

Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function

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White, Steven M., Phillip E. Constantin, and William C. Claycomb. Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function. *Am J Physiol Heart Circ Physiol* 286: H823–H829, 2004; 10.1152/ajpheart.00986.2003.—HL-1 cells are currently the only cardiomyocyte cell line available that continuously divides and spontaneously contracts while maintaining a differentiated cardiac phenotype. Extensive characterization using microscopic, genetic, immunohistochemical, electrophysiological, and pharmacological techniques has demonstrated how similar HL-1 cells are to primary cardiomyocytes. In the few years that HL-1 cells have been available, they have been used in a variety of model systems designed to answer important questions regarding cardiac biology at the cellular and molecular levels. Whereas HL-1 cells have been used to study normal cardiomyocyte function with regard to signaling, electrical, metabolic, and transcriptional regulation, they have also been used to address pathological conditions such as hypoxia, hyperglycemia-hyperinsulinemia, apoptosis, and ischemia-reperfusion. The availability of an immortalized, contractile cardiac cell line has provided investigators with a tool for probing the intricacies of cardiomyocyte function. In this review, we describe the culture and characterization of HL-1 cardiomyocytes as well as various model systems that have been developed using these cells to gain a better understanding of cardiac biology at the cellular and molecular levels.

heart muscle cell; cell culture; cell line

ALTHOUGH MANY MODELS have been used to study cardiac muscle structure and function *in vitro*, developing suitable cell culture systems has proven to be challenging. HL-1 cardiomyocytes are currently the only cells available that continuously divide, spontaneously contract, and maintain a differentiated adult cardiac phenotype through indefinite passages in culture (11). In this review we describe the derivation and characterization of HL cardiomyocyte cell lines and their culture conditions, and we discuss the various model systems in which they have been used to study various physiological and pathophysiological conditions.

Isolated embryonic and neonatal rat primary cardiomyocytes have been the most widely used models to study cardiac biology *in vitro*, but their use is somewhat limited because they lack many adult cardiomyocyte characteristics. Moreover, the myocytes become overgrown by nonmyocytes after a few days in culture, they cease to divide after the neonatal period, and genetic manipulation is difficult (10, 12). Murine embryonic stem (ES) cells and P19 embryonic carcinoma cells have also provided useful models for studying cardiomyocyte development and differentiation (2, 5, 25, 34, 37, 38). However, until recently there was no way to obtain a pure or highly enriched population of cardiomyocytes from differentiating ES cells. In 1996, the laboratory of Field (34) developed a method for obtaining essentially pure populations of cardiomyocytes from

genetically altered differentiating ES cells. Subsequently, other groups have used a similar technique with fluorescence-activated cell sorting to obtain relatively pure populations of cardiomyocytes (26, 44). Because the cardiomyocytes obtained from these selected differentiating ES cells cease to divide, obtaining enough cells for certain experiments can be problematic. Recently, Rybkin et al. (51) generated transgenic mice in which expression of the Simian virus 40 (SV40) large T antigen was conditionally controlled by the *Nkx2.5* gene promoter using Cre-lox technology. Cells isolated from ventricular tumors from these mice demonstrated voltage-dependent calcium influx, whereas other electrophysiological properties such as action potential characteristics remain undefined. Although this model may be useful for studying some aspects of cell cycle regulation, the cells obtained from these tumors do not express certain genes known to be expressed in differentiated cardiomyocytes. Moreover, these cells do not contain well-organized sarcomeres or maintain contractile activity even during electrical stimulation (51).

Development and Characterization of HL-1 Cardiomyocytes

The HL-1 cell line was derived from AT-1 cardiac myocytes, which are atrial cardiac muscle cells obtained from transgenic mice in which expression of the SV40 large T antigen was controlled by the atrial natriuretic factor (ANF) promoter (19). Although AT-1 cells maintain a cardiomyocyte phenotype and contract spontaneously in culture, they cannot be serially passaged *in vitro* or recovered from frozen stocks. AT-1 cells can only be maintained as a subcutaneous tumor lineage in syngeneic mice, and myocytes must be prepared

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from these tumors as primary cell cultures (17). A derivative of AT-1 cells that could be serially passaged while maintaining a differentiated phenotype and also be recovered from frozen stocks was designated the HL-1 cardiomyocyte line (11).

