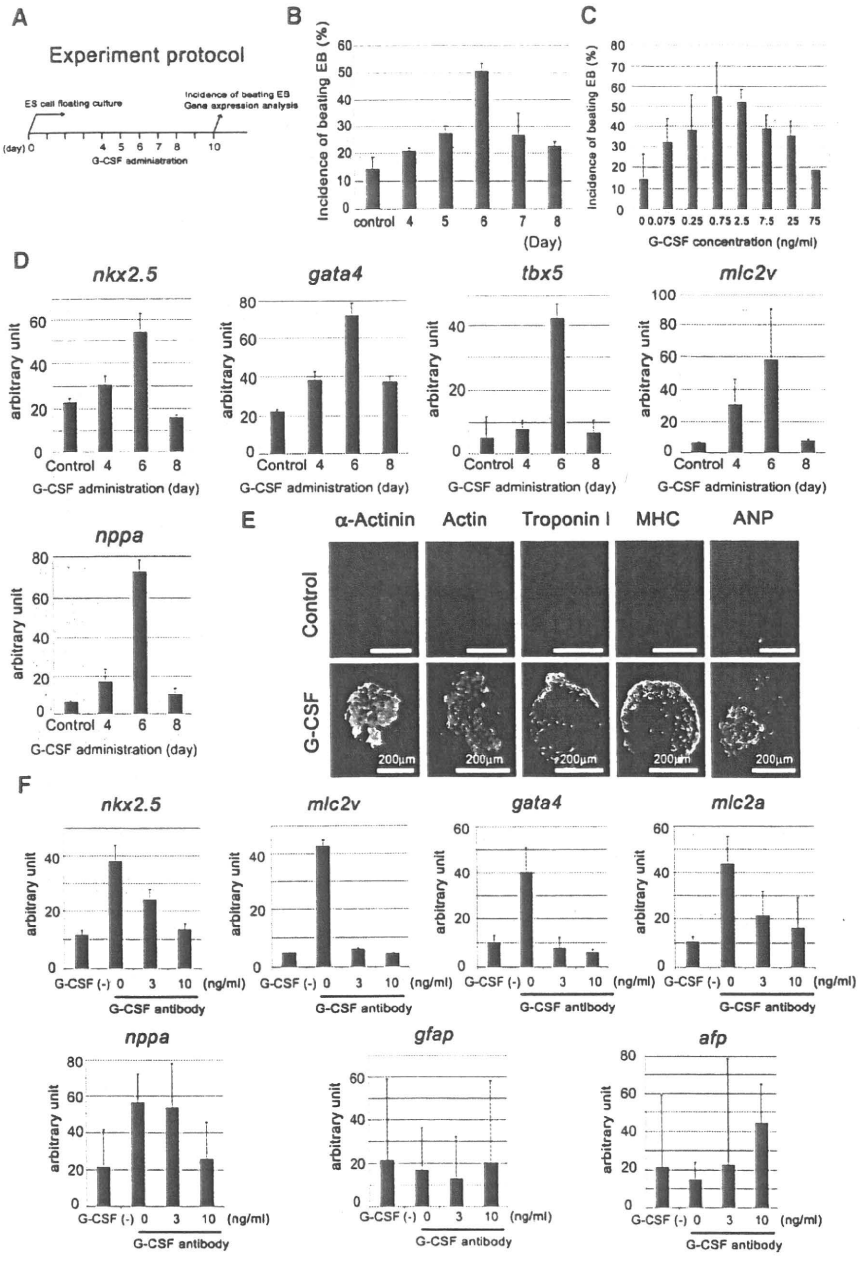

Figure 5. G-CSF Increased the Total Number of ESC-Derived Cardiomyocytes

(A) Diagrammatical representation of the experimental protocol. Differentiating EBs were stimulated with G-CSF on days 4, 5, 6, 7, and 8 after induction of differentiation. Gene expression analyses were performed on day 10.

(B) Optimal timing of G-CSF treatment of cardiomyocytes derived from ESCs. Incidence of autonomously beating EBs is indicated (n = 20, respectively).

(C) Optimal concentration of G-CSF treatment of cardiomyocytes derived from ESCs. Incidence of autonomously beating EBs is indicated (n = 20, respectively).

(D) Optimal timing of G-CSF administration for ESC-derived cardiomyocytes was investigated by quantitative RT-PCR for cardiomyocyte markers.

(E) Immunostaining for cardiac-specific proteins in G-CSF-treated ESCs.

(F) Neutralizing G-CSF antibody abrogated cardiomyocyte marker expression in differentiating ESCs in a dose-dependent manner (n = 7).

and we found that G-CSF promoted the cell proliferation of early developing cardiomyocytes through JAK2/STAT3 pathway (Figure S6). Based on these findings, we conclude that G-CSF augmented the level of cardiomyocytes in EBs by promoting their proliferation and that this effect is mediated by the JAK-STAT signaling pathway.

G-CSF Increased Primate ESC- and Human iPSC-Derived Cardiomyocytes

To test this effect in primate cardiogenesis, we investigated the beating incidence of common marmoset ESCs (CMESCs) after G-CSF administration at various time points. The optimal timing was day 6 after EB formation, and the difference between G-CSF-treated and control EBs became evident at day 14 (Figure 7A). G-CSF stimulation at earlier or later stages promoted cardiogenesis to a lesser extent than even the control levels. This suggested that the effect is specific to day 6 and that G-CSF signaling might therefore promote the differentiation of other cell lineages to decrease the beating incidence. Immunostaining showed that G-CSF increased cardiac troponin I- and Nkx2.5-positive areas in CMESC-derived EBs (Figure 7B). Next, to confirm whether G-CSF enhances the proliferation of early cardiomyocytes in human pluripotent stem cells, we examined the effect of G-CSF by using human iPSC (hiPSC) differentiation system. G-CSF administration on day 6 significantly increased the percentage of spontaneously beating EBs (Figure 7C). NKX2.5-positive cells were also significantly increased by G-CSF administration (Figure 7D). Furthermore, quantitative RT-PCR also showed that the expression levels of several cardiac markers were strongly elevated in EBs treated by G-CSF (Figure 7E). We then immunostained hiPSC-derived EBs for cardiomyocyte markers, such as α-Actinin, Troponin C, NKX2.5, and ANP. It apparently showed that G-CSF significantly increased the cardiac marker protein expression in hiPSC-derived EBs (Figure 7F). These findings indicated that G-CSF acts commonly on not only mouse but also monkey and human pluripotent stem cells and enhances the proliferation of early cardiomyocytes.

