Stem Cell Therapy for Hypoplastic Left Heart Syndrome

Mechanism, Clinical Application, and Future Directions

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Abstract: **Hypoplastic left heart syndrome is a type of congenital heart disease characterized by underdevelopment of the left ventricle, outflow tract, and aorta. The condition is fatal if aggressive palliative operations are not undertaken, but even after the complete 3-staged surgical palliation, there is significant morbidity because of progressive and ultimately intractable right ventricular failure. For this reason, there is interest in developing novel therapies for the management of right ventricular dysfunction in patients with hypoplastic left heart syndrome. Stem cell therapy may represent one such innovative approach. The field has identified numerous stem cell populations from different tissues (cardiac or bone marrow or umbilical cord blood), different age groups (adult versus neonate-derived), and different donors (autologous versus allogeneic), with preclinical and clinical experience demonstrating the potential utility of each cell type. Preclinical trials in small and large animal models have elucidated several mechanisms by which stem cells affect the injured myocardium. Our current understanding of stem cell activity is undergoing a shift from a paradigm based on cellular engraftment and differentiation to one recognizing a primarily paracrine effect. Recent studies have comprehensively evaluated the individual components of the stem cells' secretomes, shedding new light on the intracellular and extracellular pathways at the center of their therapeutic effects. This research has laid the groundwork for clinical application, and there are now several trials of stem cell therapies in pediatric populations that will provide important insights into the value of this therapeutic strategy in the management of hypoplastic left heart syndrome and other forms of congenital heart disease. This article reviews the many stem cell types applied to congenital heart disease, their preclinical investigation and the mechanisms by which they might affect right ventricular dysfunction in patients with hypoplastic left heart syndrome, and finally, the completed and ongoing clinical trials of stem cell therapy in patients with congenital heart disease.** (*Circ Res*. 2018;123:288-300. DOI: 10.1161/CIRCRESAHA.117.311206.)

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Advances in congenital cardiac surgery, interventional cardiology, and medical heart failure management have led to generally improved longevity among patients with congenital heart disease (CHD).[1] Nevertheless, certain patients continue to experience significant morbidity and decreased quality of life, especially those with single ventricle physiology. Among the single ventricle pathologies, hypoplastic left heart syndrome (HLHS) is one of the most complex and not only carries a high early mortality but is also associated with inevitable failure of the single right ventricle (RV) in spite of aggressive palliative interventions. Stem cell-based therapeutics have shown some preliminary promise in adult patients with ischemic and nonischemic dilated cardiomyopathy, and given the limitation of standard therapies for HLHS, it has become the most common form of CHD to be treated with investigational stem cell therapies.[2]

HLHS is the most common form of single ventricle CHD, with a prevalence of 2 to 3 per 10 000 live births,[3–5] and is defined by underdevelopment of the left ventricle (LV), which is unable to support adequate systemic perfusion. Untreated, this condition is universally fatal. The abnormal anatomy in HLHS can, however, be surgically modified to one that is life-sustaining. Surgical therapy commits the RV to the systemic circulation and allows systemic venous blood to drain passively through the pulmonary vasculature. Such a modification is palliative and is performed in 3 stages. The stage I palliative operation (Norwood) is performed in the first weeks of life and redirects the RV outflow to the aorta, with pulmonary blood flow being supplied via either a systemic arterial to pulmonary arterial shunt (modified Blalock-Taussig) or an RV to pulmonary arterial shunt (Sano). This configuration subjects the RV to both volume and pressure overload and is

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Nonstandard Abbreviations and Acronyms

aCPC	adult cardiac progenitor cell
ALLSTAR	Allogeneic Heart Stem Cells to Achieve Myocardial Regeneration
APOLLON	Cardiac Stem/Progenitor Cell Infusion in Univentricular Physiology
BNP	B-type natriuretic factor
CADUCEUS	Cardiosphere-Derived Autologous Stem Cells to Reverse Ventricular Dysfunction
CCNB	cyclin B
CDC	cardiosphere-derived cell
CDC25C	cell division cycle 25c
CHD	congenital heart disease
CPC	cardiac progenitor cell
ELPIS	Allogeneic Human MSC Injection in Patients With Hypoplastic Left Hearts
GDF-15	growth/differentiation factor 15
HIF	hypoxia-inducible factor
HLA	human leukocyte antigen
HLHS	hypoplastic left heart syndrome
HSF	heat shock factor
HSP	heat shock protein
LV	left ventricle
LVEF	left ventricular ejection fraction
MHC	major histocompatibility complex
MI	myocardial infarction
MSC	mesenchymal stem cell
nCPC	neonatal cardiac progenitor cell
PERSEUS	Cardiac Progenitor Cell Infusion to Treat Univentricular Heart Disease trial
pHH3	phospho-histone H3
PLK	polo-like kinase
POSEIDON	Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis
RIMECARD	Randomized Clinical Trial of Intravenous Infusion of Umbilical Cord MSCs on Cardiomyopathy
RV	right ventricle
RVEF	right ventricular ejection fraction
SCIPIO	Cardiac Stem Cell Infusion in Patients With Ischemic Cardiomyopathy
TGF	transforming growth factor
TICAP	Transcoronary Infusion of CPCs in Patients With Single Ventricle Physiology
TIME	Use of Autologous Stem Cells in Treating People Who Have Had a Heart Attack
UCB	umbilical cord blood
VEGF	vascular endothelial growth factor

followed by the stage II operation (bidirectional cavopulmonary anastomosis, Glenn) at ≈6 months of age. This operation removes the systemic or ventricular pulmonary blood supply and replaces it with a venous source by directly anastomosing the superior vena cava to the right pulmonary artery. In addition to reducing pulmonary vascular pressures, this configuration decreases RV volume overload by decreasing left-to-right shunting. Finally, the remainder of the systemic venous blood is directed to the pulmonary circulation after the stage III palliative operation (Fontan) at around 3 years of age. In this procedure, blood from the inferior vena cava is diverted to the right pulmonary artery, further decreasing the volume load on the RV, and completing the segregation of oxygenated and deoxygenated blood.