HL-1 cardiomyocytes have been characterized by using microscopic, immunohistochemical, electrophysiological, and pharmacological methods (11). According to both microscopic and immunohistochemical studies, HL-1 cells contain highly organized sarcomeres necessary for mediating contraction and intracellular ANF granules characteristic of atrial myocytes (3). Analysis of gene expression in HL-1 cells reveals that they exhibit an adult cardiomyocyte-like gene expression profile (11). HL-1 cells spontaneously depolarize and express the necessary ion channels required for generating action potentials characteristic of primary cardiomyocytes. Pharmacological studies show that HL-1 cells respond appropriately to inotropic and chronotropic agonists, indicating expression of functional receptors and intracellular signaling proteins required for these signaling pathways (11).

In order for HL-1 cells to be passaged repeatedly while maintaining a contractile cardiac phenotype, they require a fibronectin-gelatin substrate and a specially formulated growth medium (11). The recent development of the Claycomb Medium (JRH Biosciences) has provided investigators with a standardized growth medium that permits the serial passaging and maintenance of the contractile, differentiated phenotype of the HL-1 cells. The complete formulation of this medium is provided in Table 1. In addition to factors found in the normal FBS, components such as retinoic acid, norepinephrine, insulin, and essential lipids are necessary for maintaining a differentiated cardiac phenotype in vitro. However, for certain types of experiments, these cells can be maintained in reduced or even serum-free Dulbecco's modified Eagle's medium in the absence of these added components for short periods of time (up to at least 72 h) while maintaining a differentiated phenotype.

HL-1 Cardiomyocytes as Model Systems

Hypoxia. HL-1 cardiomyocytes have proven to be useful for studying many aspects of cardiac biology in vitro. Our labo-

ratory used HL-1 cells as a model system to investigate the effects of common pathophysiological conditions such as hypoxia, hyperglycemia, and hyperinsulinemia on changes in adrenomedullin gene expression in cardiomyocytes. Adrenomedullin is a vasodilatory and natriuretic peptide secreted by cardiomyocytes in response to various stressful stimuli such as hypoxia and hyperglycemia (29, 50). In the heart, adrenomedullin has been implicated in preventing pathological remodeling and the development of heart failure following myocardial infarction while also acting as a negative inotropic factor (4, 18, 43, 45, 49). In HL-1 cells, hypoxia induces an upregulation of both hypoxia-inducible factor- α and - β , which in turn leads to increased expression of the adrenomedullin gene (14, 47).

Hyperglycemia. In addition to hypoxia, we have also used HL-1 cells to study the effects of hyperglycemia and hyperinsulinemia, both of which are characteristic of Type II diabetes mellitus, on cardiac adrenomedullin gene regulation. Hyperglycemia increases vascular adrenomedullin gene expression, and increased plasma levels of adrenomedullin have been associated with diabetic conditions (24, 27, 39, 40). Using HL-1 cardiomyocytes, Collier et al. (13) found that chronic, but not acute, hyperglycemia results in a fourfold induction of adrenomedullin mRNA levels, an effect that can be repressed by insulin. These studies demonstrate that HL-1 cells are an ideal model for studying the effects of pathological conditions such as hypoxia and hyperglycemia on cardiac function in vitro.