DISCUSSION

It is well known that ESC differentiation mimics normal development. To establish an efficient system for ESC-derived

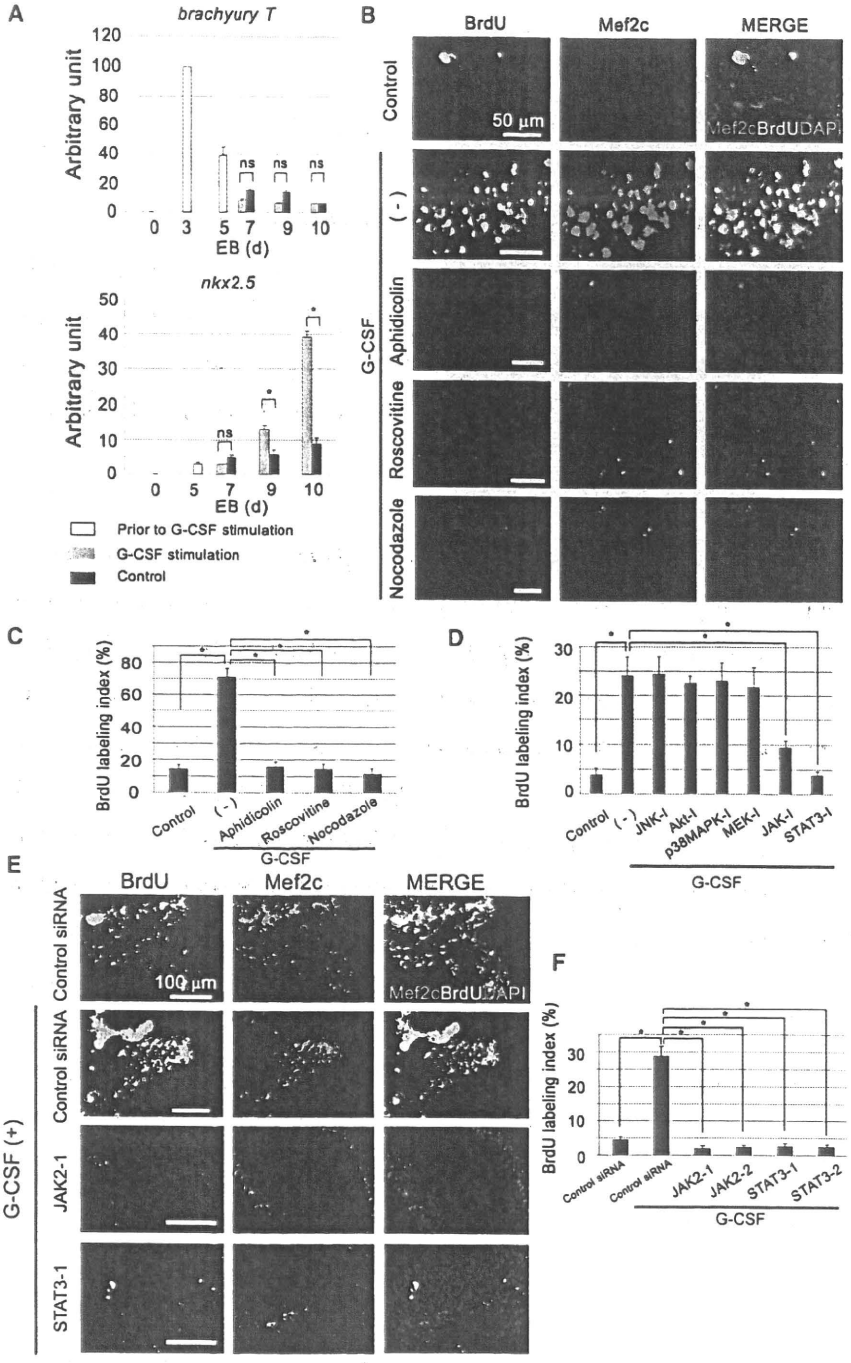

Figure 6. G-CSF Increased Proliferation in ESC-Derived Developing Cardiomyocytes via JAK/STAT Pathway

(A) Quantitative RT-PCR of *brachyury T* (mesodermal marker) and *nkx2.5* (early cardiac marker) in pre-G-CSF (white columns), G-CSF-treated (gray columns), and control (black columns) cells. Effect of G-CSF was demonstrated after cardiomyocyte differentiation.

(B and C) G-CSF administration augmented BrdU uptake and cell number in ESC-derived differentiating cardiomyocytes (n = 7), and this effect was abrogated by the mitotic inhibitors, nocodazole (n = 7), roscovitine (n = 6), and aphidicolin (n = 9). Triple immunofluorescence staining for MEF2C, BrdU, and DAPI was examined. BrdU labeling indices are shown.

(D) The effect of signal inhibitors on BrdU labeling index in G-CSF-treated cardiomyocytes was demonstrated with ESC differentiation system (n = 8). JAK and STAT3 inhibitors significantly reduced the BrdU labeling index.

(E and F) Specific silencing the expression of JAK2 and STAT3 with two different siRNAs resulted in significantly reduced the BrdU index in G-CSF-treated ESC-derived cardiomyocytes (n = 9), $^{*}p < 0.05$.

cardiomyocyte differentiation, it was necessary to determine the physiological mechanisms involved in embryonic heart development. Cardiomyocyte development is a multistep process that includes initial mesodermal induction, emergence of the cardiomyoblast, cardiomyoblast proliferation, and cardiomyocyte maturation (Srivastava, 2006). Accumulating evidence has shown that cardiomyocyte differentiation is precisely regulated by wnts (Arnold et al., 2000), wnt inhibitors (Schneider and Mercola, 2001), BMPs (Behfar et al., 2002), BMP inhibitors (Yuasa et al., 2005), hedgehog (Goddeeris et al., 2007), and notch (Grego-Bessa et al., 2007), with tightly controlled temporal and spatial patterning. Although ESC-derived cardiomyocyte differentiation methods have been designed based on these regulatory factors, none are satisfactorily efficient. To improve the efficiency of cardiomyocyte differentiation, we focused on another step of the differentiation pathway. Controlling cardiomyocyte proliferation during development was an attractive step.

