To mark the 60th birthday of *Circulation Research* (1953–2013), the editors have commissioned ***Circulation Research Classics***, a series of commentaries highlighting seminal articles published in the journal over the past six decades that have importantly shaped cardiovascular research. Written by leading experts, ***Circulation Research Classics*** are intended to describe the impact of these articles on the field by putting them in a historical perspective. The concept of "classic" is inextricably linked to time—a classic is something that maintains its value regardless of its age. Thus, an important consideration in selecting the articles to be highlighted is that they have stood the test of time, which is the most reliable indicator of the value of scientific work. By looking back at the illustrious past of *Circulation Research*, we hope to promote a deeper appreciation of the contributions of this journal to the advancement of knowledge.

Cardiac Stem Cell Biology
Glimpse of the Past, Present, and Future

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***Abstract*: Cardiac regeneration strategies and de novo generation of cardiomyocytes have long been significant areas of research interest in cardiovascular medicine. In this review, we outline a variety of common cell sources and methods used to regenerate cardiomyocytes and highlight the important role that key *Circulation Research* articles have played in this flourishing field. (*Circ Res*. 2014;114:21-27.)**

Key Words: adult stem cells ■ embryonic stem cells ■ induced pluripotent stem cells

Heart disease, whether inherited or acquired, is the leading cause of mortality in men and women worldwide, accounting for 17.3 million deaths per year.[1] The urgent need to improve existing therapies has driven researchers to seek a better understanding of the diverse but inter-related mechanistic origins of heart development and failure, with the ultimate goals of identifying novel pharmacological treatments and cell-based engineering approaches to replace damaged heart tissue. Animal models are widely used as surrogates for studying human disease**,** both in order to recapitulate the complex clinical course of human heart failure and to generate in vitro tools for studying specific aspects of tissue dysfunction.[2] Although useful insights have been gained, experimental findings from animal models have not always extrapolated to human disease presentation because of considerable species variation.[3] Here, we describe prominent routes taken toward the goal of cardiac regeneration by focusing on key contributing articles published by *Circulation Research* in the 60 years since its establishment.

Multipotent Adult Stem Cells

Multipotent adult stem cells have been the focus of most preclinical and clinical studies performed to date in the field of cardiac regeneration. They represent an attractive source of stem cells because they are relatively abundant, accessible, and autologous, and their mechanisms of action for any observed improvement in cardiac function can be potentially delineated. In 1998, Anversa and Kajstura[4] published a field-changing article challenging the notion that the myocardium is a nonregenerating tissue by describing the presence of multipotent cardiac stem cells (CSCs) in the adult myocardium that are positive for the hematopoietic progenitor marker c-kit (Figure 1). Methods for isolating functionally competent CSCs and mechanisms proving that their activation can reverse cardiac dysfunction were later published by the same group.[5,6] It was this pioneering work and the ability to adequately expand CSCs ex vivo that formed the basis for the first randomized clinical trial of CSC implant in patients with ischemic heart disease (SCIPIO trial).[7] Phase I of the trial demonstrated a sound safety profile and potential for efficacy in improving ventricular function. In 2004, Messina et al[8] were able to isolate and expand c-kit$^+$ CSCs from adult murine hearts as self-adherent clusters of progenitor cells, termed cardiospheres. This isolation technique later became feasible for human hearts and was used to test the therapeutic efficacy of cardiosphere-derived cells in the CADUCEUS trial.[9] The phase I trial demonstrated a good safety profile and potential for reducing scar size and regional function compared with controls. More recently, Dey et al[10] performed detailed characterization of multiple stem cell

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Nonstandard Abbreviations and Acronyms

CM	cardiomyocyte
CSC	cardiac stem cell
ESC	embryonic stem cell
iPSC	induced pluripotent stem cell
MNC	mononuclear cell
MSC	mesenchymal stem cell
NYHA	New York Heart Association
PSC	pluripotent stem cell

populations and concluded that c-kit$^+$ CSCs represent the most primitive population of multipotent cardiac progenitors when compared with bone marrow–derived c-kit$^+$ populations, and that cardiosphere-derived cells are more closely related to bone marrow stem cells in terms of their gene and protein expression profiles. The exact mechanistic and functional outcome implications of such differences are not yet known but may aid ongoing clinical trials in understanding the biology of these promising cell populations.

