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Supplement Material

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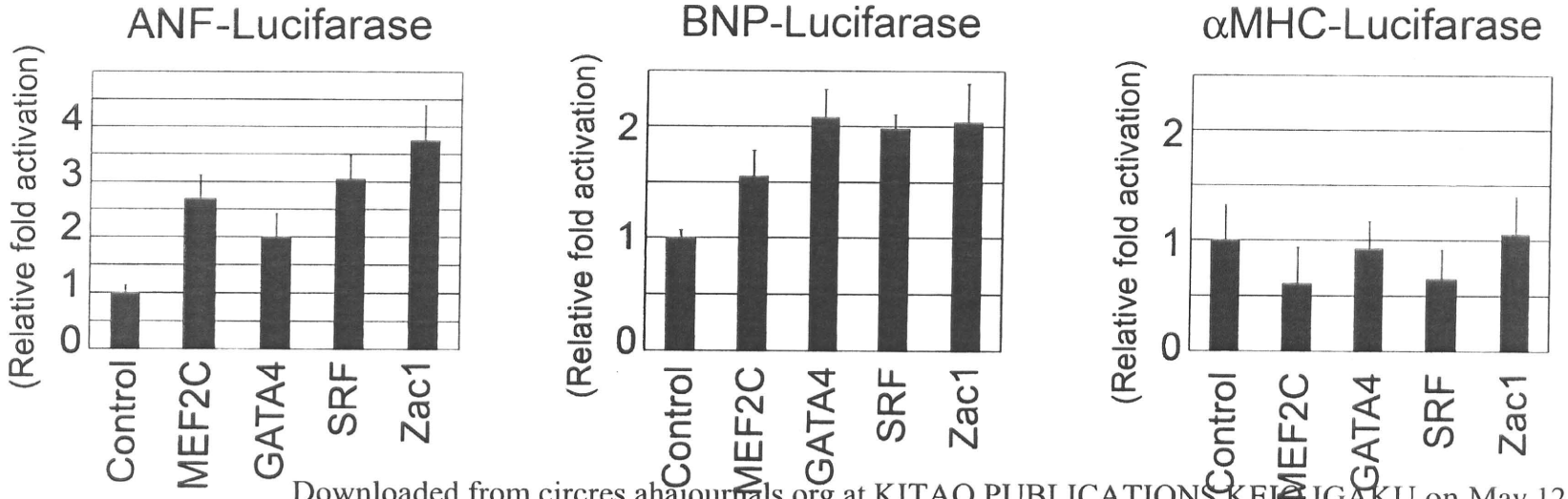

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Impacts of recent advances in cardiovascular regenerative medicine on clinical therapies and drug discovery

Mitsushige Murata, Shugo Tohyama, Keiichi Fukuda *

Department of Regenerative Medicine and Advanced Cardiac Therapeutics, School of Medicine, Keio University, Tokyo, Japan

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ABSTRACT

Although stem-cell technology holds great promise for the treatment of degenerative diseases and the development of new drugs, progress has been hindered by immune and ethical problems in association with the use of embryonic stem cells (ESCs). The recent development of reprogramming of differentiated human somatic cells to pluripotent stem cells (iPSCs) should overcome these obstacles and facilitate clinical applications of stem cells. One of the advantages of reprogramming is that it allows the establishment of patient- and disease-specific in vitro models of human hereditary diseases for pathophysiologic and developmental studies. These in vitro models can be used for drug development and testing, moving us a step closer to personalized therapies. This review outlines the current status of pluripotent stem cells and focuses on the potential applications of stem cell-derived cardiomyocytes for clinical therapies, as well as for drug development and testing.

Contents

1. Introduction

In recent years, remarkable progress has been made regarding the identification, derivation, and characterization of stem cells or progenitor cells, including embryonic stem cells (ESCs) and mesenchymal stem cells. Much attention has been focused on ESCs owing to their unique proliferation and pluripotency properties. Murine ESCs (mESCs), which were first established by Evans and Kaufman (1981), were used to establish the early developmental model systems and to generate genetically modified mice, including genetic-knockout mice. Furthermore, the establishment of human ESCs (hESCs) by Thomson et al. (1998) suggested a source of cells for regenerative therapy. Although protocols have been drawn up for the directed differentiation of stem cells into various tissue cells and for ensuring survival following transplantation, several problems need to be overcome before clinical applications are feasible (Passier et al., 2008). Ethical and legal considerations block the establishment of hESCs, as this necessitated the destruction of early human embryos, and ESCs do not display the autologous genotype of the patient (Evans & Kaufman, 1981). A potential solution to these problems that retains the characteristics of stem cells involves the reprogramming of the nuclei of differentiated cells to an ESC-like, pluripotent state. Thus, murine and human induced pluripotent stem cells (iPSCs) have been generated by reprogramming

Abbreviations: ESC, embryonic stem cell; ES-CM, ES-derived cardiomyocyte; iPSC, induced pluripotent stem cell; iPS-CM, iPS-derived cardiomyocyte; AP, action potential; TdP, Torsades de Pointes.

* Corresponding author. Department of Regenerative Medicine and Advanced Cardiac Therapeutics, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Tel.: +81 3 5363 3874; fax: +81 3 5363 3875.
E-mail address: kfukuda@sc.itc.keio.ac.jp (K. Fukuda).

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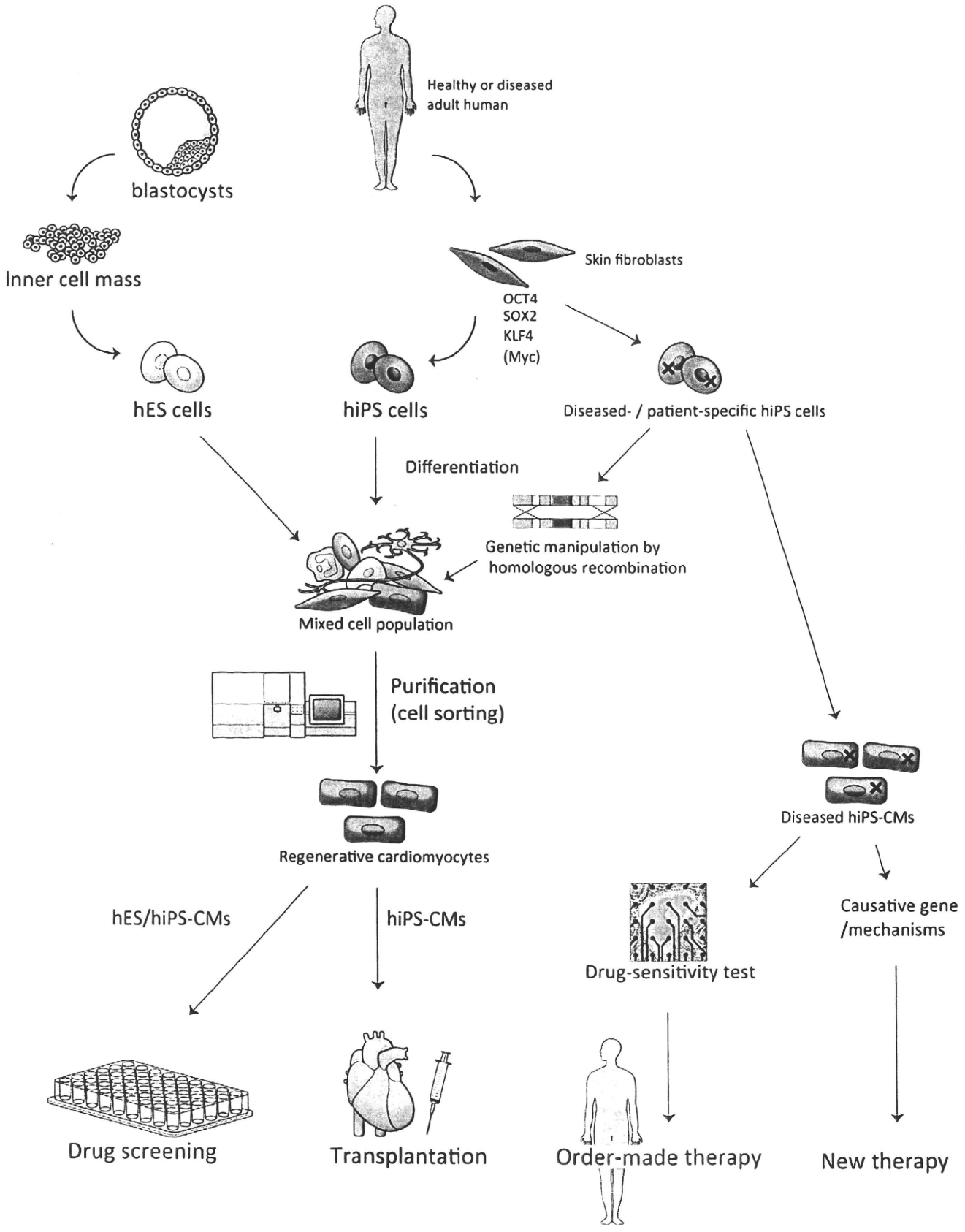
Healthy or diseased adult human
blastocysts
Inner cell mass
Skin fibroblasts
OCT4
SOX2
KLF4
(Myc)
hES cells
hiPS cells
Diseased- / patient-specific hiPS cells
Differentiation
Genetic manipulation by homologous recombination
Mixed cell population
Purification (cell sorting)
Regenerative cardiomyocytes
Diseased hiPS-CMs
hES/hiPS-CMs
hiPS-CMs
Drug-sensitivity test
Causative gene /mechanisms
Drug screening
Transplantation
Order-made therapy
New therapy

somatic cells via the overexpression of defined stem-cell transcription factors (Yu et al., 2007; Takahashi et al., 2007a,b; Nakagawa et al., 2008). iPSCs are similar to ESCs in terms of morphology, proliferation, cell surface marker patterns, and gene expression profiles. They also have the capacity to differentiate into the three germ lines, thereby providing an alternative cell source to ESCs for regenerative therapy while avoiding the problems of ethical barriers and immune rejection. Thus, advances in iPSC research have led to high expectations regarding the use of these cells both in clinical therapies and in vitro study.

Cardiovascular diseases represent important targets for regenerative medicine because they are associated with high morbidity and mortality rates. Since heart cells lack capacity for self-repair, the recovery of injured heart dysfunction requires replacement by transplantation with healthy cardiomyocytes derived from stem cells with strong proliferative capacities and reliable differentiation abilities. Recent studies have shown that human iPSCs (hiPSCs) can differentiate into cardiomyocyte-like cells that are similar in gene expression profiles and physiologic properties to native cardiomyocytes and ES-derived cardiomyocytes (ES-CMs) (Tanaka et al., 2009; Yokoo et al., 2009; Zhang et al., 2009), suggesting its potential for cardiovascular regenerative medicine. The other merit of iPSCs is that they can be used as patient- or disease-specific stem cells. Since iPSCs derived from patients retain both the original genotype and phenotype, hiPSCs provide excellent models for investigations into the mechanisms of heart disease, as well as for drug testing and toxicology (Fig. 1).

In the present review, we discuss the current state of stem-cell research, focusing on human iPSC technology and its application for regeneration therapy and for in vitro models of cardiovascular diseases.

2. Recent progress in regenerative medicine

2.1. Generation of pluripotent stem cells

hESCs are conventionally derived from the inner cell mass (ICM) of human embryos at the blastocyst stage by plating a monolayer of mitotically inactivated mouse embryonic fibroblasts (MEFs), which serve as supporting feeder cells (Thomson et al., 1998). The cells can self-renew and be maintained as undifferentiated cell lines. Furthermore, hESCs have pluripotency, which means that they have the capacity to differentiate into various types of somatic and germ cells (Reubinoff et al., 2000). Based on these unique characteristics, the use of ESCs in basic and clinical regenerative research has been proposed. However, hESCs are generated from human embryos and do not have the autologous genotype of the patient, which raises ethical issues and the potential for immune rejection following transplantation into a living human body. These problems promoted the successful development of the cell fusion and somatic nuclear transplantation techniques, which emphasize the advantage of using somatic cells from patients to generate unique ES-like cell lines (Wakayama et al., 1997; Wilmut et al., 1997; Cowan et al., 2005). Subsequent research on the mechanisms underlying the control of ESC pluripotency revealed that pluripotency is controlled by a set of transcription factors (Boyer et al., 2006). This led to the innovative discovery of iPSCs, which were generated directly from somatic cells by the introduction of defined transcription factors (Yamanaka factors: Oct3/4, Sox2, Klf4, and c-Myc) into mice (Takahashi & Yamanaka, 2006). Soon after the first report on murine iPSCs (miPSCs), four independent groups established hiPSCs from human embryonic, neonatal, and adult fibroblasts (Takahashi et al., 2007a; Yu et al., 2007; Lowry et al., 2008; Park et al., 2008b). The Thomson group used lentiviral vectors that expressed Oct3/4, Sox2, Nanog, and Lin28, while the other three groups used retroviral vectors that expressed the four Yamanaka factors. Despite the use of different combinations of reprogramming factors, the iPSCs derived by these groups were identical with respect to colony morphology, gene expression profiles, and differentiation potential. hiPSCs are very similar to hESCs in terms of morphology, proliferation, surface antigens, and gene expression (Takahashi et al., 2007b). hiPSCs express several undifferentiated ESC-marker genes, such as those for Oct3/4, Nanog (Fig. 2), Sox2, growth and differentiation factor 3 (GDF3), reduced expression 1 (REX1), fibroblast growth factor 4 (FGF4), embryonic cell-specific gene 1 (ESG1), developmental pluripotency-associated 2 (DPPA2), DPPA4, and telomerase reverse transcriptase (hTERT), at levels equivalent to or higher than those detected in the hESC line (Takahashi et al., 2007b). In addition to the murine and human iPSCs, iPSCs have been established from the rat, monkey, and pig (Shantsila et al., 2007; Lin et al., 2008; Esteban et al., 2009; Li et al., 2009). These cells should be applicable to preclinical studies.

