Biology of the cardiac myocyte in heart disease

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ABSTRACT **Cardiac hypertrophy is a major risk factor for heart failure, and it has been shown that this increase in size occurs at the level of the cardiac myocyte. Cardiac myocyte model systems have been developed to study this process. Here we focus on cell culture tools, including primary cells, immortalized cell lines, human stem cells, and their morphological and molecular responses to pathological stimuli. For each cell type, we discuss commonly used methods for inducing hypertrophy, markers of pathological hypertrophy, advantages for each model, and disadvantages to using a particular cell type over other in vitro model systems. Where applicable, we discuss how each system is used to model human disease and how these models may be applicable to current drug therapeutic strategies. Finally, we discuss the increasing use of biomaterials to mimic healthy and diseased hearts and how these matrices can contribute to in vitro model systems of cardiac cell biology.**

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INTRODUCTION

In this review, we discuss the cell biology of heart disease, using the cardiac myocyte as our focus. Although there are many cell types in the heart (myocytes, endothelial cells, fibroblasts, vascular smooth muscle cells), and each cell type contributes to cardiac function, we discuss the contractile myocytes. Myocytes constitute the majority of the heart by mass and have been shown to be major contributors to contractile dysfunction. We discuss various approaches that have been used to study myocyte cell biology in the setting of heart disease and cover recent advances.

For more than three decades, rodent models have been used to study heart disease with many different etiologies; despite divergent causes, cardiac hypertrophy is frequently a common disease indicator (Curtis *et al.*, 1987; Rockman *et al.*, 1991). In fact, pathological cardiac hypertrophy is a strong prognostic indicator of human morbidity and mortality (Kono *et al.*, 2009; Takasaki *et al.*, 2012; Shenasa *et al.*, 2015). It should be mentioned that there is also a type of cardiac hypertrophy that is physiological and caused by stimuli such as exercise and pregnancy, but this type of hypertrophy is distinct and will not be discussed here. Cardiac hypertrophy is largely the result of cardiac myocyte enlargement, and this process can be recapitulated in isolated myocytes in culture by treatment with a number of small molecules and growth factors. Myocytes either contract spontaneously or can be paced to contract and their contractile properties studied. Primary cardiomyocytes are terminally differentiated cells and are difficult to transfect (Naqvi *et al.*, 2009). Therefore some effort has been put into developing permanent cell lines, but these lines suffer from their relevance to bona fide cardiac myocytes (see later discussion). Purification of cardiomyocytes greatly reduces or eliminates other cell types in the heart, including endothelial cells and fibroblasts, making it possible to attribute findings to the cardiac myocytes rather than interactions with other cell types (Diaz and Wilson, 2006). Studying cardiomyocytes offers many inherent advantages over whole-heart models, allowing precise control of experimental conditions, including extracellular matrices, exogenous growth factors, oxygen levels, and electrical stimulation.

In addition to animal models and primary cardiac myocytes from experimental animal models, much recent work has been done to develop human cardiomyocyte model systems. These include differentiation of human embryonic stem cells (ESCs) and human induced pluripotent stem cells (iPSCs) into cardiomyocytes. These models are discussed here, and although they may ultimately be the best model for studying human cardiomyocytes, considerable research is still needed to direct these cells to a fully differentiated cardiomyocyte phenotype.

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Abbreviations used: α-SMA, alpha-skeletal muscle actin; AMVM, adult mouse ventricular myocyte; ANF, atrial natriuretic factor; Ang II, angiotensin II; ARVM, adult rat ventricular myocyte; β-MHC, beta-myosin heavy chain; BNP, brain natriuretic peptide; cAMP, adenosine 3,5-cyclic monophosphate; DCM, dilated cardiomyopathy; ESC, embryonic stem cell; ET-1, endothelin-1; HCM, hypertrophic cardiomyopathy; iPSC, induced pluripotent stem cell; ISO, isoproterenol; MLP, muscle LIM protein; NE, norepinephrine; NMVM, neonatal mouse ventricular myocyte; NRVM, neonatal rat ventricular myocyte; PE, phenylephrine; PMA, phorbol 12-myristate 12-acetate.

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PRIMARY CARDIAC MYOCYTES

Neonatal rodent ventricular myocytes

Neonatal cardiomyocytes have been the workhorse of in vitro cardiac cell biology. In comparison to adult cardiomyocytes, neonatal myocytes are relatively easy to isolate, survive in culture for relatively long periods of time, and can be transfected using nonviral methods of gene transfer. The first isolation of neonatal rat ventricular myocytes (NRVMs) occurred >50 years ago (Harary and Farley, 1963a,b), and it was clear from the first published report that these spontaneously beating, cultured cells could serve as an invaluable resource for cardiovascular research. Since the initial report, cultured neonatal cardiomyocytes have been used to study myofibrillogenesis and myofibrillar functions and to model cardiac diseases. In fact, live-cell imaging with tagged mutant and wild-type myosins has been used to follow sarcomere distribution in NRVMs, and misaccumulation of mutants has been observed (Buvoli *et al.*, 2012).