Bone marrow–derived mononuclear cells (MNCs) have also garnered considerable interest in regenerative cell therapy because they are easily accessible, autologous and require minimal expansion. Significantly, evidence of MNC mobilization after myocardial infarction in mice has supported that bone marrow cells play a role in myocardial healing after injury.[11,12] Randomized human clinical studies of injected MNCs demonstrated a modest increase in left ventricular ejection fraction and a decrease in the New York Heart Association (NYHA) functional classification system.[13] Patients with ischemic cardiomyopathy receiving MNCs also demonstrated a significant reduction in natriuretic peptide levels.[14] Notably, infusion of MNCs with higher colony-forming capacity was associated with lower mortality, raising awareness of the notion that cell viability and quality have significant impacts on therapeutic effect. Mechanistic investigations have suggested that beneficial effects of MNC therapy were a result of neovascularization and paracrine effects rather than cardiomyocyte (CM) differentiation.[15]

Studies of bone marrow–derived mesenchymal stem cells (MSCs) revealed yet another adult stem cell source thought to be suitable for cardiac regeneration. MSCs were reported to readily express phenotypic characteristics of CMs and, when introduced into infarcted animal hearts by intravenous injections, localize at sites of myocardial injury, prevent tissue remodeling, and improve cardiac recovery.[16,17] Intracoronary infusion of allogeneic mesenchymal precursors (Stro-3$^+$ subpopulation) was also shown to decrease infarct size, improve systolic function, and increase neovascularization in animal myocardial infarction models.[18] These observations led to a pilot human clinical study that confirmed the safety and tolerability of MSCs in humans, and subsequently to a phase I/II randomized trial.[19,20] More recently, additional evidence has questioned the ability of MSCs to transdifferentiate into CMs, instead attributing the mechanism of their therapeutic properties to paracrine effects, neovascularization, and activation of endogenous CSCs.[19,21]

Another class of multipotent adult stem cells of particular interest in cardiac cell therapy is cluster of differentiation-34 positive (CD34$^+$) angiogenic precursors. This interest stems from the relatively impaired angiogenesis seen in patients with ischemic heart disease as well as from findings that patients with coronary artery disease have reduced number and migratory activity of angiogenic precursors.[22] It has also been observed that CD34$^+$ cell injection ameliorates cardiac recovery in human patients with myocardial infarction by improving perfusion and by paracrine effects rather than CM differentiation.[23] In one of the largest cell therapy trials to date, Losordo et al[24] demonstrated that patients with refractory angina who received intramyocardial injections of CD34$^+$ cells experienced significant improvements in angina frequency and exercise tolerance. In a subsequent publication, the group identified that CD34$^+$ cells secrete exosomes

