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Human Stem Cells for Modeling Heart Disease and for Drug Discovery

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Abstract

A major research focus in the field of cardiovascular medicine is the prospect of using stem cells and progenitor cells for cardiac regeneration. With the advent of induced pluripotent stem cell (iPSC) technology, major efforts are also underway to use iPSCs to model heart disease, to screen for new drugs, and to test candidate drugs for cardiotoxicity. Here, we discuss recent advances in the exciting fields of stem cells and cardiovascular disease.

Cardiovascular disease is a leading cause of morbidity and mortality worldwide, with an estimated 17.3 million deaths per year (1). As this number is expected to surpass 23 million deaths annually by 2030, there is an urgent need for better treatment options (2). Current drug therapies alleviate symptoms for only 50 to 70% of cardiovascular disease patients, often with unwanted side effects (3). The availability of human stem and progenitor cells with cardiac regenerative potential has opened the door to regenerative medicine strategies for cellular cardiomyogenesis and neovascularization. Induced pluripotent stem cells (iPSCs) derived from adult somatic cells are yielding novel insights into the molecular mechanisms of heart disease, making it possible to deliver new patient-specific pharmacological, genetic, and cellular therapies for cardiovascular disease. The cardiovascular field has the potential to progress from “population medicine” toward “personalized medicine” with a new armamentarium of therapeutic and preventive strategies. Here, we highlight research progress toward the use of stem cells and progenitor cells in disease modeling, drug discovery, and cardiac regeneration.

CARDIOVASCULAR STEM CELLS FOR CARDIAC REPAIR

Adult Stem Cells and Progenitors

Multipotent adult stem cells and progenitor cells capable of cardiac repair reside within numerous human adult tissues, including the bone marrow, skeletal muscle, adipose tissue,

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peripheral blood, and the heart (Fig. 1). Adult stem cells are considered reliable and renewable cellular sources for cardiac regeneration. They have demonstrated an in vitro and in vivo ability to express cardiomyocyte-specific markers or even to differentiate toward functional cardiomyocytes, albei at very low efficiencies (4). Multipotent adult stem cells residing in the heart include c-Kit $(CD117)^+$, stem cell antigen 1 $(Sca\text{-}1)^+$, and side population (Hoechst 33342^-, $CD34^{-/low}$, c-Kit^+, and Sca-1^+) cardiac stem cells, as well as second heart field $ISL1^+$ progenitor cells (5). Specialized culture techniques have also enabled Sca-1^+ and c-Kit^+ cardiosphere-derived cell isolation from the adult human heart (6). c-Kit^+ hematopoietic stem cells do reside within the bone marrow as well as the heart but have been shown to represent a more committed cell population (7). Nevertheless, the bone marrow hosts a plethora of multipotent adult stem cells, including side population cells, mesenchymal stem cells, and mononuclear stem cells, all of which are being investigated as potential cellular therapies for cardiac regeneration (8) [see Review by Lin and Pu (9)]. $CD34^+$ cells from human peripheral blood, $CD31^+$ circulating endothelial progenitor cells, and adipose-derived stem cells have also demonstrated cardiac regeneration abilities.

Clinical trials for treatment of post-infarct patients using multipotent adult stem cells are ongoing but with mixed results for their short-term efficacy (8), and there are no current reports about their long-term efficacy. A common drawback has been the poor survival of implanted cells, irrespective of the delivery route, immunosuppression strategy, or timing. This raises questions as to the mechanisms by which adult stem cell delivery has resulted in post-infarct functional recovery. Thus far, suggested mechanisms focus on cardiac regeneration by differentiation to cardiomyocytes, fusion with endogenous cardiomyocytes, production of exosomes that might promote endogenous adult stem cell activation (10), or secretion of paracrine factors (growth factors, cytokines, or other signaling molecules) that promote neovascularization (11).

