasic mechanical stretch to generate an engineered heart tissue (EHT) [68]. Similar strategies were employed on hPSC-CMs with three general features. 1) Using force dynamics to develop advanced cardiac muscle features [69, 70];

2) Manipulation of the cardiac microenvironment by engineering heart-on-chip systems [52, 71, 72]; 3) Development of systems which have direct clinical applications, such as cell patches [73] or platforms for pharmacological screening [52, 69, 71, 74, 75]. These methods are all successful in improving upon maturation parameters such as morphology, cell size, calcium handling, contractile force and electrophysiology. However, the complexities of natural adolescent growth cannot be fully replicated, therefore most improvements are only marginal and on isolated parameters. It remains imperative to understand the overall mechanism of maturation and not overly relying on piecemeal developmental cues to inch it forward.

Biochemical Stimulation

A variety of biochemical and molecular methods have seen success in attaining moderate maturation in hPSC-CMs. I will summarize the key finds in this section.

Adrenergic receptor (AR) agonists: Adrenergic agonists have been well characterized in rodent cardiomyocytes and are well utilized models for pathological hypertrophy. However, manipulation of the adrenergic receptor does induce certain physiological changes in line with physiological maturation. Administration of the α-AR agonist phenylephrine in hPSC-CMs resulted in a 2-fold increase in cell area, 3.8-fold increase in cell number with organized sarcomere structure, and a 2-fold increase in cell volume [76].

Insulin-like Growth Factor I (IGF-1): IGF-1 is an essential regulator of cardiomyocyte proliferation, differentiation and postnatal maturation. NRVMs treated with IGF-1 has shown an increase in MLC-2 and troponin-I, significant cell size increase and an increase in protein synthesis [77]. The effect of IGF-1 on hPSC-CMs is similar, with a recently study reporting that

IGF-1 treatment increases proliferation, and together with Neuregulin-1 (NRG1) was able to induce a more mature gene expression, metabolic profile and cell size [78].

Neuregulin-1 (NRG-1): Much like IGF-1, NRG-1 is much involved in normal cardiac development. It acts as a cardioactive growth factor produced and secreted by cardiac endothelial cells only within the endocardial vessels [79]. NRG-1 not only can successfully differentiate iPSCs into cardiomyocytes, but the cardiomyocytes produced have a more ventricular phenotype and more mature electrophysiological properties[80].

Triiodothyronine (T3): Thyroid hormone plays an essential role in normal cardiac development. It promotes the maturation of fetal cardiomyocytes in sheep fetus, by reducing their proliferative capacity towards the end of gestation [81]. T3 also functions by regulating the isoform switch of key myocardial proteins such as myosin heavy chain and titin[82]. It was reported that hiPSC-CMs showed increase in size, sarcomere length, contractile force and calcium cycling ability, while decreasing in proliferation rate and cyclin-dependent kinase activity [83]. More recently, it was reported that a combination of T3 and Glucocorticoid is able to promote t-tubule development in hiPSC-CMs [84].

MicroRNA (miR): MicroRNAs have been known to regulate gene expression in development, a study performed thorough transcriptome analysis of human ESCs, hESC-CMs along with fetal human and adult human ventricular cardiomyocytes identified miR-1 as a potential modulator in cardiomyocyte maturation [85]. Overexpression of miR-1 resulted in decreased action potential duration and hyperpolarized the resting membrane potential and the maximum diastolic potential in hESC-CMs. Furthermore, miR-1 improved the immature Ca^{2+} transient amplitude and kinetics. In another similar study, transcriptome profiling revealed the let-7 family of microRNAs is required for maturation of stem cell derived cardiomyocytes. Overexpression of

let-7i and let-7g in hPSC-CMs were able to induce maturation in a myriad of parameters, including cell size, morphology, gene expression, action potential duration, force generation and fatty acid metabolic switch [86]. This represents the most convincing discovery of a global regulator of the maturation gene program in cardiomyocytes, and revealed valuable insight on the potential and the importance of molecular signals in promoting hPSC-CM maturation.

Chapter II

Maturation of Stem Cell Derived Cardiomyocytes via Alternative Splicing

Introduction

Cardiovascular disease is the leading cause of death in the world with a dearth of effective therapies. Heart undergoes a complex differentiation and maturation process throughout embryonic and post-natal stages. Intense efforts have been made in the study of cardiomyocyte differentiation, maturation and pathological remodeling [87-90]. With the aid of stem cells, investigators are able to recapitulate events in early cardiac development; with valuable insight in transcriptional regulatory networks directing early cardiomyocyte differentiation[91, 92]. However, circumstances that determine myocyte maturation in late stage development, which are especially important for understanding diseases and developing cell based clinical applications, are far less well characterized. Pluripotent stem cell derived cardiomyocytes (hPSC-CMs) are lineage committed but remain immature and fetal-like in molecular, morphological and functional characteristics [93, 94]. Their application in cell-based therapy for heart failure is limited, in part due to the lack of sufficient insight on the regulator mechanisms to promote their maturation into adult myocytes.

Alternative mRNA Splicing (AS) have emerged as a critical process in gene regulation [95, 96], it allows for fine-tuning and more subtle changes to occur in the transcriptome without impacting overall cell identify. AS events have been shown to play central roles in regulating cell cycle control, apoptosis and cell fate decisions. Furthermore, its involvement in postnatal cardiomyocyte development and health has become increasingly evident [97-99]. Indeed,

postnatal cardiomyocyte is marked by global AS transitions [98, 100-102] and key RNA splicing regulators exerted profound impact on global transcriptome programming during cardiomyocyte maturation as well as disease progression [101].

In this study, we explored the capacity of alternative splicing as a previously unknown driver of cardiomyocyte maturation *in vitro*. By analyzing large-scale transcriptomic datasets comparing neonatal and adult rat cardiac tissue, we first showed that mRNA splicing is a key difference between the immature and mature rodent heart. Furthermore, we identified a splicing "switch" that occurs during cardiomyocyte maturation which involves the induction of a conserved and muscle specific splicing factor, Rbfox1. Because Rbfox proteins has been previously associated with heart development and health [97, 103, 104], we selected it as a prime candidate for further investigation. We found that Rbfox1 is largely nonexistent in immature cardiomyocytes, and overexpression (OE) of Rbfox1 for just 1 week in neonatal rat ventricular myocytes (NRVMs) was able to significantly increase cell size, sarcomere organization, induce mature gene expression and improve contractile and calcium handling abilities. More importantly, OE of Rbfox1 in human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) for 1 week was also able produce similar changes in promoting maturation gene expression, sarcomere organization and calcium handling. Further transcriptome profiling of Rbfox1 matured cardiomyocytes and adult cardiomyocytes displayed a significant overlap of gene expression patterns, illustrating the global regulatory effect of key splicing factors. The analysis also revealed a select group of upregulated immune related genes previously uncharacterized, hinting at a previously unknown mechanism governing postnatal cardiac maturation. Altogether, our results indicate that Rbfox1 as a novel endogenous regulator that can accelerate maturation of cardiomyocytes *in vitro*.

Results

RNA Splicing is Induced Adult Cardiomyocytes. To examine the full extent of the transcriptomic changes during cardiac maturation, we employed an Illumina deep RNA sequencing platform to profile the major differential gene expression changes between day 1 neonatal rat heart and 8 weeks old adult rat heart. The analysis discovered more than 6000 differentially expressed genes with a p-value less than 0.05 (Supplemental Dataset S1). I then performed gene ontology (GO) analysis on the upregulated and downregulated genes. Top upregulated genes are associated with fatty acid beta-oxidation, mitochondrial electron transport and other key metabolic processes, while top downregulated genes are predominantly associated with cell cycle and replication. This result was unsurprising, considering cell cycle arrest and a metabolic switch as well as an increase in metabolic capacity are hallmark consequences of cardiomyocyte maturation. Surprisingly, negative regulation of mRNA splicing was also among the top enriched GO pathways in the downregulated genes (Figure 1A). This result hints at a facet of maturation that is previously uncharacterized. Furthermore, alternative mRNA splicing changes has been shown to be drivers of biological processes and critical regulators of gene expression [95, 96], therefore we further examined changes in RNA splicing factors in our dataset. Rbfox1, a conserved muscle specific splicing factor was identified to be one of the highest genes induced in adult vs. neonatal heart. Its previously reported role in cardiac health and development made it a prime target for further investigation.