During and after these staged palliative operations for HLHS, the RV is subject to systemic afterload, and eventually may become dysfunctional in spite of optimal medical management, leading to clinical heart failure. There are >14 000 hospitalizations annually related to heart failure in pediatric patients—most of whom have some form of CHD—and their mortality is ≈7%.[6] Within the pediatric heart failure population, children <1 year of age account for ≈57% of hospital admissions.[7] Mortality from HLHS is also the highest during the first year of life, when heart failure can first develop and approaches 20% to 35%.[8] Although noncardiac causes of death are common, ventricular function does impact survival. Patients with HLHS with depressed RV function after the Norwood operation have an 18-month survival of 35%, compared with 70% survival in patients with normal ventricular function.[9] After the first year of life, and especially after the stage II and stage III operations, ventricular function and survival show a period of relative stability. Even so, these patients are at increased risk of arrhythmias, thromboembolism, and overall impairment of functional status. As ventricular function ultimately declines, end-organ injury is manifested by cirrhosis and protein-losing enteropathy. Unfortunately, one third of patients with HLHS die by the age of 25 years from end-stage RV failure.[10]

The number of patients with HLHS surviving into their early twenties may increase with advances in surgical and medical care, but because no cure for this condition yet exists, this will only increase the demand for innovative approaches to the treatment of inevitable RV failure. Currently, the only option for end-stage heart failure is heart transplantation, with its attendant risks of long-term immunosuppression and graft dysfunction. Moreover, patients with HLHS who have had staged palliation have poorer survival after heart transplant than other CHD types.[11] In the absence of satisfying treatments for end-stage heart failure, an opportunity for intervention may exist much earlier in the disease process, before the onset of debilitating dysfunction. It is at such a time point that stem cell-based therapies may be the most effective. In this review, we describe the many stem cell types applied in preclinical models of CHD, how they may mechanistically address the issues of RV dysfunction in patients with HLHS, and finally discuss completed and ongoing clinical trials in patients with CHD.

Stem Cell Types

Mesenchymal Stem Cells (Bone Marrow-Derived)

Mesenchymal stem cells (MSCs) are a population of pluripotent cells that are derived from bone marrow stromal cells and are capable of differentiating into mesodermal tissues, including bone, cartilage, muscle, tendon, ligament, and adipose tissue.[12] MSCs are clonogenic,[13] and their identity was initially confirmed in culture by observing specific differentiation in response to controlled environmental stimuli. Contemporary cell surface marker–based identification of MSCs relies on the expression of CD105, CD73, CD90, CD29, and CD166, with the

absence of CD45, CD34, CD14, CD11b, CD79α, CD19, and HLA-DR (human leukocyte antigen-antigen D-related).[12,14] MSCs can also be isolated from other sources, such as umbilical cord blood (UCB; discussed in the next section), adipose tissue, and peripheral blood samples.[15] MSCs are unique immunologically, because they have reduced expression of MHC (major histocompatibility complex) class I and lack MHC class II and costimulatory molecules CD80 (B7-1), CD86 (B7-2), and CD40.[12] These unique immunomodulatory properties have allowed in vivo evaluation of both autologous and allogenic MSC preparations and likely contribute to their promotion of favorable cardiac remodeling after myocardial infarction (MI) in animal models.[16,17]

Since their most comprehensive description in the 1990s, there have been >20 000 publications in the field of MSC biology and clinical application. To date, 32 clinical trials have been undertaken using MSCs to treat a variety of adult heart conditions, including acute MI, ischemic cardiomyopathy, and nonischemic dilated cardiomyopathy. The majority of the adult trials use bone marrow-derived MSCs; however, several trials have derived MSCs from other sources, including UCB. Initial clinical trials tested MSCs expanded from autologous tissue, but the 4 to 6 weeks required to culture a therapeutic dose significantly limited their general application and spurred the investigation of allogeneic MSC preparations. In a phase I, double-blind, placebo-controlled Safety Study of Adult MSCs to Treat Acute MI (NCT00114452), 53 patients underwent intravenous administration of allogeneic MSCs or placebo within 10 days after acute MI.[18] Although the study was designed to demonstrate safety, it also revealed improved functional outcomes in the allogeneic MSC-treated patients, including a reduction in malignant ventricular arrhythmias, improved pulmonary function, improved ejection fraction in the subset of patients with anterior MI, and improved quality of life at 6 months. The efficacy of allogenic MSCs was further evaluated in adults with either ischemic or nonischemic cardiomyopathy in the phase I/II POSEIDON-Pilot trial (Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis, NCT01087996) and POSEIDON-DCM trial (NCT01392625). In each of these trials, MSCs were administered transendocardially into the LV myocardium. The POSEIDON-Pilot trial (ischemic disease) demonstrated acceptably low rates of adverse events for the both the allogenic and autologous MSC groups. Although patients who received autologous MSCs demonstrated some improvement in 6-minute walk test distance and Minnesota Living With Heart Failure Questionnaire scores, neither group showed a significant change in cardiac function.[19] The later POSEIDON-DCM trial (nonischemic disease) demonstrated that allogeneic MSCs significantly increased left ventricular ejection fraction (LVEF) and 6-minute walk test distance in comparison with the autologous group,[20] though 1 patient of the 19 in the allogeneic cohort did develop donor-specific antibodies. Taken together, these early clinical trials with allogeneic MSCs have demonstrated safety and clinically meaningful efficacy.