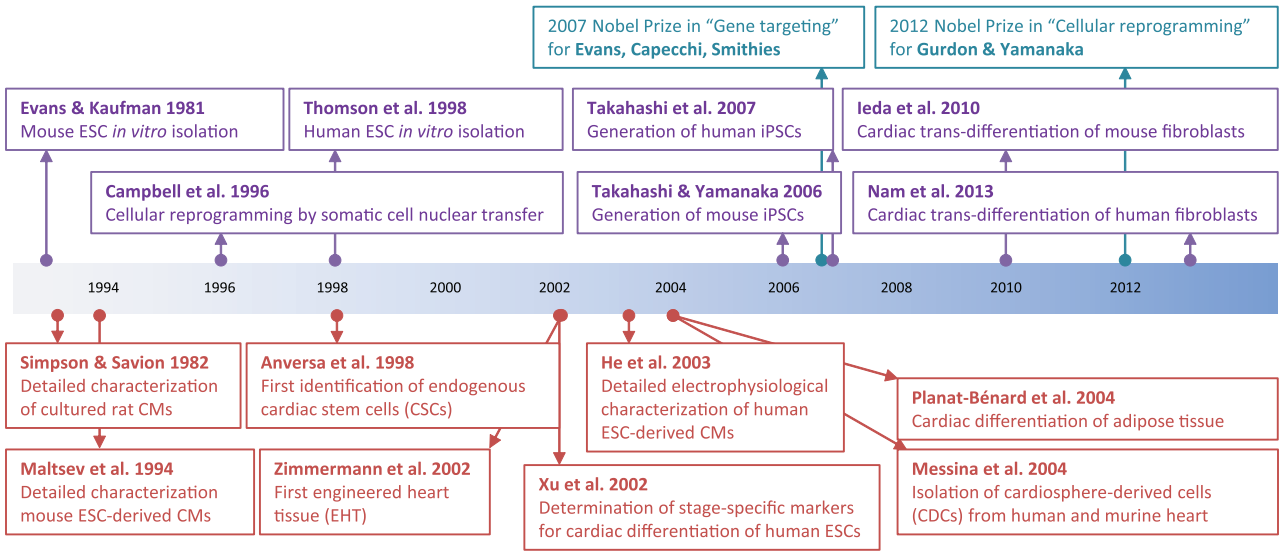

Figure 1. Historic landmarks in the field of cardiac stem cell biology. Timeline of important discoveries contributing to the field of stem cell cardiac differentiation and characterization (purple and green boxes), including the key *Top 100 Circulation Research* articles discussed in this review (red boxes). CMs indicate cardiomyocytes; ESC, embryonic stem cell; and iPSC, induced pluripotent stem cell.

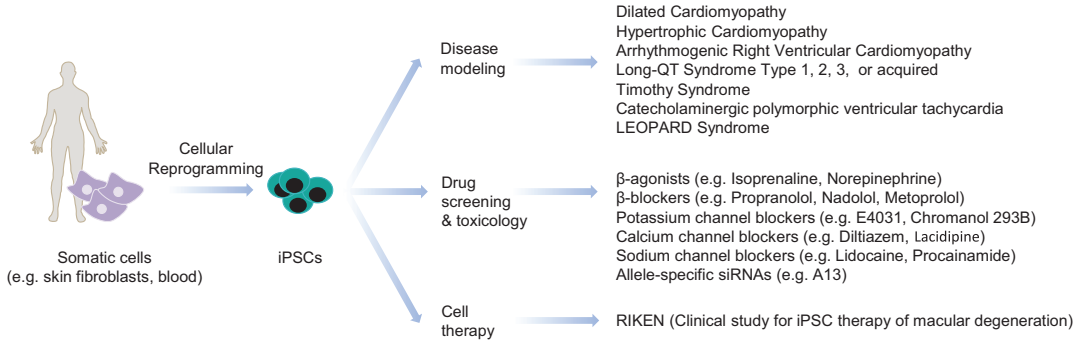

Figure 2. Uses of induced pluripotent stem cells (iPSCs). iPSCs are ideal cellular models for providing a renewable source of cardiomyocytes for in vitro disease modeling, pharmacological testing, and therapeutic applications in regenerative medicine.

that might account for some of the improved phenotypes.[25] The benefit of $CD34^+$ cells was also shown for nonischemic cardiomyopathy, when intracoronary injections resulted in a small, but significant, improvement in ventricular function and survival.[26] More importantly, this study demonstrated that higher intramyocardial homing was associated with better cell therapy response, providing support to previous observations regarding MNCs that cell delivery method and quality play significant roles in their therapeutic efficacy.

Finally, adipose-derived stem cells abundantly available from liposuction surgeries have been considered as potential sources of CMs. In 2004, Planat-Bénard et al[27] reported potential derivation of CMs from human adipose-derived stem cells by treatment with transferrin, interleukin (IL)-3, IL-6, and vascular endothelial growth factor, although at a low event rate (Table). Ongoing trials are evaluating the efficacy of this cell population in regeneration of ischemic myocardium and, although complete results have yet to be published, preliminary data are encouraging (trial identifier: NCT00426868).