Rbfox1 Induction is Conserved in Human Cardiomyocyte Maturation. To examine whether Rbfox1 induction is conserved in human cardiomyocyte maturation, we used long term culture of hiPSC-CMs as a model to simulate maturation [56]. We found that much like rodent

cardiomyocytes (Figure 1B), hiPSC-CMs show increased RBFOX1 expression during after 9 days in culture (Figure 1C). However, the overall levels of RBFOX1 remain extremely low in these hiPSC-CMs, and they remain largely fetal-like. Interestingly, Rbfox2, a ubiquitously expressed member of the Rbfox family of splicing factors is downregulated as Rbfox1 is induced, suggesting a coupled alternative splicing switch between the two proteins.

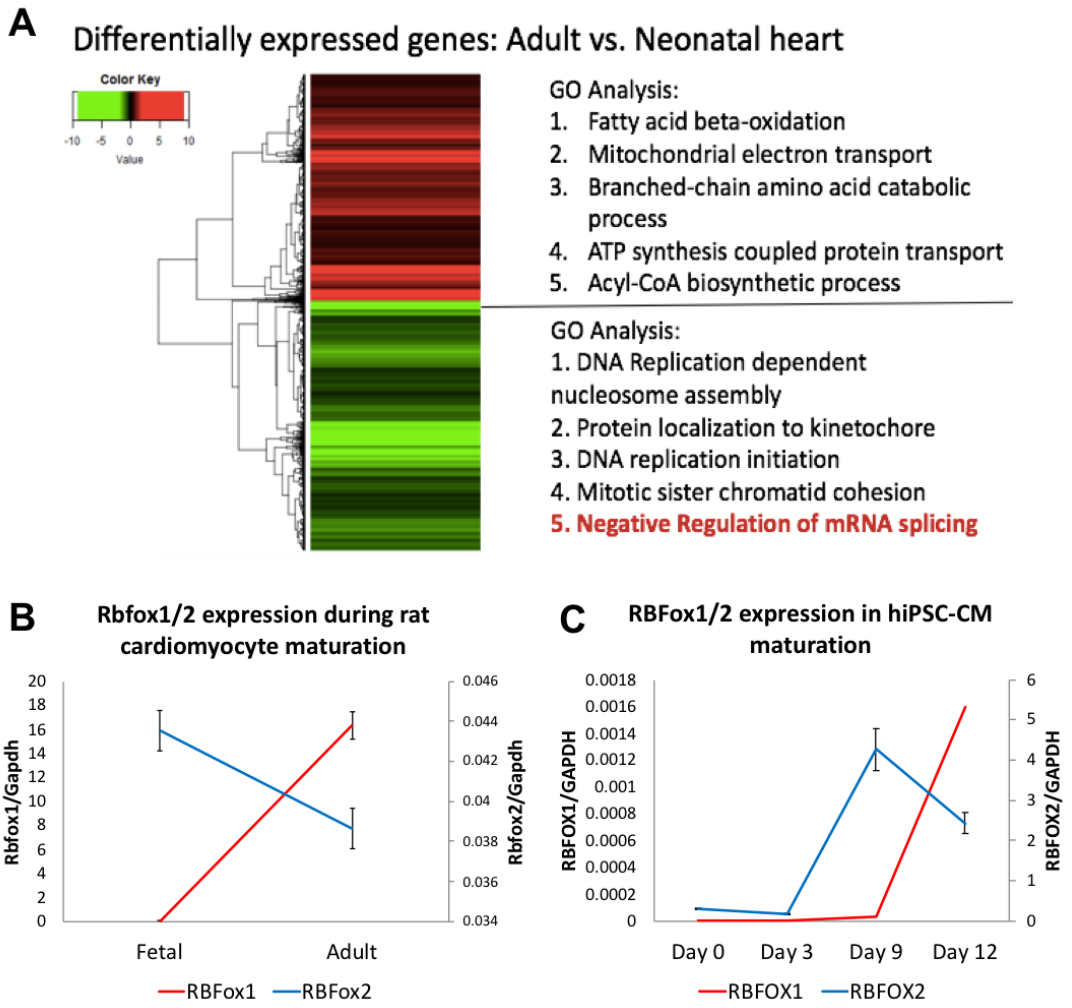

Figure 1. Rbfox1 Induction in Cardiomyocyte Maturation is Conserved.
A) RNA-Seq analysis of immature vs adult rodent heart reveals mRNA splicing is potentially a driver of cardiomyocyte maturation. B) Rbfox1 expression is induced only in adult rat heart, while Rbfox2 expression is downregulated. C) Similar pattern of Rbfox1 induction coupled with Rbfox2 downregulation is seen in long-term culture of hiPSC-CMs, a common model of maturation.

Rbfox1 Overexpression Promotes Maturation in NRVMs. To test if Rbfox1 can promote maturation in cardiomyocytes *in vitro*, I employed adenovirus expressing Rbfox1 (Adv-Rbfox1) as a method for ectopic OE. NRVMs were infected with Adv-Rbfox1 alongside control adenovirus (Adv-GFP) and cultured for 7 days (Figure 2A). At the end of day 7, cells were tested for various maturation parameters. By day 7, Rbfox1 OE NRVMs displayed significantly more mature morphology, greater cell size, increased beating and more organized sarcomere (Figure 2B-E). Further investigation into gene expression by qPCR showed upregulation of a cohort of known maturation markers, including critical genes involved calcium handling and energetics (Figure 2F-H). To further understand gene expression changes by Rbfox1 OE in NRVMs we utilized Illumina RNA sequencing to elucidate its effects in cardiomyocytes. Strikingly, Rbfox1 promoted global changes in alternative mRNA splicing and accounted for 21% of all maturation associated splicing events (Figure 3A) and 23.4% of differential gene expression associated with maturation (Figure 3B). More importantly, the effect of Rbfox1 promoted maturation is distinct from phenylephrine (PE) induced hypertrophy commonly used to boost cardiomyocyte function *in vitro*, but is well documented as an agent for pathological hypertrophy (Figure 3C). These data suggest Rbfox1 is able to control a distinct facet of the cardiomyocyte maturation process.