Cardiomyocyte proliferation is one of the most important physiological steps in heart development and is regulated by several growth factors and cytokines (Olson and Schneider, 2003). Insulin-like growth factor-1 (IGF-1) and its receptor (Kajstura et al., 1994; Reiss et al., 1996), as well as several fibroblast growth factors and their receptors (Lavine et al., 2005), regulate myocardial proliferation during midgestational heart development. Retinoic acid produced by the epicardium is also a critical regulator of cardiac growth, and $RXR\alpha^{-/-}$ mice die during embryogenesis from a failure in the proliferative expansion of ventricular cardiomyocytes (Sucov et al., 1994; Kastner et al., 1997). BMP10 is specifically expressed in the ventricular trabecular myocardium from E9.0 to 13.5 and $BMP10^{-/-}$ mice have a cardiac growth defect (Chen et al., 2004). These factors synergistically regulate cardiomyocyte proliferation under normal physiological conditions. Therefore we attempted to use these factors to achieve cardiomyocyte proliferation during ESC differentiation, but we failed to establish an efficient cardiomyocyte

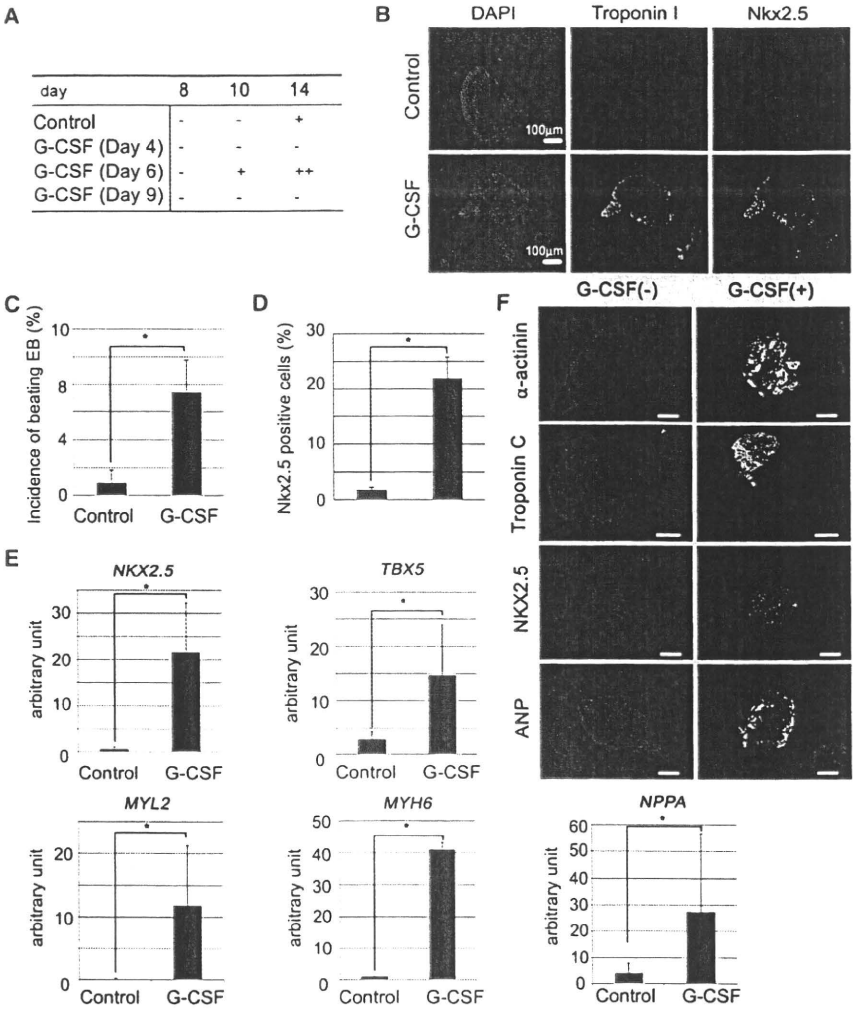

Figure 7. G-CSF-Augmented Cardiomyocyte Content in Common Marmoset Monkey ESCs and Human iPSCs

(A) Panel shows the incidence of spontaneously beating cells. G-CSF administration on day 6 increased beating cells compared with the control.

(B) Immunofluorescent staining shows G-CSF increased the expression of cardiac troponin I and nkx2.5 in G-CSF-treated CMESCs compared to the nontreated control.

(C) Effect of G-CSF on the incidence of the spontaneous beating EBs in hiPSCs. G-CSF administration significantly increased the incidence of spontaneous beating EBs compared with the control (n = 12).

(D) Effect of G-CSF on the percentage of NKX2.5-positive cells in hiPSCs (n = 5).

(E) Quantitative RT-PCR for cardiac markers such as *NKX2.5*, *TBX5*, *MYL2*, *MYH6*, and *NPPA*. Note that all cardiac markers showed strong enhancement of there expression levels (n = 7).

(F) Immunofluorescent stainings of the cardiac marker proteins including α-Actinin, Troponin C, NKX2.5, and ANP in the control and G-CSF-treated hiPSC-derived EBs. Note that cardiomyocyte marker-positive cells were increased in G-CSF-treated EBs.

*$p < 0.05$.

proliferation protocol. We thought these factors would synergistically regulate cardiomyocyte proliferation with other factors. However, the requirement for multiple factors has hampered research into regenerative medicine approaches to controlling cardiomyocyte proliferation. We therefore had to search for other potent and useful cardiomyocyte proliferating factors.