UCB-Derived Cells

UCB is a source of both hematopoietic and nonhematopoietic precursors that were initially used in the treatment of hematologic disorders.[21] The isolation of MSCs from UCB (UCB-derived MSCs) is a more recent development, and these cells differentiate into classical MSC lineages (bone, cartilage, and fat), as well as hepatocyte-like cells, neuroglial-like cells,[22] respiratory epithelial cells,[23] and cardiomyocytes.[24] In preclinical studies, transplanted UCB-derived MSCs improve ventricular structure and function after left ventricular MI[25] and after right ventricular pressure overload,[26] with tissue-level evidence of increased angiogenesis and decreased myocardial fibrosis.[27] The only completed clinical trial using UCB-derived MSCs is the phase I/II, double-blind, controlled RIMECARD trial (Randomized Clinical Trial of Intravenous Infusion of Umbilical Cord MSCs on Cardiomyopathy, NCT01739777).[28] This trial examined the effect of intravenous administration of allogeneic UCB-derived MSCs in adults with chronic, stable heart failure with reduced ejection fraction (New York Heart Association class I–III and LVEF ≤40%). UCB-derived MSC treatment was associated with a significant increase in LVEF at 1 year compared with control (+7% versus +2%), and a decrease in heart failure symptoms as assessed by New York Heart Association class and Minnesota Living With Heart Failure Questionnaire score. Of note, 7 of the 15 cell-treated patients were evaluated for the development of antibodies against the allogeneic cells; no such antibodies were detected in any member of this subset.

Cardiosphere-Derived Cells

Cardiospheres are heterogeneous, self-assembling spherical cellular clusters that arise from myocardial tissue cultured on poly-D-lysine.[29] These cardiac-derived cells are comprised of a core of undifferentiated cells surrounded by a shell of cardiac-committed cells, defined by a phenotypic profile of $CD105^+$ >90% and $CD45^-$ <1%.[30] Cardiosphere-derived cells (CDCs) are produced by expanding cardiospheres on fibronectin-coated plastic. The myocardial tissue needed for isolation and expansion for this resident cell type is most commonly obtained by endomyocardial biopsy but may be derived from any cardiac tissue explants obtained during surgical interventions on the heart. Transplanted CDCs differentiate in vivo into cardiomyocytes, smooth muscle cells, and endothelial cells within the injured myocardium but at very low frequencies.[31] Similar to MSCs, allogeneic CDC transplantation is safe and has been shown to promote cardiac regeneration and improve cardiac function in animal models of MI.[32,33]

CDCs were first used clinically in the CADUCEUS trial (Cardiosphere-Derived Autologous Stem Cells to Reverse Ventricular Dysfunction, NCT00893360).[34] Seventeen patients with LV dysfunction after recent MI were treated by intracoronary CDC administration and then compared with 8 similar patients who did not receive cells.[34] The treated cohort was free of treatment-related arrhythmias and other safety end points and exhibited mild improvement in LVEF with significant reduction in myocardial scar formation. More recently, an allogeneic preparation of CDCs was tested in the ALLSTAR trial (Allogeneic Heart Stem Cells to Achieve Myocardial Regeneration, NCT01458405).[35] This 142-patient phase I/II, randomized, double-blind, placebo-controlled clinical trial was designed to detect a decrease in infarct size at 12 months

post-MI but was stopped after a planned 6-month interim analysis identified a low probability of demonstrating a significant difference in this end point. Looking only at the partial trial data, the treatment group reported a borderline-significant (P=0.05) reduction in mean LV end-diastolic volume, as well as a trend toward reduction of mean LV end-systolic volume.

C-kit$^+$ Cardiac Progenitor Cells

Cardiac progenitor cells (CPCs) are one of the best-studied resident cardiac stem/progenitor cell types because of their promising preclinical and early clinical performance.[36] CPCs are characterized by their expression of c-kit—a surface receptor tyrosine kinase. Unlike hematopoietic cells, however, CPCs lack expression of CD45 or Lin (general hematopoietic markers) or tryptase (mast cell-specific marker). Several studies have shown that the transplantation of CPCs is effective in attenuating LV dysfunction in animal models of both acute and chronic myocardial ischemia.[37]

In one of the largest and most systematic characterizations of cardiac stem cells in CHD patients, their natural density in the myocardium and their performance in functional assays has been clearly delineated.[38,39] In studying right atrial appendage samples from young patients undergoing surgery for various CHD diagnoses, the authors found that CPC density within the myocardium decreased with advancing age, falling from 9% in neonates to ≈ 3% in older children.[38] Of note, the presence of ongoing heart failure (New York Heart Association class III or IV) was associated with preservation of neonatal CPC density within the myocardium regardless of age, as seen ≤14 years. CPC densities in other regions of the myocardium are known to be dramatically lower than those seen in the right atrium, so these numbers likely represent an overestimate of the prevalence of this cell type.[40]

There is now evidence that donor age and environmental effects, including hypoxia, affect the functional capacity of the CPCs. After isolating and expanding human CPCs from donors of different ages, neonatal CPCs were shown to be functionally superior to adult CPCs after transplantation into a rat MI model,[41] in which they preserved LVEF at 7 (71% versus 63%) and 28 days (69% versus 60%) postinjection. At the tissue-level, there was less peri-infarct inflammation and fibrosis in the group that received neonatal CPCs.[41] These findings indicate that age directly impacts the biology of CPCs by affecting their density within the myocardium and functional efficiency in promoting recovery after myocardial injury and have been validated by other preclinical studies demonstrating that both donor age and environmental influences affect functional efficacy. One such study examined the efficacy of CPCs in recovering RV function in a rat pulmonary arterial banding model.[42] In this study, CPCs were isolated from human donors of different ages (neonates [0–1 month], infants [1 month to 1 year], and children [1–5 years]), then intramyocardially administered at the time of banding. Two weeks postoperatively, neonatal CPC-treated rats demonstrated improved RV function (tricuspid annular plane systolic excursion and right ventricular ejection fraction [RVEF]) compared with controls, and this improvement was maintained to 4 weeks. The infant- and child-derived CPCs did not demonstrate improved function at 2 weeks but did show improvement of tricuspid annular plane systolic excursion, but not RVEF, at 4 weeks compared with controls. Another study demonstrated that along with donor age, lower environmental oxygen levels significantly improved the efficacy of pediatric human CPC-derived exosomes when tested in a different rodent model of MI.[43] With such promising preclinical data, neonatal and hypoxia-conditioned CPCs may be the most regenerative CPC types, with potential for functional recovery surpassing adult-derived CPCs.