Direct Transdifferentiation to Cardiomyocytes and Progenitors

The ability to induce transdifferentiation of adult skin or cardiac fibroblasts toward functional cardiomyocytes either in vitro or in vivo was first described in 2010 (Fig. 1) (12). Transdifferentiation was achieved by viral overexpression of cardiac transcription factors (Gata4, Mef2c, and Tbx5), resulting in the formation of induced cardiomyocytes that activate expression of sarcomeric markers and exhibit cardiomyocyte-like electrophysiological and calcium handling properties. However, transdifferentiation protocols remain elaborate and time consuming, often requiring coculture with rodent myocytes (13, 14). Some studies have disputed the ability of lineage-committed fibroblasts to generate induced cardiomyocytes (15), suggesting that experimental artifacts such as incomplete transgene silencing or cell fusion events might explain induced cardiomyocyte formation. Further work is needed to validate the direct transdifferentiation technology and make protocols more amenable for future application in cardiac regeneration.

An alternative approach toward cardiac transdifferentiation has been described more recently, in which fibroblasts first undergo partial reprogramming by expression of exogenously supplied pluripotency-associated genes (*Oct4*, *Sox2*, and *Klf4*) for a 4- to 6-day

period, followed by differentiation to cardiomyocytes without formation of pluripotent intermediates (16). The possibility that partially reprogrammed cells could undergo proliferation in vitro has raised hopes that sufficiently large numbers of cells primed for cardiac differentiation could be created for regenerative medicine applications.

Pluripotent Stem Cell–Derived Cardiomyocytes and Cardiac Progenitors

Cardiovascular precursor cells have also been derived from pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and iPSCs. Both of these PSC types have the ability to generate cell lineages from all three embryonic germ layers, but whereas ESC isolation requires donation of human embryos, iPSCs can be derived from adult tissue (e.g., skin, adipose cells, keratinocytes, or peripheral blood) by overexpressing key pluripotency-associated genes (*OCT4*, *SOX2*, *KLF4*, or *c-MYC*), thus alleviating the need for human embryonic tissue (17). PSC-derived cardiovascular progenitors include human $HCN4^+$ populations that later become specified as first heart field cardiomyocytes (18). A population of human ESC-derived $ROR2^+/CD13^+/KDR^+/PDGFR\alpha^+$ cells has also been identified to generate cardiomyocytes, endothelial cells, and vascular smooth muscle cells in vitro (19). Aside from isolation of precursor cells based on surface marker expression, PSC-derived cardiovascular precursor cells have been generated by treatment with small molecules such as nicotinamide, which was sufficient to induce cardiac mesoderm specification and trigger progression to beating cardioblasts from ESCs with high efficiency (20). In a separate study, a combination of bone morphogenetic protein 4 (BMP4), the glycogen synthase kinase 3 inhibitor CHIR99021, and ascorbic acid was also sufficient to rapidly convert human PSCs into cardiovascular precursor cells (21). A key advantage of cardiovascular precursor cells is their ability to self-renew and to retain the potential for efficient in vitro specification toward cardiovascular lineages. Therefore, this cell population represents a powerful means for understanding the mechanisms of early cardiovascular development and may provide a new source of cells for cardiac regenerative medicine.

Contracting cardiomyocytes can also be generated directly from human PSCs by using one of three methods: (i) coculture with END2 mouse endoderm-like cells, (ii) embryoid body formation, or (iii) monolayer culture. END2 coculture is restricted by its reliance on animal cells. Methods to make embryoid bodies involve the suspension of PSC colonies in fetal bovine serum, enabling the formation of three-dimensional (3D) aggregates that are thought to recapitulate the growth factor gradients and cell-cell interactions that normally occur in the human embryo (22). Although initial protocols produced contracting embryoid bodies with only 5 to 15% efficiency (23), subsequent optimization with the timely addition of growth factors (such as BMP4, fibroblast growth factor 2, Activin A, and WNT3A) during early differentiation (day 0 to day 4), coupled with WNT inhibition during later stages of differentiation (day 4 to day 8), could improve efficiency to >70% (24). Monolayer differentiation (25), which involves simple serum-free and scalable protocols, has largely replaced embryoid body formation. Meanwhile, Activin A and BMP4 growth factors have been replaced by CHIR99021, a small molecule that can be reproduced more reliably than growth factors. Maintaining undifferentiated PSCs in a defined biologics-free culture system that allows faithful expansion and controllable direct differentiation will be crucial for their therapeutic application.