Cellular signaling. HL-1 cells have also been useful for studying cellular signaling pathways and various types of receptors in cardiomyocytes. In addition to β -adrenergic receptors, HL-1 cells express α -adrenergic receptors, which modulate numerous intracellular signaling events (42). Endothelin signaling in the heart is associated with physiological as well as pathophysiological conditions such as apoptosis and heart failure (58). Kitta and coworkers (30) demonstrated that endothelin-1 induces phosphorylation and subsequent activation of the cardiac transcription factor GATA-4 in HL-1 cells, implying functional endothelin receptor-mediated signaling. Opioid receptors are also expressed by primary cardiomyocytes and have been shown to confer cardioprotective effects following ischemia-reperfusion injury (20, 41, 48, 54, 55). Neilan and coworkers (46) demonstrated that HL-1 cells express functional δ -opioid receptors, demonstrating that HL-1 cells may be useful for studying the cardiac effects of opioids. Additionally, Seymour and coworkers (56) demonstrated that δ -opioid preconditioning in HL-1 cells is dependent on protein kinase C- and ATP-sensitive K (K_{ATP}) channel-mediated signaling. Other investigators have used HL-1 cells to examine such cardiomyocyte characteristics as membrane transport (8) and regulation of metabolic pathways (57). All of these studies highlight the potential importance of HL-1 cardiomyocytes as a cell system to develop novel cardiac pharmacological agents.

Electrophysiology. Two important characteristics of HL-1 cells are the spontaneous action potentials and contractions they display in culture. Until recently, expression of the inward delayed rectifier current (I_{Kr}), characteristic of cardiomyocytes, was the only published data regarding the electrophysiology of the HL-1 cells (11). Akhavan et al. (1) have used HL-1 cardiomyocytes to study mutations in the human *ether-a-go-go*-related gene (HERG), which encodes the α -subunit of I_{Kr} .

Table 1. Formulation of Claycomb Medium

Total protein	261 mg/l
Bovine albumin	48.85 mg/l
Nonessential amino acids	0.1 mM
Fetuin	165 mg/l
Transferrin	31.8 mg/l
Retinoic acid	300 μ g/l
Human insulin (recombinant)	15 μ g/l
Long R ³ IGF-1 (recombinant)	0.1 μ g/l
Long EGF (recombinant)	0.1 μ g/l
Cholesterol	1.96 mg/l
Linoleic acid	0.78 mg/l
γ -Oleyl- β -pal- α -phosphatidylcholine	1.96 mg/l
Ascorbic acid	0.3 mM
Norepinephrine	100 μ M
L-Glutamine	2 mM
Penicillin-streptomycin (optional)	100 U/ml-100 μ g/ml
Fetal bovine serum	10%

The basal medium for Claycomb Medium is Dulbecco's modified Eagle's medium. This complete formulation for Claycomb Medium was provided by JRH Biosciences, Lenexa, KS.

HERG mutations have been associated with long QT syndrome, which is characterized by abnormal cardiac repolarization and prolongation of the QT interval in the electrocardiograms (1). Understanding the molecular mechanisms of how specific HERG mutations lead to alterations in I_{Kr} function is critical for treating and preventing abnormal cardiac repolarization. These investigators (1) found that a mutation in the carboxy-terminal region resulted in defective maturation, intracellular trafficking, and plasma membrane insertion of HERG. More recently, Kupersmidt and coworkers (35) used HL-1 cells to demonstrate how KCR1, a novel HERG channel binding protein, decreases the sensitivity of HERG channels to antiarrhythmic agents. These experiments further demonstrate how HL-1 cells can be effectively manipulated in culture to study molecular mechanisms of cardiac function.

In 2002, Sartiani and coworkers (53) published an extensive electrophysiological characterization of HL-1 cells by studying calcium cycling, action potential characteristics, and expression of a hyperpolarization-activated, cyclic nucleotide-gated "pacemaking" current. Pacemaking or conducting cardiomyocytes spontaneously depolarize due to activation of a hyperpolarization-activated inward cation current termed the "funny" current (I_f). The genes encoding subunits of the channels responsible for the I_f current are the hyperpolarization-activated, cyclic nucleotide-gated channel (HCN) genes. Sartiani and coworkers (53) used the calcium-sensitive dye Fluo 3-AM