CPCs were introduced clinically in the phase I, randomized, open-label SCIPIO trial (Cardiac Stem Cell Infusion in Patients With Ischemic Cardiomyopathy, NCT00474461), in which CPCs were isolated from right atrial tissue collected from adult patients with ischemic cardiomyopathy (LVEF, ≤40%) undergoing coronary artery bypass grafting. After sufficient expansion, the autologous CPCs were infused into the vessel(s)/graft(s) supplying areas of infarcted myocardium.[44] LVEF improved over the course of the year after cell administration (28% to 41%), and the infarct size decreased by an average of ≈40%—the largest clinical improvements ever reported for cell-based therapies. Although the SCIPIO trial was not designed to demonstrate efficacy, these results provide the rationale for larger trials aimed at confirming the functional effect of the transplanted CPCs.

Even in the face of these promising preclinical and clinical results, skepticism remains regarding the utility of CPCs as therapeutic agents. This arises in large part from disagreements about their differentiation potential in rodent models. Using powerful mouse genetics, there is now strong evidence that CPCs have low or no capacity to differentiate into mature cardiomyocytes.[45,46] Although it is important to acknowledge the differences between mouse and human cardiomyocytes, and the imperfect correlation of mouse findings with their human equivalents, such studies call into question the ability of CPCs to engraft and differentiate into new myocytes. The apparent discordance of the CPCs' genetic potential and functional capacity may, however, be evidence that the secretome is actually the functional unit of the CPC.[41,44] The importance of paracrine activity is fundamental to the understanding of CPC-based cell therapy and is discussed in subsequent sections of this review. With regard to CPCs, carefully designed, adequately powered clinical trials will better resolve their clinical efficacy, and continued basic research will better identify the cellular mechanisms underlying the clinical phenomena seen in the SCIPIO trial triggered by the CPCs.

Differences Between the RV and LV

The distinct characteristics of the RV and LV arise from the physiological requirements of each chamber, its gene expression, and its embryological origins. In the normal adult mammalian heart, there is a complete muscular septum dividing the RV from the LV. In conjunction with the interatrial septum, this leads to essentially separate pulmonary and systemic circulatory systems, and each ventricle is morphologically and functionally coupled to the demands of its particular outflow vasculature. The LV is required to pump across the relatively high resistance systemic vascular bed, which is facilitated by thick walls and a conical shape with the inlet and outlet on the same side. This LV configuration is robust in the setting of high wall stress[47] and is supported by blood flow

from all 3 coronary arteries. The RV, however, is required to pump across the relatively low resistance of the pulmonary vascular bed. This low resistance flow across the pulmonary vasculature is aided by the LV, when present, during diastole. During diastolic relaxation, the LV creates a negative pressure across an open mitral valve, which is enhanced with lower LV end-diastolic volume. This myocardial suction is transmitted across the pulmonary vascular bed, further decreasing RV afterload.[48] The RV's crescent shape with thin walls and notable separation of the inlet and outlet is able to support low-pressure flow and is perfused primarily by the right coronary artery, with some septal contribution from the left anterior descending coronary artery. The RV is adept at accommodating wide variations in inflow due, for example, to respiration or position changes, but it is relatively intolerant to a significant elevation of outflow resistance.[49] This becomes particularly problematic when the RV is permanently committed to systemic outflow resistance after surgical palliation for single ventricle physiology. HLHS patients, inherently lacking a functioning LV, have an RV that is even less equipped to deal with the strain of systemic flow because it no longer has systolic support of strong LV-dominated interventricular contraction.[50]

Such stress elicits cardiomyocyte hypertrophy and angiogenesis, which though initially beneficial, eventually wane or become maladaptive, and ventricular fibrosis and contractile dysfunction ensue.[10] Maladaptive responses are atypical in cases of systemic RV repairs with an intact LV as seen in patients with transposition of the great arteries after surgical atrial baffling. Moreover, the maladapted RV in CHD is uniquely hypertrophied, and in addition to being susceptible to systolic dysfunction, is particularly prone to diastolic dysfunction marked by fibrosis and poor compliance. The subepicardial and subendocardial layers of both ventricles are continuous. However, the LV has a thick concentric middle layer that the RV lacks. This was first seen in patients with repaired tetralogy of Fallot and is believed a contributing factor to the especially poor compliance of the hypertrophied RV.[51]

Although a component of the RV's pressure intolerance is certainly related to its general structure, there may be interchamber differences in gene expression that further impair its adaptation. This difference in gene expression begins with the different origins of the RV and LV. The developing heart at mouse embryonic day (E)6.5 begins to develop with the anterior migration of nascent mesodermal cells. By E7.5, these cells have differentiated and are split into 2 heart fields. The first heart field, identified by Nkx2-5$^+$HCN4$^+$ markers, gives rise to the LV and parts of both atria. The second heart field, identified by Nkx2-5$^+$Isl1$^+$ markers, gives rise to the RV, the outflow tracts, and parts of both atria.