Pluripotent Stem Cell–Derived Cardiomyocytes for Drug Screening

In vitro modeling of human heart disease has been hampered by inaccessibility and lack of proliferative capacity of primary human cardiac tissue. Although recent methods now enable in vitro maintenance of human cardiac tissue for at least 1 month (26), donors are rare and tissue is limited, thus restricting the extent of molecular and drug-testing studies that can be performed. Animal models, primarily mice, are extensively used to study heart disease, offering valuable mechanistic insights. However, their extrapolation to human cardiac disease is poor because of considerable species variation in heart beat rate [mouse, 500 beats per min (bpm); human, 60 bpm], electrocardiogram duration (mouse, 50 to 100 ms; human, 450 ms), cardiac repolarization currents (mouse, I_{to}, $I_{K,slow1}$, $I_{K,slow2}$, I_{SS}; human, I_{Ks} and I_{Kr}), phospholamban regulation of calcium homeostasis (necessary for myocardial relaxation), and expression of surface and structural genes (27). Transgenic Chinese hamster ovary cells and human embryonic kidney cells overexpressing the I_{Kr} protein hERG (human Ether-à-go-go-Related Gene) are used extensively by the pharmaceutical industry to evaluate the cardiotoxicity of new drugs. However, these platforms compare poorly with the complex multichannel or structural phenotype of functional cardiomyocytes (Fig. 2), resulting in unsafe drugs being released to the market. Numerous drugs against aches (propoxyphene), weight loss (sibutramine), inflammatory disease (rofecoxib), and gastrointestinal disease (cisapride) were recently withdrawn from the market owing to structural damage or apoptosis of cardiomyocytes, induction of arrhythmias, seizures, or sudden cardiac death (28). With research and development costs per drug averaging $1.5 billion, drug withdrawals impose a huge socioeconomic and healthcare burden that fuels the need for using functional human cardiomyocytes in drug screening assays (29).

PSC-CMs have been thoroughly characterized in vitro by use of assays for cross striation alignment, sarcomere length, and density measurements, as well as ultrastructural and electrophysiological responses (30). They also demonstrate the ability to form mixed populations of ventricular, atrial, and nodal subtypes and exhibit most cardiac-specific ionic currents (31). However, a major drawback is their immaturity—their closer resemblance to fetal rather than adult cardiomyocytes. Specifically, PSC-CMs display an immature sarcomeric architecture characterized by the absence of H zones, I bands, and M lines; high proliferation rates (~17%); spontaneous contraction due to high pacemaker currents; immature sarcoplasmic reticulum; and round/oval rather than rod-like shapes (32). Several approaches have been used to encourage maturation of PSC-CMs, including differentiation on microfabricated polydimethylsiloxane 3D pillar array patches to enhance expression of genes involved in cardiac contractile function and to promote cardiomyocyte elongation, orientation, alignment, and conduction velocities (33). Platforms that facilitate cycles of mechanical stretching in engineered heart muscle (in vitro tissue models of the human myocardium) as well as continued electrical stimulation have also been considered for enhancing cardiomyocyte maturation (34). Combinations of these approaches, together with defined hormonal cues, are likely to be necessary to render PSC-CMs comparable with adult human cardiomyocytes.