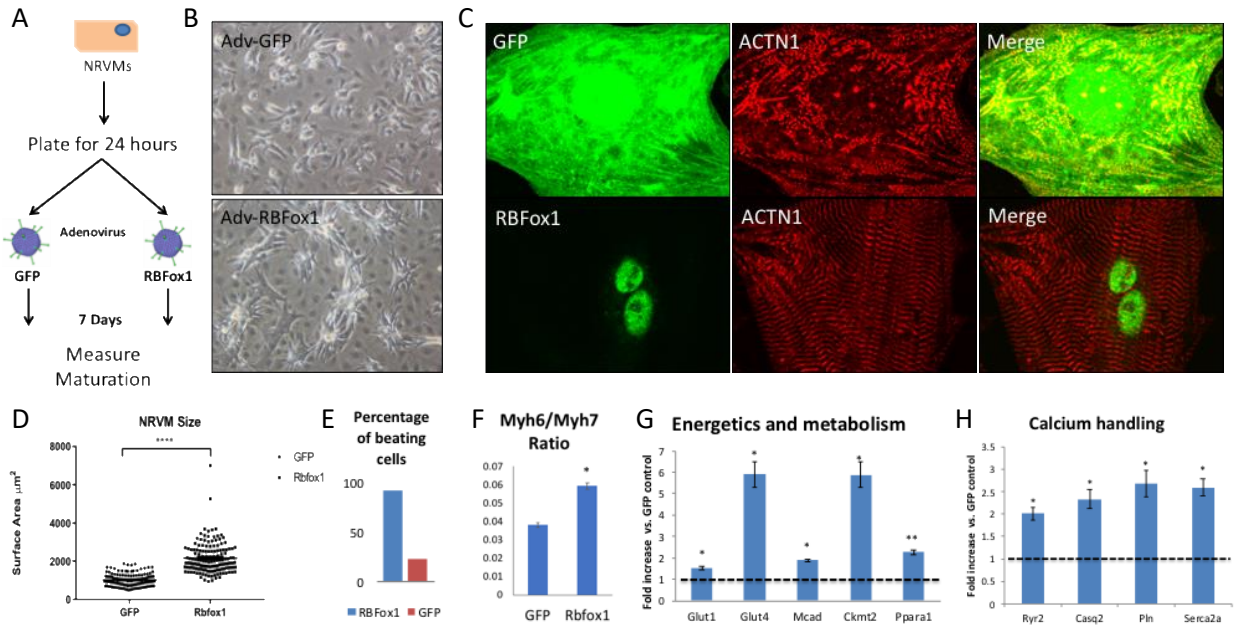

Figure 2. Rbfox1 promotes maturation in NRVMs. A) Schematic overview of Rbfox1 OE and culture in NRVMs. (B) Rbfox1 OE in NRVMs for 7 days in culture is able to promote mature morphology. Cells become more elongated and rod-like in shape. C) Sarcomere Organization is enhanced after Rbfox1 OE. Cells were stained with alpha-actinin which marks the sarcomere. Percentage of cells with organized sarcomere in Rbfox1 OE vs. control are 78% to 2%, respectively. D) NRVM cell size was measured using ImageJ after staining with WGA, 150 cells were measured for Adv-Rbfox1 and Adv-GFP each and Rbfox1 significantly increases NRVM cell size by an average of more than 70%. **** $p<0.0001$ (D) Dramatic rhythmic beating was observed in Adv-Rbfox1 treated NRVMs, where close to 100% of the cells beat in sync (F-H) Gene expression was measured by qPCR and Rbfox1 promotes a cohort of maturation associated gene expression as well as Myh6/Myh7 ratio, which is a hallmark for cardiomyocyte maturation. * $p<0.05$, ** $p<0.01$

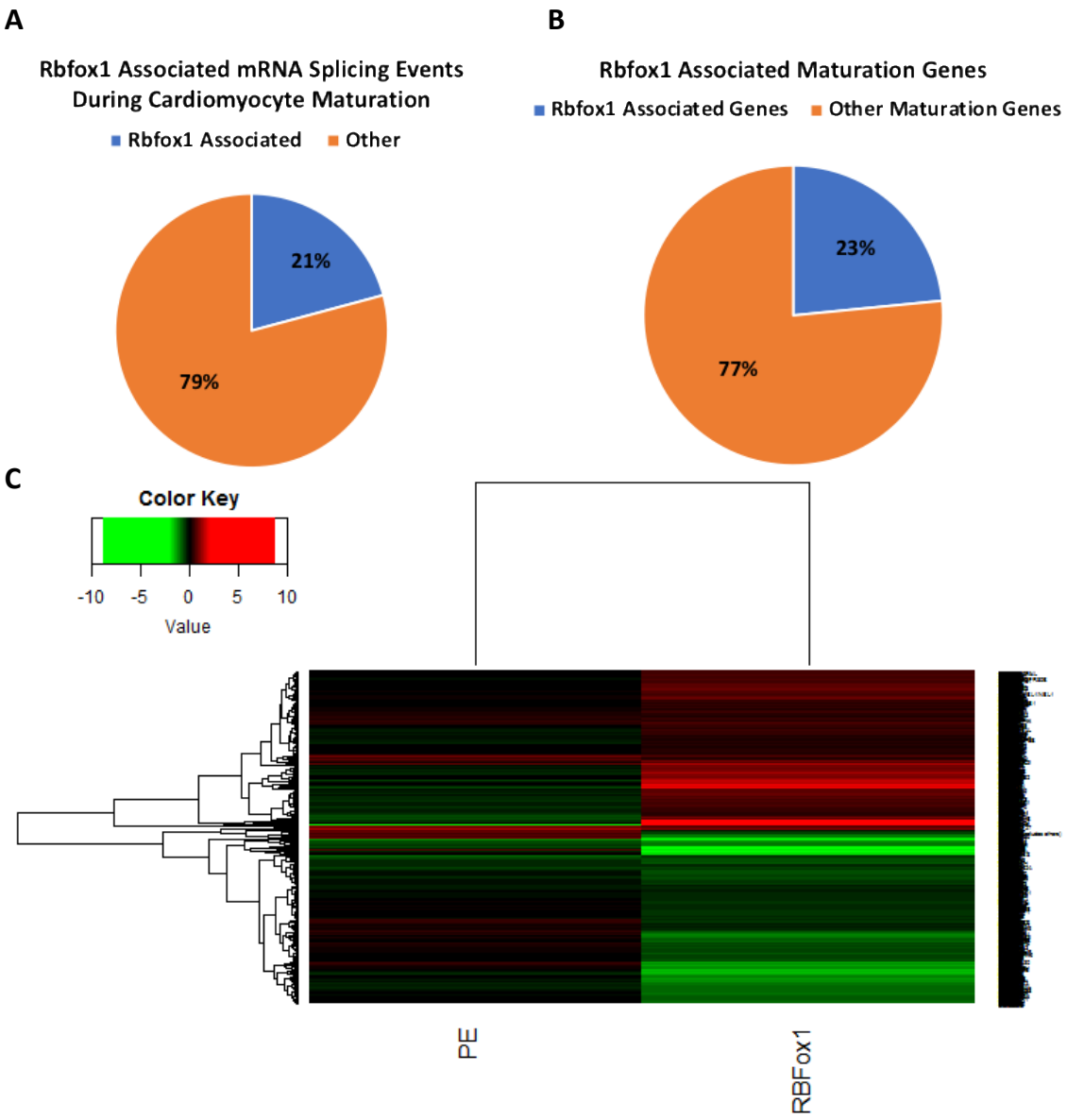

Figure 3. Rbfox1 induces global transcriptome alterations in line with maturation and is a distinct pathway from PE induced pathological hypertrophy. A) Rbfox1 OE in NRVMs is responsible for 21% of all observed maturation associated mRNA splicing events and B) 23% of all maturation associated differential expressions. C) Rbfox1 OE operates in a distinct gene regulatory system than PE, and is not associated with PE induced pathological hypertrophy.

Rbfox1 Improves Contractile and Calcium Handling Functions in NRVMs. To test whether the improvements made by Rbfox1 have functional outcomes in NRVMs we constructed a platform to test contractile and calcium handling properties (Figure 4A). The platform is made from a hydrogel mattress at 10kPa in stiffness [59, 60] and coated with Matrigel. The mattress is then loaded onto a platform culturing well with graphite electrodes attached. This allows live cells to be paced up to 2 Hz and be recorded via a bright field or fluorescence microscope. Using this apparatus, we studied the contractile capabilities of Rbfox1 matured NRVMs vs. GFP control. NRVMs were again infected with Adv-Rbfox1 and Adv-GFP and kept in culture for 7 days on the Matrigel mattress, they were loaded onto the pacing apparatus and paced at 0.6 Hz, results were recorded, and the video was analyzed by an in-house software program measuring the movement of the cells while paced. This allowed us to gauge if the NRVMs are able to contract properly while being paced at a fix frequency. Rbfox1 OE NRVMs showed rhythmic, on pace contraction throughout the recording, while GFP OE NRVMs started with contractions in line with the electrical stimulus but soon deteriorated (Supplemental Video S1, Figure 4B). This shows that Rbfox1 is able to improve contraction of cardiomyocytes.