G-CSF was cloned in 1986 (Souza et al., 1986) and was considered to be a hematopoietic cytokine that stimulated the growth of neutrophil colonies (Welte et al., 1996). *csf3r*$^{-/-}$ mice have chronic neutropenia, granulocyte and macrophage progenitor cell deficiencies, and impaired neutrophil mobilization (Lieschke et al., 1994). To date, research has not focused on the role of G-CSF in development because although approximately 50% of *csf3r*$^{-/-}$ mice die during embryogenesis because of heart defects, the surviving mice grow to adulthood. The heart defects seen in *csf3r*$^{-/-}$ mice indicate that G-CSF might play a critical role in cardiomyocyte proliferation, although there is a possibility that lack of G-CSFR in the hematopoietic or other type of cells may indirectly affect its results. Interestingly, G-CSF also affects the adult heart. It promotes adult cardiac regeneration by mobilizing bone marrow mesenchymal stem cells (Kawada et al., 2004) and prevents left ventricular remodeling after myocardial infarction by reducing cardiomyocyte apoptosis (Harada et al., 2005).

Based on our previous results in the developing heart, we investigated the possible role of G-CSF in cardiac development and ESC differentiation. Our results demonstrated that both G-CSF and its receptor are strongly expressed in the embryonic heart and that G-CSF is critically involved in cardiomyocyte proliferation during development. G-CSF increased the number of ESC-derived cardiomyocytes in not only mouse but also common marmoset monkey ESCs, indicating that this phenomenon is applicable to primate ESCs and is potentially universal. Human ESCs are not easy to handle. We propose that efforts in regenerative medicine with human ESC-derived cardiomyocytes might therefore benefit from G-CSF treatment to boost cardiomyocyte yield from ESCs, possibly in combination with other cardiomyocyte differentiation protocols.

EXPERIMENTAL PROCEDURES

Whole-Mount In Situ Hybridization

Murine embryos were removed from the wild-type ICR pregnant mice on embryonic days 7.5, 8.5, 9.5, and 10.5. Whole-mount in situ hybridization was performed as described (Yuasa et al., 2005) utilizing ULTRAhyb (#8670, Ambion, Austin, TX). The full-length cDNAs for murine *nkx2.5* (accession number NM_008700) were obtained by RT-PCR and subcloned into pBluescript plasmid, and the probes were generated with T3 or T7 RNA polymerase. A plasmid subcloning murine *csf3r* (*g-scfr*) was a kind gift from Prof. S. Nagata (Department of Genetics, Osaka University) (Fukunaga et al., 1990). All experiments were approved by the Keio University Ethics Committee for Animal Experiments.

ESC Culture

Undifferentiated murine ESCs (EB3) were maintained on gelatin-coated dishes in GMEM containing 10% FBS (Equitechbio, Ingram, TX), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, and 2000 U/ml murine LIF (Chemicon International, Temecula, CA). EB3 cells were a kind gift from H. Niwa (Riken, Japan) (Niwa et al., 2000). EB3 cells carry the blasticidin S-resistance gene, which is activated by the Oct3/4 promoter to eliminate differentiated cells. EB3 cells were maintained in medium containing 20 μg/ml blasticidin S. EB3 is a subline derived from E14tg2a ESCs and was generated by targeted integration of the Oct3/4-IRES-BSD-pA vector into the Oct3/4 allele.

Differentiation of ESCs

Maintained undifferentiated ESCs were trypsinized and cultured with a three-dimensional culture system in α-MEM containing 10% FBS and 0.1 mM 2-mercaptoethanol on uncoated Petri dishes to induce embryoid bodies (EBs) from a single cell. Recombinant murine G-CSF (414-CS, R&D Systems, Minneapolis, MN) was added on prescribed days. Signal inhibition analysis was performed by administering G-CSFR neutralizing antibody (AF-414-NA, R&D Systems) 5 days after the differentiation induction. In the mitosis inhibition assay, 0.2 μg/ml of nocodazole, 30 μg/ml of roscovitine (487928 and 557360, respectively, Calbiochem, Tokyo, Japan), and 5 μg/ml of aphidicolin (Wako, Osaka, Japan) was administered 6 days after the differentiation induction, simultaneously with the addition of G-CSF.

csf3r$^{-/-}$ Mice

csf3r$^{-/-}$ mice (G-CSFR KO mice) were a kind gift from Dr. Daniel C. Link (Washington University School of Medicine, St. Louis) (Richards et al., 2003).

Morphological Analysis

G-CSF or PBS (negative control) was injected into the uterus of the pregnant wild-type and *csf3r*$^{-/-}$ mice at E9.5 (n = 10, respectively). Mice were sacrificed at E16.5 (n = 5) or P1 (n = 5) and the hearts were removed and analyzed by sectioning and staining with hematoxylin and eosin.

Common Marmoset ESC Culture

The common marmoset ESC (CMESC) line (#20) (Sasaki et al., 2005) was used in this study. CMESCs were maintained on a layer of mitotically inactivated mouse embryonic fibroblasts (MEF) in Knockout DMEM (GIBCO) supplemented with 20% Knockout Serum Replacement (KSR; GIBCO), 1 mM L-glutamine (GIBCO), 0.1 mM MEM nonessential amino acids (GIBCO), 0.1 mM β-mercaptoethanol (2-ME; Sigma), 10 ng/ml basic fibroblast growth factor (bFGF; Invitrogen), and 10 ng/ml human leukemia inhibitory factor (hLIF; Alomone labs). CMESCs were passaged every 5 days to maintain their undifferentiated state.

Differentiation of CMESCs

Undifferentiated CMESCs were removed from the MEF feeder layer, dissociated into small clumps with dissociation solution for human and monkey ESCs (ReproCELL, JAPAN), and cultured to form EBs in suspension culture with HydroCell culture plates (CellSeed, JAPAN) in culture medium without bFGF and hLIF.

Human iPSC Culture

The hiPSC line 201B7 (Takahashi et al., 2007), into which Oct3/4, Sox2, Klf4, and c-Myc were retrovirally transfected, was maintained in the undifferentiated state on a mitomycin C-inactivated MEF feeder layer in DMEM/F12 medium (Invitrogen) supplemented with 20% Knock-out Serum Replacement (KSR), 1 mM l-glutamine, 1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, and 4 ng/ml basic fibroblast growth factor (bFGF), as described previously (Tanaka et al., 2009). The hiPSC culture medium was changed daily, and the cells were passaged every 5–6 days.