Drug Discovery and Toxicity Assays

Despite their immature characteristics, extensive pharmacological studies have shown that PSC-CMs could accelerate drug screening, enable more accurate prediction of human cardiac response to pharmacotherapy, and provide tools for personalized drug screening assays to timely and accurately predict a patient's drug response. Clinically relevant drugs ranging from adrenergic and muscarinic receptor agonists to anti-arrhythmic agents and ion channel modulators exert chronotropic, inotropic, and lusitropic effects on PSC-CMs (35). In addition, PSC-CMs can accurately predict cardiototoxic drug-induced side effects (36). Good correlations between clinical and PSC-CM toxicity have been demonstrated for drugs known to prolong the QT interval (quinidine and sotalol), whereas low arrhythmogenic drugs (terfenadine and sertindole) prolong the field potential duration only at high doses in PSC-CMs (37). Engineered heart muscle that facilitates cardiomyocyte characterization in a 3D tissue-like format also demonstrates dose-dependent responses to proarrhythmic compounds such as E4031, quinidine, procainamide, and cisapride (38). Moreover, cell sheet and microfabricated stamp engineering technologies enable the generation of increasingly sophisticated cardiac tissues that can be combined with vascular beds to create truly representative predictive models of human myocardium drug responses (39). Thus, consolidated evidence indicates that PSC-CMs offer the pharmaceutical industry a refined, human, physiologically relevant tool for validating new drug candidates in early drug-development stages.

Beyond the ability of PSC-CMs to enable investigation of experimental drug therapies, these cells also offer exceptional opportunities to explore genetic therapies (40) and to refine drug response-to-genotype correlations. These studies are most successful when applied to pharmacogenetic outcomes rather than clinical phenotypes because drug responses are more easily quantifiable. Therefore, personalized medicine approaches in which a patient's own clinical, genetic, and in vitro cellular characteristics can fine-tune decisions regarding the diagnosis, treatment, and prevention of cardiovascular disease are starting to take shape, thanks to in vitro PSC-CM models.

IN VITRO MODELING OF CARDIOVASCULAR DISEASE

Cardiac Channelopathies

The discovery of iPSC technology has opened new avenues into in vitro modeling of human cardiovascular disease (Fig. 2). The most extensively studied models are those of arrhythmic disorders caused by mutations in cardiac ion channels (channelopathies). Electrophysiological assessment with multi-electrode arrays and patch-clamp techniques has enabled the identification of prolonged field potentials and action potentials in iPSC-CMs from patients with long-QT syndrome (LQTS) type 1 and type 2 mutations in potassium ion channels (41, 42). Upon β-adrenergic stimulation, LQTS iPSC-CMs developed arrhythmias, which could be corrected by treatment with β-blockers (propranolol and nadolol). Such findings demonstrate that iPSC-CMs have comparable drug responses with those of LQTS patients.

LQTS type 3, caused by gain-of-function mutations in the sodium ion channel–encoding gene *SCN5A*, was also recapitulated in iPSC-CMs by decreased voltage-dependent inactivation of sodium channels (43, 44). Faster pacing combined with the sodium channel–blocker mexiletine corrected sodium current irregularities. This regimen mirrored the patient's therapy in the clinic, further reinforcing the ability of iPSC-CMs to serve as appropriate models for human disease. LQTS type 8 (LQT8; Timothy syndrome) caused by mutations in the L-type calcium channel Ca(v)1.2 also has been modeled by iPSC-CMs (45). These cells showed prolonged action potentials, developed arrhythmias, showed excess Ca^{2+} influx, and abnormal Ca^{2+} transients. Roscovitine, a Ca(v)1.2 activator, restored the electrical and Ca^{2+} signaling properties of LQT8-CMs.

Last, iPSC-CMs from patients with catecholaminergic polymorphic ventricular tachycardia (CPVT), a hereditary arrhythmic disorder caused by mutations in the ryanodine receptor (RYR2) or cardiac calsequestrin (CASQ2), showed increased susceptibility to arrhythmic episodes in the form of delayed after-depolarizations that typically interrupt phase 4 of the action potential (46). Flecainide, a clinically used pharmacological agent for CPVT, eliminated the iPSC-CM arrhythmias. Analysis of the molecular mechanism demonstrated that increasing intracellular calcium ion concentrations by treatment with thapsigargin, a sarcoplasmic reticulum calcium adenosine triphosphatase (SERCA2a) pump inhibitor, abrogated intracellular calcium stores and eliminated delayed after-depolarizations, demonstrating that increased diastolic calcium can lead to the development of arrhythmias (47). These studies show that iPSC-CMs can help to elucidate the molecular and cellular mechanisms of cardiac arrhythmias in humans and serve as robust platforms for developing new drugs for clinical therapy.