Robust calcium handling is an important aspect of cardiomyocyte maturation, while Rbfox1 can increase the expression of calcium handling genes it was still unknown if it has a functional impact. We tested the ability of Rbfox1 to improve calcium handling in NRVMs with the same Matrigel mattress and pacing apparatus described above. Fluo-4 calcium dye was used to visualize calcium cycling, the video was recorded with a Nikon fluorescence microscope and calcium transient was analyzed with ImageJ plugin: Intensity vs. Time Plot and cardiomyocyte synchrony is visualized with the ImageJ surface plot function. Rbfox1 OE resulted in significantly more robust calcium cycling capabilities at both frequencies of 0.8 Hz and 2 Hz.

When compared to lacZ OE (Used because Fluo-4 and GFP conflicted in fluorescence), Rbfox1 OE NRVMs displayed a homogenous population of paced cardiomyocytes with better ability to both influx and clear calcium from the cytoplasm (Figure 4C, 4D, Supplemental video S2-S5). To further validate the improvements to synchronization and calcium cycling, western blots against Connexin 43 and SERCA2a was performed. Connexin 43, or Gap junction alpha-1 protein, is a gap junction associated protein which facilitates intercellular communication and synchronized contraction of the heart [105]. SERCA2a is the essential Ca^{2+} ATPase on the SR for moving Ca^{2+} from the cytosol to the lumen of the SR, it serves a critical role in regulating intracellular calcium cycling. In Rbfox1 OE NRVMs, both Connexin 43 and SERCA2a observed a significant increase (Figure 4E, 4F), explaining the functional maturation observed. Taken together, these data suggest that Rbfox1 OE has a direct functional impact on the maturation of immature cardiomyocytes *in vitro*.

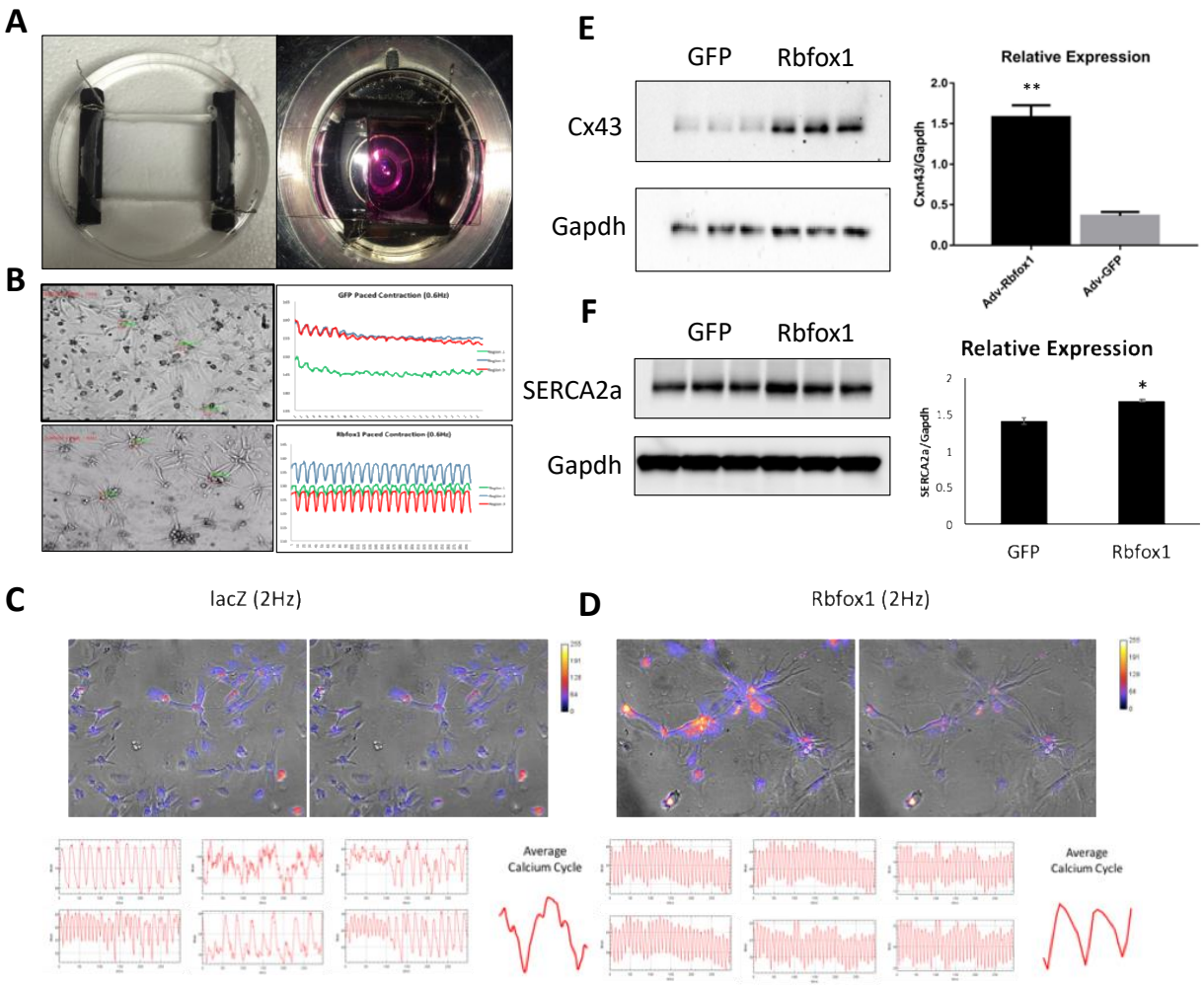

Figure 4. Rbfox1 promotes improved contraction and calcium handling in NRVMs. A) Schematic of pacing apparatus used to measure cardiomyocyte function. B) Rbfox1 OE allows for continuous rhythmic contraction while paced at 0.6 HZ. C-D) Rbfox1 OE induces mature calcium handling. Calcium transient measurements obtained shows a greater synchronization and robust calcium handling capabilities. E-F) Rbfox1 promotes more expression of gap junction protein Connexin 43 which is critical for cardiomyocyte synchronization and SERCA2a, the Ca^{2+} ATPase pump essential for calcium cycling through the SR.

Rbfox1 Regulates a Novel Maturation Gene Program. To understand how Rbfox1 is driving maturation and implementing a vast gene expression change we utilized GeneAnalytics™ to analyzed which GO pathways are most prevalent among the differentially expressed genes in Rbfox1 OE that also correlated with expression patterns in Adult rat heart. The results showed that correlated down-regulated genes were enriched for heart cellular proliferation and early heart development, this is in line with our expectations of cardiomyocyte maturation. However, correlated up-regulated genes were heavily enriched in immune pathways. The list includes antigen presenting and processing, Interferon I signaling, protection from natural killer cell and Il-1 secretion (Dataset S8). This immune regulatory signature that exists in late stage cardiomyocyte development has yet to be explored and deciphered. The data suggests that Rbfox1 utilizes this previously unknown mechanism as an important facet of the maturation gene program.