2.2. Mechanism underlying iPSC derivation

Investigation of the expression dynamics of pluripotency markers revealed that the fibroblast-specific gene *Thy1* is initially down-regulated, and this is followed by the up-regulation of the expression of alkaline phosphatase and the ESC surface marker SSEA during the initial phase of reprogramming. In contrast, activation of endogenous Oct4, Nanog, and Sox2 occurs during the late phase of reprogramming, while the continuous expression over 10–12 days of exogenous Yamanaka factors (Oct3/4, Sox2, Klf4, and c-Myc) is essential for iPS induction (Brambrink et al., 2008; Stadtfeld et al., 2008). Furthermore, several studies have revealed the genome-wide binding sites of these reprogramming factors, as well as other transcription factors that are associated with pluripotency. Oct4, Nanog, and Sox2 interactions regulate the expression of genes that are essential for the maintenance of pluripotency or differentiation (Boyer et al., 2005; Loh et al., 2006; Jiang et al., 2008; Kim et al., 2008). Moreover, c-Myc is crucial for the suppression of fibroblast-specific genes during the initial stage of reprogramming, which suggests distinct roles for these transcription factors in the reprogramming of somatic cells to ES-like cells. However, reprogramming cannot be regulated exclusively by transcriptional regulation, since not all somatic cells that express the Yamanaka factors can be reprogrammed into iPSCs. Inhibitors of DNA methyltransferase and histone deacetylase (HDAC), such as 5-azacytidine and valproic acid, improve reprogramming efficiency (Huangfu et al., 2008a,b; Shi et al., 2008). Furthermore, the biosynthesis of microRNAs that are related to differentiation may be suppressed by Lin28, which is one of the four reprogramming transcription factors used by Yu et al. (2007), and other microRNAs, including miR-302s, miR-291-3p, miR-294, and miR-295, may activate genes that are related to pluripotency (Lin et al., 2008; Judson et al., 2009). This suggests that the reprogramming of epigenetic information is one of the major components of iPSC generation. Overall, the induction of iPSCs can be regarded as: 1) the reprogramming of

Fig. 1. Application of human stem cell-derived cardiomyocytes. hESCs can be derived from human blastocysts. Inner cell mass (ICM) cells are isolated from blastocysts, plated on the feeder cell layer, and propagated for the generation of hESCs. hiPSCs are generated from healthy or diseased human fibroblasts by transduction with defined transcription factors. To induce cardiomyocyte differentiation, hESCs or hiPSCs are cultivated in suspension, whereby they show three-dimensional aggregation and form embryoid bodies (EBs). The differentiation cell clusters are composed of mixed cell populations, which include differentiated cardiomyocytes, other differentiated cells, and undifferentiated cells. These cells are subjected to FACS analysis, and the stem cell-derived cardiomyocytes are purified for appropriate studies. Both hESCs and hiPSCs are applicable to drug screening. In contrast, hiPSCs are preferred for cell transplantation, as they avoid ethical problems and immune rejection. hiPSCs can also be generated from patients. Although disease-specific iPSCs retain the genetic dysfunction of the patient, they can be converted to normal stem cells by homologous recombination, thus becoming a source of cells for transplantation into the patient. Without genetic manipulation, disease-specific hiPSCs can be used for drug-sensitivity testing, screening for genetic disorders, and understanding disease mechanisms. Drug-sensitivity testing of individual patients may lead to customized therapies, and the new knowledge of pathogenesis may lead to novel therapeutic approaches.

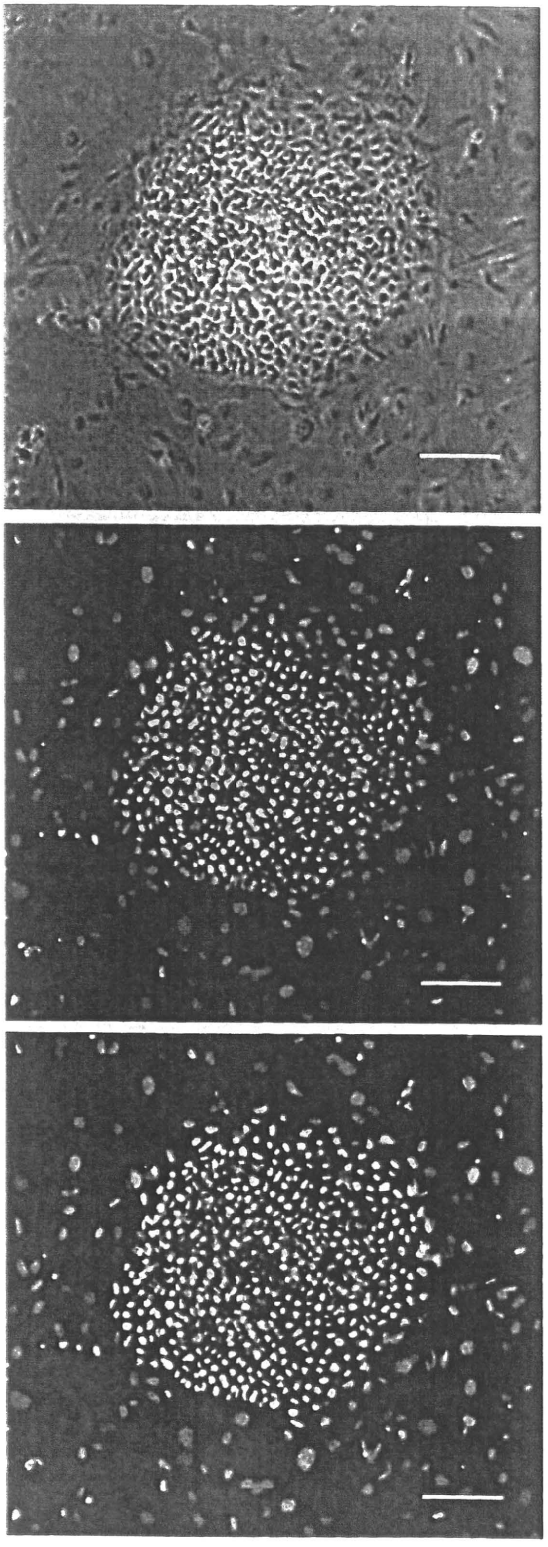

Fig. 2. Characterization of undifferentiated hiPSCs. Immunocytochemical staining for the representative ES markers Oct4 (green) and Nanog (red) in hiPSCs. Nuclei are stained with DAPI (blue). Scale bar, 100 μm.

epigenetic and expression profiles by a few transcription factors; and 2) the reversal of the differentiated state of a somatic cell into a pluripotent state.

2.3. Optimization of pluripotent stem-cell induction

The critical issues associated with current methods of iPSC generation are the extremely low efficiency of the process and safety problems related to virus integration and tumorigenicity. The low efficiency of iPSC generation negatively affects its clinical and basic applications. To enhance reprogramming efficiency, new reprogramming factors or small molecules have been investigated. Inhibitors of DNA methyltransferase and HDAC enhance the derivation of iPSCs (Huangfu et al., 2008a,b). Furthermore, genetic knockdown of p53 and the overexpression of UTF1 in addition to the four Yamanaka factors increase 100-fold iPS derivation (Zhao et al., 2008b). The tumorigenicity of iPSCs, which is related to viral integration or reactivation of the oncogenic gene *c-Myc*, needs to be resolved before clinical applications are possible. Recently, to reduce tumorigenicity due to viral integration, methods for the transduction of reprogramming factors using a plasmid vector, adenoviral vector, transposons, and recombinant proteins were reported to be safer techniques for iPS derivation (Okita et al., 2008; Stadtfeld et al., 2008; Woltjen et al., 2009; Yu et al., 2009). Regarding the use of an oncogenic gene, several groups succeeded in the generation of iPSCs without using c-Myc. Subsequent studies overcame the reduced efficacy of iPSC derivation due to the lack of c-Myc using a combination of transcription factors and signal modification or small molecules (Marson et al., 2008; Zhao et al., 2008a). Furthermore, the donor cell type influences the tendency of miPSCs to form tumors (Aoi et al., 2008). Therefore, to ensure safety, the use of an oncogenic transcription factor and integration of the viral vector should be avoided in the induction of iPSCs, even if the outcome is reduced efficiency. Further studies are needed to identify novel methods to improve the efficiency of iPS generation without the use of an oncogenic gene.

3. Human induced pluripotent stem cell-derived cardiomyocytes

3.1. Cardiac differentiation of hiPSCs

ESCs and iPSCs can self-renew indefinitely and can differentiate into the cellular derivatives of all three germ layers. However, the efficiency of cardiac differentiation is typically low. To induce cardiac differentiation, many approaches using ESCs have been tested to date. The most commonly used method involves the formation of embryoid bodies (EBs) in suspension culture. In general, the differentiation of ESCs into any cell lineage is based on the mechanism of normal early development. Various signaling protein families, including the BMPs, Wnts, and FGFs, are thought to be involved in cardiomyocyte induction from ESCs (Dell'Era et al., 2003; Kawai et al., 2004; Kwon et al., 2007; Terami et al., 2004; Ueno et al., 2007). Several studies have shown that various combinations of BMPs, activin, and Wnts induce mesoderm or endoderm development from ESCs (Laflamme et al., 2007; Sumi et al., 2008; Vijayaragavan et al., 2009). We have speculated that the context-dependent differential action of BMPs in cardiomyocyte induction is explained by the local action of Noggin and other BMP inhibitors, and we have developed a protocol to induce cardiac differentiation of mESCs through transient administration of Noggin (Yuasa et al., 2005). In addition, the visceral endoderm is known to play a key role in the differentiation of the cardiac precursors that are present in the adjacent mesoderm during development (Nascone & Mercola, 1995). Mummery et al. (2003) previously reported that hESCs effectively differentiate into cardiomyocytes when co-cultured with mouse visceral endoderm-like (END-2) cells. Moreover, many other methods have been described for the induction of cardiac differentiation, e.g., the addition of ascorbic acid and the elimination of serum or insulin (Takahashi et al., 2003; Passier et al., 2005; Freund et al., 2008).

For cardiomyocyte induction from hiPSCs, most studies have used methods that have been already been established for ESCs, such as the

addition of 5-azacytidine, BMPs, ascorbic acid, and VEGF (Gai et al., 2009; Moretti et al., 2009). Zhang et al. (2009) reported that hiPSC lines tended to show lower efficiencies for the formation of contracting EBs than those typically observed for hESC lines when induction was carried out in low-serum conditioned medium. These results are consistent with the lower efficiencies of formation of contracting EBs from miPSCs, as compared with those formed from mESCs (Mauritz et al., 2008). These outcomes may be related to the continued expression of the transgenes in hiPSC lines during differentiation. However, it is difficult to discuss the difference in cardiomyocyte induction efficiency between hiPSCs and hESCs, given the significant variability in efficiency of cardiac differentiation previously described for hESCs, which was attributed to genetic or epigenetic heterogeneity (Adewumi et al., 2007; Moore et al., 2008; Osafune et al., 2008). Therefore, in terms of future clinical applications, more information is needed with respect to the selection of appropriate cell lines and the efficiency of cardiac differentiation.