Cardiomyopathies

Another class of cardiac disorders that can be modeled in vitro are cardiomyopathies, which are associated with deterioration in myocardial function and are linked to heart failure and cardiac sudden death (48). One such disorder is dilated cardiomyopathy, characterized by ventricular dilation and systolic dysfunction. iPSC-CMs derived from patients with familial dilated cardiomyopathy (DCM) carrying a cardiac troponin T (cTnT) mutation exhibit irregular organization of sarcomeres, a scattered distribution pattern of Z bodies, reduction in contractile force, altered regulation of Ca^{2+}, and decreased tolerance to β1-adrenergic challenge (49). Notably, chronic treatment with the β1-adrenergic blocker, metoprolol, or transgenic overexpression of SERCA2a ameliorated the disease phenotypes. Another effort to model DCM involved a lamin A/C mutation, in which iPSC-CMs demonstrated accelerated nuclear senescence and apoptosis under electrical stress that was diminished by blockade of the ERK1/2 signaling pathway (50). These findings suggest that iPSC-CMs could closely recapitulate the morphological and functional phenotypes of dilated cardiomyopathy.

Familial hypertrophic cardiomyopathy (HCM)—characterized by thickening primarily of the left ventricular wall—has also been efficiently modeled by using iPSC-CMs. Lan *et al.* (51) generated iPSCs carrying a thick myofilament myosin heavy chain 7 (MYH7) mutation and demonstrated that the iPSC-CMs had increased myofibril content and were enlarged as

compared with those derived from healthy control patients. The MYH7 mutated iPSC-CMs also had contractile arrhythmias such as delayed after-depolarizations, Ca^{2+} cycling perturbations, and increased intracellular Ca^{2+}. Adrenergic stimulation exacerbated Ca^{2+} transient irregularities and arrhythmias, mirroring the development of arrhythmias in HCM patients with activation of the sympathetic nervous system because of stress. These abnormalities were counteracted by the β-adrenergic blocker, propranolol, or the L-type Ca^{2+} channel blocker, verapamil, thus supporting the notion that Ca^{2+} cycling perturbations and intracellular Ca^{2+} elevation are central mechanisms for disease development at the cellular level.

A separate study of LEOPARD syndrome—a multi-organ condition in which 80% of patients present with hypertrophic cardiomyopathy as the most life-threatening aspect—showed that iPSC-CMs had an increased median surface, sarcomeric organization, and localization of nuclear factor of activated T cells (NFATC4) to the nucleus (46). The authors also demonstrated that phosphorylation perturbations in proteins involved in RAS-MAPK signal transduction might be implicated in development of disease pathophysiology. These features correlate well with the hypertrophic state observed in patients with HCM, further highlighting the potential of iPSC-CMs to reveal molecular mechanisms underlying the pathology of this disease.

A third type of cardiomyopathy modeled by iPSC-CMs is arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C), which is characterized by right ventricular fibrofatty accumulation and aneurysms that in familial cases are linked to desmosome protein mutations. ARVD/C iPSC-CM modeling recapitulated disease pathologies (lipogenesis and apoptosis) after induction of adultlike metabolic energetics from glycolysis to fatty acid oxidation (52). More recently, the developmental heart defect known as hypoplastic left heart syndrome (HLHS), which is characterized by severe underdevelopment of the left ventricle, has been recapitulated in vitro. Using iPSC-CMs, Jiang *et al.* (53) found decreased differentiation efficiencies and myofilament organization, as well as induction of fetal gene expression profiles, calcium transient abnormalities, and increased sensitivity to caffeine and stimulation by β-adrenergic antagonists in iPSC-CMs. They suggested that HLHS might activate alternative mechanisms for Ca^{2+} release from the sarcoplasmic reticulum via the inositol triphosphate receptor rather than the RYR2, thus providing evidence that iPSC-CMs can be used for mechanistic investigations of cardiac developmental diseases.