Rbfox1 OE Matures hiPSC-CMs Similarly to NRVMs. To examine if the phenomenon of maturation via Rbfox1is applicable to hiPSC-CMs we used a similar protocol used in NRVMs for Rbfox1 OE in hiPSC-CMs (Figure 5A). Rbfox1 OE had minimal effect on cell size but did exhibit more organized sarcomere (Figure 5B), and mature gene expression (Figure 5C). Calcium handling parameters investigated using the Matrigel mattress showed that Rbfox1 matured hiPSC-CMs are able to handle calcium properly at 2 Hz, while lacZ OE control failed to keep pace, and is cycling calcium at a reduced frequency (Figure 5D, Supplemental Video S6, S7). We also measured time to peak and time to baseline for Rbfox1 OE vs. lacZ OE hiPSC-CMs calcium transients using the Ionoptix system. This serves as a quantification of calcium influx and clearance capabilities, and a meaningful measurement of maturation. Rbfox1 OE cells

displayed a significant reduction in time to baseline (Figure 5E), and a clear trend of reduced time to peak. Taken together, we believe that the splicing factor Rbfox1 is sufficient to drive cardiomyocyte maturation *in vitro*, and that alternative mRNA splicing is a novel mechanism in regulating postnatal heart development.

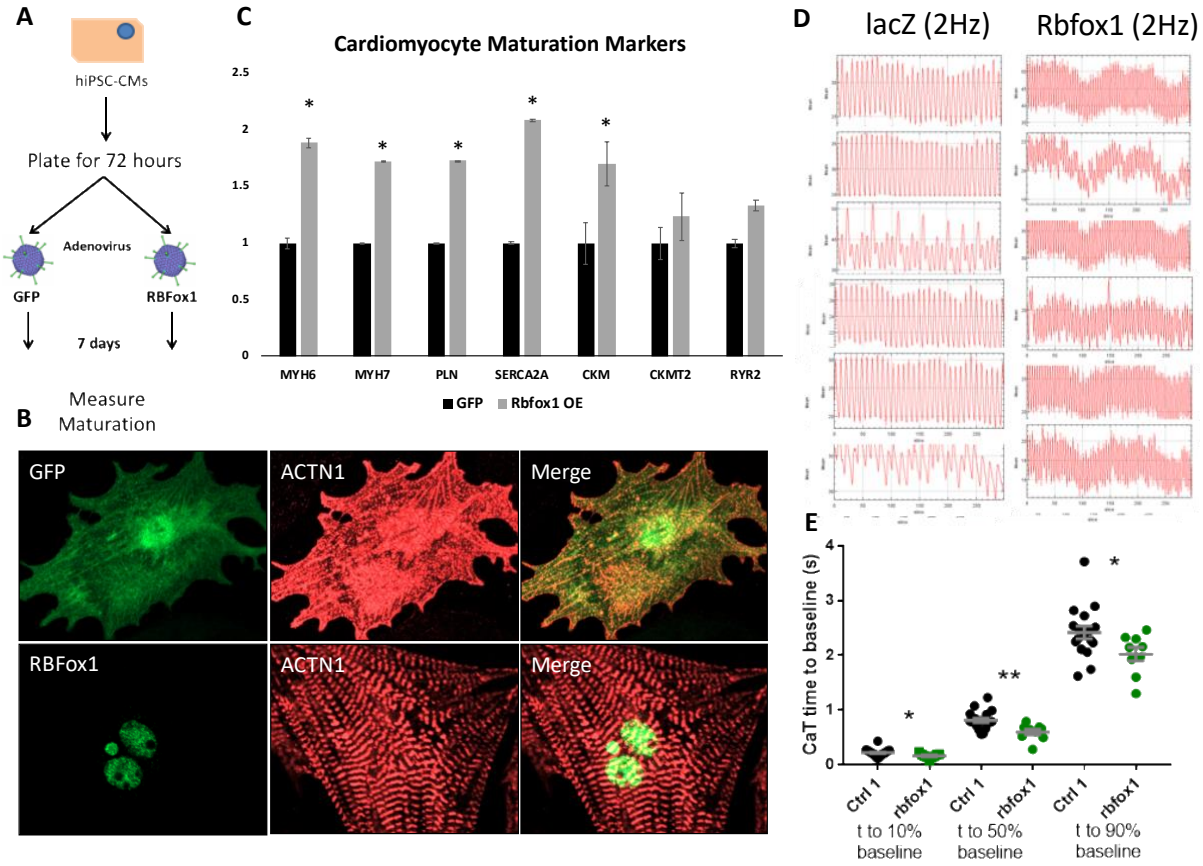

Figure 5. Rbfox1 induces maturation in hiPSC-CMs. A) Schematic overview of Rbfox1 OE in hiPSC-CMs. B) Rbfox1 promotes sarcomere organization. hiPSC-CMs were stained for ACTN1 and FLAG (FLAG tagged Rbfox1). C) Rbfox1 OE in hiPSC-CMs promotes mature cardiomyocyte gene expression. Results were observed by qPCR. *, $p<0.05$. D) Calcium transient recordings analyzed by ImageJ showed a striking difference between lacZ OE and Rbfox1 OE hiPSC-CMs. Rbfox1 matured hiPSC-CMs displayed a more robust calcium handling capability, able to keep pace at 3 Hz electrical stimulation. E) Calcium transient measurements via Ionoptix showed a significant increase in time to baseline in hiPSC-CMs. Suggesting a more robust ability for calcium clearance.

Rbfox1 Maturation Involves Global Transcriptome Changes. To examine the overall impact of Rbfox1 on hiPSC-CM transcriptome, we employed Illumina RNA sequencing to understand which genes were differentially expressed during Rbfox1 OE, and if it is in line with what we see in the morphological and functional studies performed. Rbfox1 was responsible directly or indirectly for 23,089 gene changes. GO analysis revealed that genes involved in muscle fiber assembly, sarcomere assembly, force of heart contraction, sodium/potassium homeostasis, cardiac muscle action potential, conduction and contraction were upregulated. Interestingly, the unique immune gene signature was also present in the form of inflammatory response, cytokine-mediated signaling, monocyte/neutrophil/lymphocyte chemotaxis. Taken together, this further validates the morphological and functional maturation we see in both NRVMs and hiPSC-CMs, and shed light on a possible novel pathway in cardiomyocyte maturation.

Discussion

In this study, we are the first to demonstrate that manipulation of an alternative splicing factor is sufficient for the maturation of cardiomyocytes *in vitro.* The induction of Rbfox1 is able to promote changes in a wide variety of properties related to cardiomyocyte maturation. RNA-Seq results has shown that Rbfox1 promoted maturation is completely different than PE induced functional changes and is not related to pathological hypertrophy. Furthermore, OE of Rbfox1 accelerates the maturation process and induces a distinct immune expression signature that is shared in normal postnatal mouse heart development.

Maturation of cardiomyocytes is complex and is likely to be regulated by multiple pathways in a coordinated manner. This convoluted process has been extremely hard to define, and the overall mechanism of cardiomyocyte maturation has yet to be understood and defined. In this context,

Rbfox1 has shed light on an important mechanism of maturation, one that has impact on global gene expression changes. However, further investigation on the immune system associated genes need to be made. In conclusion, our discovery of a splicing factor with the ability to promote cardiac maturation in a variety of different parameters provides unique insights for development strategies for generating mature tissue types in the advancement of stem cell technology and precision medicine.

Chapter III

Epigenetic Regulation of Cardiomyocyte Maturation

Background

In this section I will discuss preliminary data on a continuing project. In Chapter II I have described the profound effect of Rbfox1 on the maturation of cardiomyocytes. However, Rbfox1 only represent a portion of the overall mechanism governing cardiomyocyte maturation. The question remains, if the onset of Rbfox1 expression right after birth governs a subset of maturation gene expression, then what controls the timing of Rbfox1 expression? Using Rbfox1 as a case study for maturation, I begin to look for upstream regulators that usher in the first wave of strategic gene expression changes at the early stages of postnatal heart development. These regulators could very well be the master drivers for the entire maturation expression program. Much like the overall cardiac maturation process, no transcription factor is known to control Rbfox1 expression. How Rbfox1 is first expressed in postnatal heart is largely a mystery. To

shed more light on this problem, I looked to survey the epigenetic landscape around the Rbfox1 gene.