3.2. Characterization of hiPS-CMs

It has been reported that hESCs can be differentiated into cardiomyocytes using the EB method (Kehat et al., 2001; Xu et al., 2002) or other induction methods (Laflamme et al., 2007; Mummery et al., 2003), so that the derived cells show unambiguously the cardiac phenotype. Thus, the human ES-CMs (hES-CMs) express the genes and proteins for sarcomeric organization, e.g., α-actinin, cardiac troponins, myosin heavy chain, myosin light chain, desmin, and tropomyosin (Kehat et al., 2001; Xu et al., 2002; He et al., 2003; Mummery et al., 2003; Lev et al., 2005; Xu et al., 2006), cardiac-specific transcription factors, e.g., Nkx2.5, GATA4, myocyte enhancer factor 2C (MEF2C), Tbx5, and Tbx20 (Kehat et al., 2001; Xu et al., 2002; Snir et al., 2003; Tamargo et al., 2004; Lev et al., 2005; Norstrom et al., 2006; Xu et al., 2006), and gap junctions (Kehat et al., 2001; Xu et al., 2002; Mummery et al., 2003; Cui et al., 2007).

Mauritz et al. (2008) reported immunocytochemical, electrophysiologic, and calcium imaging studies revealing that miPS-CMs had similar features to mES-CMs. These data suggested that there were no difference between mES-CMs and miPS-CMs at the molecular, structural, and functional levels. In addition, Narazaki and colleagues succeeded in directing the differentiation of miPSCs to cardiovascular cells by sorting Flk1 (vascular endothelial growth factor receptor-2)-expressing cardiovascular progenitor cells, which was previously established as a method for mESCs (Yamashita et al., 2000; Narazaki et al., 2008). In that study, some of the miPS-CMs expressed HCN4 and the T-type calcium channel CACNA1G, which are localized to the sinoatrial node and play important roles in the automaticity of mES-CMs (Yanagi et al., 2007), in addition to sarcomeric formation factors and cardiac-specific transcription factors (Narazaki et al., 2008).

Zhang et al. (2009) were the first to describe how cardiomyocytes derived from hiPSCs, which were generated from fetal and newborn fibroblasts transduced with *Oct4*, *Sox2*, *Nanog*, and *Lin28*, expressed the myofilament proteins and sarcomeric organization proteins (e.g., α-actinin, myosin light chain, and cardiac troponins) with the same expression profiles as cardiomyocytes derived from hESCs. They also reported that hiPS-CMs showed a marked reduction in proliferation, similar to hES-CMs (Snir et al., 2003; McDevitt et al., 2005), and that the proliferative activity of hiPS-CMs tended to be slightly lower than that of hES-CMs owing to the continued expression of transgenes, as was the case for miPS-CMs (Narazaki et al., 2008; Zhang et al., 2009). We showed that cardiomyocytes derived from hiPSCs, which were generated from adult fibroblasts transduced with *Oct4*, *Sox2*, *Klf4* and *c-myc*, also expressed the genes and proteins for sarcomeric organization (e.g., α-actinin, myosin heavy chain, and tropomyosin), cardiac-specific transcription factors (e.g., Nkx2.5 and GATA4), and chamber-specific proteins (e.g., ANP) (Fig. 3). These data indicated that hiPS-CMs had similar characteristics regardless of somatic cell source or transcription factor used, although the important problem of

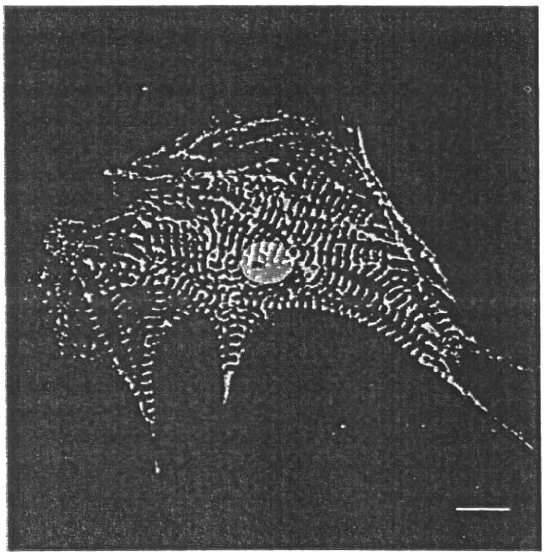

Fig. 3. Cardiomyocyte differentiation from hiPSCs. Immunocytochemical staining for the cardiac-specific transcription factor Nkx2.5 (green) and sarcomeric protein α-actinin (red) in hiPS-CMs. Scale bar, 10 μm.

continued transgene expression persisted. In addition, we showed that hiPS-CMs expressed the genes for: 1) the sodium channel α-subunit SCN5A, which determines cardiac excitability and conduction velocity; 2) the L-type calcium channel α-subunit CACNA1C, which contributes to cardiac contraction; and 3) the rapidly activating delayed rectifier potassium channel KCNH2 (I_{kr}), which contributes to action potential (AP) repolarization. Furthermore, Zwi et al. (2009) reported that, in addition to CACNA1C and KCNH2, hiPS-CMs expressed the genes for the slowly activating delayed rectifier potassium channel KCNQ1 (I_{ks}), which also contributes to AP repolarization, and for the hyperpolarization-activated cyclic nucleotide gated potassium channel HCN2, which is responsible for the I_f pacemaker current.

In summary, hiPS-CMs show unambiguously the cardiac phenotypes, in similarity to hES-CMs. However, a major concern is that continued expression of transgenes affects the efficiencies of differentiation and proliferation of iPS-CMs. In the near future, improved methods for reprogramming are expected, and it will then be necessary to examine whether there are real differences between iPS-CMs and ES-CMs.

3.3. Purification of stem cell-derived cardiomyocytes

Several studies have demonstrated the directed differentiation of human, simian, and murine ESCs into cardiomyocytes (Mummery et al., 2003; Yuasa et al., 2005; Nemir et al., 2006), and the efficiencies of these differentiation processes have been improved using specific growth factors. However, a large proportion of differentiating cells do not become cardiomyocytes owing to the heterogeneous cell mixture within EBs. Transplantation of undifferentiated stem cells may lead to the formation of teratomas, and in vitro drug test using cells with heterogeneous phenotypes may result in unstable and unreliable effects of the drugs. Therefore, it is necessary to establish purification strategies for stem cell-derived cardiomyocytes. ESC lines with various combinations of cardiomyocyte-specific reporters can be used to purify ES-CMs. Klug et al. (1996) achieved >99% pure cardiomyocyte cultures using G418 antibiotic selection after stable transfection of a fusion gene that consisted of the α-cardiac myosin heavy chain promoter and a cDNA that encoded aminoglycoside phosphotransferase. Muller et al. (2000) generated mESCs that overexpressed the CMV enhancer and the MLC-2v promoter, which drove the expression of

green fluorescent protein (GFP). Subsequent sorting of the GFP-positive cells gave a high level of purity of ES-CMs. However, these methods require genetic modification of the cells, which may restrict clinical applications. With regard to non-genetic methods, discontinuous Percoll density gradient centrifugation may be used to enrich for murine and hES-CMs (Olson, 2001; Xu et al., 2002), although the degree of purity achieved with this method may not be sufficient for clinical purposes. We recently reported an innovative non-genetic purification method for ES-CMs that uses a mitochondrial fluorescent dye (Hattori et al., 2010). A major obstacle to cardiomyocyte purification has been the lack of cardiac-specific cell marking systems. We found that a fluorescent dye that labels mitochondria could be used to mark selectively rat cardiomyocytes, as well as murine, marmoset, and human stem cell-derived cardiomyocytes, so that the cells could be enriched (to >99% purity) subsequently using fluorescence-activated cell sorting. This method does not require genetic modification of the cells, so there is no concern regarding tumor formation. Indeed, the ES-CMs purified using this method did not induce teratoma formation in either the heart or testes. Furthermore, this method is likely to be widely applicable beyond these species, given that an abundance of cellular mitochondria is a common characteristic of cardiomyocytes, despite the reduced mitochondrial content with cell age. Overall, this method should increase the success rate for using stem cell-derived cardiomyocytes including ES-CMs and iPS-CMs in basic and clinical applications.

3.4. Electrophysiologic properties of hiPS-CMs

Previous studies have revealed that hES-CMs display action potentials and a variety of cardiac-like morphologies (atrial-, ventricular-, and nodal-like), the ion channel expression patterns of which are similar to those of native cardiomyocytes (He et al., 2003; Mummery et al., 2003; Satin et al., 2004; Sartiani et al., 2007). Most of the cardiac ion currents underwent developmental maturation in regards to current density and properties, despite the fact that the gene expression patterns for each ion channel differed (Sartiani et al., 2007). The KCNH2, HCN1, 2, 4 and CACNA1C were expressed in the undifferentiated hESCs and hES-CMs, whereas the transient outward and inward rectifier potassium channels (I_{to} and I_{K1}, KCND2 and KCNJ2) were expressed only in the hES-CMs. The observed variabilities in channel expression and function may reflect the physiologic maturation of cardiomyocytes. The electrophysiologic properties of iPS-CMs were first described in mice (Mauritz et al., 2008; Narazaki et al., 2008). miPS-CMs display spontaneous beating, and the I_f channel HCN4 and T-type Ca^{2+} channel $Ca_v3.2$, which are expressed in nodal tissues and are responsible for pacemaker activity, co-exist in miPS-CMs with nodal-like action potential configuration. Furthermore, I_{K1}, which is responsible for maintaining the resting membrane potential, was expressed in miPS-CMs with atrial- and ventricular-like AP configurations. These results indicate that miPS-CMs have ion channel expression profiles typical of cardiomyocytes. Recently, the electrophysiologic properties of hiPS-CMs were reported by us and other groups (Tanaka et al., 2009; Zhang et al., 2009; Zwi et al., 2009). hiPS-CMs express functional ion channels, including SCN5A, CACNA1, KCNE1, HCN4, KCNJ2, and KCND2, as evidenced by PCR-based gene expression analyses and positive responses to ion channel inhibitors. hiPS-CMs also display typical AP configurations with nodal-, atrial-, and ventricular-like configurations. However, it has not been clarified whether the ion channel expression patterns and AP configurations of hiPS-CMs change during culturing, given that hES-CMs achieve more mature phenotypes during 3 months of in vitro culturing. Maturation of the physiologic phenotypes of hiPS-CMs may be critical for determining which cellular phase should be used for cell transplantation and drug screening, since more homogeneous cell sources are preferred. Further investigations are needed to resolve these issues.

3.5. Intracellular Ca^{2+} homeostasis in hiPS-CMs

For a hiPS-CM to become a working cardiomyocyte that can be used for cell transplantation therapy, it needs to possess functional contractile proteins that will allow the formation of the appropriate excitation–contraction (EC) coupling. Apart from their intracellular and extracellular electrical activities, miPS-CMs display intracellular $[Ca^{2+}]_i$ transients and appropriate chronotropic responses to adrenergic and cholinergic drugs, consistent with the responses of native cardiomyocytes (Mauritz et al., 2008; Schenke-Layland et al., 2008). The hES-CMs exhibit the AP-initiated $[Ca^{2+}]_i$ transient and local Ca^{2+} events (Ca^{2+} sparks), and contain the sarcoplasmic reticulum (SR) calcium release channels, ryanodine receptor 2, and inositol-1,4,5-triphosphate (IP3) receptor (Satin et al., 2008). Furthermore, hiPS-CMs display the same functional intracellular Ca^{2+} handling as hES-CMs (data not shown). However, the expression profiles of Ca^{2+} handling proteins and precise mechanism for their coupling with membrane excitation in hiPS-CMs remain to be elucidated. Further investigations are needed to clarify these issues before hiPS-CMs can be used as a source of working cardiomyocytes.