CONCLUSIONS AND FUTURE OUTLOOK

Autologous or allogeneic adult stem cells— including mesenchymal stem cells, cardiac stem cells, hematopoietic stem cells, mononuclear cells, adipose-derived stem cells, and cardiosphere derived cells—have provided exciting avenues for promoting heart tissue repair (8). Several labs worldwide have also joined the race to demonstrate in vivo transplantation of PSC-CMs in preclinical models that will eventually lead to phase 1 clinical trials. In the meantime, PSC-CMs are increasingly considered an appropriate platform for drug discovery and toxicology studies. However, their immature phenotype and inability to undergo directed subtype specification are still major hurdles. Cardiac tissue engineering may deliver a means to promote cardiomyocyte maturation, and it is anticipated

that in combination with next generation sequencing and other high-throughput or computational approaches (54), these advances will bridge our knowledge regarding molecular mechanisms of cardiovascular disease and will increase confidence in the use of PSC-CMs as accurate models for prediction of human clinical response to pharmacotherapy. Genome-edited pluripotent stem cell lines whose cardiovascular disease-associated mutations/variants are engineered into the same genetic background by endonuclease (ZFN or TALEN) or palindromic repeat (CRISPR) approaches will also be instrumental for generating libraries of disease-specific cardiomyocytes for drug testing and other applications (55).

Most major pharmaceutical companies have adult and pluripotent stem cell programs or are closely collaborating with academic groups to explore the potential of these cell populations in predictive pharmacology, toxicology, and personalized medicine (28). The development cost of a typical new drug from research to the clinic has reached an astronomical $1.5 billion and is expected to continue rising. A mere 1% improvement in predictive drug assays could save up to $100 million for the industry. Therefore, in the pursuit of regenerative and personalized cardiovascular medicine, rationally designed therapies informed by human PSC-based disease models may be more effective, leading to fewer off-target effects than current therapeutic approaches (56). Importantly, advances in stem cell–based medicine are expected to benefit pharmaceutical development by reducing the rate of drug withdrawal in late-stage clinical trials as well as improve cardiovascular patient care by increasing confidence in safety and efficacy of administered drugs.