Results

A Muscle Specific Enhancer Exists Near Rbfox1. It is known that chromatin states can have profound impact on gene regulation [106-109] by controlling the epigenetic landscape of critical elements such as enhancers [110-113]. To see if such an element exists for Rbfox1 I examined public ChIP-Seq dataset uploaded onto ENCODE by the Ren Lab at UCSD. The dataset included known histone marks for gene activation: Histone 3 lysine 27 acetylation (H2K27ac) and Histone 4 lysine mono-methylation (H3K4me1) as well as known marks for gene repression: Histone 3 lysine 27 tri-methylation (H3K27me3). All ChIP-Seq experiments were done in both adult and fetal mouse heart, and allowed me to examine and compare the epigenetic landscape of Rbfox1 in both mature and immature heart. Interestingly, we see a putative enhancer signature ~10kb upstream of the Rbfox1 transcription start site (TSS). The approximately 1.2kb DNA element of interest was marked by H3K27ac and H3K4me1 in adult heart, displaying the characteristics of an active enhancer which enhances the gene expression of associated gene(s) [113, 114]. While in fetal heart, marked by a lack of H3K27ac and H3K4me1, an example of a primed enhancer, which does not facilitate gene expression but is in a state of awaiting activation [113-115] (Figure 1A). Additional investigation on ChIP-Seq data of adult mice heart revealed colocalization of P300 and Pol2 with the enhancer element. To see if the enhancer presence is marked in skeletal muscle where the muscle isoform of Rbfox1 is expressed, I examined DNase-Seq in C2C12 myotubes, and discovered that chromatin is open at the location of the enhancer (Figure 1B). furthermore, adult brain tissue which does not express the cardiac and skeletal

muscle-specific isoform of Rbfox1 does not have such histone marks at the enhancer element (Figure 1C), suggesting that this enhancer is muscle specific and correlates with Rbfox1 expression and the onset of the maturation gene program.

At the center of this 1.2kb element a sequence of 277 base pairs showed high conservation in humans. It is likely that this conserved sequence is the true enhancer, and exists as a regulatory element that functions to control cardiomyocyte maturation in both human and rodent heart.

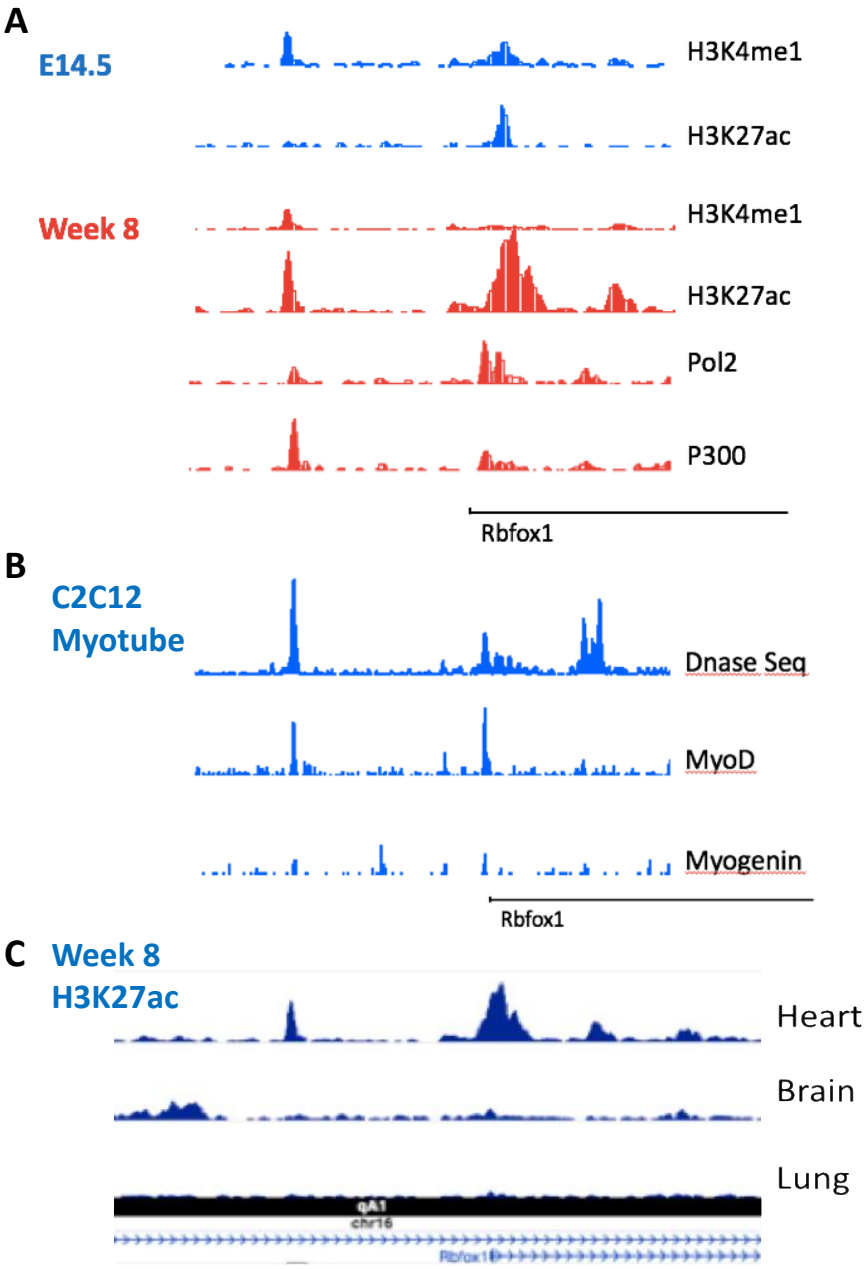

Figure 1. There is a clear enhancer signature in the region 10kb up. A) The enhancer region is marked by H3K4me1, H3K27ac, Pol2 and P300 in adult mouse ventricle. However, in E14.5 only H3K4me1 exists. B) The enhancer mark is also present in C2C12 myotubes, making it a usable model to study its function. C) The enhancer signature is tissue specific, as it is none existent in adult mouse brain and lung tissue.

Enhancer is Active During Myoblast Maturation. We then tested the enhancer's function during muscle maturation in C2C12 cells using a variety of vectors. I first cloned tdTomato into a pcDNA5 plasmid (PCDNA-Td), and removed its CMV enhancer and promoter using BglII and HindIII. In the place of CMV enhancer and promoter, I cloned 1) full length 1.2kb enhancer sequence alone (PCDNA-EN), 2) full length enhancer with FGF minimal promoter (PCDNA-EN-FGF), 3) full length sequence with endogenous Rbfox1 promoter (PCDNA-EN-Rbfox1) (Figure 2A). I then transfected these vectors along with the intact PCDNA5-tdTomato plasmid into different C2C12 cells plates, induced myotube differentiation and observed fluorescence activity. After 3 days in culture, I was able to visualize myotube formation and found that only the positive control and PCDNA-EN-Rbfox1 had tdTomato expression (Figure 2B). To further examine if the conserved sequence was necessary and sufficient to induce tdTomato, I then replaced the 1.2kb enhancer sequence with 1) the 277bp conserved sequence (PCDNA5-277-Rbfox1), 2) the sequence 1kb upstream from the conserved sequence (PCDNA5-Up-Rbfox1), 3) The sequence 1kb downstream from the conserved sequence (PCDNA5-Down-Rbfox1) (Figure 2A). I then performed the same experiment described above by transfecting the plasmids into C2C12 and induce myotube differentiation. PCDNA5-277-Rbfox1 had robust tdTomato expression similar to the full-length enhancer, while no expression was seen in PCDNA5-Up-Rbfox1 and minimal expression for PCDNA5-Down-Rbfox1 (Figure 2B). We conclude that the 277bp conserved enhancer sequence is a functional enhancer that responds during muscle maturation by inducing gene expression of downstream genes when paired with a compatible promoter. We termed this enhancer maturation transcription upregulation response element (MATURE).