Although many protocols exist for isolation of NRVM and neonatal mouse ventricular myocyte (NMVM) isolation, the fundamental aspects of the protocol are essentially the same as described many years ago (Karliner *et al.*, 1985). One of the greatest advantages of using NRVMs and NMVMs is the ability to study cardiac hypertrophic responses using compounds that mimic states seen in heart disease, such as adrenergic stimulation. A number of compounds and growth factors have been shown to induce pathological cardiac myocyte hypertrophy in NRVMs and NMVMs, including phenylephrine (PE; Zobel *et al.*, 2002; Huang *et al.*, 2015; Nakaoka *et al.*, 2015), norepinephrine (NE; Simpson *et al.*, 1982; Bishopric and Kedes, 1991), angiotensin II (Ang II; Menaouar *et al.*, 2014), endothelin-1 (ET-1; Menaouar *et al.*, 2014), and the diacylglycerol mimetic phorbol 12-myristate 12-acetate (PMA; Reid *et al.*, 2016) (see Table 2 later in this paper). Cardiac myocytes can increase in both volume and cell surface area by as much as 150% within 48 h of treatment with PE, for example (Figure 1A; Simpson, 1983; LaMorte *et al.*, 1994). In addition to increases in myocyte size, it has been shown that PE stimulation causes significant ($p < 0.05$) changes in the expression of between 600 and 3000 genes, so the effects that cause/accompany changes in size are substantial (Frank *et al.*, 2008; Riquelme, Heimiller, Barthel, and Leinwand, unpublished observations). In addition, increases in cell area and volume are positively correlated with sarcomeric organization, determined by sarcomeric disarray, in response to PE (Bass *et al.*, 2012; Buvoli *et al.*, 2012). Further, consistent with what is seen in intact hearts, the cells show increases in the speed and force of contraction when treated with PE (Zobel *et al.*, 2002). This response undoubtedly represents the initial compensatory phase of adrenergic stimulation, and this has limitations in terms of studying the longer decompensation and failure phases. Common downstream readouts of induction of pathological cardiac hypertrophy, both in vivo and in vitro, include reactivation of a "fetal gene program." Such genes include atrial natriuretic factor (ANF), brain natriuretic peptide, α-skeletal muscle actin, and β-myosin heavy chain (Chien *et al.*, 1991; Harvey and Leinwand, 2011). In addition, activation of Fak and MEK1/2/ERK1/2 signaling pathways are hallmarks of pathological cardiac remodeling (Bass *et al.*, 2012; Huang *et al.*, 2015).

Given the desire to develop agents that that blunt or block cardiac cellular hypertrophy, rodent myocytes represent an opportunity to conduct high-throughput screens for agents that block cellular hypertrophy in vitro. Although NRVMs have used utilized in targeted drug screening assays (Dolinsky *et al.*, 2015; von Lueder *et al.*, 2015), the lack of a high-throughput imaging technology that can reproducibly detect cardiac hypertrophy has prohibited the use of these cells in large-scale analysis (Zhang *et al.*, 1999). The use of primary cardiac cells in high- throughput screens could potentially lower the high attrition rates of lead compounds because compounds that cause decreased cell viability could be excluded by this initial assay. An automated, high-throughput NRVM imaging technique has been successfully used to objectively quantify multiple hallmarks of cardiac hypertrophy: myocyte size, elongation, circularity, and sarcomeric organization (Bass *et al.*, 2012). A similar approach was used to quantify both morphology and gene expression profiles of NRVMs in response to 15 hypertrophic agonists (Ryall *et al.*, 2014). A high-throughput screen was recently used to identify microRNAs with the ability to regulate NRVM hypertrophy (Jentzsch *et al.*, 2012). Further expansion of this high-throughput imaging approach was used in a recent study that measured cell size and ANF induction in NRVMs exposed to PE and PMA (Reid *et al.*, 2016). Using this high-content imaging approach, the authors were able to successfully identify compounds that successfully blocked two distinct NRVM hypertrophic models (Reid *et al.*, 2016). This seminal study provides the first example of an in vitro drug-screening assay using primary cardiomyocytes, which, it can be hoped, will reduce the time from drug discovery to in vivo drug trials.

The availability of genetically modified mouse models also provides an opportunity to analyze the structural and functional roles of specific proteins or modifications of those proteins hypothesized to play a role in cardiac disease. Isolation of myocytes from genetically

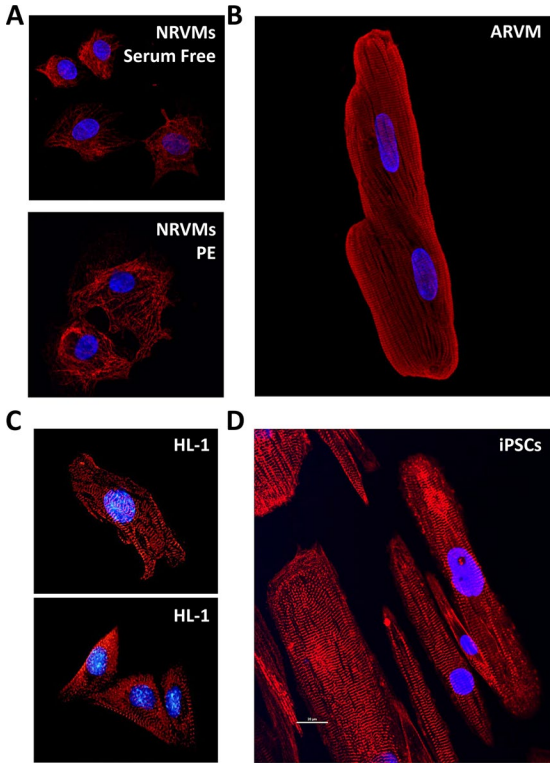

FIGURE 1: Morphology and sarcomeres in primary cardiac myocytes and the HL-1 cell line. Blue, 4′,6-diamidino-2-phenylindole for nuclei. (A) Mononuclear NRVMs either untreated (top, serum free) or treated with PE (bottom) and stained for myosin heavy chain (red). (B) Binuclear ARVM stained for myosin heavy chain (red). (C) HL-1 cells stained for myosin (top) or titin (bottom). Published with permission from White *et al.* (2004). (D) iPSC-derived cardiomyocytes cultured on nanopatterned surfaces for 80 to 100 d postdifferentiation induction and stained for α-actinin (image provided by the Michael Regnier laboratory).