Transdifferentiation of Committed Cells

Early attempts at inducing cardiac regeneration involved transplant of skeletal myoblasts or fetal CMs to infarcted canine, rat, as well as human hearts. Unfortunately, these studies ultimately disappointed the field because myoblasts remained firmly committed to form mature skeletal muscle in the heart and led to induction of arrhythmias,[28] whereas extensive cell death coupled with limited proliferation after transplant prevented fetal CMs from repairing injury.[29] Transplantation of noncontractile committed cells such as fibroblasts and smooth muscle cells into infarcted rat hearts was then briefly thought to enhance heart function, possibly because of aforementioned paracrine effects.[30] More recently, several studies have demonstrated in vitro[31] and in vivo[32] transdifferentiation of mouse fibroblasts into seemingly functional CMs by overexpressing combinations of the cardiac transcription factors Gata4, Mef2c, Tbx5, Hand2, and Nkx2.5. Mouse CMs generated by direct transdifferentiation are positive for CM-specific sarcomeric markers and exhibit electrophysiological and gene expression profiles similar to those of fetal CMs, although this was disputed by other investigators.[33] In vitro transdifferentiation toward CM-like cells was also reported for human fibroblasts, albeit by more time-consuming and less efficient protocols that generated mostly partially reprogrammed CMs.[34] Current efforts in this research area focus on optimizing transdifferentiation efficiency and CM maturation, further characterizing derived CMs, and validating that in vitro and in vivo transdifferentiation occur in the absence of experimental artifacts, which can include incomplete silencing of transgene expression from Cre-lox systems, cell fusion events, and the possibility of retrovirus transfecting not only dividing fibroblasts but also nondividing CMs in vivo. For this technology to be fully applied in the clinic, a greater understanding of the following issues that have plagued the field must be reached: (1) the potential consequences of depleting endogenous cardiac fibroblasts to replenish CMs; (2) the ability to transfect bystander cells such as smooth muscle and endothelial cells with cardiac transcription factors; and (3) the challenge of triggering immune response against the host cells transfected with viral versus nonviral vectors.

Pluripotent Stem Cells

Embryonic Stem Cells

The isolation by Evans and Kaufman of mouse embryonic stem cells (mESCs) in 1981[35] and the generation of human embryonic stem cells (hESCs) by Thomson in 1998[36] allowed new opportunities for in vitro generation of CMs. Many protocols have been developed during the years to maximize the yield and efficiency of pluripotent ESC differentiation to CMs.[37] One of the most used methods has been the formation of 3D aggregates named embryoid bodies, within which cardiac differentiation occurs. In 2002, Xu et al[38] were among the first to optimize cardiac differentiation protocols for hESCs by using DNA demethylating agent 5-azacytidine and enrichment with Percoll separation gradients to obtain ≤70% pure CM populations (Table). Later, rigorous protocol standardization and the use of key signaling factors such as bone morphogenetic protein 4 (BMP4) and Activin A enabled conversion of hESCs to CMs with >90% efficiency.[39] Consequently, the formation of 3D aggregates, a labor-intensive process, has now been largely replaced by differentiation in monolayer cultures, which are more amenable to scale-up and automation.[40]

Induced Pluripotent Stem Cells

The discovery of induced pluripotent stem cell (iPSC) technology,[41] based partly on principles highlighted by early somatic cell nuclear transfer experiments,[42] has meant that mature somatic cells such as skin fibroblasts and peripheral blood mononuclear cells can be reprogrammed with relative ease to acquire an