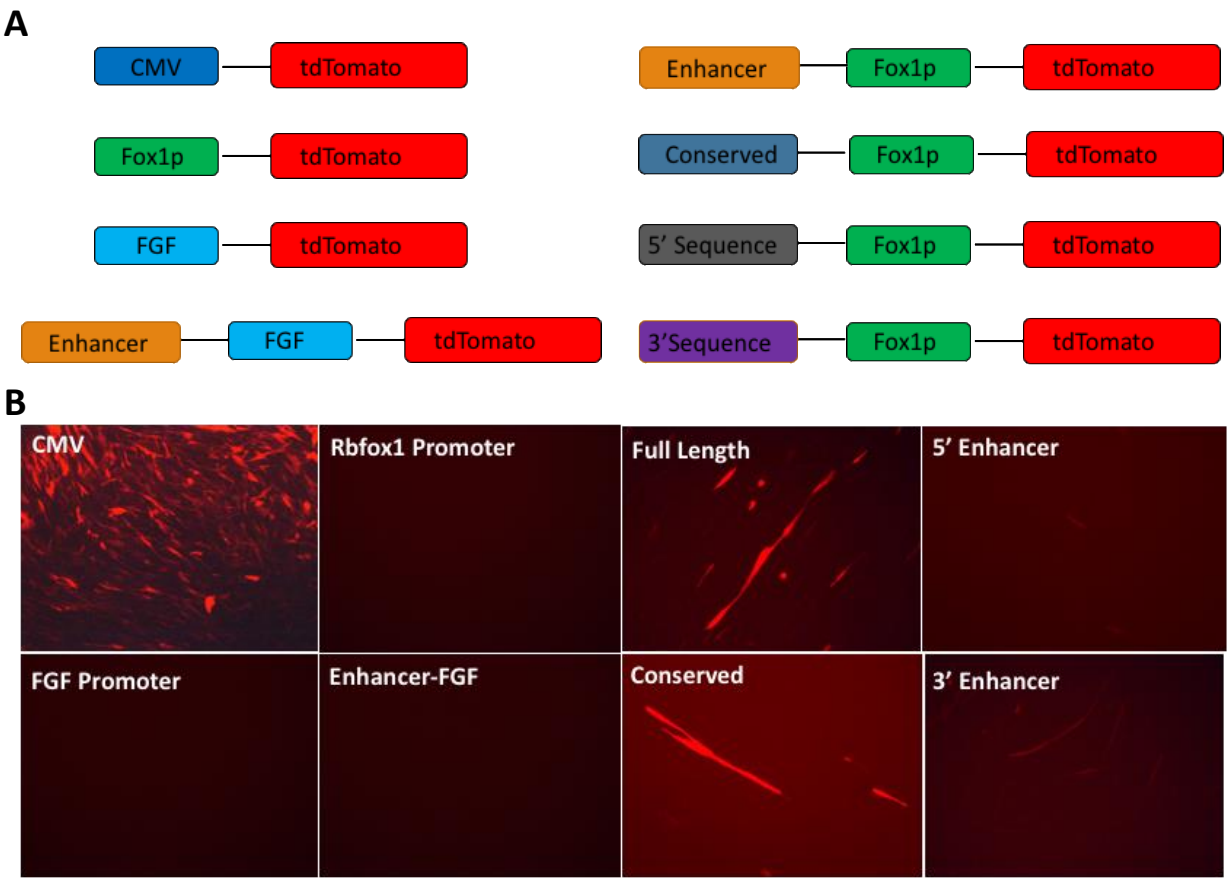

Figure 2. A) Schematic overview of all enhancer constructs. B) Only when the enhancer is paired with the endogenous Rbfox1 promoter does it functions during myotube differentiation.

MATURE is Regulated by a Maturation Associated lncRNA: CARMER. To investigate the potential regulators acting through MATURE we examined potential transcription factor (TF) binding on the conserved sequence. We found a myriad of TFs (Figure 3A) that can potentially bind to MATURE and is currently using molecular biology techniques to investigate further. Long non-coding RNAs (lncRNA) are known to play crucial roles in gene regulation, often acting as scaffolds recruiting the necessary gene regulatory proteins to the specific DNA target [116-120]. In many cases, lncRNAs have also been shown to be involved in the maintenance and control of enhancer sequences [120-127]. We examined the conserved sequence and observed that it is a part of a non-coding transcript identified through BLAST, we termed the lncRNA Cardiac Maturation Enhancer RNA or CARMER. RNA fractionation and quantitative PCR confirms CARMER expression is restricted to the nucleus (Figure 3B), and its induction overlaps that of Rbfox1 in both neonatal heard maturation and in C2C12 myotube differentiation (Figure 3C). In addition, siRNA mediated knockdown (KD) of CARMER performed before C2C12 myotube differentiation blunts Rbfox1 expression and reduces expression of MyoD and Myogenin (Figure 3E). Although CARMER is shown to have an impact on Rbfox1 and ultimately maturation. It is still unclear whether CARMER acts only to regulate Rbfox1, or does it have other targets. Hi-C data done in TAC and sham adult mice where Rbfox1 expression greatly decreased in TAC mice, show that the enhancer position is located in distinct regions and have many more distal genomic interactions when it is active. In addition, the interacting partners of CARMER is yet to be identified. The continuing study of this previously undiscovered lncRNA and its interacting partners could uncover a novel understanding of the mechanism of maturation in cardiomyocyte and yield valuable insight into development and disease.