to measure intracellular calcium oscillations. They found cesium-sensitive, spontaneous, rhythmic calcium oscillations that correlated with the spontaneous action potential frequency for these cells. Using whole cell current-clamp conditions, Sartiani and coworkers (53) observed that the membrane capacitance, which is indicative of cell size, for the HL-1 cells ranged from ~ 5 to 50 pF. Additionally, the cells had a maximal diastolic potential of -68.8 ± 1.6 mV with an overshoot of $+15.3 \pm 1.9$ mV and an action potential waveform characteristic of atrial myocytes (53). They then demonstrated the presence of a pacemaking I_f current with a half-maximal activation potential ranging from -50 to -60 mV and showed that the I_f current could be blocked with extracellular cesium, which is a characteristic of this current. They also observed that activating adenylyl cyclase, thereby increasing intracellular cAMP, caused a positive shift in the I_f activation potential, also characteristic of this current. Additional studies revealed that the percentage of the cells expressing the I_f current and the characteristics of the current did not change significantly with confluency or passage number. Finally, these investigators determined that HL-1 cells express all four HCN gene isoforms, with HCN-1 and HCN-2 being expressed at much higher levels than the other two isoforms. The results of these studies highlight how very similar HL-1 cells are to primary atrial cardiomyocytes with regard to their electrophysiological properties. Because the electrophysiological properties of