Table. Cell Sources for Cardiac Repair

Category	Cell Type	Advantages	Disadvantages	Published Clinical Trials	Radiographic Improvement	Symptomatic Improvement	Unpublished/ Ongoing Clinical Trials
Adult stem cells	ADSCs	Relatively abundant	Limited proliferation potential	None to date			(ACELLDream)
	$CD34^+$	Accessible by minimally invasive procedures	Inefficient in vitro or in vivo cardiac differentiation	ACT34-CMI	NA	+	NOGA-DCM, RENEW PreSERVE-AMI
		Autologous cell population	Modest improvements in cardiac function observed to date	NOGA-DCM	+	+	
	MSCs			POSEIDON	+/–	+	PROMETHEUS POSEIDON-DCM NCT00644410 CHART-1, ixCELL-DCM
				C-CURE	+	+	
				TAC-HFT	+	+	
	MNCs			FOCUS-HF,	+/–	+	
				FOCUS	–	–	
				Swiss-AMI	–	NA	
				LateTIME,	–	–	
				TIME	+	NA	
				TOPCARE-CHD	+	+	
				ASTAMI	–	+/–	
				BOOST	–	NA	
	CSCs	Adequately expanded ex vivo Autologous cell population	Procured by relatively invasive procedures	SCIPIO	+	+	SCIPIO
	CDCs			CADUCEUS	+	+	ALLSTAR
Committed cells	DFs CFs	Potential for in vivo direct transdifferentiation in humans Bypass need for stem cell progenitors	In vitro cardiac transdifferentiation extremely inefficient, mostly generating partially reprogrammed cardiomyocytes	None to date			None to date
Pluripotent stem cells	ESCs	Indefinite self-renewal Efficient in vitro cardiac differentiation	Ethically problematic Allogeneic transplant requires immunosuppression Immature fetal-like differentiated cells	None to date			(GERON, ACT)
	iPSCs	Additional potential for autologous transplant compared to ESCs	Immature fetal-like differentiated cells	None to date			(RIKEN)

Advantages and disadvantages of the various cell sources used for cardiac regeneration studies, with examples of clinical trials in which cells were used for cardiac regeneration or, in parentheses, other conditions. ACELLDream indicates Adipose Cell Derived Regenerative Endothelial Angiogenic Medicine; ACT, advanced cell technology; ACT34-CMI, Autologous CD34-Chronic Myocardial Ischemia; ADSCs, adipose-derived stem cells; ALLSTAR, Allogeneic Heart Stem Cells to Achieve Myocardial Regeneration; AMI, acute myocardial infarction; ASTAMI, Autologous Stem Cell Transplantation in Acute Myocardial Infarction; CADUCEUS, Cardiosphere-Derived Autologous Stem Cells to Reverse Ventricular Dysfunction; CDCs, cardiosphere-derived cells; CFs, cardiac fibroblasts; CHD, coronary heart disease; CSCs, cardiac stem cells; DFs, dermal fibroblasts; ESCs, embryonic stem cells; HF, heart failure; iPSCs, induced pluripotent stem cells; MNCs, mononuclear cells; MSCs, mesenchymal stem cells; NA, not available; NCT, number of clinical trial; NOGA-DCM, NOGA Mapping-Dilated Cardiomyopathy; POSEIDON, Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis; POSEIDON-DCM, Percutaneous Stem Cell Injection Delivery Effects On Neomyogenesis in Dilated Cardiomyopathy; PROMETHEUS, Prospective Randomized Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery; SCIPIO, Stem Cell Infusion in Patients With Ischemic Cardiomyopathy; Swiss-AMI, Swiss Multicenter Intracoronary Stem Cells Study in Acute Myocardial Infarction; and TAC-HFT, Transendocardial Autologous Cells in Ischemic Heart Failure.

ESC-like phenotype. iPSCs retain the same capacity for high-efficiency cardiac differentiation as ESCs, with the added advantages of avoiding ethical debates related to use of human embryos and enabling autologous transplantation of CMs without the need for immunosuppression. These characteristics make iPSCs ideal cellular models to provide a renewable source of CMs for basic research, pharmacological testing, and cell therapy (Figure 2).[43]

Characterization of Pluripotent Stem Cell–Derived Cardiomyocytes

The use of pluripotent stem cell–derived cardiomyocytes (PSC-CMs), which include both hESC-CMs and iPSC-CMs, for downstream applications requires that their properties be physiologically analogous to human CMs in vivo. Assays for CM characterization, such as assessment for cross striations, ultrastructure, and chronotropic drug response, were established decades ago for primary rodent myocytes and published in a highly cited *Circulation Research* article by Simpson and Savion in 1982.[44] In 1994, Maltsev et al[45] were able to apply the same assays for extensive characterization of mESC-CMs. In addition, rigorous experimental optimization enabled them to identify internal and external solutions for patch-clamp electrophysiological analysis to confirm that CM populations comprised ventricular, atrial, and nodal subtypes and exhibited most

basic cardiac-specific ionic currents (L-type, ICa, INa, Ito, IK, IK1, IK, ATP, IK, Ach, and If). In 2003, He et al[46] were among the first to perform similar characterizations of hESC-CMs.