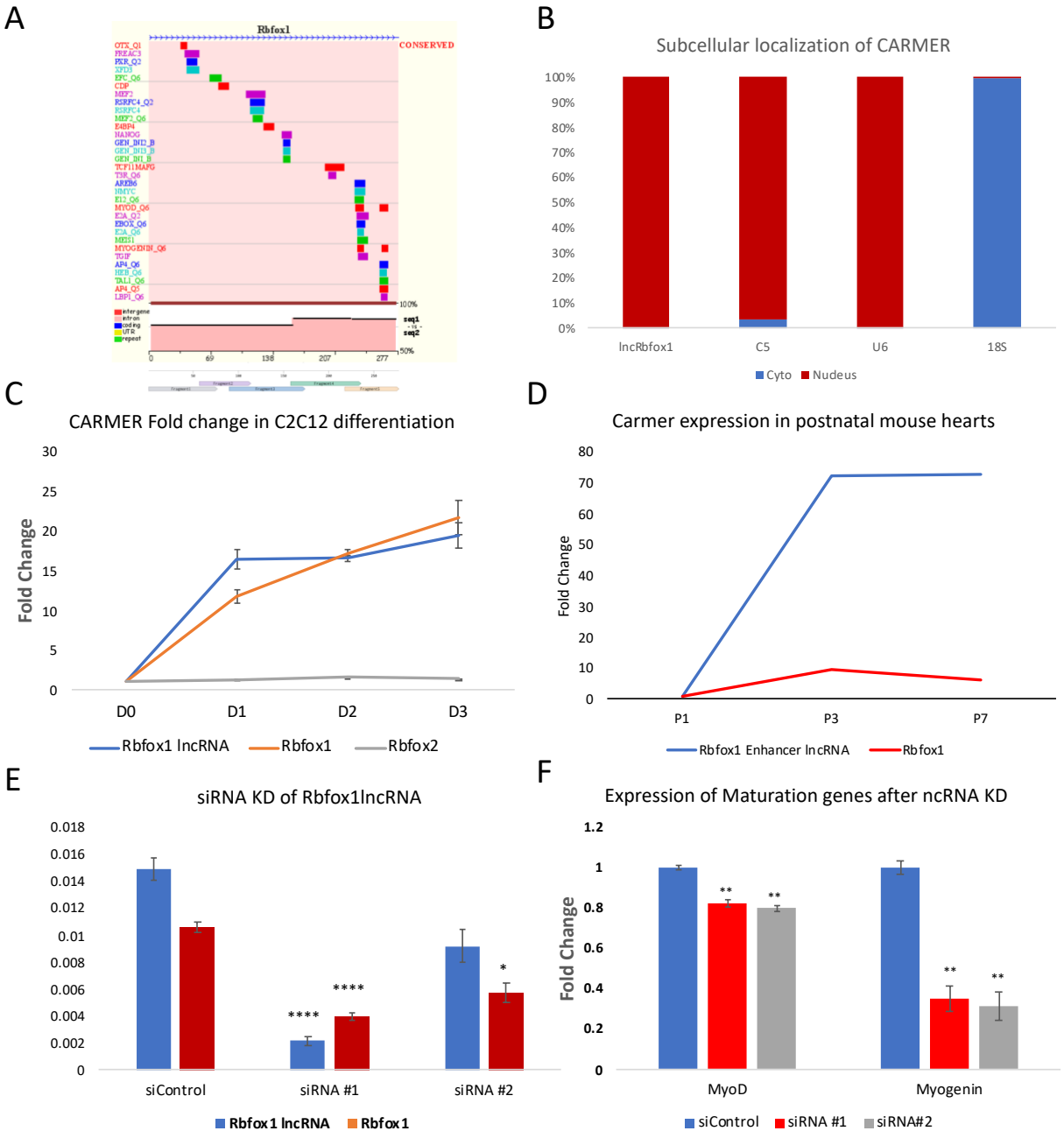

Figure 3. MATURE is found to be regulated by potential TFs (A) and a novel lncRNA CARMER. B) CARMER is found to be localized in the nucleus cardiomyocytes. C, D) CARMER induction correlates with Rbfox1 expression in both C2C12 myotube differentiation and postnatal cardiomyocyte maturation. KD of CARMER leads to decreased Rbfox1 expression in C2C12 cells and blunts myotube differentiation by suppressive key transcription factors MyoD and Myogenin.

Development of a high-throughput phenotyping platform for cardiomyocyte maturation.

Utilizing MATURE as a screening tool for maturation I plan to develop iPSC-CMs carrying a tdTomato driven by MATURE and the Rbfox1 endogenous promoter. Upon differentiating iPSCs into iPSC-CMs due to the fetal nature of the cardiomyocytes, the tdTomato should remain inactive. The cells then can be used to screen for compounds that promote maturation via a high-throughput platform that combines visualization of tdTomato and contractile function measurements as confirmation. The platform takes advantage of previously described methods [128, 129] and is illustrated in Figure 4.

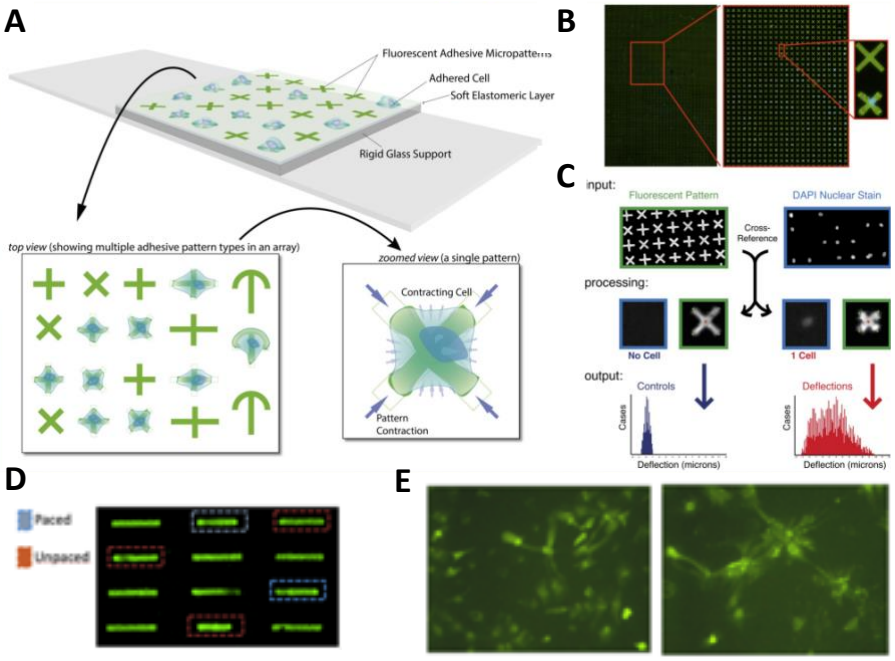

Figure 4. We will partner with our collaborators at Forcyte Biotechnologies Inc, to design a novel high-throughput screening platform for mature hiPSC-CMs. A) Schematic of the screening platform. B, C) Force generation of each plated cell will be measured via contraction length and stiffness of micropatterns. D, E) The platform can also be adapted to measure pacing and calcium handling

Discussion

Postnatal maturation of cardiomyocytes, particularly that which takes place in adolescence has never been understood. In this study, we used Rbfox1 as a case study to better understand the overarching regulatory mechanism in cardiomyocyte maturation. We were able to show, for the first time that mRNA splicing is a driver of cardiomyocyte postnatal maturation, and manipulation of a single splicing factor is able to markedly advance the *in vitro* maturation state of both NRVMs and hiPSC-CMs with a wide range of morphological, transcriptional and functional changes. This adds a layer of understanding to the complex process of maturation accompanying adolescence, and serves as a reference point for similar strategies to be used in other tissues. The more nuanced changes in which mRNA alternative splicing can alter the transcriptome without changing cellular identity significantly contributes to the phenomenon observed. However, we were surprised to see how powerful of a transcriptional driver a single splicing factor truly is, able to significantly affect more than 20,000 genes, many in critical pathways affect cardiomyocyte function, health and development. Further investigations into downstream gene expression changes is needed, to pinpoint the exact pathways and expression changes that is the main driver of phenotypes observed. In particular the group of genes identified as "immune related" and retained during natural heart maturation should be examined carefully. Our study has so far focused on ventricular cardiomyocytes, it would be fascinating to see if other cardiomyocytes such as those that reside in the atrium also express Rbfox1 during postnatal development, and if OE of this factor can also facilitate those cells towards maturation. Using Rbfox1 as a case study for the maturation gene program, we also discovered a short, conserved enhancer sequence upstream of the Rbfox1 promoter which responds to muscle maturation. The identification of the mechanism and players involved in regulating this enhancer

could shed valuable light onto the facets of maturation previously undiscovered. Among the possible methods of enhancer regulation, a lncRNA covering the enhancer was shown to express in coordination with muscle maturation. KD of this lncRNA in C2C12 cells via siRNA blunted Rbfox1 expression as well as key transcription factors in myotube differentiation. More detailed studies on the enhancer MATURE as well as the lncRNA CARMER is needed, but the discovery could reveal a novel regulatory circuit previously uncharacterized, and reveal a larger, more fundamental network of postnatal gene regulation which is initiated by chromatin architecture. Taken together, we have uncovered another layer in the complex yet elegant regulation of postnatal maturation of cardiomyocytes, and future endeavors will hopefully shed more light on this mysterious and coordinated process.

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