4. Pharmacologic testing using stem cells

4.1. Drug-induced QT prolongation and Torsades de Pointes

Many cardiac-acting and non-cardiac-acting drugs prolong AP duration and give rise to acquired long QT syndrome (LQTS), which may cause a fatal life-threatening arrhythmia called Torsades de Pointes (TdP). Several potassium channels contribute to the process of AP repolarization (Snyders, 1999; Tamargo et al., 2004). The early repolarization of the ventricular AP is attributable to I_{to1}, while late repolarization is associated with I_{K1}. Furthermore, both I_{Kr} and I_{Ks} contribute to repolarization beyond the plateau phase, which suggests that dysfunction of either of these channels leads to delayed repolarization as well as QT prolongation. Excessive delayed repolarization is linked to arrhythmogenesis following the development of early after-depolarizations (EADs) (January et al., 1988; January & Riddle, 1989; January & Moscucci, 1992) and exacerbation of transmural dispersion of repolarization (TDR) (Antzelevitch, 2005; Lankipalli et al., 2005; Shantsila et al., 2007). EADs may account for R-on-T ventricular extrasystoles, which trigger the initiation of TdP. In addition, increased TDR leads to increased heterogeneity of tissue refractoriness, which produces a substrate for re-entrant arrhythmias. Since the AP duration of the mid-myocardium is usually longer than that of the endocardium or epicardium due to lower expression of I_{Ks} (Burashnikov & Antzelevitch, 2002), the effect of I_{Kr} suppression may be more prominent in the mid-myocardium than in the other regions of the ventricles, which implies that I_{Kr} blockade increases TDR. Thus, many drugs which block I_{Kr} may cause LQTS and TdP. In addition to the inhibitory effects of these drugs on the hERG channel, several factors predispose to drug-induced TdP, including female gender, abnormal electrolytes (low K^+ and Mg^{2+} plasma levels), bradycardia, and heart disease. Pharmacologic interactions between these drugs and co-administered drugs may also interfere with the biotransformation and excretion of these drugs, leading to arrhythmogenic exacerbation. For example, the antifungal agent ketoconazole interferes with the biotransformation of the antihistamine drug terfenadine into a metabolite that does not prolong the AP duration. Thus, co-administration of these two drugs results in a high concentration of terfenadine, which leads to acquired LQTS (Zechnich et al., 1994; Priori et al., 2001). This type of interaction applies to many drugs that inhibit cytochrome P450 enzymes. LQTS can be acquired as an adverse effect of drug therapy or an electrolyte abnormality that alters the electrochemical conditions needed for normal cardiac excitability. Furthermore, genetic variations and mutations in KCNQ1, KCNH2,

KCNE2, and SCN5A result in the modified susceptibilities of these ion channels to drug interactions (Paulussen et al., 2004).

4.2. High-throughput model systems for pharmacologic testing

The common denominator in most in vitro drug discovery applications is the biological component for which the functionalities and responses are being assayed. There is a substantial need for physiologically relevant cell models, particularly for efficacy and safety studies. Several in vitro models are currently used for drug development, testing, and toxicity screening (Carlsson, 2006; Kannankeril & Roden, 2007; Caspi et al., 2008). The establishment of hESC lines might overcome the lack of an in vitro human cardiac tissue model. In the last decade, drug-induced prolongation of the QT interval, which may lead to the induction of TdP, has become the single most common reason for the withdrawal of drugs in development (Fermini & Fossa, 2003; Roden, 2004). Thus, it is essential that pro-arrhythmic risk is identified at an early stage in the drug development process, so as to define an unacceptable safety profile and to avoid unproductive costs. Since a long QT interval predisposes an individual to an increased risk of TdP, surrogate markers of TdP tend to relate to the drug-induced changes in the AP duration. As the I_{Kr} (hERG) channel contributes to AP repolarization, preclinical drug safety tests have focused on the effects on hERG current inhibition in native cardiomyocytes or in recombinant cells that overexpress the hERG channel (Finlayson et al., 2004; Joshi et al., 2004; Bass et al., 2005; Ducroq et al., 2007). However, the reduction of hERG current per se is not a good predictor of AP prolongation (Martin et al., 2004) and other currents, such as I_{Ks}, I_{Na}, and I_{Ca}, may also be related to the QT interval (Belardinelli et al., 2005). Therefore, the provision of a predictive, high-throughput, cell-based, in vitro QT assay system is highly desirable for cardiotoxicity screens. Although the patch clamp technique is a well-accepted way to study AP parameters, it is time-consuming and requires a skilled operator. Considering these issues, a combination of multielectrode arrays (MEAs) and stem cell-derived cardiomyocytes (hES-CMs or hiPS-CMs) may represent the best system to measure the surface electrogenic activities of cell clusters. The MEAs may be useful for recording the electrical activities of the various derivatives of hESCs and hiPSCs (Harding et al., 2007; Tanaka et al., 2009). Importantly, MEAs are easy to operate and can be adapted to automated high-throughput systems. In addition, MEAs permit stable and long-duration recordings, which are necessary to evaluate the relationships between dose-dependency and induction of side-effects for new drugs (Kaneko et al., 2007; Reppel et al., 2007; Tanaka et al., 2009). At present, MEAs record the electrical activities of contracting EBs, which consist of heterogeneous cell populations, resulting in potentially unstable drug effects. Therefore, it is essential to purify the hES-CMs or hiPS-CMs from the differentiating cell population or non-cardiac cells, as described in Section 3.3.

However, the risk of TdP induction by a drug cannot be based solely on an assessment of AP duration, since EADs and increased TDR, which provides a re-entrant substrate, are essential for TdP induction, as described in Section 4.1. Thus, for predicting the risk of TdP, innovative assays of cell-to-cell connections and conduction velocities are essential. This goal might be achieved using the on-chip agarose microchamber cell microcultivation system (Kojima et al., 2003, 2004, 2005, 2006). This system, which enables the generation of an artificial anatomic re-entrant substrate, is a more realistic in vitro screening assay. However, there remains a limitation with regard to the electrophysiologic phenotypes of available cell sources, which have embryonic characteristics and differ from those of adult cardiomyocytes. Therefore, it needs to be elucidated whether stem cell-derived cardiomyocytes mature during culturing and can be used as a source of adult-like cardiomyocytes.

5. Pluripotent stem-cell derivatives as models of cardiac disease

5.1. Genetic cardiovascular disease

Cardiovascular diseases are major sources of morbidity and mortality. Genetic cardiovascular diseases include channelopathies and cardiomyopathies, which are related to abnormal electrophysiology and impaired contractility (Fig. 4). Genetic alterations that lead to dysfunctional cardiac ion channels are referred to as cardiac channelopathies (Marban, 2002). Cardiac channelopathies, which include long QT syndrome (LQTS), Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia (CPVT), and short QT syndrome, are inherited arrhythmogenic diseases caused by mutations in the genes that encode the ion channels or their related proteins. The electrical instability inherent to channelopathies (i.e., QT prolongation and triggered activity) increases the risk of fatal arrhythmias, which may lead to sudden cardiac death. The other genetic cardiovascular disease, cardiomyopathy, is a heterogeneous disease caused by structural and functional abnormalities of heart muscle, and the etiology includes both extrinsic and intrinsic factors (Maron et al., 2006). Cardiomyopathies caused by intrinsic factors are defined as idiopathic cardiomyopathies, the phenotypes of which include hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM). The major intrinsic factors are mutations in the genes that encode the cytoskeletal and sarcomeric proteins of cardiomyocytes. Importantly, the same disease-related genes overlap across the different clinical phenotypes of primary cardiomyopathy. Functional analyses of disease-related mutations have revealed characteristic functional alterations that may be associated with clinical phenotypes, such as increased or decreased Ca^{2+} sensitivity and stiffness of the sarcomere (Kimura, 2008).

5.2. Patient- and/or disease-specific hiPSCs

There are several advantages to creating patient- or disease-specific hiPSCs. First, hiPSCs can be generated from patients with genetic diseases, which means that the hiPS-CMs derived from these patients possess the same genetic disorders as the patients themselves (disease-specific hiPSCs). Second, the genetic manipulation of disease-specific hiPSCs increases the potential of applying cell therapy to patients with the disease. Hanna et al. (2007) recently demonstrated proof-of-concept for an iPSC-based treatment in combination with genetic repair in a mouse model of sickle cell anemia. Disease-specific iPSCs were generated from these mice, and subsequent homologous recombination and correction of the genetic defect by the wild-type human variant rescued the phenotype, demonstrating the potential application of a combination of hiPSCs and gene therapy to clinical therapy.

5.3. Creation of in vitro models for cardiac diseases

The generation of a transgenic cell line is valuable for the characterization of a single-gene disorder. Over the years, many genetically modified animal models of cardiac diseases have been generated. Despite the importance of these animal models, they are unlikely to be applicable to drug-sensitivity testing or the development of new therapies. In contrast, disease-specific hiPSCs represent an in vitro tool for studying the pathogenesis of a genetic disease. In the case of LQTS, which is the most common channelopathy, following the identification of the first three LQTS genes (LQT1, LQT2, and LQT3) (Curran et al., 1995; Wang et al., 1995; Wang et al., 1996), 13 genes have been shown to be associated with LQTS. From the epidemiologic standpoint, LQT1, LQT2, and LQT3 account for more than 90% of all genotyped LQT patients. However, no genetic abnormality related to causative ion channels or related proteins has been found in approximately 40% of LQTS patients. In addition, the genetic mutations in approximately 80%

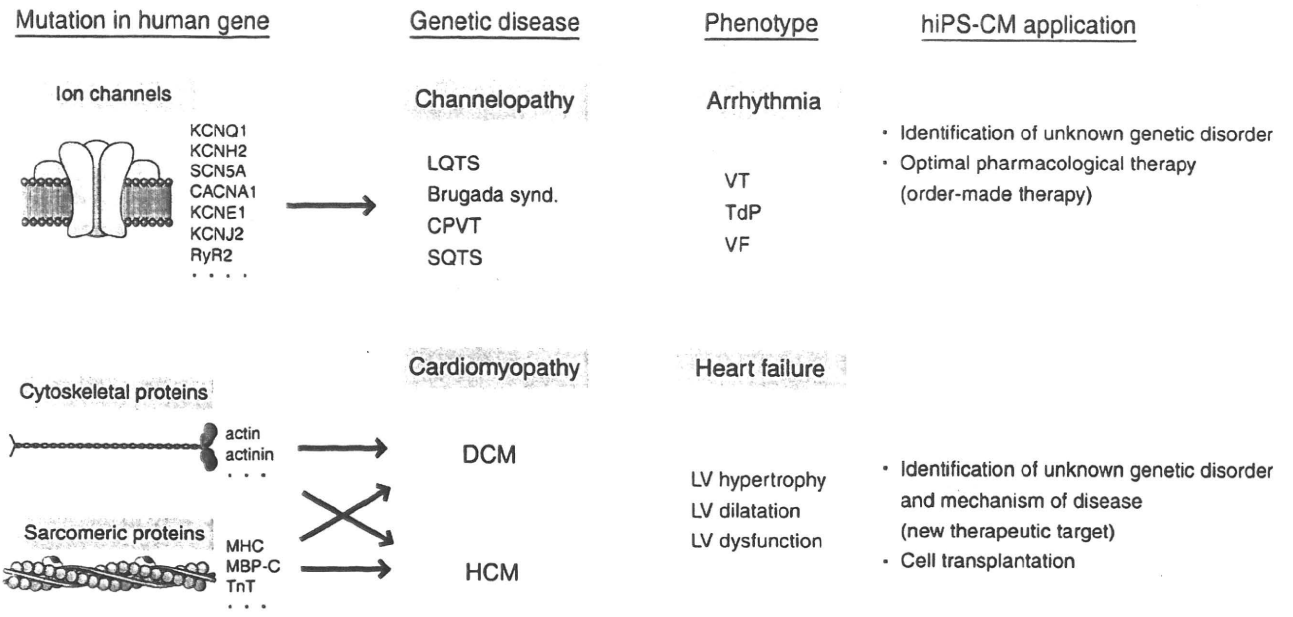

Fig. 4. Cardiac genetic diseases and human iPSC applications. Cardiac genetic diseases mostly comprise the mutations in ion channels (channelopathy) and in cytoskeletal protein and sarcomeric protein (cardiomyopathy). LQTS, long QT syndrome; CPVT, catecholaminergic polymorphic ventricular tachycardia; SQTS, short QT syndrome; TdP, Torsades de Pointes; VF, ventricular fibrillation; MHC, myosin heavy chain; MBP, myosin binding protein; TnT, troponin T.

of Brugada syndrome patients have not been identified, suggesting that there are many unknown genetic abnormalities that cause channelopathies. In this regard, drug-sensitivity testing or genetic screening of disease-specific hiPS-CMs might lead to the identification of mutations that could be targeted in new medical treatments. Furthermore, the generation of reproducible cell populations of patient-specific hiPS-CMs that have the phenotypic characteristics of the cardiomyocytes of the donor patient would enable the establishment of patient-specific drug screening systems, and might lead to customized therapies.