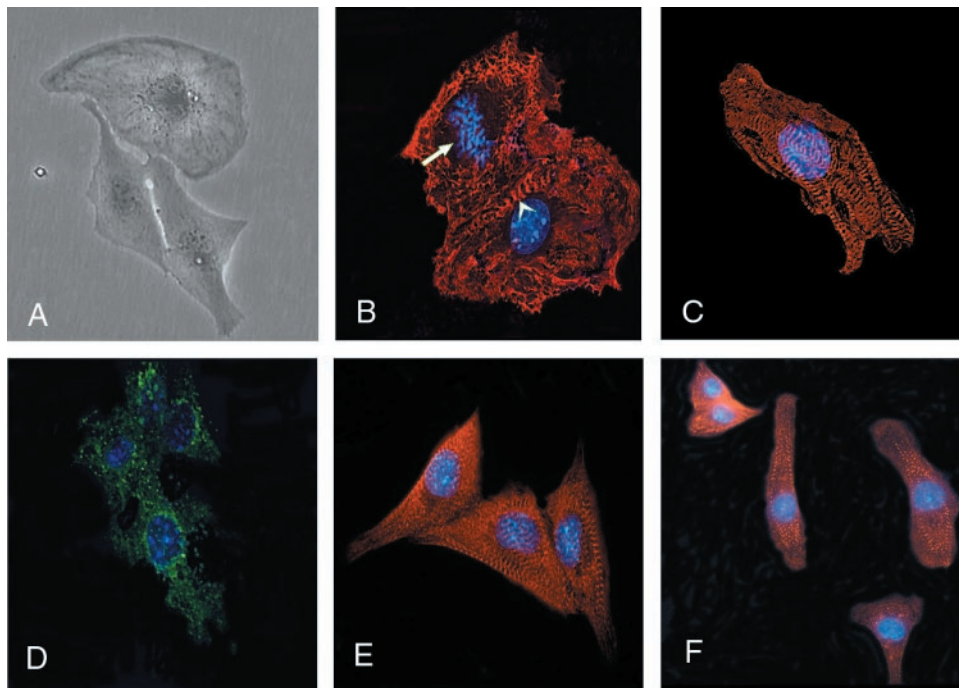


Fig. 1. Images of HL cells demonstrating morphology and expression of proteins representative of a cardiomyocyte phenotype. *A*: a phase-contrast image of two HL-1P cardiomyocytes that have just completed cytokinesis adjacent to a contracting myocyte. *B–F*: all indirect immunofluorescent images (all nuclei are stained blue with Hoechst dye). *B*: two HL-2 cardiomyocytes stained with the sarcomeric myosin antibody MF-20 (Developmental Studies Hybridoma Bank), one of which is in metaphase of mitosis (arrow indicates metaphase chromosomes) adjacent to a nondividing myocyte containing organized sarcomeres (arrowhead). Sarcomeric structure in these cells becomes disorganized during cell division and quickly reorganizes following cytokinesis similar to what occurs in mitotically active embryonic cardiomyocytes in the developing heart. *C*: an HL-2 cardiomyocyte containing highly organized sarcomeres also stained with the MF-20 antibody for myosin. *D*: localization of atrial natriuretic factor (ANF) (Peninsula Laboratories) expression in HL-2 cardiomyocytes (note the intensely stained perinuclear ANF-containing granules). *E*: highly organized sarcomeric structure in HL-2 cardiomyocytes stained for titin (Developmental Studies Hybridoma Bank). *F*: HL-1 cells stained for the sarcomeric protein α -actinin (Sigma).

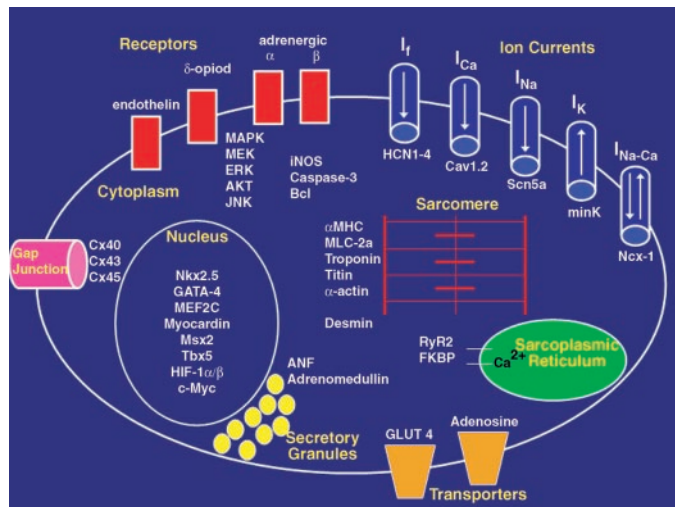


Fig. 2. Schematic depiction of characteristic features of a typical HL-1 cardiomyocyte. Although HL-1 cells possess additional components, only those that have been published are shown in this diagram. The components listed are grouped according to their function and location within the cell. Cx, connexin; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase (MAPK); ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; JNK, Jun kinase; MHC, myosin heavy chain; MLC, myosin light chain; GLUT, glucose transporter; RyR, ryanodine receptor; FKBP, FK506 binding protein; I_f , pacemaking or “funny” current; I_{Ca} , calcium current; I_{Na} , sodium current; I_K , potassium current; $I_{Na/Ca}$, sodium-calcium exchange current. The following genes expressed in HL-1 cells are shown under the current with which they are associated in vivo: the hyperpolarization, cyclic nucleotide-gated channels (HCN), the voltage-gated L-type Ca^{2+} channel (Cav1.2), the voltage-gated sodium channel (Scn5a), modulatory β -subunit for the inward rectifier potassium current (minK), and the sodium/calcium exchanger (Ncx).

HL-1 cells are so similar to those of primary cardiomyocytes, they have been used to develop a portable cell-based biosensor system capable of monitoring the effects of chemical and biological agents on cardiac function outside of the laboratory (16, 22).

Calcium handling. In addition to membrane depolarization, efficient intracellular calcium handling is necessary to maintain rhythmic contractions in cardiomyocytes. Altered intracellular calcium handling in cardiomyocytes is associated with the development of arrhythmias and the progression of heart failure. Therefore, the development of cellular model systems to study intracellular calcium handling is important for developing novel therapeutic pharmaceutical agents. George and coworkers (21) used HL-1 cells to study three ryanodine receptor (RyR) mutations associated with stress-induced ventricular tachycardia in humans. Of the three known RyR isoforms, RyR2 is the isoform expressed in primary cardiomyocytes and is also the isoform expressed by HL-1 cells. These investigators transiently transfected HL-1 cells with expression vectors encoding recombinant GFP-tagged human RyR2 (wild-type) and with each of the three single amino acid RyR2 mutations known to be associated with stress-induced ventricular tachycardia. George and coworkers (21) demonstrated correct localization of the receptors to the endoplasmic reticulum and colocalization with FK506 binding protein, which modulates RyR activity. Intracellular calcium release was significantly augmented in HL-1 cells expressing mutant RyR2 after the addition of agonists or β -adrenergic stimulation (21). This

paper provides an example of how HL-1 cells can be used to study cardiac pathological conditions at the molecular level.