altered rodents allows investigation of sarcomere integrity, force, and rate of contraction ex vivo. Screens for therapeutics could potentially be performed on cardiac myocytes modified to mimic human genetic cardiovascular disease. For example, loss of the muscle LIM protein (MLP) in mice leads to a dilated cardiac phenotype (Arber *et al.*, 1997; Heineke *et al.*, 2010). Isolated NMVMs from these mice show disrupted myofibrillar architecture after 5 d in culture, suggesting that these spontaneously beating myocytes have impaired resistance to mechanical stress. Myofibrillar disruption in MLP-null NMVMs was rescued by transfection of MLP-null NMVMs with an MLP overexpression vector, thus conclusively showing MLP expression is essential for myofibrillar function and resistance to contraction-induced stress in cardiomyocytes. Following the initial murine MLP-knockout study, genetic analysis in a large patient cohort, including patients diagnosed with nonfamilial dilated cardiomyopathy and nonfamilial hypertrophic cardiomyopathy (HCM), uncovered three unrelated HCM patients, each carrying a unique MLP missense mutation. Further analysis revealed cosegregation of clinically affected individuals within each of the families of the index patients with each of the respective mutations in MLP (Geier *et al.*, 2003). Another example in which the study of NMVMs led to insight into the molecular mechanisms behind the development of a cardiac phenotype is the Hspa4-knockout mouse model (Mohamed *et al.*, 2012). As a molecular chaperone, HSPA4 was found to be up-regulated in murine hearts subjected to pressure overload and in failing human hearts. In Hspa4-null mice, cardiac hypertrophy along with enhanced activation of gp130-STAT3, CaMKII, and calcineurin-NFAT signaling pathways was observed in the intact myocardium. Because this was not a cardiomyocyte-specific knockout, it was unclear which cell type within the heart was primarily responsible for these signaling alterations and the development of hypertrophy. NMVMs isolated from neonatal Hspa4-null mice had a significant increase in cross-sectional area and increased expression of hypertrophic markers, suggesting that the hypertrophy of the intact heart was likely the result of primary defects in cardiomyocytes themselves. Hence NMVMs have the advantage of the abundance of genetic alterations in the mouse (Table 1).

Adult ventricular myocytes

Adult cardiomyocytes have been isolated from many different organisms for the purpose of detailed study of cardiac function and the cellular basis of heart disease. Early studies often used relatively large animals, such as cats or rabbits, yielding many cells from a single animal (Jacobson and Piper, 1986; Mitcheson *et al.*, 1998). Dogs and guinea pigs have also proven to be useful for isolation of adult cardiomyocytes (Horackova *et al.*, 1996; Maltsev *et al.*, 2008). The current standard for adult cardiomyocytes is the adult rat ventricular myocyte (ARVM), in large part due to the relative ease and cost-effectiveness of the rat as an animal model. Even though the first isolation of ARVMs occurred 40 years ago (Powell and Twist, 1976), no single, universal method is currently used for isolation of ARVMs. Instead, investigators follow protocols that are similar to one another in principle (i.e., retrograde perfusion of the heart with an enzymatic solution) but differ widely in details (e.g., apparatus, enzyme(s), dissociation methods; Mitcheson *et al.*, 1998; Louch *et al.*, 2011). Thus the reproducibility of results from studies using ARVMs suffers from this lack of consistency.

Primary cardiomyocytes are commonly prepared from adult mice, as well as from rats (O'Connell *et al.*, 2007). Adult mouse ventricular myocytes (AMVMs) have many characteristics in common with ARVMs, with a few important differences. The isolation procedure for AMVMs is much the same as for ARVMs but yields fewer cells per animal and requires different perfusion pressure and cannula size. For pathologies for which transgenic mouse models are available, the decreased efficiency of cell isolation is offset by the opportunity to study these animal models at the cellular level. It is important to note that AMVMs isolated from transgenic or otherwise diseased mice have been exposed to the disease conditions over a period of days to months. Therefore the phenotypes observed may be due to chronic or long-term changes, including altered gene expression, fibrosis, and tissue remodeling. The same can be said for cardiomyocytes isolated from rats and mice on which surgical procedures, such as induction of pressure overload, have been performed (Waring *et al.*, 2014). For example, myocytes isolated from a transgenic mouse model of familiar HCM show numerous structural and functional abnormalities, but these mice also have interstitial fibrosis even at the younger ages studied, and the older mice have systolic dysfunction (Olsson *et al.*, 2004). Thus the cellular phenotypes may be a secondary consequence of the advanced disease progression. For observation of acute effects, in vitro treatment with hypertrophic agonists or infection of ARVMs with viruses (typically adenovirus or adeno-associated virus) carrying genes of interest is more effective (Zhou *et al.*, 2000; Louch *et al.*, 2011). Virus-mediated expression of sarcomeric proteins in ARVMs enables not only the study of the gross cellular phenotype of disease-associated mutations, but also detailed study of sarcomere dynamics (Thompson and Metzger, 2014). The two approaches (surgical/transgenic and drug/virus) can also be combined to analyze signaling pathways downstream of pathogenic hypertrophic stimuli (Métrich *et al.*, 2008; Feest *et al.*, 2014).