Differentiation of Human iPSCs

The hiPSC colonies were dispersed into cell aggregates that contained 2000–5000 cells via CTK dissociation solution. For differentiation, hiPS colonies of appropriate size were chosen with a combination of 40 μm and 100 μm cell strainers (Becton-Dickinson), which also facilitated the complete removal of feeder cells. The cell aggregates were cultured in suspension in ultralow attachment cell culture dishes in hiPS differentiation medium (DMEM/F12 supplemented with 20% FBS, 1 mM l-glutamine, 1 mM nonessential amino acids, and 0.1 mM β-mercaptoethanol), as described previously (Tanaka et al., 2009). The EBs were incubated at 37°C in 5% CO_2. During differentiation, the medium was replaced every 3–4 days. Recombinant human G-CSF (Wako 074-04841) was administrated at a concentration of 2.5 ng/ml on day 6, and assay was performed on day 30.

Immunofluorescence

EBs were fixed in 4% paraformaldehyde for 30 min and embedded for frozen sectioning with Tissue-Tek OCT (Sakura Finetek, Chuoku, Tokyo) on day 10. In some experiments, undifferentiated ESCs were fixed in 4% paraformaldehyde for 30 min and immunostained as described below. The samples were incubated with Triton X-100 for 5 min at room temperature, then washed and incubated with primary antibodies: anti-Nkx2.5 (N-19, 1:50), anti-MEF2C (C-21, 1:100), anti-Troponin C (E-7, 1:50), anti-ANP (FL-153, 1:50), anti-G-CSFR (H-176, 1:50), and anti-GCSF (E-19, 1:50) purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-α-Actinin (EA-53, 1:800) from Sigma (St. Louis, MO); anti-ANP (1:100) from CHEMICON (Temecula, CA); anti-Actin (HNF35) from NeoMarker (Fremont, CA); and anti-myosin heavy chain (MHC) (MF20), a gift from David Bader. After overnight incubation, bound antibodies were visualized with a secondary antibody conjugated to Alexa488 or Alexa546 (Molecular Probes, Minato-ku, Tokyo). Nuclei were stained with 4′,6-diamidine-2-phenylidole dihydrochloride (DAPI; Sigma) or TO-PRO3 (Molecular Probes).

Quantitative RT-PCR

Total RNA was extracted with Trizol reagent (GIBCO, Minato-ku, Tokyo). RT-PCR was performed as described previously with the following primers (Yuasa et al., 2005). *gapdh* was used as an internal control. For quantitative analysis of *brachyury T* and *nkx2.5* expression, cDNA was used as the template in a TaqMan real-time PCR assay with the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. All samples were run in triplicate. Data were normalized to *gapdh*. The primers and TaqMan probe for murine *brachyury T*, *nkx2.5*, *csf3*, *csf3r*, *gata4*, *mlc2v*, *tbx5*, and *nppa* rat *csf3r* were Mm00436877_m1, Mm00657783_m1, Mm00438334_m1, Mm01179739_m1, Mm00484689_m1, Mm00440384_m1, Mm00803521_m1, Mm011255747_g1, and Rn01516131_m1 (Applied Biosystems), and primers and probes for human *NPPA*, *TBX5*, *NKX2.5*, *MYH6*, and *MYL2* were Hs00383230_g1, Hs01052563_m1, Hs00231763_m1, Hs_00411908m1, and Hs_00166405m1, respectively.

Proliferation Assay

Pregnant mice were injected directly into the uterus with 7.5 ng of murine G-CSF (414-CS, R&D Systems) on embryonic day 9.0. After G-CSF injection, pregnant mice were injected intraperitoneally with BrdU (BrdU labeling kit; 1296 736 Roche, Basel, Switzerland) at embryonic day 9.5. Mice were sacrificed at embryonic day 12.5, when embryos were removed and prepared for fresh frozen sectioning. After antigen retrieval with histoVT One (L6F9587 Nacalai Tesque, Nakagyoku, Kyoto) and blocking, BrdU was stained as described in the protocol. Phospho-histone H3 staining was also performed with a specific primary antibody (#9701, Cell Signaling Technology, Danvers, MA) as described above. MEF2C and BrdU double staining was performed with MEF2C antibody (C-21, Santa Cruz; 1:100). The MEF2C signal was amplified with biotinylated anti-rabbit antibody (Jackson Immunoresearch, 711-065-152; 1:500), VECTASTAIN Elite ABC kit (PK-6200, VECTOR laboratories; 1:100), and TSA Fluorescence systems (NEL702, 1:50).

Small Interfering RNAs and Transfections

Knockdown of proteins was performed with Silencer Select Pre-designed siRNA (Applied Biosystems) with the following IDs: JAK2 siRNA1, s68539; JAK2 siRNA2, s68540; STAT3 siRNA1, s74451; and STAT3 siRNA2, s20848. Transfection of siRNAs was performed with siPORT *NeoFX* Transfection agent (Applied Biosystems).

Explant Culture

E9.0 embryonic hearts were removed and cultured in serum-free α-MEM. Recombinant murine G-CSF, mitotic inhibitors, and BrdU were added at the

start of culture as described above. The proliferation assay was performed after 2 days of culture. Mitotic inhibitors used in the assay were 50 μM of JAK2 inhibitor AG490 (658401, Cosmobio, Kotoku, Tokyo), 100 μM of STAT3 inhibitor peptide, 5 μM of MEK1/2 inhibitor, 10 μM of JNK inhibitor, 20 μM of Akt inhibitor, and 10 μM of p38 inhibitor SB203580 (573096, 444939, 420119, 124005, and 559389, respectively, Calbiochem, Meguroku, Tokyo). Immunostaining of BrdU was performed as described above.

TUNEL Assay

G-CSF was injected in to the uterus of pregnant mice at E9.0, and embryos were removed at E12.5 or E16.5. TUNEL staining was performed with the in situ apoptosis detection kit (TAKARA, MK500) according to the manufacturer's instruction.

Purification of Cardiomyocytes

Purification of cardiomyocytes was performed as described previously (Hattori et al., 2010).