6. Conclusions

We have discussed the recent advances in cardiac regenerative medicine and the potential for future applications. In recent years, much attention has been focused on the development of iPS technology for two distinct applications: 1) in vitro use of iPSCs for the development of disease models, drug screening, and toxicology; and 2) regenerative medicine. Applications for in vitro use are just around the corner. Disease-specific iPSCs have already been generated from patients suffering from a variety of diseases, including amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), familial dysautonomia (FD), and Parkinson's disease, some of which appear to display the pathologic phenotypes seen in the patients (Park et al., 2008a; Xu et al., 2008; Dimos et al., 2008; Ebert et al., 2009; Lee et al., 2009). Furthermore, hiPS-CMs are likely to be principally applicable to drug-induced arrhythmia screening using extracellular potential recording systems (Tanaka et al., 2009; Yokoo et al., 2009). This system would contribute to the identification of compounds that prolong the QT interval and cause subsequent fatal arrhythmia (TdP), before costly preclinical studies. Although the establishment of high-throughput drug screening systems requires further optimization of the method, including a sufficient supply of purified hiPS-CMs and the development of automated phenotype recording systems, these are purely technical problems that are likely to be solved in the near future. In contrast, the application of hiPS-CMs to regenerative medicine is still some years off, since safety issues and the low efficiencies of hiPSC derivation and differentiation remain as significant hurdles. To avoid transgene-initiated tumor formation, the generation of iPSCs without transgene integration has been reported. However, the efficiency of integration-free iPSC generation remains low. Therefore, it is necessary to elucidate the mechanism of iPSC derivation, so as to increase the efficiency of this process to a more practical level. Moreover, to avoid teratoma formation, we need to know why distinct iPS clones have different proportions of undifferentiated cells after differentiation. One strategy to prevent teratoma formation is to exclude undifferentiated cells from in vitro differentiated cell clusters and to purify the specific cell types. Although the differentiation efficiency of hiPS-CMs is extremely low, the development of purification method for cardiomyocytes has led to the successful avoidance of teratoma formation (Hattori et al., 2010). This type of technique should be used to create hiPS-CM grafts for safer cell transplantation. Although it seems likely that iPSCs will eventually replace ESCs for most applications, recent reports have claimed that there are significant differences between ESCs and iPSCs, and the possibility exists that some iPSCs will have side-effects not exhibited by ESCs. Therefore, a comprehensive molecular comparison of ESCs and iPSCs and an assessment of their full differentiation potentials should be undertaken before their clinical use.

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Cell

A Global In Vivo *Drosophila* RNAi Screen Identifies *NOT3* as a Conserved Regulator of Heart Function

G. Gregory Neely,[1,16] Keiji Kuba,[2,16,*] Anthony Cammarato,[3,16] Kazuya Isobe,[2,4] Sabine Amann,[1] Liyong Zhang,[7] Mitsushige Murata,[8] Lisa Elmén,[3] Vaijayanti Gupta,[9] Suchir Arora,[9] Rinku Sarangi,[9] Debasis Dan,[9] Susumu Fujisawa,[2] Takako Usami,[5] Cui-ping Xia,[1] Alex C. Keene,[10] Nakissa N. Alayari,[3] Hiroyuki Yamakawa,[8] Ulrich Elling,[1] Christian Berger,[1] Maria Novatchkova,[1] Rubina Koglgruber,[1] Keiichi Fukuda,[8] Hiroshi Nishina,[6] Mitsuaki Isobe,[4] J. Andrew Pospisilik,[1] Yumiko Imai,[2] Arne Pfeufer,[11,12] Andrew A. Hicks,[13] Peter P. Pramstaller,[13,14,15] Sai Subramaniam,[9] Akinori Kimura,[5] Karen Ocorr,[3] Rolf Bodmer,[3,*] and Josef M. Penninger[1,*]

[1]Institute of Molecular Biotechnology (IMBA) of the Austrian Academy of Sciences, Dr. Bohr Gasse 3-5, A-1030 Vienna, Austria
[2]Department of Biological Informatics and Experimental Therapeutics, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan
[3]Development and Aging Program, Del E. Webb Neuroscience, Aging, and Stem Cell Research Center, Sanford-Burnham Medical Research Institute, La Jolla, CA 92037, USA
[4]Department of Cardiology
[5]Department of Molecular Pathogenesis, Medical Research Institute
[6]Department of Developmental and Regenerative Biology, Medical Research Institute
Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113-8510, Japan
[7]Division of Cardiology, Toronto General Hospital, University Health Network, University of Toronto, Canada
[8]Department of Regenerative Medicine and Advanced Cardiac Therapeutics, Keio University School of Medicine, 160-8582 Tokyo, Japan
[9]Strand Life Sciences, 237 C V Raman Avenue, Rajmahal Vilas, 560024 Bangalore, India
[10]Biology Department, New York University, 100 Washington Square East, New York, NY 10003, USA
[11]Institute of Human Genetics, Technische Universität München, D-81675 Munich, Germany
[12]Institute of Human Genetics, Helmholtz Center München, D-85764 Neuherberg, Germany
[13]Institute of Genetic Medicine, European Academy Bozen/Bolzano, 39100 Bolzano, Italy
[14]Department of Neurology, General Central Hospital, 39100 Bolzano, Italy
[15]Department of Neurology, University of Lübeck, 23538 Lübeck, Germany
[16]These authors contributed equally to this work
*Correspondence: kuba@med.akita-u.ac.jp (K.K.), rolf@burnham.org (R.B.), josef.penninger@imba.oeaw.ac.at (J.M.P.)
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SUMMARY

Heart diseases are the most common causes of morbidity and death in humans. Using cardiac-specific RNAi-silencing in *Drosophila*, we knocked down 7061 evolutionarily conserved genes under conditions of stress. We present a first global roadmap of pathways potentially playing conserved roles in the cardiovascular system. One critical pathway identified was the CCR4-Not complex implicated in transcriptional and posttranscriptional regulatory mechanisms. Silencing of CCR4-Not components in adult *Drosophila* resulted in myofibrillar disarray and dilated cardiomyopathy. Heterozygous *not3* knockout mice showed spontaneous impairment of cardiac contractility and increased susceptibility to heart failure. These heart defects were reversed via inhibition of HDACs, suggesting a mechanistic link to epigenetic chromatin remodeling. In humans, we show that a common *NOT3* SNP correlates with altered cardiac QT intervals, a known cause of potentially lethal ventricular tachyarrhythmias. Thus, our functional genome-wide screen in *Drosophila* can identify candidates that directly translate into conserved mammalian genes involved in heart function.

INTRODUCTION

Cardiovascular diseases are the most common cause of death in North America and Europe (Yusuf et al., 2001) killing more than 860,000 people annually in the United States (A.H.A., 2005; Lloyd-Jones et al., 2009). Moreover, 80 million people in the United States are estimated to suffer from cardiovascular diseases (A.H.A., 2005; Lloyd-Jones et al., 2009). Known or associated causes of cardiovascular disease include diabetes mellitus, inflammation, high cholesterol, hypertension, overweight and obesity, physical inactivity, or smoking (A.H.A., 2005; Lloyd-Jones et al., 2009). Although there have been great advances in the understanding of heart failure in recent decades (Mudd and Kass, 2008), there is still a gap in understanding the genetic causes and an unmet need for better therapies. In particular, the complex interplay of lifestyle, genetic susceptibilities,

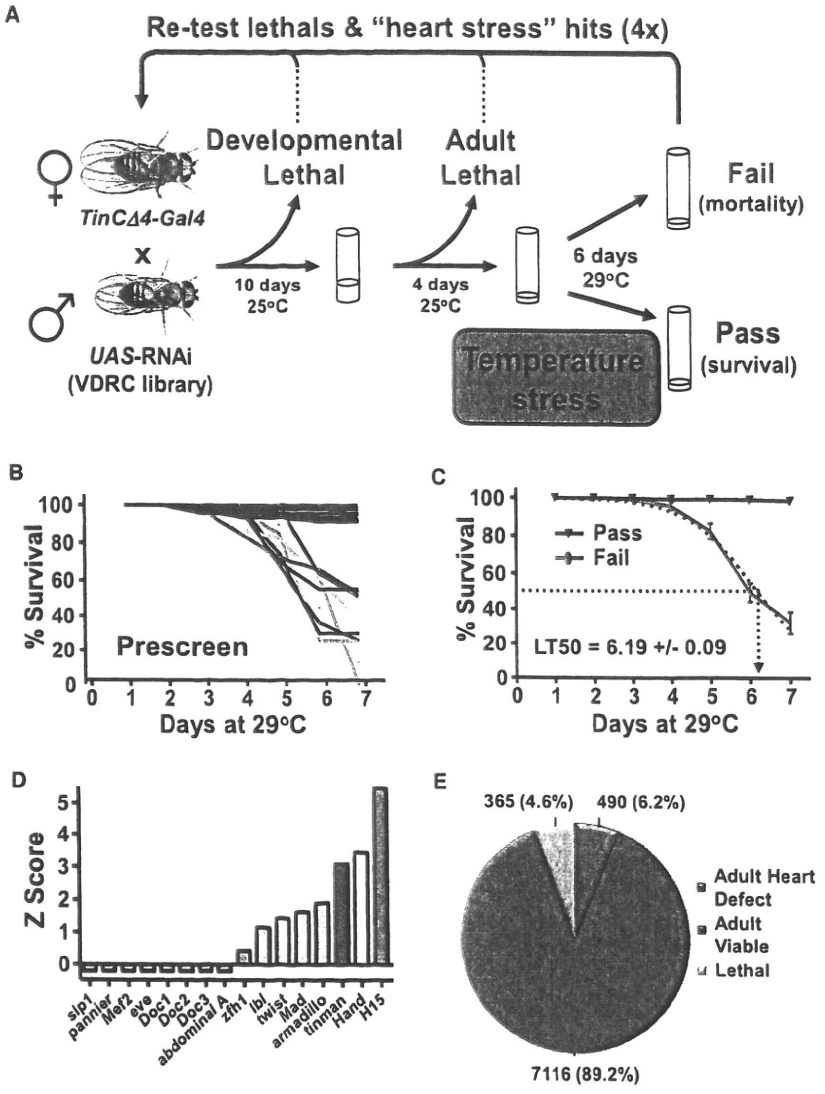

Figure 1. Genome-wide Screen for Conserved Heart Genes

(A) Schematic for screen setup. *TinΔ4-Gal4*, a cardiac tissue specific driver, was used to drive conserved *UAS*-RNAi hairpins in the developing heart. Developmental lethality and baseline adult viability was scored. Viable adult flies were then given a heart stress (continued exposure to 29°C) and survival was scored on day 6. Fly lines showing a potential developmental or heart function phenotype were then retested to confirm the candidate gene.

(B) Eighty randomly selected *UAS*-RNAi lines were crossed to *TinCΔ4-Gal4* and evaluated for adult lethality after an increase in ambient temperature as a cardiac stressor. Lines were either viable (black) or died starting around day 3. Data from individual lines are shown as percent survival on the indicated days.

(C) Mean responses from viable and failing (death after exposure to 29°C) flies revealed an average lethal time at which 50% of failing flies died (LT50) of 6.19 days.

(D) Efficacy of *TinCΔ4-Gal4* x *UAS*-RNAi lines to knock down transcription factors known to play a role in heart formation.

(E) With this system, a genome-wide screen was performed to search for conserved candidate genes for adult heart function under conditions of cardiac stress; 4.6% *TinCΔ4-Gal4* x *UAS*-RNAi lines were developmental lethal. Among the 7971 viable lines, 490 transformant lines exhibited significantly increased death (Z score >3, determined on day 6 after shifting the ambient temperature to 29°C).

See also Figure S1 and Tables S1 and S2.

diseases, and aging have made it difficult to understand the underlying pathogenic principles (Yusuf et al., 2001). In addition to large-scale genetic mapping and phenotyping in humans (Gordon et al., 1977; Morita et al., 2005; Nabel, 2003), a genetic dissection of the cardiovascular system in less complex model organisms would greatly facilitate the understanding of basic controls of cardiac physiology and mechanisms of disease.

Multiple proteins that control contraction in cardiomyocytes are highly conserved between species. For instance, the fly heart is capable of spontaneous rhythmic activity required for the circulation of hemolymph, and the same genes control heart rhythm in humans and flies (Ocorr et al., 2007a). In aging flies, the heartbeat becomes irregular with increased episodes of arrhythmias (Ocorr et al., 2007b), reminiscent of increased atrial fibrillation and heart failure in older humans (Lakatta and Levy, 2003). Moreover, genes involved in specification and differentiation of the heart are also conserved between *Drosophila* and mammals (reviewed in Bodmer, 1995; Cripps and Olson, 2002; Ocorr et al., 2007a). Mutations in subunits of repolarizing voltage gated potassium channels I_{Kr} (*eag, KCNH2*) and I_{Ks} (*kvLQT1, KCNQ1*) perturb heart function in *Drosophila* and in vertebrates can cause long QT syndrome (Ocorr et al., 2007b; Sanguinetti and Tristani-Firouzi, 2006). Moreover, the sarco-endoplasmic reticulum Ca^{2+}-ATPase (*serca2a*, *ATP2A2*) and the Ca^{2+}-channel *Cacophony* control heart function also in *Drosophila* (Ray and Dowse, 2005; Sanyal et al., 2006). Thus, *Drosophila* has become a powerful genetic model system to identify conserved genes involved in heart function.