Apoptosis. HL-1 cells have been widely used to study mechanisms involved in cardiac apoptosis. Growth factors are released in the myocardium following injury to stimulate cellular growth or survival. Kitta and coworkers (31, 33) have shown that hepatocyte growth factor (HGF) is capable of protecting HL-1 cardiomyocytes from oxidative stress-induced apoptosis. This same group investigated the role of GATA-4 in the cardioprotective effects of HGF because GATA-4 can induce cell survival. Using HL-1 cells, they demonstrated that HGF induces GATA-4 phosphorylation and DNA binding activity through the mitogen-activated protein kinase/extracellular signal-regulated protein kinase (ERK) kinase (MEK) signaling pathways. Moreover, phosphorylation of GATA-4 induces expression of anti-apoptotic Bcl-x_L (32). It was further demonstrated that GATA-4 is critical for cell survival and that suppression of GATA-4 expression in HL-1 cells leads to apoptosis (28). HL-1 cells have also been used to study the effects of the antihypertensive agent doxazosin on cardiomy-

Table 2. Types of studies that have utilized HL-1 cardiomyocytes as a cell culture model

Type of Study	Reference
Apoptosis	Carlson et al. (6)
	Cicconi et al. (9)
	Gonzalez-Juanatey et al. (23)
	Kim et al. (28)
	Kitta et al. (30)
	Kitta et al. (31)
	Kitta et al. (32)
Cell cycle	Lanson et al. (36)
	Zandstra et al. (61)
Electrophysiology	Akhavan et al. (1)
	Claycomb et al. (11)
	DeBusschere and Kovacs (16)
	Gilchrist et al. (22)
	Kupersmidt et al. (35)
Oxidative stress	Sartiani et al. (53)
	Cicconi et al. (9)
	Kitta et al. (30)
	Kitta et al. (31)
	Nguyen and Claycomb (47)
	Sanders et al. (52)
Signal transduction	Suzuki et al. (58a)
	Chaudary et al. (8)
	Cicconi et al. (9)
	Gonzalez-Juanatey et al. (23)
	Kim et al. (28)
	Kitta et al. (30)
	Kitta et al. (32)
	McWhinney et al. (42)
	Neilan et al. (46)
	Sanders et al. (52)
Seymour et al. (56)	
Transcriptional regulation	Soltys et al. (57)
	Wu et al. (60)
	Collier et al. (13)
	Dai et al. (15)
	Kim et al. (28)
	Kitta et al. (30)
Cellular transplantation	Kitta et al. (32)
	Nguyen and Claycomb (47)
	Watanabe et al. (59)

ocyte apoptosis. Doxazosin, an α_1 -adrenoceptor antagonist, is associated with an increased risk of heart failure in hypertensive patients. However, the cellular mechanisms responsible for the development of heart failure in these patients are unknown. Gonzalez-Juanatey and coworkers (23) showed that the mechanism of cellular injury induced by doxazosin is independent of its blockade of α_1 -adrenoceptors, thereby implying that the drug has cellular effects other than those mediated by these receptors. Other investigators (6, 52) have also used HL-1 cells as models for studying cardiomyocyte apoptosis.