Statistical Analysis

Data were analyzed with StatView J-4.5 software. Values are reported as means ± SD. Comparisons among groups were performed by one-way ANOVA. Scheffe's F test was used to determine level of significance. The probability level accepted for significance was $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.stem.2010.01.002.

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

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Drug discovery

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.

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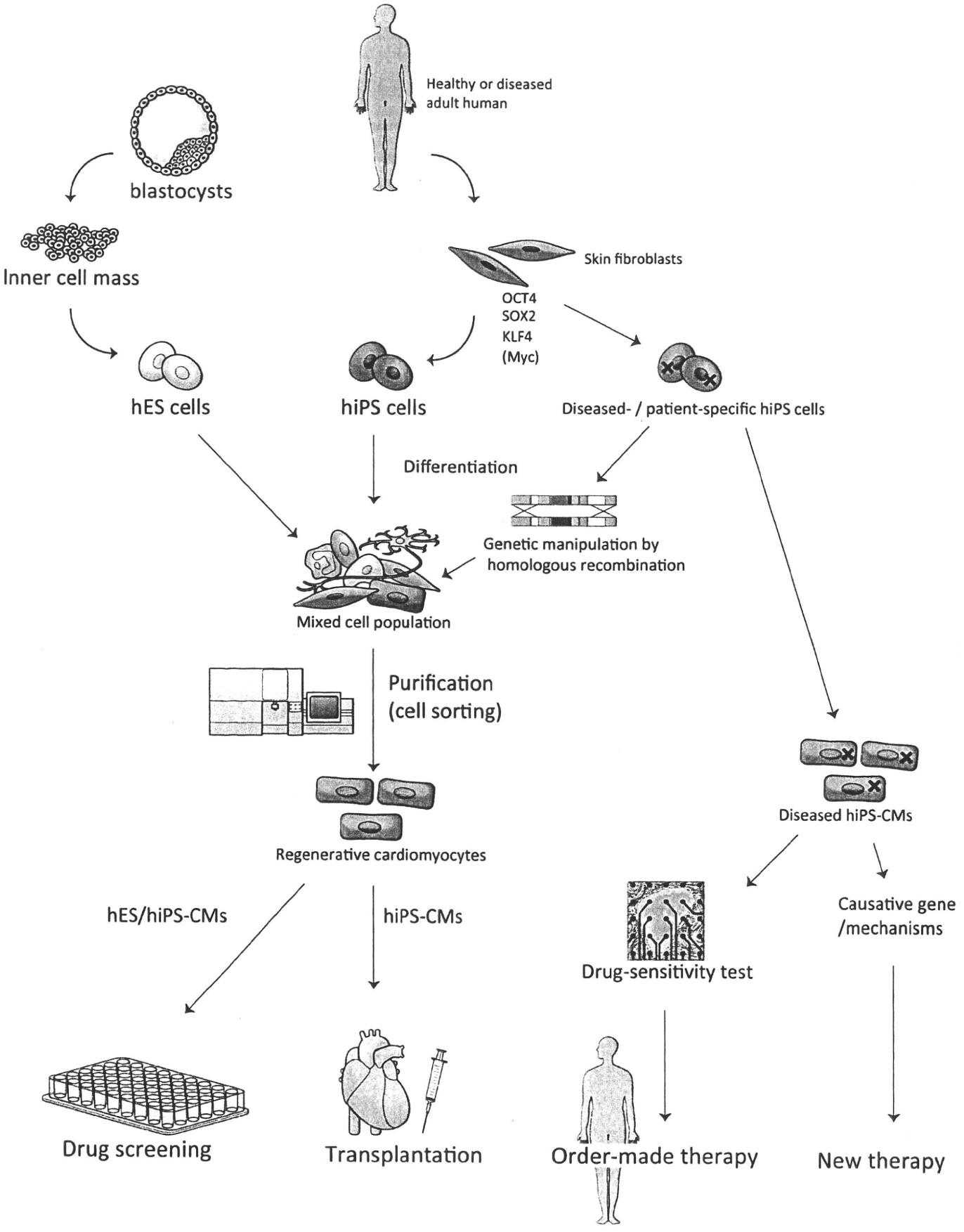
Healthy or diseased adult human
blastocysts
Inner cell mass
Skin fibroblasts
OCT4
SOX2
KLF4
(Myc)
hES cells
hiPS cells
Diseased- / patient-specific hiPS cells
Differentiation
Genetic manipulation by homologous recombination
Mixed cell population
Purification (cell sorting)
Diseased hiPS-CMs
Regenerative cardiomyocytes
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.