The greatest advantage of primary adult cardiomyocytes for cellular modeling of heart disease may be in the similarity of the morphology and behavior of the isolated cells to those of cardiomyocytes in intact tissue (Table 1). ARVMs, AMVMs, and adult cardiomyocytes from other animal models are rod shaped and binucleated and have well-organized sarcomeres throughout the cell body (Figure 1B; Zhou *et al.*, 2000; Louch *et al.*, 2011). Healthy adult cardiomyocytes contract regularly when paced with an electrical stimulus. Pacing the cells in culture not only permits a great deal of control over experimental variables (e.g., duration, intensity, and frequency of stimulation), but also may serve to maintain the cells in more physiological state (Berger *et al.*, 1994; Joshi-Mukherjee *et al.*, 2013). As with any experimental system, the use of isolated adult cardiomyocytes has some significant limitations. The morphology and functional characteristics of adult cardiomyocytes change over time in culture (Banyasz *et al.*, 2008; Hammer *et al.*, 2010; Louch *et al.*, 2011), abrogating one of the key advantages of these cells. Therefore most investigators perform their analyses within 24 h of isolation, although some studies are carried out over the course of several days (Communal *et al.*, 2000; Miyashita *et al.*, 2001; Snabaitis *et al.*, 2005; Mu and Harvey, 2012). This limits the utility of adult cardiomyocytes for investigation of phenotypes other than those that present acutely upon application of the experimental stimulus. Several approaches have been tested to lengthen their usable time in culture, including electrical pacing (Holt *et al.*, 1997; Joshi-Mukherjee *et al.*, 2013), drug treatments (Tian *et al.*, 2012), and low temperatures (Abi-Gerges *et al.*, 2013). The cells are also very difficult to transfect and are not as infectable as other cell types, such as NRVMs or immortalized cell lines (Louch *et al.*, 2011).

As with neonatal cells, many different treatments have been used to cause hypertrophy in primary adult cardiomyocytes. These stimuli include PE (Sowah *et al.*, 2014), NE (Thandapilly *et al.*, 2011), Ang II (Sowah *et al.*, 2014), isoproterenol (Li *et al.*, 2011), and mechanical loading (Clark *et al.*, 1993; Leychenko *et al.*, 2011; Table 2).

Cell type	Advantages	Disadvantages
Neonatal cardiomyocytes (NRVMs/NMVMs)	Relatively easy isolation Cost-effective Greatest ratio of cell number per animal Spontaneously beat in culture Can be maintained in a serum-free culture medium Can be maintained up to 28 d in culture after isolation Large number of genetic models available Small, circular cells can be analyzed by automated cell systems (fluorescence-activated cell sorting, Coulter, etc.) Easily transfectable with lipid or electroporation methodologies Respond to hypertrophic stimuli	Immature phenotype
Adult cardiomyocytes (ARVMs/AMVMs)	Cost-effective Mature sarcomeric structure is ideal for patch-clamp/contractility studies Presence of mature ion channels are ideal for Ca^{2+} imaging studies Can be maintained in a serum-free culture medium Large number of genetic models available Respond to hypertrophic stimuli	Technically challenging isolation Must be transfected with viral vectors Can only be maintained for a short time in culture after isolation Do not spontaneously beat in culture
HL-1 cardiomyocytes	Immortalized Homogeneous Rapid expansion Easily manipulated Contract spontaneously in ideal culture conditions	Derived from AT-1 atrial tumor cell lineage and do not recapitulate ventricular cells in culture Have to be maintained in medium containing a cardioprotective agent, a hypertrophic stimulus, and an atrial differentiation factor
ANF-T-antigen cardiomyocytes	Immortalized Homogeneous Rapid expansion Contract spontaneously	Derived from atrial tumor cell lineage and do not recapitulate ventricular cells in culture Unknown response to pathological stimuli
H2C9 myoblasts	Immortalized Homogeneous Rapid expansion Easily manipulated Ventricular origin Respond to hypertrophic stimuli	Immature Do not spontaneously beat in culture Media must be supplemented with an atrial differentiation factor in order to differentiate in cardiomyocytes and express cardiomyocyte lineage markers
Human embryonic and pluripotent stem cells (ESCs)	Immortalized Rapid expansion Easily manipulated Ventricular origin Respond to hypertrophic stimuli Contract spontaneously in ideal culture conditions	Expensive Technically challenging Numerous protocols for differentiation make it difficult to compare data across different studies Immature unless maintained in culture for 12–15 wk Heterogeneous

TABLE 1: Advantages and disadvantages of the most commonly used in vitro cardiomyocyte model systems.

Similar readouts to hypertrophy of NRVMs are also present, including activation of fetal gene program (Li *et al.*, 2011; Sowah *et al.*, 2014) and increase in the rate of protein synthesis, total protein levels, and abundance of myosin heavy chain protein (Clark *et al.*, 1993; Thandapilly *et al.*, 2011; Sowah *et al.*, 2014). Owing to their elongated shape, adult cardiomyocytes are not compatible with flow-based cell volume measurement methods and are typically measured using image-based morphometric analysis (Clark *et al.*, 1993; Métrich *et al.*, 2008; Thandapilly *et al.*, 2011; Sowah *et al.*, 2014). The shape of cardiomyocytes is closely related to contractile

Cell type	Pathogenic hypertrophic stimuli	Methodologies used to determine cell hypertrophy	Contractility after hypertrophic stimuli	Fetal gene reexpression with hypertrophic stimuli
Neonatal cardiomyocytes (NRVMs/NMVMs)	PE NE Ang II ET-1 Diacylglycerol mimetic PMA	Coulter counter (diameter and volume) Protein content normalized to DNA content Cell area determined by image analysis	Action potentials show increases in the speed and force of contraction when treated with PE (Zobel *et al.*, 2002)	Yes
Adult cardiomyocytes (ARVMs/AMVMs)	Ang II ISO NE PE Mechanical loading	Morphometry (3)H-phenylalanine incorporation Total protein content Myosin heavy chain content Time course of activation of protein synthesis	Action potentials show increases in shortening amplitude and force of contraction when treated with ISO (Jiang *et al.*, 2012)	Yes
HL-1 immortalized cardiomyocytes	cAMP Ang II ET-1 ISO	Cell area determined by image analysis	Ang II treatment induces fibrillations (Tsai *et al.*, 2011)	Yes
ANF-T-antigen cardiomyocytes	Unknown	Unknown	Unknown	Unknown
H2C9 myoblasts	Ang II ET-1 ISO	Cell area determined by image analysis	Unknown	Yes, brain natriuretic peptide
Human embryonic and pluripotent stem cells (ESCs)	ISO PE	Undetermined	Action potentials mimic those seen in isolated human fetal ventricular tissue (Mummery *et al.*, 2003)	Undetermined

Ang II, angiotensin II; ET-1, endothelin-1; ISO, isoproterenol; NE, norepinephrine; PE, phenylephrine; PMA, phorbol 12-myristate 12-acetate.