RESULTS

A *Drosophila* High-Throughput Assay to Identify Candidate Heart Genes

To identify candidate genes for heart development and heart function (Figure 1A), we used cardiac tissue-specific RNA interference (RNAi) silencing of all genes that we identified as showing possible conservation between mammalian species and *Drosophila melanogaster* (Table S1, part A, available online).

TinCΔ4-Gal4 specifically drives expression in cardioblasts (Lo and Frasch, 2001) and has been previously used to study genes involved in heart function of the adult fly (Qian et al., 2008). Because RNAi-mediated downregulation of gene expression in many cases permits the circumvention of lethality commonly associated with classical mutations (Dietzl et al., 2007), cardiac tissue-specific *TinCΔ4-Gal4* RNAi-mediated gene silencing therefore allowed us to assay the functional roles of the respective target genes in adult flies. Since elevated ambient temperature results in an increase in *Drosophila* heart rate (Paternostro et al., 2001; Ray and Dowse, 2005), we combined cardiac tissue-specific RNAi knockdown with an increased ambient temperature to reveal cardiac phenotypes under conditions of stress. Elevated temperature also enhances the activity of the UAS/GAL4 system, without affecting survival within the timeframe of the experiment (Figure S1A).

To evaluate the efficacy of this experimental setup (Figure 1A), we performed a prescreen with 80 randomly selected genes that were targeted by *TinCΔ4-Gal4* RNAi (Table S1, part B). Whereas ~10% of these *TinCΔ4-Gal4* RNAi lines started to die at the increased ambient temperature, the vast majority survived for more than 7 days (Figure 1B). From these pilot experiments, we calculated an average time of 6.19 days at which 50% of flies among the susceptible lines had died (lethal time 50 [LT50]) (Figure 1C). Thus, our large-scale genome-wide screen was carried out at 29°C and lethality was recorded for each line at day 6. As a control, *TinCΔ4-Gal4* RNAi knockdown of known cardiogenic transcription factors resulted in viable lines at 25°C (data not shown), but a shift to 29°C resulted in increased death of nearly half of the transcription factor RNAi lines tested, including *Tinman*, *Hand*, and *H15* (*neuromancer-1/Tbx20*) (Figure 1D). Cardiac knockdown of *pannier/Gata4* and the *Doc* genes (*Tbx5/6*) did not cause premature lethality at 29°C, even though they are known to contribute to adult heart function (Qian and Bodmer, 2009; Qian et al., 2008). As negative controls, we used RNAi lines targeting *eve* and *zfh-1*, which are not expressed in the myocardium targeted by *TinCΔ4-Gal4* (Figure 1D). Thus, we have set up a model system that allows for efficient high-throughput screening and has the capacity to pick up known heart genes.

A Genome-wide In Vivo Fly RNAi Screen for Conserved Genes

In total we screened 8417 transgenic RNAi lines corresponding to 7061 conserved genes for potential developmental and adult heart functional defects (Table S1, part C). We only included 7971 lines representing 6751 genes that fit the previously defined criteria of specificity (Dietzl et al., 2007) for further analyses, i.e., only lines with an S19 score ≥ 0.8 and having six or fewer CAN repeats were considered specific (Table S1, part D). Progeny of each RNAi line crossed to *TinCΔ4-Gal4* were first monitored for viability (reared at 25°C). Among these 7971 RNAi lines, 365 lines resulted in lethality (Figure 1E and Table S1, part E), indicating that many of these genes function in heart development. Developmental lethality was further staged as lethal (embryonic lethal or we never observed any offspring), larval lethal, pupal lethal, or early adult (within 4 days after eclosion) lethal (Table S1, part F).

To identify candidate genes for adult heart function, we assayed 7804 adult *TinCΔ4-Gal4* RNAi progeny (Dietzl et al., 2007) for survival after shifting the flies to 29°C (Figures S1B–S1D). To categorize our hits from the screen, we used the Z score, which is a measure of the distance in standard deviations of a sample from the mean. All RNAi lines with a Z score of 2 in the primary screen were tested on average 4.18 independent times (an average of 90 flies per genotype) using in some cases second RNAi transformants to control for transgenic insertion effects and second independent RNAi hairpins to target a different region of the gene (Table S2). After repeated screening, we identified 498 genes that passed the more stringent Z score of 3 (Figure 1E and Table S2), indicating that these hits exhibit a death score of three standard deviations from the mean. Using gene ontology (GO) annotations, our candidate hits were classified according to their predicted biological processes (BP), molecular functions (MF), and cellular components (CC). Of the classified genes, those involved in signaling, ion transporter activity, metabolism and mitochondrial structure, development and morphogenesis, transcriptional regulation, or nucleic acid binding were highly represented among the entire data set (Figure S2 and Table S4, part A). To remove any artificial bias in the gene list created by the ad hoc Z score cutoff >3, we performed a gene set analysis (GSA) to confirm enrichment of selected GO terms (Table S4, part B). In addition, 121 candidate heart genes had no annotated function by GO. With panther (http://www.pantherdb.org/), we were able to functionally annotate 116 of these genes (Table S4, part C).

Given that the RNAi library screened is known to generate a level of false negative phenotypes because of inefficient targeting of genes to levels required to reveal phenotypes (Dietzl et al., 2007), and based on the assumption that our candidate heart hits perform some of their functions in protein complexes, we next identified first-degree binding partners (Table S4, part D). Using this list of primary heart hits and their binding partners, we performed fly KEGG pathway analyses. Moreover, we included developmental lethal hits to generate a global interaction network. KEGG analyses showed enrichment of multiple pathways, such as mTOR signaling and PI3K/AKT, amino acid metabolism, JAK-STAT signaling, ErbB signaling, the Wnt, Notch, hedgehog, or TGFβ pathways, protein degradation, VEGF signaling, DNA repair, and calcium homeostasis (Table S3 and Table S4, part E). Besides the identification of multiple known genes, our screen has also revealed hundreds of candidate genes and pathways that have not been previously associated with heart function.

A Global View of Heart Function

To extend our *Drosophila* results to mammalian systems, we used the power of data mining and bioinformatics at a global systems level. Potential mouse and human orthologs of our candidate heart screen hits were evaluated for GO enrichment. The GO analyses of the human and mouse orthologs showed marked enrichment of genes involved in PIP3 and calcium signaling, ion transporter activity, metabolism, development, fatty acid metabolism, and muscle contraction (Table S4, part F). We next performed KEGG pathway as well as Broad Institute C2 gene set analysis on the mouse and human orthologs and

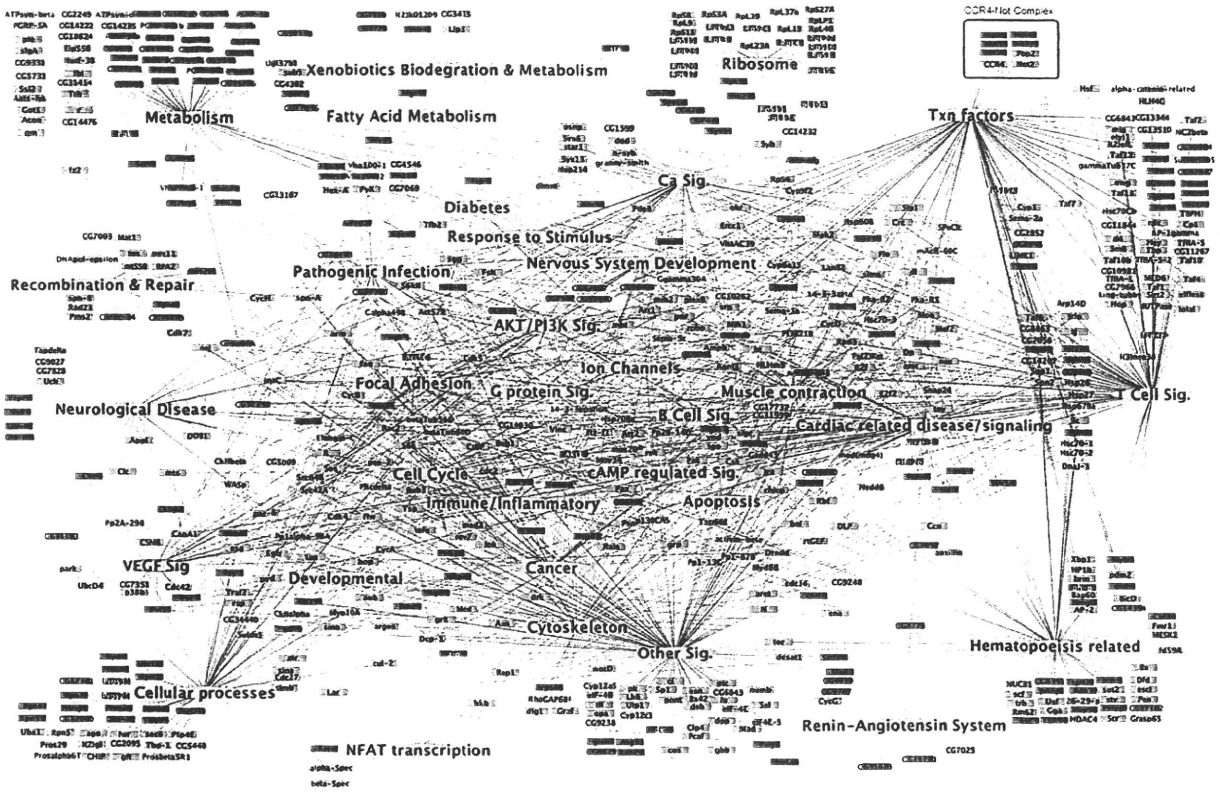

Figure 2. A Global Network of Heart Function

The systems network includes data from the significantly enriched *Drosophila* KEGG and mouse and human KEGG and C2 data sets. Pathways and gene sets from the same biological processes were grouped into common functional categories. Orange nodes represent statistically enriched functional categories of pathways, red nodes represent direct primary fly RNAi hits, green nodes represent their first degree binding partners, and blue nodes indicate genes that were scored as developmentally lethal in our *Drosophila* heart screen. Lines indicate associations of the genes to the appropriate functional category. All KEGG pathways and selected C2 gene sets have been represented in the systems map. See also Tables S3, S4, and S5.

their first-degree binding partners. Based on the mammalian KEGG (Table S4, part E) and C2 (Table S4, part G) analyses, we found significant enrichment for gene sets involved in signaling, metabolism, ion channels, inflammation, aging, and transcription.

To generate a network map that includes our functional data in *Drosophila*, their human and mouse orthologs, and first-degree binding partners, we combined KEGG pathways from *Drosophila*, mouse, and human with relevant gene sets from the Broad Institute C2 annotations (Table S4). A combined systems map and the interactions between the individual genes in the indicated nodes are shown in Figure 2 and Table S4, part H. A systems map using only direct screening hits was also generated, yielding a comparable network map (Table S3). Importantly, by using this network approach, we identified multiple pathways known to play key roles in heart function and cardiovascular disease. For instance, we found significant enrichment in NFAT transcription, AKT activation, and PI3K signaling, calcium signaling and muscle contraction, GPCR- and cAMP signaling, ion channels and proton-transporting ATPase complexes, and transcription. We also found associations with the renin-angiotensin system, a key pathway involved in cardiovascular function in humans (Figure 2 and Table S4, part H). In support of our network approach, advanced data mining revealed that 171 of our primary fly hits and their first-degree binding partners corresponded to mouse knockouts with known cardiovascular phenotypes (Table S5). Thus, our genome-wide screen for candidate heart genes and in silico analyses provides a first attempt at a global roadmap of essential molecular components and key pathways potentially involved in heart function and cardiac failure.