The exact mechanism by which differentiation of first heart field and second heart field progenitor cells occurs is still an object of active investigation.[52] What is known is that the development of specific cardiac tissues is based on the molecular milieu bathing these primordial cells, as well as their anatomic location. In a mouse study, tissue from the migrating mesodermal tissue at E6.5 was transplanted to another location, and the transplanted tissue took on the characteristics of the tissue into which it was engrafted. However, when transplanted to a noncardiac location, cells taken from the heart fields at E7.5 became cardiac tissue.[53] This commitment to cardiac lineage when cells are within the heart fields suggests that there are molecular cues from the surrounding heart field tissue, perhaps the endoderm. This creates a more complex model for cardiac lineage development that combines temporal dependence, molecular sensitivity, and subsequent gene expression. Unsurprisingly, specific genetic mutations (such as those often found in CHD) change the path of cardiac development with resultant anatomic abnormalities.[54]

These fundamental differences in ventricular origins and gene expression patterns have been further validated through several animal and human studies. The Tbx (T-box) family of genes have well-defined roles in embryonic patterning, and Tbx5 has been shown to be an important differentiator of RV and LV development in chicks. Normally present in the precardiac mesoderm, Tbx5-expressing tissue eventually develops into the LV, with the interventricular septum forming the boundary between Tbx5-expressing and nonexpressing myocardium. Ubiquitous misexpression leads to a single LV with no septum, whereas attenuation of the Tbx5-positive region leads to development of a small LV.[55] A right ventricular counterpart has been identified in Tbx20, which is completely repressed by Tbx5 expression. Knockout of Tbx5 in mice is associated with arrest of cardiac development at E9.5 and is lethal by E10.5.[56] Haploinsufficiency leads to forelimb and cardiac abnormalities (atrial septal defects, ventricular septal defects, and other complex abnormalities) that are similar to those observed in Holt-Oram syndrome, an autosomal dominant condition caused by a heterozygous mutation in the *TBX5* gene on chromosome 12.[57] Nevertheless, haploinsufficiency is not sufficient in this particular condition to totally abolish development of 2 distinct ventricles. Other transcription factors, such as Hand1 and Hand2, have been implicated in chamber differentiation in rodent models.[58] Deletion of Hand2 results in the absence of RV regions of the heart, whereas elimination of Hand1 (or its regulator, Nkx2.5) results in the absence of LV tissue.

Notch1 expression has also been shown to be important in cardiogenesis, though most of its influence is likely exerted indirectly via control of ventricular inflow and outflow tract development.[59] Notch1 knockout in mice is lethal during embryogenesis and is characterized by impaired valve patterning and development.[60] Notch1 heterozygous loss-of-function mutations in mice are associated with bicuspid aortic valve and accelerated aortic valve calcification,[61] with clinically significant aortic valve disease present in mice with simultaneous Notch1 heterozygosity and knockout of Nos3.[62] Furthermore, Notch1has been shown to promote the transcription of Nkx2.5 in CPCs, in turn promoting their differentiation into cardiomyocytes during development.[63]

The importance of these pathways during the abnormal cardiogenesis characteristic of HLHS has been most successfully elucidated using induced pluripotent stem cells derived from HLHS patients. Prior cohort genetic analyses have identified certain chromosomal abnormalities of interest[64] but are limited by the rarity of the disease and the multiple genetic variants that may contribute to its development.[65] By generating and analyzing induced pluripotent

stem cells from the right atrium-derived CPCs of HLHS patients, Kobayashi et al[66] demonstrated reduced levels of TBX2, NKX2.5, NOTCH1, and HAND in these cells with associated with impaired SRE (serum response element) and TNNT2 (cardiac troponin-T) transcription. Transfection of the HLHS-induced pluripotent stem cells with NKX2.5, HAND1, and NOTCH1 restored normal promoter activation. Although treatment for established HLHS may continue to demand that we address not only cardiomyocyte function but also existing anatomic malformations, induced pluripotent stem cell-based study of the disease may lead to a better understanding of its underlying cellular mechanisms that may become informative in the development of novel stem cell-based therapeutics.

After embryonic development, chamber-specific maturation continues in the perinatal period. The Wnt signaling pathways have been demonstrated to be fundamental to proper RV and LV differentiation during this period of major hemodynamic changes.[67,68] A chamber-specific transcriptome analysis in neonatal mice at postnatal days 0, 3, and 7 better defined the mechanisms by which Wnt signaling drives chamber differentiation.[69] Although many expression patterns were shared (eg, the switch from carbohydrate to fatty acid metabolism), some differed in magnitude and kinetics, particularly at postnatal day 3. Genes involved in the polo-like kinase mitotic pathway (*plk1* [polo-like kinase], *ccnb1* [cyclin B1], *ccnb2*, and *cdc25c*) were abruptly suppressed in the RV at day 3, but their suppression in the LV was much more gradual. A similar pattern was observed with cell cycle regulators, with corresponding increases in the levels of pHH3 (phospho-histone H3), Ki67, and aurora kinase B in LV myocytes compared with RV myocytes at the same time point. In their analyses of human tissue from patients with cyanotic congenital heart conditions (specifically, tetralogy of Fallot and double outlet RV), Wnt11 activity was suppressed, and cell cycle activation was enhanced. These phenomena were recapitulated by exposing neonatal mice to hypoxic conditions, and the authors demonstrated the induction of cell proliferation was stronger in the RV than the LV, providing a clinically relevant example of differential response to stress at the cellular level.

Potential Mechanisms of RV Dysfunction and Stem Cell Therapy in HLHS

Although the majority of preclinical and clinical investigations addresses LV dysfunction after MI, it is the RV that becomes dysfunctional in HLHS. This dysfunction arises from chronic pressure overload, with a diminishing component of volume overload as the sequence of single ventricle palliative operations and the eventual requirement of the RV to pump against systemic pressures. These abnormal conditions activate a cascade of compensatory pathways, beginning with increased cardiomyocyte hypertrophy, increased angiogenesis, increased production of antioxidative enzymes, and a reversion to fetal gene expression.[70,71] The angiogenic response to pressure overload is reduced in the RVs of HLHS patients,[72] limiting the delivery of oxygen and nutrients to cardiomyocytes, thus resulting in myocardial dysfunction.[73] Other evidence from pulmonary hypertension models has suggested that RV dysfunction develops because of increased myocardial fibrosis, diminished antioxidant response, cardiomyocyte apoptosis, and a shift from oxidative mitochondrial metabolism to less energy efficient glycolytic metabolism.[74–76] Established medical treatment for the LV does not directly address the issues for the RV, and thus more innovative treatment options using stem cell-based therapies are needed.