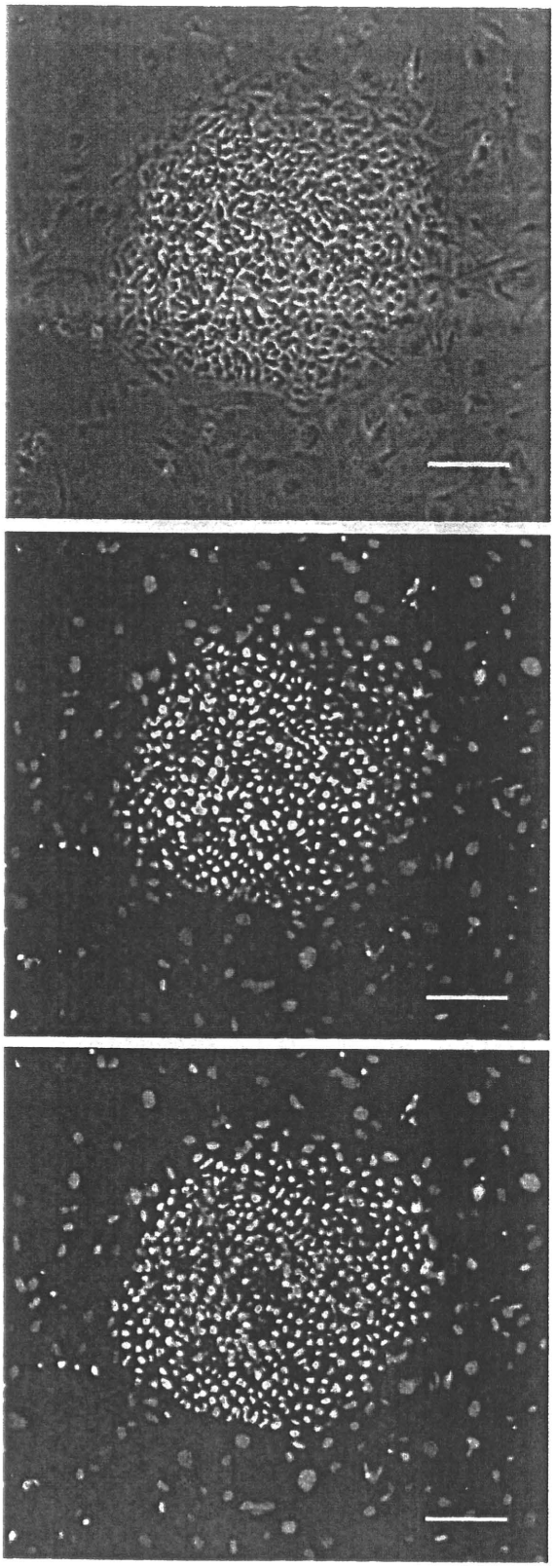

Fig. 2. Characterization of undifferentiated hiPSCs. Immunocytochemical staining for the representative ES markers Oct4 (green) and Nanog (red) in hiPSCs. Nuclei are stained with DAPI (blue). Scale bar, 100 μm.

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 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.

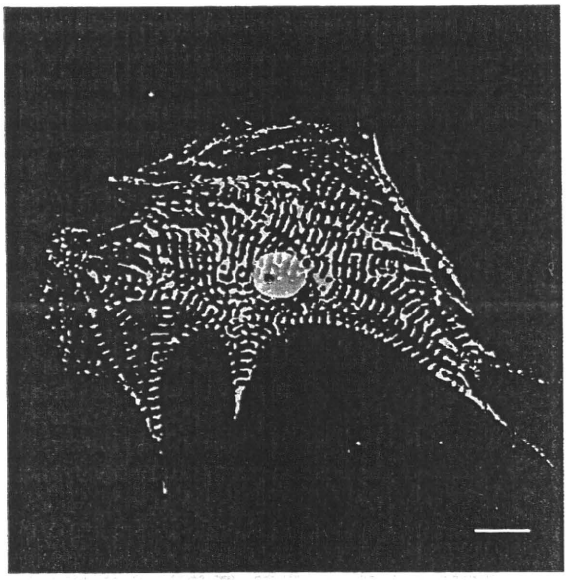

Fig. 3. Cardiomyocyte differentiation from hiPSCs. Immunocytochemical staining for the cardiac-specific transcription factor Nkx2.5 (green) and sarcomeric protein α-actinin (red) in hiPS-CMs. Scale bar, 10 μm.

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%

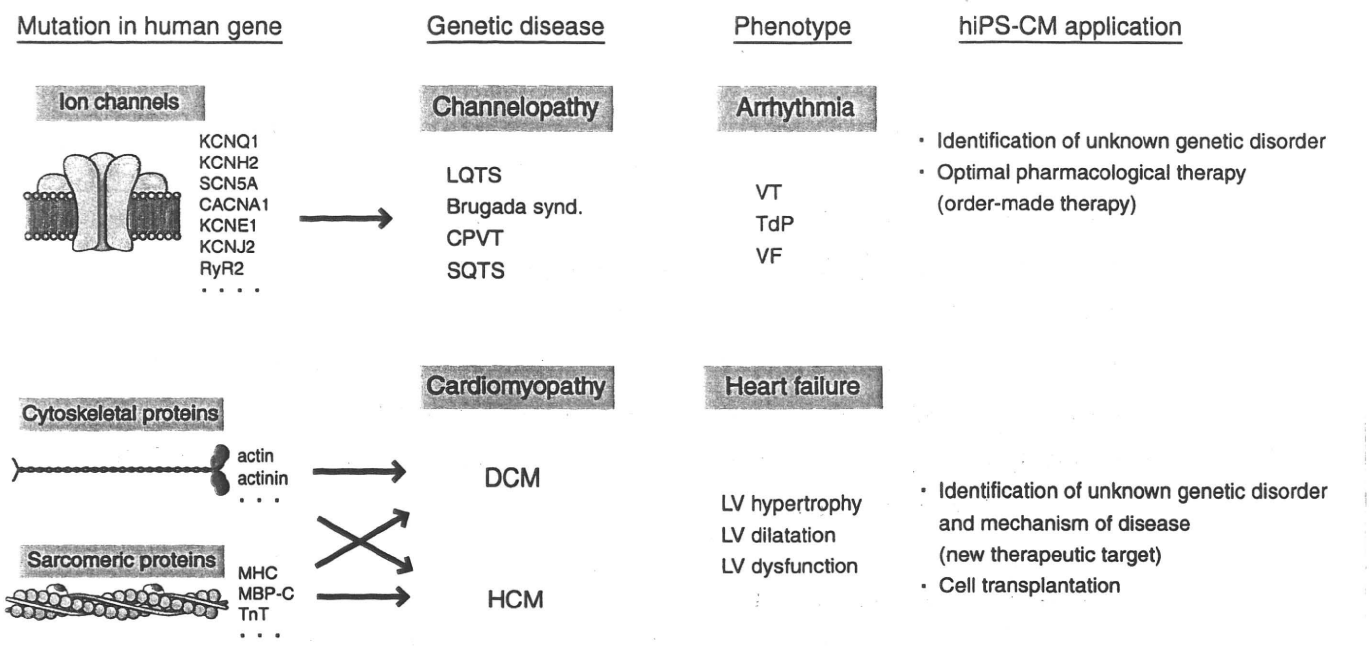

Fig. 4. Cardiac genetic diseases and human iPSC applications. Cardiac genetic diseases mostly comprise the mutations in ion channels (channelopathy) and in cytoskeletal protein and sarcomeric protein (cardiomyopathy). LQTS, long QT syndrome; CPVT, catecholaminergic polymorphic ventricular tachycardia; 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