RNAi Silencing of *not3* and *UBC4* Results in Dilated Cardiomyopathy in *Drosophila*

One of the pathways we found in our global network analysis was the CCR4-Not complex (Figure 2 and Table S3). Intriguingly, among the eight members of this complex assayed, we hit the subunits *not1*, *not3* (*not3/5* in fly), *not4*, *UBC4*, and *Hsp83* (Figure 3A). In addition, the subunits *not2* and *CG8759* were "weak" hits (Figure 3A). The CCR4-Not complex was first identified in yeast (Denis, 1984) and is highly conserved in evolution (Albert et al., 2000). Components of the CCR4-Not complex

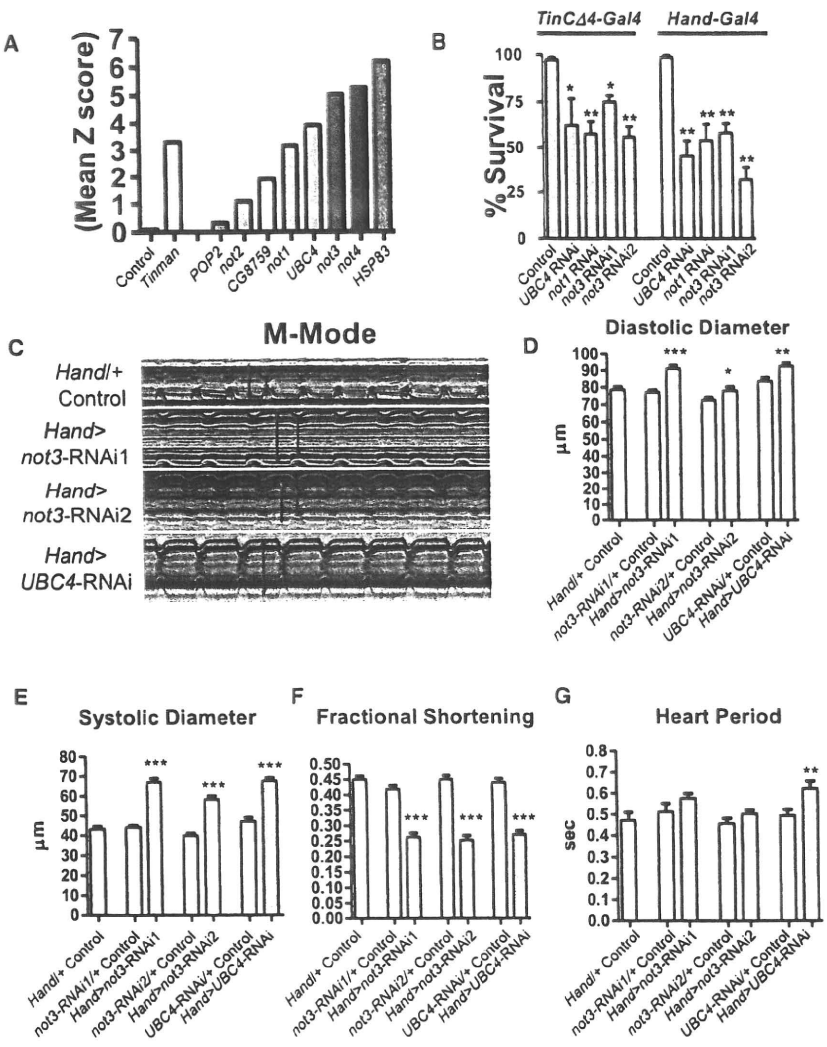

Figure 3. The CCR4-Not Complex Is a Central Regulator of Adult Heart Function, and Loss of not3 Results in Dilated Cardiomyopathy in *Drosophila*

(A) Mean Z scores for *TinCΔ4-Gal4* x *UAS*-RNAi lines targeting the indicated members of the fly CCR4-Not complex. A negative control (w^{1118} [isogenic to the RNAi library] X *TinΔ4-Gal4*) and the positive control *Tinman* RNAi line are shown.

(B) *not1*, *not3*, and *UBC4* are essential for proper adult heart function in both *Tinman*- and *Hand*-expressing cells. Data are shown as mean ± SEM for at least three replicates. RNAi1 and RNAi2 indicate different transgenic hairpins targeting *not3* . *p < 0.05, **p < 0.01 by ANOVA.

(C) One-week-old adult flies with *Hand-Gal4* driving *not3* or *UBC4* cardiac-specific knockdown exhibit impaired heart function. M modes provide traces of the heart contractions to document the movements in a 1 pixel wide region of the heart tube over time. *HandG4/+* control are the progeny of *Hand-Gal4* crossed to w^{1118}. *Hand>Not3* complex flies are the progeny of *Hand-Gal4* crossed to either *UAS-not3*-RNAi (−1 or −2) or to UAS-*UBC4*-RNAi lines. Fly heart analysis was performed with a MatLab-based image analysis program (Fink et al., 2009; Ocorr et al., 2007b). M modes of the RNAi knockdown hearts reveal dilated diastolic and systolic diameters (double-headed red arrows) and reduced shortening properties (difference between diameters) when compared to M modes of control hearts. Each trace represents a 5 s recording.

(D–G) *Not3* or *UBC4* heart-specific knockdown perturbs several indices of cardiac performance. Progeny of *Hand-Gal4* crossed to two different *UAS-not3*-RNAi lines or an *UAS-UBC4*-RNAi line (experimental) and w^{1118} crossed to *UAS*-RNAi or *Hand-Gal4* driver (controls) were used for these experiments as in (C). *not3* and *UBC4* knockdown led to significantly wider diastolic (D) and systolic (E) diameters, and as a result significantly depressed (F) fractional shortening in all experimental lines relative to controls. *not3* knockdown trended toward a slight lengthening in the heart period (time between consecutive diastolic intervals), while *UBC4* knockdown led to a significant increase in heart period (G). Mean values ± SEM are shown for each group (n = 29–40). Unpaired t tests were performed between each *Hand-Gal4>UAS*-RNAi and each corresponding *UAS*-RNAi/+ control (progeny of w^{1118} crossed to *UAS*-RNAi line). Additionally, one-way ANOVAs with Bonferroni multiple comparison tests revealed no significant differences between the *HandG4/+* control and all *UAS*-RNAi/+ control lines, for any cardiac parameter measured.

*p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S3 and Movie S1.

have not yet been associated with cardiovascular function. We therefore retested components of this pathway using *TinCΔ4-Gal4*-driven knockdown in the heart, which confirmed the phenotype (Figure 3B). Moreover, use of a second heart driver, *Hand-Gal4*, which is expressed with high specificity in myocardial and pericardial cells throughout development and in the adult fly heart (Han and Olson, 2005), showed that silencing of *not1*, *not3*, and *UBC4* resulted in early death when adult flies were shifted to 29°C (Figure 3B).

Since *not3* RNAi gave a strong phenotype with two different *UAS*-RNAi lines (Figure 3B), we focused on the CCR4-Not component *not3*. Cardiac-specific knockdown of *not3* with two different RNAi lines (*Hand>not3*-RNAi: progeny from *Hand*-Gal4 crossed to *UAS-not3*-RNAi) significantly increased both diastolic and systolic diameters and resulted in reduced systolic fractional shortening relative to control flies (Figures 3C–3F and Movie S1). Hearts with cardiac *not3* knockdown also showed slight increases in heart periods (Figure 3G); however, this was not statistically significant. Fluorescent microscopy revealed that *not3* RNAi lines exhibit marked myofibrillar disarray, especially in the conical chamber (Figures 4A–4D). Heart-restricted *not3*-RNAi-mediated knockdown was confirmed by qRT-PCR (Figure S3). In addition, we observed transcriptional downregulation of the Sarcoplasmic/endoplasmic reticulum calcium ATPase

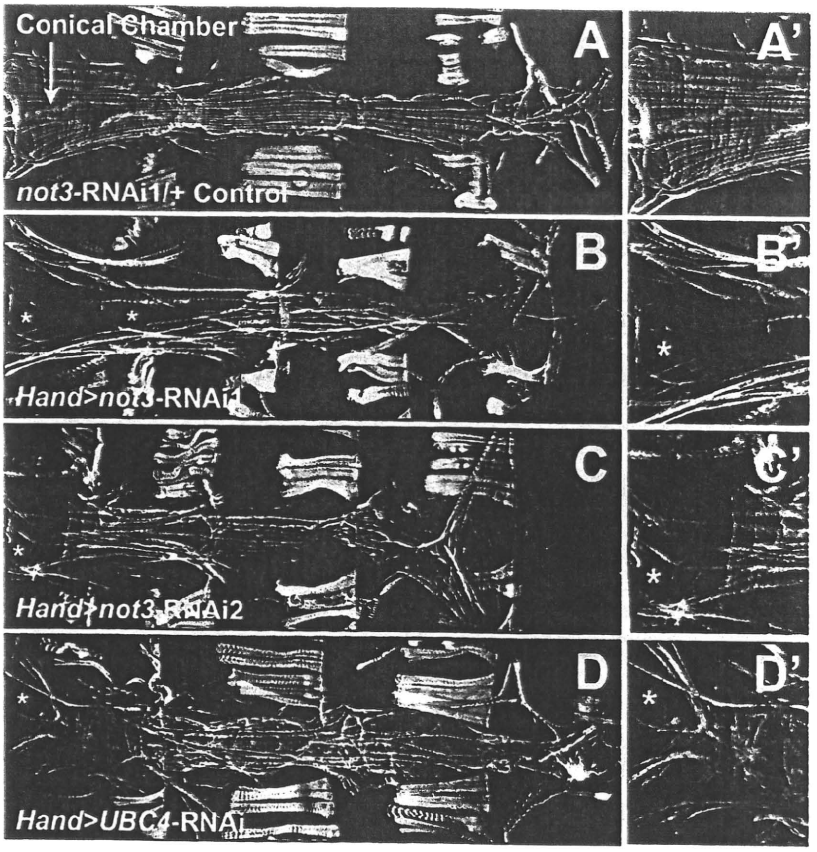

Figure 4. *not3* and *UBC4* Cardiac-Specific RNAi Knockdown Substantially Perturb Myofibrillar Organization and Content

(A) Alexa584-phalloidin staining of control *Drosophila* cardiac tubes reveals typical spiraling myofibrillar arrangements within the cardiomyocytes. The fibers, especially those in the conical chamber, located anteriorly, are densely packed with F-actin.

(B–D) Relative to control hearts, *not3* or *UBC4* RNAi knockdown severely disrupts myofibrillar organization and leads to an apparent loss of myofilaments as noted by large gaps in F-actin staining (*) as well as by a lack of myosin heavy chain transcripts (Figure S3F).

(A′–D′) Enlarged images of the conical chambers from (A)–(D), respectively, which illustrate the high degree of myofibrillar disarray and large gaps in F-actin staining within the cardiomyocytes of *not3* and *UBC4* RNAi knockdown hearts. Original images were taken at 10× magnification with a Zeiss Imager Z1 fluorescent microscope.

(*Serca2a*, *ATP2A2*), myosin heavy chain (*mhc*, *MYH7*), and the potassium channel *KCNQ* (*kcnq1*, *KCNQ1*) (Figure S3) involved in heart rhythm control. Cardiac-specific knockdown of *not3* increased the number of flies exhibiting contractile irregularities (Figures S3H and S3I), a finding similar to what is seen in response to cardiac-specific knockdown of the *KCNQ* K^+ channel (Figure S3I) and what has been reported for *KCNQ* mutant flies (Ocorr et al., 2007b). Of note, a *not3 P* element mutant was developmentally lethal, exhibiting a late stage defect in embryonic heart tube organization, which could be rescued by *P* element excision (Figure S3C).

The CCR4-Not complex component *UBC4* was also a major hit identified by our heart screen. Moreover, *UBC4* expression was reduced after *not3* knockdown (Figure S3B). *Hand-Gal4>UAS-UBC4*-RNAi flies also exhibited significantly longer heart periods and showed dramatically altered diastolic and systolic diameters and reduced fractional shortening relative to control hearts (Figures 3C–3G). Fluorescent imaging again revealed severe myofibrillar disarray (Figure 4D) that was strikingly similar to that observed in *not3* knockdown hearts. Further, we observed similar structural and functional phenotypes in *not1* cardiac-specific knockdown flies (A.C., G.N., J.P., and R.B., unpublished data). Thus, knockdown of different components of the CCR4-Not complex result in abnormal heart structure and severely impaired cardiac function indicative of dilated cardiomyopathy.

Functional Assessment of Additional *Drosophila* Heart Hits

To extend our confirmations beyond the CCR4-Not complex, we assayed heart function in adult flies with heart-specific knockdown of four additional candidates identified in our heart screen (Figure S3). One candidate heart gene tested was *CG1216* (*Mrityu*), which encodes a mesoderm-expressed BTB-POZ domain-containing protein (Rusconi and Challa, 2007). Cardiac knockdown of *CG1216* resulted in a significant increase in systolic diameter. Another candidate heart gene, *CG8933* (*extradenticle*), encodes a PBX-family transcription factor. Cardiac knockdown of *CG8933* resulted in increased systolic diameter and reduced fractional shortening. Cardiac knockdown of *CG33261* (*Trithorax-like*) resulted in significantly altered diastolic and systolic diameters as well as impaired fractional shortening. Finally, knockdown of *CG7371*, which encodes a Vps52 domain-containing protein predicted to participate in Golgi trafficking, resulted in a marked increase in heart period and affected the diastolic diameter. These data further demonstrate that our screen indeed has the capacity to identify novel factors involved in and required for normal adult heart function.