Some preclinical studies have tested stem cell-based therapies in more relevant CHD animal models that induce pressure or volume overload to create RV dysfunction. Human MSCs and c-kit$^+$ CPCs were evaluated in a juvenile porcine model of pressure-induced RV failure achieved by banding the main pulmonary artery.[77,78] One million cells were injected intramyocardially into the RV free wall. Both transplanted cell types decreased RV dilation and preserved RV function relative to controls as measured by RV fractional area change and RV strain. Histological analysis revealed reduced myocardial fibrosis, increased angiogenesis, and increased cardiomyocyte and endothelial cell proliferation with cell treatment. The mechanism for this myocardial remodeling with MSC treatment was associated with activation of GDF-15 (growth differentiation factor 15)—a secreted member of the TGF (transforming growth factor)-β superfamily that antagonizes the hypertrophic response to pressure overload. In a similar neonatal ovine pulmonary artery banding model, human UCB-derived mononuclear cells improved end-systolic elastance and preload-recruitable stroke work compared with banded controls.[79] In a model of volume overload-induced RV dysfunction generated by surgical creation of pulmonary insufficiency, transplanted autologous UCB-derived mononuclear cells improved RV diastolic function and increased angiogenesis relative to controls.[26] These early results highlight the potent efficacy of different transplanted stem cell types in models of RV dysfunction relevant to single RV CHD.

Although the use of stem cells has been shown in early preclinical research to be effective in regeneration, remodeling, and renewing of injured myocardium, the engraftment/differentiation of exogenous stem cells is exceptionally low, and the magnitude of functional improvement is disproportionate to the number of exogenous cell-derived cardiomyocytes observed after transplantation.[80] Rather than engraftment and differentiation, there is growing evidence that the primary therapeutic benefit of stem cells is attributable to secreted products that promote neovascularization, favorable remodeling, and activation of endogenous stem cells and cardiomyocytes, ultimately leading to improvement in overall function.[15]

In a rodent MI model, the potency of the secretome was shown to be directly related to the age of the stem cell donor.[39,41] CPCs were isolated from neonates (<1 month of age, neonatal CPCs [nCPCS]) and adults (>40 years of age, adult CPCs [aCPCs]), and although the 2 CPC types were similar in their cell surface markers, nCPCs were highly clonogenic, maintained their telomere length, and improved cardiac function after transplantation significantly more than aCPCs. This myocardial recovery occurred despite negligible retention of either cell type, identified by highly sensitive methods, such as reverse transcription-polymerase chain reaction, thus

strengthening the hypothesis that CPCs mediate endogenous recovery through a paracrine mechanism. Administration of the secretomes derived from nCPCs or aCPCs was found to be at least as effective as live cell transplantation in recovering the injured myocardium. For nCPC-derived secretome, beneficial effects persisted for the 28-day duration of the study.[41]

In-depth proteomics, coupled with literature-based software analysis, revealed that this in vivo efficacy is associated with several cardioprotective pathways that are exclusively modulated by the secretome of nCPCs, including the heat shock pathway via differential expression of HSF1 (heat shock factor 1).[41] This was confirmed in vitro by knocking down HSF1 in nCPCs, and overexpressing HSF1 in aCPCs. Quantitative polymerase chain reaction analysis showed that HSF1 knockdown in nCPCs reduced the expression of HIF (hypoxia-inducible factor)-1α, VEGF (vascular endothelial growth factor), HSF2, HSP (heat shock protein)90AB, HSP70, HSPD1 by >50%, whereas HSF1-overexpressing aCPCs showed a 2- to 3-fold increase in the levels of these factors. Although nCPCs are typically more robust and demonstrate more potent therapeutic effects than their adult-derived counterparts, this molecular manipulation essentially reversed their phenotype. Modified nCPCs became less resistant to oxidative stress and exhibited decreased metabolic activity and impaired growth, whereas the opposite effect was observed in the modified aCPCs. The same pattern was demonstrated in the total conditioned media of the modified cells, with increased angiogenic potential and increased exosome production in HSF1-overexpressing aCPC total conditioned media compared with HSF1-deficient nCPC total conditioned media. These experiments underscore how the cellular environment directly and strongly affects the stem cell secretome, thereby modulating its effects on myocardial function.

Clinical Trials of Stem Cell Therapy in Children

In comparison with the many completed and ongoing adult trials, there are relatively few emerging clinical trials for CHD patients, mostly targeting patients with single ventricle physiology. These trials are summarized in the Table, and implement different stem cell types, target different patient populations, and vary in administration routes for delivery of the stem cells but with a common goal of improving RV function. One of the first such endeavors was the TICAP trial (Transcoronary Infusion of CPCs in Patients With Single Ventricle Physiology, NCT01273857) a phase I nonrandomized, controlled trial aimed at confirming the feasibility of intracoronary delivery of CDCs in postpalliation single ventricle physiology patients.[81] In this study, autologous CDCs were isolated, expanded, and administered via intracoronary delivery 4 to 5 weeks after the stage II palliative surgery or the stage III palliative surgery. Cardiac function was measured by echocardiography at 24, 30, and 36 weeks, and efficacy assessment with cardiac magnetic resonance imaging at 36 weeks. In addition, hemodynamic parameters were measured by cardiac catheterization at 30 to 36 weeks. No adverse events in the form of procedural complications, life-threatening arrhythmia, myocardial necrosis, or sudden death were reported in the 7-patient CDC-treated cohort. At 18 months of follow-up, the CDC-treated patients demonstrated an improvement in RVEF from an average baseline value of 46.9±4.6% to 54.0±2.8% and a significant reduction in tricuspid valve annulus diameter, whereas control patients showed little improvement in RVEF (46.7±4.4% to 48.7±6.7%) and no change in diameter of the tricuspid valve annulus, despite volume reduction after the stage II and III palliative operations. CDC-treated patients also showed significant reductions in RV free wall mass and indexed end-systolic and end-diastolic volumes at 18 months. Interestingly, the somatic growth of CDC-treated patients significantly improved from baseline to 18 months, as indicated by an increase in *z* scores for height and weight, whereas there was no change in the control group.