Disease Modeling, Drug Screening, and Cell Therapy With PSC-CMs

In vitro–derived PSC-CMs have been assessed as potential screening platforms for drug discovery and toxicology studies. Despite their immature fetal phenotype, extensive pharmacological validation confirms their potential use in drug evaluation.[47] Clinically relevant drugs (eg, adrenergic receptor blockers, calcium channel blockers) have been shown to exert chronotropic and inotropic effects on PSC-CMs. In addition, experimental drugs have been used for in vitro amelioration of diseased phenotypes in human iPSC models of cardiovascular diseases[48] and prediction of cytotoxic drug-induced side effects.[49,50] Accumulated evidence suggests that PSC-CMs can offer the pharmaceutical industry a valuable physiologically relevant tool for validation of novel drug candidates and identification of potential cardiotoxic effects in early drug development stages, thereby easing the huge associated economic and patient care burdens.[51,52]

The most successful and widely acknowledged use of PSCs-CMs has, to date, been in disease modeling. The development of disease models by genome editing of mESCs, a technology that led to award of the Nobel Prize in 2007 for Sir Martin Evans, Mario Capecchi, and Oliver Smithies (Figure 1), has offered new tools for in vivo mechanistic investigation into cardiac illnesses. The discovery of induced pluripotency technologies, which likewise led to the Nobel Prize in 2012 for Sir John Gurdon and Shinya Yamanaka, allowed the generation of patient-specific iPSC-CMs for studying human disease models of familial hypertrophic cardiomyopathy,[53] familial dilated cardiomyopathy,[54] long QT syndrome,[55] Timothy syndrome,[56] arrhythmogenic right ventricular dysplasia,[57] and others[47] (Figure 2). Beyond the potential ability of these models to reveal insights into pathological disease mechanisms, they also offer unique opportunities to explore promising new genetic therapies[58] and to identify loci or pathways related to predisposition toward cardiac disorders, thus enabling refinement of phenotype-to-genotype correlations to improve risk stratification and disease management.

The use of PSC-CMs has also expanded to in vivo applications, with transplantation shown to improve cardiac function in rat and guinea pig models of acute myocardial infarction.[59,60] Effective strategies to deplete potential tumorigenic cells,[61,62] induce immunotolerance,[63,64] and enhance cell survival[65] are being sought. Novel tissue engineering approaches to create engineered heart tissues for aiding cell delivery, survival, alignment, and functionality of transplanted PSC-CMs are being developed in parallel.[66] Notably, these technologies were pioneered by Thomas Eschenhagen's group, who published one of the first engineered heart muscle articles in *Circulation Research* in 2002.[67]

Conclusions

Extensive progress has been made in the field of cardiac stem cell biology to promote heart tissue repair by introduction of exogenous stem cells, such as MSCs, MNCs, adipose-derived stem cells, $CD34^+$ cells, c-kit^+ CSCs, and cardiosphere-derived cells, as evidenced by recent early phase clinical trials shown to reduce infarct size in patients (Table). New clinical trials are underway to validate the efficacy of these therapies. Investigation into identifying ideal patient populations, cell delivery timing,[68] cell delivery route,[69] and efficacy end points[70] will certainly be needed to optimize their full potential. At the same time, hESCs and iPSCs are progressively being used to reliably generate de novo CMs. A major hurdle, however, is their closer resemblance to fetal rather than adult CMs.[71] Combination of increasingly efficient CM generation protocols[40] and next-generation sequencing technology,[72] as well as other high-throughput screening assays, such as single-cell PCR,[73] can lead to identification of molecular markers to further enhance CM maturation. Taken together, these advances in adult and PSC biology during the past decades may herald a new area of cardiovascular regenerative and personalized medicine in upcoming years.

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J.C. Wu is a consultant for Merck and Novartis and is a cofounder of Stem Cell Theranostics.

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