TABLE 2: Hypertrophic stimuli and markers of cardiac hypertrophy in commonly utilized in vitro model systems.

function. NRVMs plated on substrates patterned to constrain the cells in a specific length:width ratio had the best contractile function at ratios similar to those of cells in a healthy adult heart and performed poorly at ratios similar to those of myocytes in failing hearts (Kuo *et al.*, 2012). Unfortunately, the changes observed in the shape of hypertrophic cardiomyocytes in vivo and in vitro are not necessarily consistent. In early stages of hypertrophy, cells become wider, as reflected by an increase in the cross-sectional area (Kehat and Molkentin, 2010). In the later stages of hypertrophy, heart failure is occurring, and cells typically appear elongated. Therefore it is not surprising that in vivo models, in which hypertrophy is chronic, report an increase in left ventricular myocyte length (~30%; Gerdes *et al.*, 1996), whereas adult cardiomyocytes treated acutely with hypertrophic stimuli in vitro have similar increases in cell width instead (Kehat *et al.*, 2011).

For all of the cell types thus far discussed, in vitro study of the effects of hypertrophy-inducing compounds (e.g., isoproterenol, PE) or potentially antihypertrophic drugs is technically straightforward. The simplicity of treating cells in culture is one of the primary advantages of an in vitro model relative to more physiologically relevant in vivo models. However, concerns have arisen about the applicability of drug screening in animal models (in vitro or in vivo) to human medicine (Tzatzalos *et al.*, 2016). The development of new cellular models that more accurately recapitulate hypertrophic disease in humans has been a focus for the past decade or more.

PERMANENT "CARDIAC" MYOCYTE CELL LINES

Immortalized cardiac myocytes

Because cardiac myocytes are terminally differentiated and thus do not divide in culture, many attempts have been made to derive immortalized cardiac cell lines. Four such lines are AT-1 (Claycomb and Palazzo, 1980; Delcarpio *et al.*, 1991; Lanson *et al.*, 1992; Kline *et al.*, 1993; Yang *et al.*, 1994), HL-1 (Claycomb *et al.*, 1998), ANF-T-antigen (Steinhelper *et al.*, 1990), and H9C2 (Kimes and Brandt, 1976) cells. These cell lines, derived from either mouse atrial cardiomyocyte tumors (AT-1, HL-1, and ANF-T-antigen) or embryonic rat ventricular tissue (H9C2), do not precisely recapitulate the structural and functional physiology of acutely isolated ventricular cardiomyocytes (Figure 1C and Table 2).

Although AT-1, HL-1, and ANF-T-antigen cells have the same origin, they differ in culturing techniques. Unlike AT-1 cells, HL-1 cells can undergo freeze–thaw cycles; however, HL-1 cells need to be maintained in medium containing adenosine (a known cardioprotective agent), retinoic acid (a reagent used to induce or maintain atrial cardiomyocyte differentiation; Zhang *et al.*, 2011), and norepinephrine (a hypertrophic stimulus; Simpson *et al.*, 1982) to maintain a differentiated phenotype and stimulate beating. These requirements

vastly limit their usefulness in developing models of cardiac disease (Claycomb *et al*., 1998). Although HL-1 cells do express connexin 43, the dominant connexin in ventricular cells, they also express high levels of connexin 40, predominantly expressed in atrial cells (Dias *et al*., 2014). In addition, HL-1 cells do not express connexin 45, which is expressed by both atrial and ventricular cells localized in the atrioventricular node and is essential for cardiac conduction and embryonic development (Kumai *et al*., 2000). Thus the absence of connexin 45 makes HL-1 cells less myocyte-like compared with primary cell lines. Because these studies did not compare the protein expression of each connexin isoform in HL-1 cells with primary myocytes, it is difficult to determine how closely HL-1 cells recapitulate the connexin expression profile of ventricular myocytes. Hence altered expression of connexins in conjunction with immature or absent sarcomere formation (Claycomb *et al*., 1998) and an overall heterogeneous cellular phenotype limit the utility of these cells for modeling ventricular or arrhythmogenic cardiac diseases. A recent study found that increased expression of cAMP was sufficient to induce cell hypertrophy and fetal gene expression in HL-1 cells (Fang *et al*., 2015). However, sarcomeric organization was not thoroughly investigated, nor were the results compared with a primary cardiac myocyte. In a separate study analyzing isoproterenol (ISO)- and ET-1–mediated hypertrophic responses, the authors did not see increases in cell size or fetal gene activation, although they did see some downstream changes in hypertrophic protein profiles in treated cells (Hong *et al*., 2011).

Similar to HL-1 cells, ANF-T-antigen–derived cardiomyocytes spontaneously beat in culture, can undergo freeze–thaw cycles, and can be passaged. Spontaneously beating cardiomyocytes can be obtained for at least four serial passages without appearing to undergo any dedifferentiation, but serial passaging of these cells does appear to decrease the rapid expansion of the cells. However, because these cells possess the ability to undergo subcutaneous propagation and tumor formation when reintroduced into syngeneic hosts, they may express markers reflective of their tumor lineages. In addition, these cells have yet to be analyzed after treatment with hypertrophic stimuli (Steinhelper *et al*., 1990). Thus, AT-1, HL-1, and ANF-T-antigen cell lines do spontaneously beat in culture and utilize technically challenging protocols to maintain their contractile properties through serial passages. Combined with their atrial conduction phenotype and their immature sarcomeres, their use in contractility studies and models of ventricular hypertrophy is limited.