The TICAP trial was followed by the PERSEUS trial (Cardiac Progenitor Cell Infusion to Treat Univentricular Heart Disease, NCT01829750), a phase II randomized controlled trial designed to follow-up on the TICAP trial. Again, CDCs were administered via the coronary arteries after either the stage II or III palliative operations.[82] The primary end point was change in ejection fraction from baseline to 3 months and was measured by cardiac magnetic resonance imaging, echocardiography, and ventriculogram. The CDC-treated group showed greater improvement in RV function at 3 months than controls (+6.4% versus +1.3%; P=0.003), and at 1 year continued to exhibit augmented RV function and quality of life. Follow-up analysis at 36 months demonstrated an improvement in RVEF among patients receiving CDCs (+8.0% versus +2.2%; P=0.03) even at this late time point.[83] Stem cell therapy was also associated with decreased BNP (B-type natriuretic factor) levels and higher age to weight z score. This trial further reinforced that the clinical benefits imparted by CDCs are not only consistent, but the delivery of stem cells beyond the stage II palliative operation is safe and feasible.

Beyond these 2 reported studies, several additional studies evaluating stem cell therapy in single ventricle patients are underway (Table). The APOLLON (Cardiac Stem/Progenitor Cell Infusion in Univentricular Physiology, NCT02781922) phase III trial is a continuation of the TICAP and PERSEUS trials using autologous CDCs delivered via intracoronary injection in single ventricle patients. Other trials using autologous stem cell preparations include UCB-derived mononuclear cells intervening at the time of stage II palliative operation in HLHS patients and bone marrow–derived mononuclear intervening after stage III palliative operation in single ventricle patients. Up to this point, clinical trials involving CHD patients focused on the transplantation of autologous cell preparations, but the time, expense, and tissue requirement behind these autologous therapeutics limits their application. The ELPIS (Allogeneic Human MSC Injection in Patients With Hypoplastic Left Hearts, NCT02398604), phase I/II trial is an ongoing clinical trial that investigates the safety and feasibility of intramyocardially injected allogeneic MSCs as opposed to an autologous preparation at the time of the stage II operation in HLHS patients (Figure).[84] This trial may lay the groundwork for an off-the-shelf, MSC-based product for CHD patients

Table. Clinical Trials of Stem Cell Therapy for Single Ventricle Congenital Heart Disease

Trial	Year	Design	Sponsor and Collaborators	Cell Type	Route	Timing	Status
TICAP	2011	Nonrandomized, controlled Phase I	Okayama University National Cerebral and Cardiovascular Center, Japan	CPC (autologous)	IC	Stage II or III operation	Reported
PERSEUS	2013	Randomized, controlled Phase II	Okayama University Translational Research Informatics Center, Kobe	CPC (autologous)	IC	Stage II or III operation	Reported
Safety Study of Autologous Umbilical Cord Blood Cells for Treatment of Hypoplastic Left Heart Syndrome	2013	Single treatment group Phase I	Mayo Clinic University of Oklahoma	UCB-derived MN cells (autologous)	IM	Stage II operation	Ongoing
ELPIS	2015	Randomized, controlled Phase I/II	University of Miami University of Maryland	MSC (allogeneic)	IM	Stage II operation	Ongoing
Phase I Safety and Feasibility Study of Intracoronary Delivery of Autologous BM-Derived MN Cells	2015	Single treatment group Phase I	Mayo Clinic	BM-derived MN cells (autologous)	IC	After stage III operation (with ventricular dysfunction)	Ongoing
APOLLON	2016	Randomized, controlled Phase III	Japanese Regenerative Medicine, Co, Ltd	JRM-001 (cardiac stem cells)*	IC	Stage II or III operation	Ongoing
Mesoblast Stem Cell Therapy for Patients With Single Ventricle and Borderline Left Ventricle	2017	Randomized, controlled Phase I/II	Boston Children's Hospital	MPC (allogeneic)	IM	Stage II or LV recruitment operation	Not yet enrolling

All data were obtained from http://clinicaltrials.gov and verified on September 12, 2017. Stages refer to palliative procedures for single ventricle CHD. APOLLON indicates Cardiac Stem/Progenitor Cell Infusion in Univentricular Physiology; BM, bone marrow; CHD, congenital heart disease; CPC, cardiac progenitor cell; ELPIS, Allogeneic hMSC Injection in Patients With Hypoplastic Left Heart Syndrome; IC, intracoronary; IM, intramyocardial; JRM, Japanese regenerative medicine; MPC, mesenchymal precursor cell; MN, mononuclear; MSC, mesenchymal stem cell; NIH, National Institutes of Health; PERSEUS, Cardiac Progenitor Cell Infusion to Treat Univentricular Heart Disease; TICAP, Transcoronary Infusion of CPCs in Patients With Single Ventricle Physiology; and UCB, umbilical cord blood.

*A proprietary agent, described as such in the NIH listing. Okayama University is a participating site under the direction of Dr Hidemasa Oh.

Exploring Clinical Application

Timing of Intervention

When describing the clinical efficacy of stem cell therapy, there is an important distinction to be made as to whether the transplanted cells' effects are either protective or regenerative. Certain myocardial effects, such as the modulation of the inflammatory responses postinfarction[85] or maintenance of cardiac function, despite prolonged exposure of pressure overload,[77] are indeed protective in that the cell-based therapy modifies the immune and inflammatory processes to prevent ventricular functional decline. As mentioned previously, preclinical studies have shown that the activation of GDF-15 in cardiomyocytes exposed to transplanted MSCs may be one such protective mechanism. Transplanted stem cells also regenerate the myocardium by stimulating cardiomyocyte proliferation, endogenous stem cell proliferation,[86] and neovascularization,[87] which results in lasting changes to myocardial structure and function.