Cell cycle regulation. In addition to the use of HL-1 cells for the examination of cardiomyocyte apoptosis, they can also be very useful for studies of the cardiomyocyte cell cycle. Because HL-1 cells have been immortalized by using the SV40 large T antigen, which binds and alters the function of the tumor suppressors pRb and p53, they can serve as a useful model for studying how cells become immortalized while maintaining a differentiated phenotype. We have immunoprecipitated proteins from HL-1 cells by using antibodies to T antigen and p53 to determine the identity of other proteins involved with this T antigen-dependent immortalization. Three proteins that were isolated and sequenced following immunoprecipitation were identified as MRE11, NBS1, and RAD50, which are known to act together in a complex that participates in detecting and repairing DNA double-strand breaks and is required for a functional S-phase checkpoint (7, 36). Therefore, part of the mechanism of immortalization by the SV40 large T antigen can be explained by the binding of T antigen to the MRE11-NBS1-RAD50 complex in addition to pRb and p53, thereby ablating this cell-cycle S-phase checkpoint in cardiomyocytes (36). The altered cell cycle in HL-1 cells makes them a useful tool for studying cellular immortalization, but this fact should also be considered when data are interpreted from other types of experiments. Although HL-1 cells are immortalized with an oncogenic viral protein, we used them for cellular transplantation studies in a porcine model of myocardial infarction and no tumors were found 3 mo after the engraftment. Interestingly, electron microscopy demonstrated that some of these transplanted HL-1 cells actually formed cellular junctions with the host cardiomyocytes while inducing substantial local angiogenesis (59).

Genetic manipulation. HL-1 cardiomyocytes are also amenable to genetic manipulation using various techniques. Cationic lipid reagents typically yield a transfection efficiency of ~75–80%. Treatment of HL-1 cells with replication-deficient adenoviruses gives a transduction efficiency of close to 100%. Therefore, cardiac genetic knock-in and knock-out studies may be performed initially in these cells without having to generate transgenic animals. HL-1 cells are also used in studies of cardiac gene promoter function or identifying novel genetic regulatory elements (15). They have also been used as a positive control for assessing the degree of differentiation of murine embryonic stem cell-derived cardiomyocytes (61). Because HL-1 cells express the same receptors as primary cardiomyocytes, they can be useful for studying the effects of novel cardiac pharmacological agents. As with most cell culture models, cellular confluency and frequency of passaging must be considered when designing experiments. Therefore, the use of HL-1 cells at similar densities for a series of experiments is critical for obtaining reproducible results.

Derivation of Additional HL Cell Lines

Subsequent to the development of the HL-1 cardiomyocyte line, additional HL cell lines (HL-1P, HL-2, and HL-5) have been derived in our laboratory from cultured AT-1 cells. Morphological, genetic, and immunohistochemical analyses (Fig. 1) of these HL cell lines show that they all exhibit essentially the same adult cardiomyocyte phenotype as do HL-1 cells. The genes expressed in these HL cell lines include those coding for transcription factors, sarcomeric proteins, ion channels, gap junction proteins, and various other genes characteristic of cardiomyocytes as depicted diagrammatically in Fig. 2. The HL-5 cell line has already been used to study intracellular processing of atrial natriuretic peptide by using RNA interference technology (60). Additionally, HL-5 cells have proven useful for characterizing apoptotic signaling mechanisms in an in vitro model of ischemia-reperfusion (9).

In summary, HL-1 cardiomyocytes and similarly derived HL cell lines have been shown by us and others to be useful models for studying many features of cardiac physiology and pathophysiology because they demonstrate characteristics of differentiated cardiomyocytes while continuously proliferating in culture. Although HL-1 cells were originally derived from atrial myocytes, they have proven to be useful as a general model for studying contracting (working) cardiomyocytes because of their organized structure and ability to contract in culture. Table 2 provides an overview of the various types of studies that have utilized HL-1 cells as a model system, and Fig. 2 provides a summary of the characteristics of HL-1 cells that make them such a useful model for studying cardiomyocyte physiology. Many of the studies described in this review demonstrated that similar results were obtained when HL-1 and isolated primary cardiomyocytes were used simultaneously. Therefore, the availability of HL cells provides investigators with a simple, reproducible cell culture system that can be used as models to develop a better understanding of the intricate cellular and molecular regulation of cardiac function.

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