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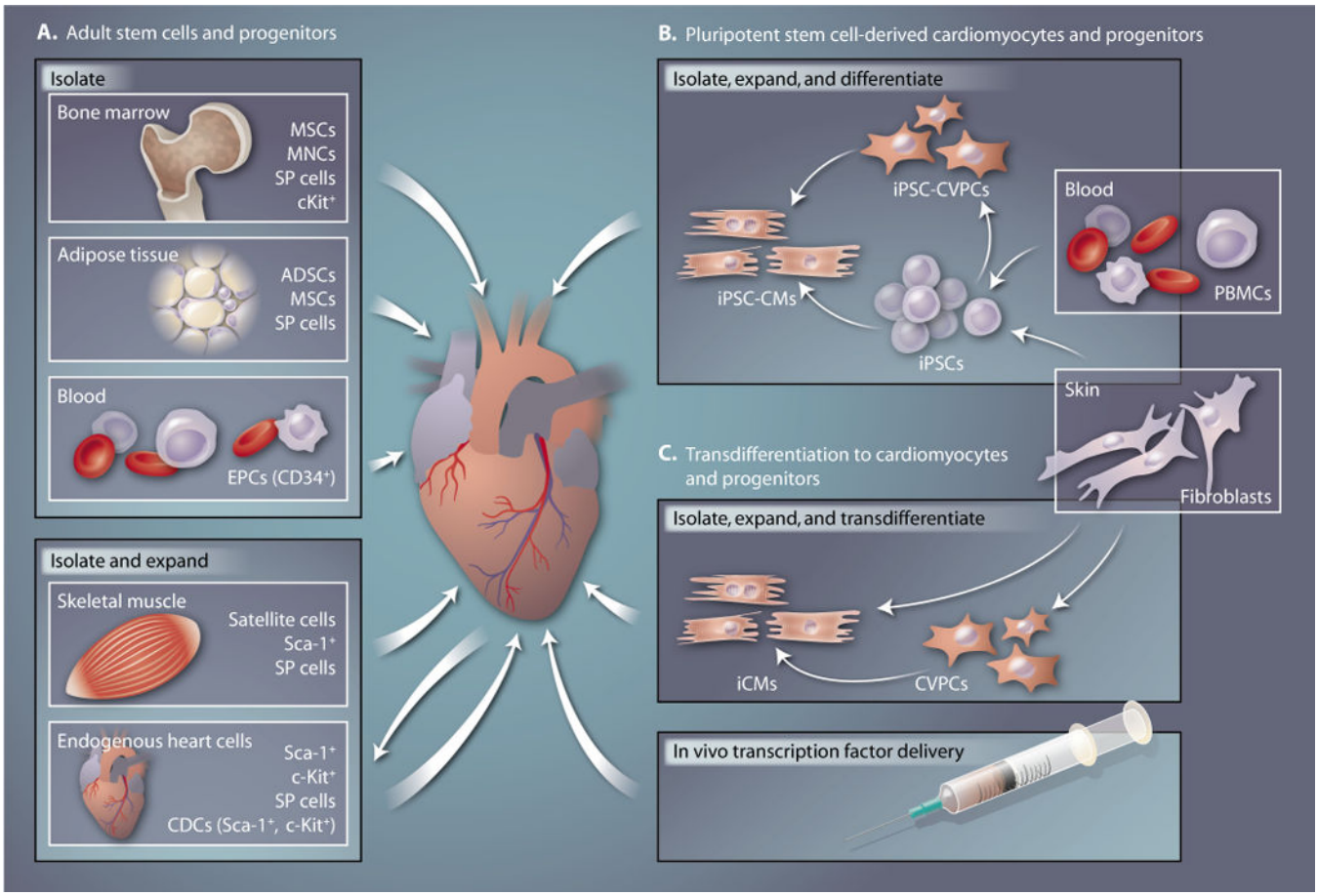

Fig. 1. Adult and pluripotent stem cells for cardiovascular tissue repair

Shown are stem cell–based therapies for regenerative medicine that (**A**) are currently in clinical trials or (**B** and **C**) have potential as therapeutic strategies in the future. Adult stem cells and progenitors isolated from the bone marrow, adipose tissue, and blood can be transplanted into the heart without the need for expansion, whereas skeletal muscle and cardiac-derived stem cells require in vitro expansion before transplantation. Pluripotent stem cell and transdifferentiation strategies require both expansion and conversion to cardiomyocytes before transplantation, unless transdifferentiation takes place in vivo through delivery of transcription factors. ADSCs, adipose-derived stem cells; MSCs, mesenchymal stem cells; MNCs, mononuclear cells; S P, side population; CDCs, cardiosphere-derived cells; PBMCs, peripheral blood mononuclear cells; iPSCs, induced pluripotent stem cells; CMs, cardiomyocytes; CVPCs, cardiovascular progenitor cells; EPCs, endothelial progenitor cells.