Although H9C2 cells were initially isolated from embryonic mouse ventricular tissue, they must undergo a "differentiation" procedure involving the addition of retinoic acid in low-serum (1%) medium in order to express cardiac differentiation markers (Menard *et al*., 1999). Without the addition of retinoic acid, H9C2 cells will differentiate into an "adult" skeletal muscle phenotype in low-serum media. The cell surface area of H9C2 cells increased approximately threefold in response to Ang II and ET-1 whereas NRVMs increased approximately twofold in the same study (Watkins *et al*., 2011; Table 1). Fetal gene activation was also present in H9C2 cells at approximately the same induction level as for NRVMs; however, distinct sarcomere organization in H9C2 cells was absent after hypertrophic stimulation (Watkins *et al*., 2011). Thus, H9C2 cells have been shown to respond to hypertrophic stimuli, but they do not display mature sarcomeric organization, nor do they spontaneously beat in culture as do primary neonatal cardiac myocytes. Therefore, H9C2 cells have limited functionality in contraction-based studies. However, unlike HL-1 cells, H9C2 cells possess β-tubulin II, a mitochondrial isoform of tubulin that plays an important role in mitochondrial function and regulation and may contribute to the decreased cell viability of H9C2 cells in response to hypoxia–reoxygenation injury compared with HL-1 cells (Kuznetsov *et al*., 2015). However, because this study did not compare HL-1 or H9C2 cells with primary cells, it is difficult to gauge their response to hypoxic environments with primary cells. Although these cells were reportedly isolated from a ventricular origin, their morphology and gene expression patterns are more similar to skeletal muscle myoblasts than to primary cardiomyocytes (Menard *et al*., 1999). Thus, although these cells are commonly used in place of primary cardiomyocytes, one should use caution when interpreting data obtained from these cells and applying them to other in vitro or in vivo cardiomyopathy model systems.

iPSC/ESC cardiac myocytes

One of the biggest hurdles facing researchers in the pharmaceutical industry is the development of an in vitro assay using human cells to test potential pharmaceutical compounds for cardiotoxicity in a high-throughput manner. For such assays, it is critical that the cardiac cells be as "adult" human as possible, given that the mechanism for cardiac repolarization after contraction can differ greatly among species and levels of maturity (Astashkina *et al*., 2012). ESCs and iPSCs, particularly those derived from patients with genetic diseases, are increasingly regarded as invaluable tools for disease modeling and drug-screening assays (Drawnel *et al*., 2014; Hashem *et al*., 2015). iPSCs taken from patients with a genetic disease are particularly valuable because they capture the precise genetic mutation and background from the individual from whom they are derived. Consequently, the use of iPSCs could pave the way to personalized cardiomyocyte therapeutics in a way that no other cell model has done.

Although directed cell differentiation is still difficult for some cell lineages, several protocols exist for differentiation of both iPSCs and ESCs into cardiomyocytes. For 15 years, ESCs have been used to generate cardiomyocytes (Kehat *et al*., 2001, 2002; Mummery *et al*., 2002, 2003). Many early studies examining cardiomyocyte differentiation from ESCs struggled with low cardiomyocyte yields (Kehat *et al*., 2001). In addition, although the spontaneously beating ESC-derived cardiomyocytes expressed early cardiomyocyte lineage markers, they remained phenotypically immature, appearing more similar to NRVMs than ARVMs. Recent advances in cell culture techniques have increased the percentage of cardiomyocytes in these cultures, even increasing the yield of cardiomyocytes to a level that could be used to potentially regenerate the infarcted human myocardium (Niebruegge *et al*., 2008; Chong *et al*., 2014). However, it is clear from studies in which ESC-derived cardiomyocytes were injected into an ischemic primate model that the ESC-derived cardiomyocytes used were not fully mature and increased the incidence of cardiac arrhythmias (Chong *et al*., 2014). The increased incidence of arrhythmias is not too surprising, given that prior patch-clamp electrophysiology and subsequent adrenergic receptor stimulation, by both PE and ISO, showed that ESC-derived cardiomyocytes displayed action potentials similar to those of human fetal ventricular cells isolated at 16 wk of gestation. In addition, staining for junctional proteins such as connexin 43 showed that ESC-derived cardiomyocytes were immature, although Ca^{2+} imaging showed electrical coupling between adjacent cells (Mummery *et al*., 2003). Thus, although ESCs have been used successfully in vitro to mimic contractility responses to PE and ISO (Mummery *et al*., 2003; Braam *et al*., 2010), these cells are clearly not fully functional, differentiated cardiomyocytes and need to be viewed with the same limitations as the neonatal animal models.