Generation of *not3* Knockout Mice

We next tested whether our data on *Drosophila* can be directly translated into a mammalian species. The mouse and human NOT3 proteins (official gene name *Cnot3*) share 60% identity with the *Drosophila not3* ortholog. Expression of human and mouse *not3* messenger RNA (mRNA) transcripts can be found in the majority of tissues analyzed. Although *not3* is evolutionarily conserved from yeast to mammals, essentially nothing is known about the in vivo role of mammalian *not3*. We therefore generated *not3* knockout mice.

We disrupted the *not3* gene in murine embryonic stem cells (ESCs) using a targeting vector in which nucleotides encompassing exons 2 through 9 are deleted (Figure 5A and Figure S4A). Both $not3^{+/-}$ male and $not3^{+/-}$ female mice are viable and exhibit normal fertility. We never obtained viable $not3^{-/-}$ newborn mice, indicating that loss of *not3* results in embryonic lethality. We staged embryonic development but failed to recover *not3* null embryos from placental implantations (Figures S4B and S4C). We therefore assayed early embryogenesis and observed that $not3^{-/-}$ blastocysts can develop. These mutant blastocysts have a normal appearance (Figure S4D), occur at Mendelian frequencies (Figures S4E and S4F), and express key markers of early embryonic differentiation at normal levels (Figure S4G). *not3* mRNA transcripts and NOT3 protein were undetectable in $not3^{-/-}$ blastocysts by RT-PCR and immunostaining (Figures S4F and S4G). In $not3^{+/+}$ and $not3^{-/-}$ epiblast cultures (Figure S4H), trophoblast cells started to spread and supported the outgrowths of the inner cell mass (ICM). While the ICM of $not3^{+/+}$ blastocysts continued to grow, $not3^{-/-}$ ICM cells exhibited a severe outgrowth defect. Thus, complete loss of mouse *not3* results in early embryonic death at the implantation stage.

not3 Haploinsufficiency Results in Impaired Heart Function

We speculated that similar to RNAi-mediated downregulation of *not3* in *Drosophila*, *not3* haploinsufficiency might also reveal a role in mammalian heart function. In *not3* heterozygote mice, *not3* expression is indeed downregulated in the heart (Figure 5B). We failed to observe overt structural changes in the hearts of $not3^{+/-}$ mice. However, both male and female *not3* haploinsufficient mice exhibited a reduction in cardiac contractility as determined by decreased left ventricle fractional shortening and increased left ventricular diameter in systole (Figures 5C and 5D).

To address whether the defects in cardiac function are intrinsic to the heart per se or whether the observed impairment of contractility was secondary because of haploinsufficiency of *not3* in other tissues, we subjected explanted hearts from wild-type and $not3^{+/-}$ littermate mice to Langendorff perfusion, assessing ex vivo heart function (Joza et al., 2005). When isoproterenol was used to activate β-adrenergic receptors, $not3^{+/-}$ hearts exhibited severe contractile abnormalities as defined by impaired generation of left ventricular pressure (LVP) (Figure 5E and Figure S5A). Hemodynamic measurements confirmed that all functional heart parameters such as dP/dT_{max} or dP/dT_{min}, indicative of generated contractile pressure, were markedly reduced in $not3^{+/-}$ hearts (data not shown). Moreover, when explanted hearts were electrically stimulated, $not3^{+/-}$ hearts exhibited a striking defect in contractility (Figure 5F). Thus, downregulation of *not3* expression in *not3* haploinsufficient mice results in an intrinsic impairment in heart function.

Yeast strains mutant for components of the CCR4-Not complex, including *not3*, display reduced acetylation levels of lysine residues on histone tails (e.g., H3K9) (Peng et al., 2008) and/or reduced trimethylation of H3K4 (Laribee et al., 2007). H3K9 acetylation and H3K4 trimethylation are indicative of transcriptionally active states of chromatin. Moreover, promoter regions of NOT3 target genes were shown to recruit trimethylated H3K4 in mouse ESCs (Hu et al., 2009), suggesting that NOT3 may regulate chromatin modifications. Our gene expression and bioinformatic analyses of mouse *not3* knockout cells revealed that histone deacetylases (HDACs) and mRNA metabolisms are localized central in gene networks (K.K., unpublished data). We therefore assessed the state of histone modifications in hearts from $not3^{+/-}$ mice. Histone extracts of whole hearts from *not3* haploinsufficient mice showed a slight but significant reduction in active histone marks such as acetylation of H3K9 and trimethylation of H3K4 (Figures 5G and 5H and Figure S5). H3K27 trimethylation was not changed (Figure 5I and Figure S5). Treatment of $not3^{+/-}$ hearts with the HDAC inhibitor VPA restored the reduced acetylation of H3K9 and H3K4 trimethylation to that of wild-type levels (Figures 5G–5I and Figure S5). Most importantly, administration of HDAC inhibitors rescued the impairment in heart function in $not3^{+/-}$ mice; i.e., ex vivo heart functions of VPA treated mice were similar to control mice in response to both isoproterenol (Figure 5J) and electrical stimulation (Figure 5K). These data were confirmed with TSA, a second HDAC inhibitor (Figures S5H and S5I). Together, these data show that $not3^{+/-}$ mice exhibit a spontaneous and intrinsic defect in cardiac function that can be rescued with HDAC inhibitors.

$not3^{+/-}$ Mice Develop Severe Cardiomyopathy in Response to Cardiac Stress

We next exposed control and $not3^{+/-}$ littermates to chronic pressure overload by surgical constriction of the aorta (transverse aortic constriction, TAC). Three weeks after TAC, heart weight/body weight ratios (HW/BW) increased in both $not3^{+/+}$ and $not3^{+/-}$ mice, although this increase was significantly larger in the $not3^{+/-}$ mice (Figure 6A). Cardiac hypertrophy was also seen by histology (Figure 6D and Figure S6A). Aortic banding of $not3^{+/-}$ mice resulted in severe heart failure characterized by decreased fractional shortening (Figure 6B) and a dilation of the left ventrical as determined by echocardiography (Figure 6C). In addition, $not3^{+/-}$ mice develop severe cardiac fibrosis after TAC, as shown by Masson-trichrome staining of hearts 3 weeks after TAC (Figure 6D and Figure S6B). Thus, $not3^{+/-}$ mice develop severe symptoms of heart failure in response to cardiac stress.

We next assessed whether HDAC inhibitors can also rescue stress-induced heart failure. HDAC inhibitor treatment could indeed block the augmented loss of cardiac function observed in $not3^{+/-}$ mice after TAC (Figures 7A and 7B). In vivo treatment of $not3^{+/-}$ mice with HDAC inhibitors also blocked the exaggerated induction of heart failure markers such as *ANF* (Figure S6C) and *βMyhc* (Figure S6D). Moreover, treatment with an HDAC inhibitor restored the observed histone alterations in $not3^{+/-}$ mice to that of wild-type littermates (Figure 7C and Figures S6E and S6F). Thus, *not3* haploinsufficiency results in exaggerated heart failure that can be rescued by HDAC inhibition in vivo.

A Common Genomic Variant in the *NOT3* Promoter Correlates with Cardiac Repolarization Duration in Humans

Using an in silico search to identify NOT3 target genes, we found that NOT3 has been shown to bind to the *Kcnq1* promoter in

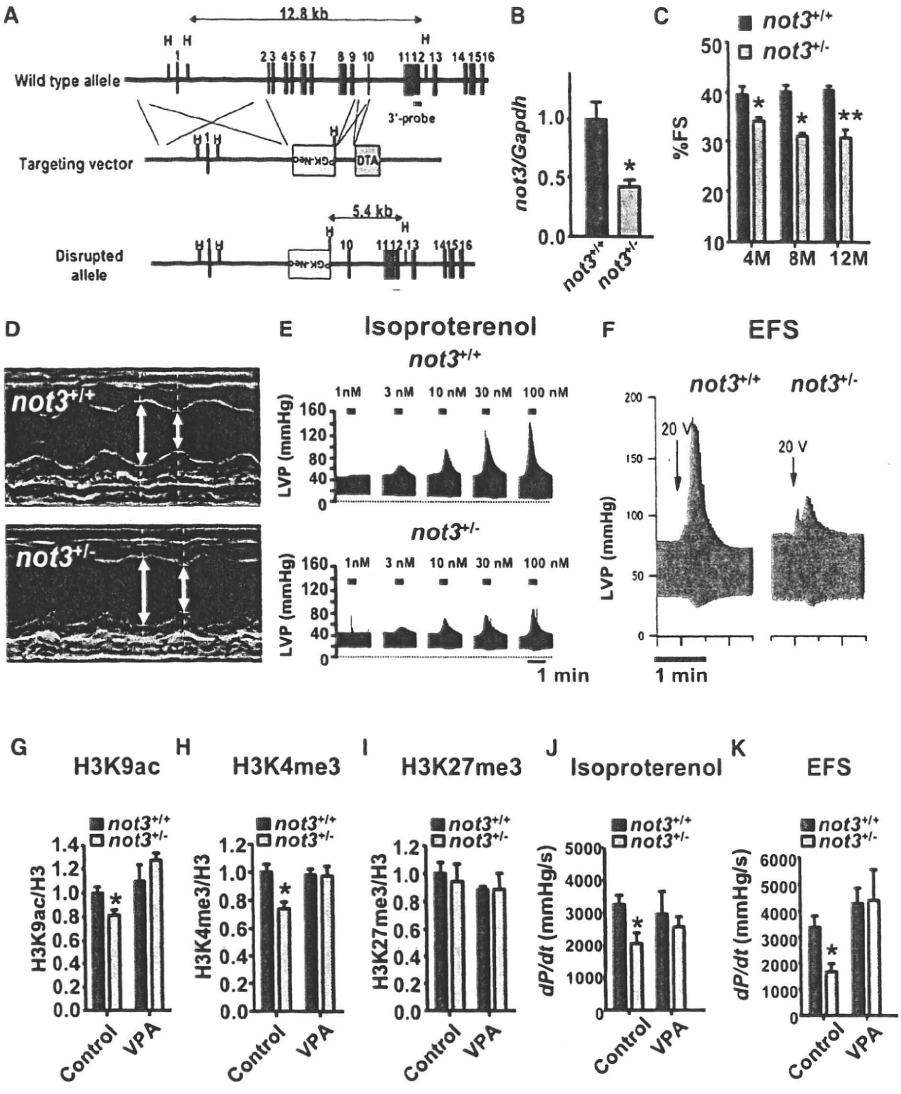

Figure 5. *not3*$^{+/-}$ Mice Exhibit Reduced Heart Contractility, Ex Vivo Function, and Histone Modifications that Can Be Rescued by Treatment with HDAC Inhibitors

(A) Gene targeting strategy. Exons 2 to 9 of the *not3* gene (official symbol *cnot3*) were replaced with a PGK-Neo cassette by homologous recombination in A9 ESCs. The wild-type allele, targeting vector, mutant allele, and PGK-Neo and DTH selection cassettes are shown. Blue boxes indicate exons.

(B) Real-time PCR analyses for *not3* mRNA expression in 3-month-old wild-type and *not3*$^{+/-}$ hearts. Values were normalized to *gapdh* mRNA expression. n = 6 mice per group.

(C) *not3*$^{+/-}$ mice display a significant reduction in percent fractional shortening at 4 months of age, which became more pronounced with age. n = 6–8 mice per group. Fractional shortening was determined by echocardiography.

(D) Representative M mode echocardiography for wild-type and *not3*$^{+/-}$ mice at 8 months of age.

(E) Left ventricular pressure (LVP) measurements in isolated ex vivo *not3*$^{+/-}$ and *not3*$^{+/+}$ hearts under isoproterenol perfusion. *not3*$^{+/-}$ hearts from 4-month-old mice showed impaired contractile responses to different doses of isoproterenol perfusion in the retrograde Langendorff mode as compared to age-matched controls.

(F) Impaired contractile response of ex vivo *not3*$^{+/-}$ hearts to electrical field stimulation (EFS) compared with littermate *not3*$^{+/+}$ hearts. Representative data for left ventricular pressure (LVP) at 20 V stimulation are shown.

(G–I) H3K9 acetylation (H3K9ac) (G), H3K4 trimethylation (H3K4me3) (H), and H3K27 trimethylation (H3K27me3) (I) levels were analyzed by western blot for acid-extracted histones from whole heart ventricles of wild-type and *not3*$^{+/-}$ mice treated with vehicle or VPA (0.71% w/v in drinking water for 1 week) Band intensities were normalized to total H3 levels.

(J and K) Treatment (1 week) with the HDAC inhibitor VPA rescue impaired ex vivo heart contractility of *not3*$^{+/-}$ hearts to isoproterenol (100 nM) perfusion or 25 V EFS.

All values are mean ± SEM. *$p < 0.05$; **$p < 0.01$. n = 5–12 per group. See also Figures S4 and S5.