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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,

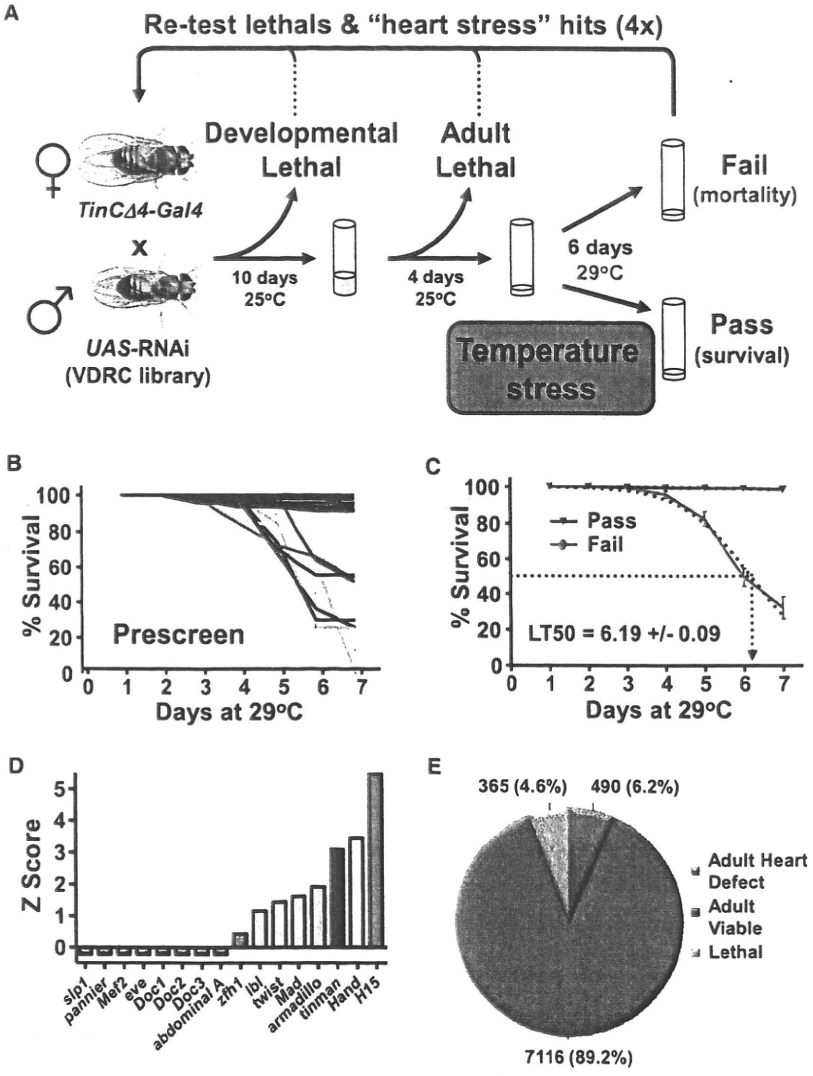

Figure 1. Genome-wide Screen for Conserved Heart Genes

(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

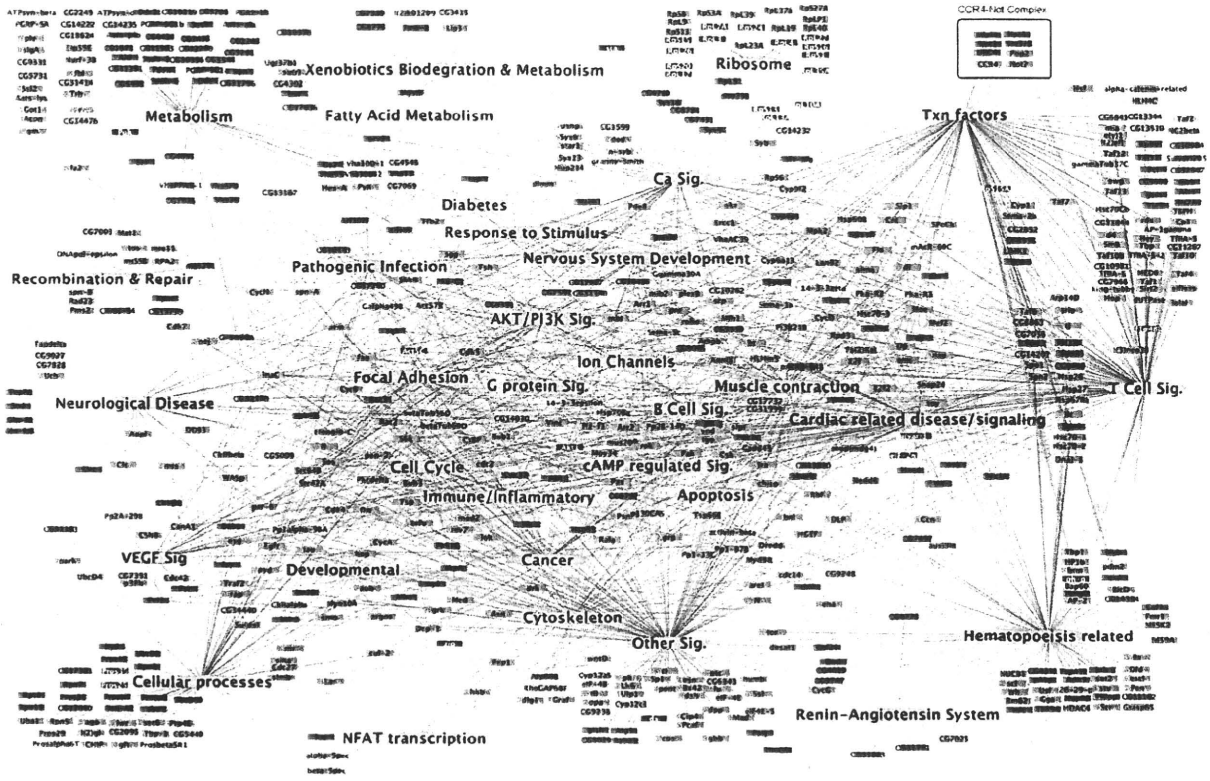

Figure 2. A Global Network of Heart Function

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