iPSCs, possibly the most innovative stem cell discovery in the past decade, are generated directly from somatic cells by the introduction of transcription factors (Takahashi and Yamanaka, 2006). Like ESCs, iPSCs can self-renew and differentiate into cellular derivatives of all three germ layers (Takahashi and Yamanaka, 2006; Murata *et al.*, 2010). The first method used for generating iPSC-derived cardiomyocytes was the embryoid body (EB) suspension protocol (Kehat *et al.*, 2001; Fujiwara *et al.*, 2011; Muller *et al.*, 2012). Although the EB method does produce differentiated myocytes, the efficiency is low (Braam *et al.*, 2010), hampering the use of this protocol in high-throughput drug screens. Although directed, growth-factor dependent methods of cardiomyocyte differentiation have been developed (Paige *et al.*, 2010; Kattman *et al.*, 2011), their reliance on recombinant growth factors significantly increases their variability, technical difficulty, and the cost associated with these protocols. Cost-saving protocols using small molecules as opposed to recombinant human cytokines show great promise and may reduce the cost of these cultures substantially (Karakikes *et al.*, 2014; Zanella *et al.*, 2014; Zanella and Sheikh, 2016). Although many improvements have been made regarding cardiomyocyte differentiation in stem cells, the efficiency of proliferation and differentiation remains lower for iPSCs than for ESCs (Mauritz *et al.*, 2008). A study found that complete return to pluripotency might not be necessary in order to generate cardiomyocytes, reducing time to differentiation and thus overall cost (Efe *et al.*, 2011). In addition, commercially available iPSC-derived cardiomyocytes from sources such as Cellular Dynamics (iCell Cardiomyocytes, Madison, WI) and Axolbio (Little Chesterford, Cambridgeshire, UK) increase the accessibility of these cells to labs that lack the funding, resources, or experienced personnel necessary to develop these types of cells de novo. As mentioned earlier, the greatest advantage to using iPSCs over any other cell type mentioned thus far is the ability to use cells from patients with known genetic mutations. As with ESCs, iPSCs have also been used to study PE- and ISO-induced cardiac hypertrophy (Braam *et al.*, 2013; Liang *et al.*, 2013; Table 2). However, unlike ESCs, in which genetic manipulation is necessary in order to recapitulate disease-causing mutations, iPSCs derived from patients with known mutations associated with HCM have been used to recapitulate hallmarks of cardiomyocyte dysfunction associated with cardiac hypertrophy in vitro (Carvajal-Vergara *et al.*, 2010; Moretti *et al.*, 2010; Lan *et al.*, 2013; Ma *et al.*, 2013, 2015; Han *et al.*, 2014). As an example, a study in which iPSC-derived cardiomyocytes generated from a 10-member family cohort carrying a hereditary HCM missense mutation (Arg663His) in the Myh7 gene was able to recapitulate cellular hallmarks of HCM in vitro (Lan *et al.*, 2013). iPSCs carrying this missense mutation exhibited cellular enlargement, contractile arrhythmia at the single-cell level, and dysregulation of calcium cycling. Of interest, pharmacological restoration of calcium homeostasis prevented the development of hypertrophy and electrophysiological irregularities seen in untreated cells. This study highlights the importance of using these cells as both disease models and tools for drug screening.

It is also worth noting that, whereas ESCs and iPSCs remain the best models for human cardiomyocytes, the mechanisms and pathways involved in differentiation are ambiguous, and there are numerous conflicting reports on the purity and maturity of these cells. Most current studies investigating these cells were performed within the first 2–3 wk after differentiation, a time at which these "cardiomyocyte" cells resemble an immature, embryonic-like phenotype (Hartman *et al.*, 2016). More recent studies, aimed at producing more mature cardiomyocytes, have examined cells 12–15 wk after differentiation induction. The cells in these studies are characterized by longer, more rectangular architecture (Snir *et al.*, 2003; Földes *et al.*, 2011; Lundy *et al.*, 2013), the appearance of mature Z-, A-, H-, and I-bands, and more tightly packed, parallel-oriented myofibril arrays (Figure 1D; Kamakura *et al.*, 2013). Combining longer differentiation procedures (80–100 d postdifferentiation) and nanopatterned surfaces has reduced the fragility of iPSC-derived myofibrils, allowing measurements to be performed similar to those conducted in ARVMs (Pioner *et al.*, 2016). Thus, whereas iPSCs and ESCs remain a good in vitro model system for analyzing cardiotoxicity for future therapeutics, the immaturity of these cells at shorter time points and the length of time it takes to make them phenotypically more similar to differentiated cardiomyocytes make them less than ideal for applications in which fully differentiated myocytes are essential. Because other model systems, such as the ARVMs and NRVMs discussed earlier, are easy to establish and cost-effective and have relatively low variability, animal-based cardiomyocyte in vitro model systems remain the most commonly used cells in cardiac research (Table 1).

ALTERNATIVE AND ENGINEERED MATRICES

There is a major thrust in cardiac myocyte biology to develop culture conditions that more closely mimic the native niche in the heart. The development of cardiac tissue in vivo is dependent on a wide range of physical stimuli (Lindsey *et al.*, 2014). Therefore it is not surprising that alterations in the physical environment in which cardiac cells are cultured can have a dramatic effect on their phenotype. The majority of studies on cultured cardiac myocytes are done on tissue culture polystyrene or glass, the elastic modulus of which is 10^6-fold greater than that of a normal heart. This can affect the structure and function of the cells in many ways. For example, embryonic cardiomyocytes on stiff substrates show a progressive loss of rhythmic beating and have fewer striated myofibrils than cells plated on a softer substrate (Engler *et al.*, 2008). Furthermore, it is well known that the physical environment (e.g., substrates, force loading, electrical stimulation) affects the phenotype and differentiation of stem cells (Discher *et al.*, 2009). Given the importance of these factors for differentiation and maturation of cardiomyocytes in vivo (Turnbull *et al.*, 2014; Zhu *et al.*, 2014), manipulation of substrate elasticity, composition, and geometry is a logical place to begin optimizing differentiation and culture protocols for both primary and iPSC/ESC-derived cardiomyocytes.

For primary neonatal cardiomyocytes, the goals of alternative culture methods include increased longevity in culture, induction of an adult gene expression profile, and organization of myofibrils. Modification of substrate stiffness is a common starting point for cell and tissue engineering, but the effects of alterations in substrate elasticity on cellular phenotype of primary cardiomyocytes are not clear. In contrast to embryonic chick cardiomyocytes, neonatal rat and mouse cardiomyocytes show increased myofibrillar organization on stiff substrates (20–34 kPa or 2 MPa) relative to soft ones (1–5 kPa; Engler *et al.*, 2008; Yahalom-Ronen *et al.*, 2015). The differences could be due to the cell type (e.g., embryonic vs. neonatal) or to the substrates and extracellular matrices (e.g., collagen-coated polyacrylamide vs. fibronectin-coated polydimethylsiloxane). Primary cardiomyocytes are very sensitive to the matrix on which they are plated; cells plated on a surface coated with an integrin-binding arginine-glycine-aspartic acid (RGD) peptide have a less mature phenotype than those plated on whole RGD-containing matrix proteins such as laminin and collagen (LaNasa and Bryant, 2009). One way to create a tissue scaffold with biologically relevant extracellular matrix composition is to use decellularized hearts or heart tissues that can then be repopulated with myocytes or progenitor cells

(Ott *et al.*, 2008; Lu *et al.*, 2013). These scaffolds retain the three-dimensional (3D) structure of an intact heart but, like any system, have drawbacks, such as reduced control over the differentiation program of cells seeded within them.