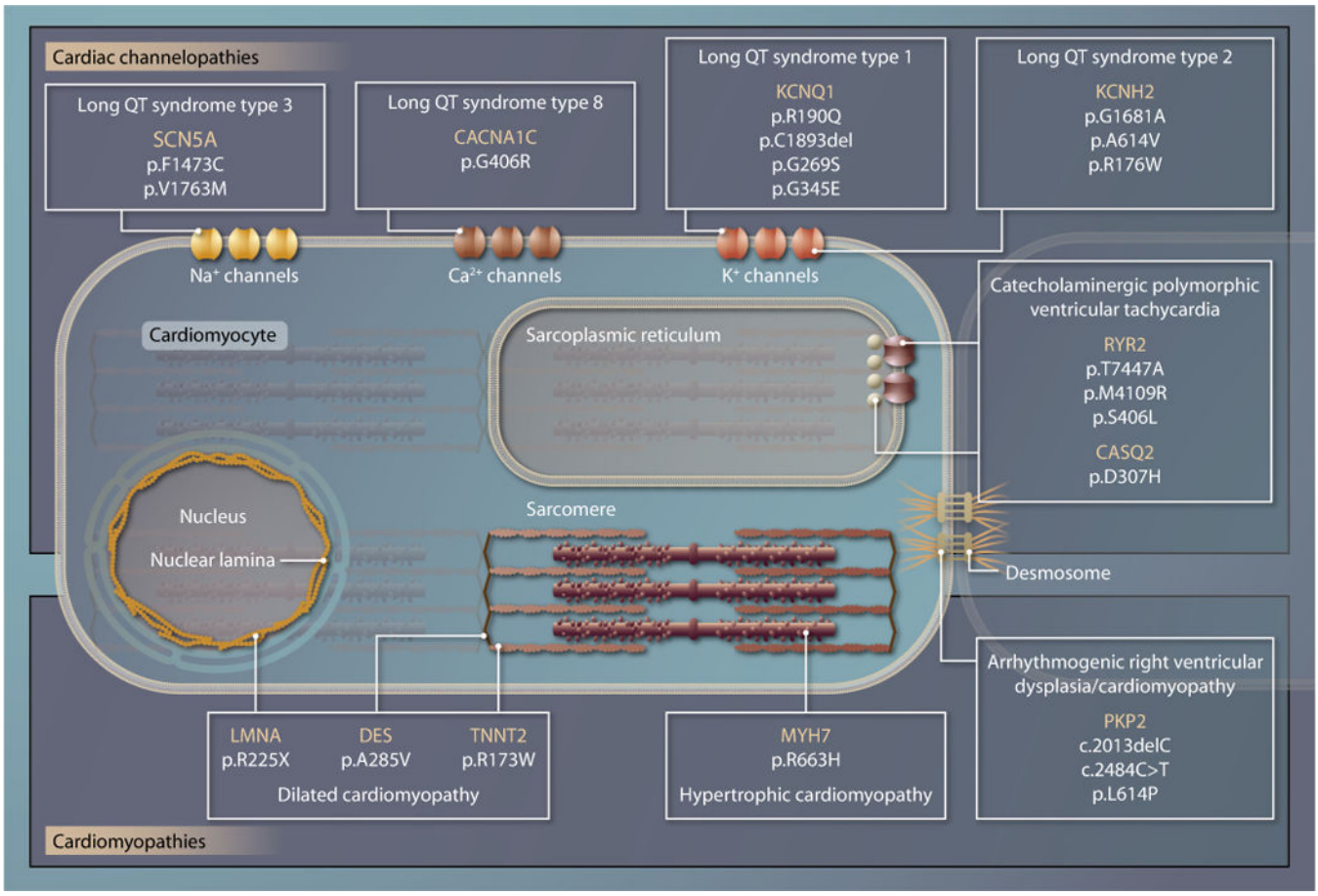

Fig. 2. iPSC-derived cardiomyocytes for drug screening and toxicity testing

Because of the sensitivity of the hERG potassium ion channel to drug-induced cytotoxicity, the safety of new candidate drugs is tested by looking at their effects on hERG electrophysiology. This assay is routinely performed in Chinese hamster ovary cells or human embryonic kidney cells engineered to express hERG, which do not exhibit the complex physiology of human cardiomyocytes. Human cardiomyocytes derived from induced pluripotent stem cells (iPSCs) are promising new tools for drug discovery and have already permitted drug testing and toxicology studies for a variety of human cardiac disorders. These cardiac disorders, caused by a variety of mutations, include LQTS, CPVT, DCM, HCM, and arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C).