It is important to consider that the cellular environment in vivo is dynamic (Li *et al.*, 2014). Therefore culture conditions that mimic the changes in substrate stiffness or patterning may provide a more physiological environment. For example, hydrogels that stiffen over time promote maturation of embryonic chicken cardiomyocytes, as determined by myofibril orientation and expression of mature cardiac markers such as troponin T (Young and Engler, 2011). The 3D nature of the physiological environment is also a key consideration. The expression of cardiac-specific biomarkers and cellular responses to hypertrophic stimuli have been improved in both primary cardiomyocytes and ESC-derived cardiomyocytes by culture within 3D hydrogels (Shapira-Schweitzer *et al.*, 2009). Multidimensional micromolded gelatin surfaces produce a longer period of active contraction in culture and improved metabolic function in neonatal cardiomyocytes and also increase the longevity of iPSC-derived cardiomyocytes (McCain *et al.*, 2014).

There are many possible approaches to the principal problem of cardiac myocyte maturation with iPSC- and ESC-derived cardiomyocytes (Yang *et al.*, 2014). The simplest approach is time; when iPSC derived cardiomyocytes are kept in culture for a long enough period (days to months), the cells reach a more mature state (Lundy *et al.*, 2013). However, this does not fully recapitulate the phenotype of adult cardiomyocytes and is clearly inefficient. It appears that optimal differentiation occurs on substrates with an elasticity that is similar to that of the intact adult heart (Hazeltine *et al.*, 2014). Of interest, iPSCs are sensitive to this mechanical parameter only during the early specification stage. Improvements in myofibril alignment and mechanical output have been achieved with a combination of tunable polyacrylamide substrates and defined geometry of adhesion (Ribeiro *et al.*, 2015). Alternatively, culture of iPSC-derived cardiomyocytes on a thick Matrigel "mattress" for 5–7 d after initial differentiation improves the contractile function of the cells to a level typically only seen after 80–120 d in culture (Feaster *et al.*, 2015). The contractile kinetics of these cells is similar to that of freshly isolated adult rabbit cardiomyocytes, and there is an elevation in the level of cardiac troponin I. A shift from expression of the slow skeletal troponin I to the cardiac troponin I has been identified as a useful marker of the maturity of iPSC-derived cardiomyocytes (Bedada *et al.*, 2014). The identity of the substrate on which the cells are cultured also has a significant effect on maturity of the resulting phenotype. Screening of a library of combinatorial polymers yielded a substrate that produces cells with improved contractility, mitochondrial function, and, most notably, a switch to expression of cardiac troponin I (Chun *et al.*, 2015).

These pioneering methods produced iPSC- or ESC-derived cardiomyocytes that are similar in structure and function to adult cells, increasing their utility for the study of hypertrophy and other cardiac disorders. They also lengthened the duration of stable phenotypes and increased the maturity of neonatal cardiomyocytes. These approaches are useful for studying responses of individual cells, but many aspects of disease depend on interactions between multiple cells of the same or different cell types. There are many groups devising approaches to culturing cardiomyocytes in a defined architecture to create microtissues (Thavandiran *et al.*, 2013; Tiburcy and Zimmermann, 2014; Tzatzalos *et al.*, 2016; Zhang *et al.*, 2016). A combination of defined architecture or mechanical load and the addition of support cells results in improvements in the organization, electrophysiology, and calcium handling of iPSC-derived cardiomyocytes (Tulloch *et al.*, 2011; Nunes *et al.*, 2013). Engineered heart tissues generated from neonatal rat cardiomyocytes have higher levels of binucleation, improved sarcomere assembly, and a more physiological response to hypertrophy than neonatal cells in standard culture conditions (Tiburcy *et al.*, 2011). Such engineered heart tissues are being used to model HCM and elucidate pathogenic mechanisms of disease-causing mutations (Wijnker *et al.*, 2016). Cardiac microtissues, including those with cocultured fibroblasts and vascular cells, may be able to mimic disease progression and predict drug efficacy more accurately than single-cell assays (Ou *et al.*, 2011; Mathur *et al.*, 2015; Tzatzalos *et al.*, 2016). In this case, the presence of multiple cells and additional cell types is both a benefit, because it is more physiological, and a drawback, due to the limitations in types of assays that can be performed (e.g., difficulty in measuring the size of individual cardiomyocytes) and the confounding effects of multiple cell types.

CONCLUSIONS

The study of cardiac hypertrophy in vitro has yielded a great many insights into the signaling pathways activated on exposure of cardiomyocytes to various hypertrophic stimuli. Although primary cardiomyocytes remain the tool of choice for many cell biologists seeking to uncover the mechanistic underpinnings of cardiac hypertrophy, alternate model systems, including iPSC- and ESC-derived cardiomyocytes, are gaining ground. The advent of engineered culture conditions has vastly improved the maturity of iPSC/ESC-derived cardiomyocytes, and this model has reached the stage at which biologically interesting investigations can be performed. These new methods do not completely abrogate concerns about using induced cells (e.g., purity, toxicity, completeness of differentiation) but do add to the available toolbox. Remaining challenges in optimizing in vitro model systems for understanding cardiac hypertrophy include consistency between in vivo and in vitro models, development of standardized protocols to allow direct comparisons among studies, and refinement of techniques to reduce the technical hurdles associated with culture of primary or induced cardiomyocytes.

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