Whether protective, regenerative, or a combination of both, it is presumed that early intervention in single ventricle CHD patients will halt ventricular deterioration and may promote myocardial healing, with supporting evidence from preclinical and clinical studies. In a rat model, direct intramyocardial administration of 2 million allogeneic MSCs immediately after MI limited the postinfarction reduction in LVEF to 8%, whereas LVEF decreased by 18% if the same dose was administered 1 week after MI.[88] At the myocardial level, this may be explained by time-dependent changes in cellular activity and consequent differences in the levels of stem cell activation. This has been shown to be true for endogenous human CPCs, which proliferate immediately after MI but exhibit less activity with advancing time.[89] In the TIME (Use of Autologous Stem Cells in Treating People Who Have Had a Heart Attack, NCT00684021) randomized, controlled, double-blind clinical trial, adult patients who underwent successful percutaneous coronary intervention for ST-segment–elevation myocardial infarction received autologous bone marrow–derived mononuclear cells by the intracoronary route either 3 or 7 days after intervention. Unfortunately, there was no difference demonstrated between treatment and the placebo groups at 6-month follow-up.[90] The TIME trial was followed by The LateTIME trial (Use of Autologous Stem Cells in Treating People 2 to 3 Weeks After Having a Heart Attack, NCT00684060), in which cell administration was delayed cell until 2 to 3 weeks postintervention. Again, no treatment effect was demonstrated.[91]

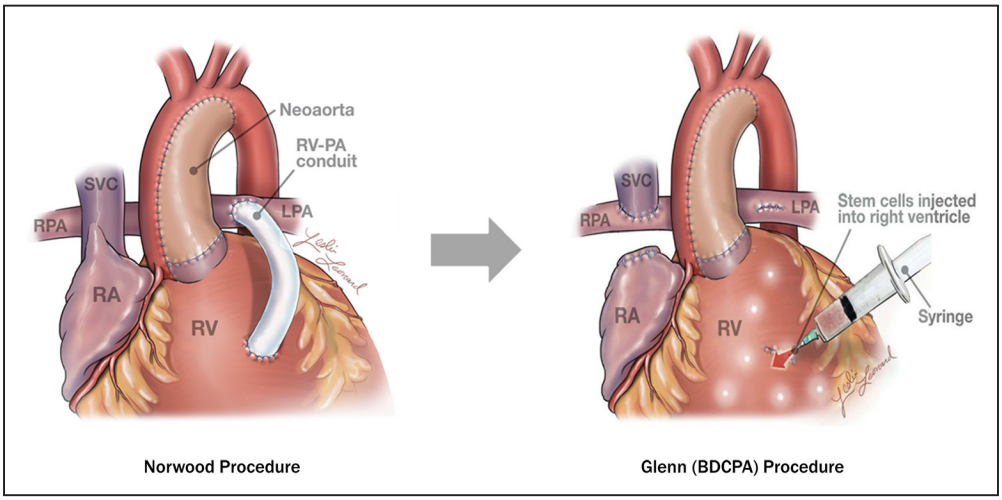

Figure. Illustrations of cardiac anatomy following the Norwood (stage I) operation and Glenn (bidirectional cavopulmonary anastomosis [BDCPA], stage II) operation for hypoplastic left heart syndrome. Currently, clinical trials of stem cell therapy for single ventricle congenital heart disease have offered treatment after the stage II or stage III (Fontan, not shown) operations. The ELPIS trial (Allogeneic Human Mesenchymal Stem Cell Injection in Patients With Hypoplastic Left Hearts) of mesenchymal stem cell therapy offers treatment at the time of the stage II operation and involves intramyocardial cell administration. LPA indicates left pulmonary artery; PA, pulmonary artery; RA, right atrium; RPA, right pulmonary artery; RV, right ventricle; and SVC, superior vena cava. Reprinted from Wehman et al[102] with permission. Copyright ©2015, Cambridge University Press.

However, both studies were performed with bone marrow–derived stem cells, not other stem cell types, and so it is possible that fundamental differences in biology of the injected cells limited the final outcome of the trials.

In patients with single ventricle CHD, the TICAP and PERSEUS trials showed that younger age at treatment was associated with greater improvement in ventricular ejection fraction (10%–15% at age 1, versus ≈5% at age 3).[83] Both of these trials, however, provided stem cell treatment at the time of the stage II or III palliative operations, and it is possible that additional benefits may have been realized if cell therapy was provided earlier. From the perspective of clinical trial design, 1 potential disadvantage of treatment between stage I and stage II is the relatively high morbidity and mortality in this time period. Indeed, the majority of mortality from single ventricle CHD patients occurs in the interval between the stage I and stage II operations, and although cardiovascular etiologies are most common, a significant minority of deaths is from noncardiac causes.[92] It might, therefore, be more difficult to tease out the safety and efficacy of novel cell-based treatments if administered at an earlier time point, though this will be an important age group to address after stem cell therapy becomes better established.

Children: The Best Candidates for Stem Cell Therapy?

The benefits of stem cell therapy for adults with ischemic heart disease have been modest and inconsistent.[93] Children, however, may be more receptive to the biological cues offered by the various stem cell types, and their myocardium may respond more favorably to stem cell therapy. Such unique developmental properties of the pediatric myocardium have been demonstrated in several studies. Using carbon-14 dating, cardiomyocyte turnover has been shown to be at a postnatal maximum of ≈1% per year just after birth, with a decline to a sustained level of 0.45% later in childhood.[94] This confinement of significant myocyte proliferation to childhood has been supported by histone phosphorylation analysis, from which it is estimated that cardiomyocyte cell cycle activity falls to low levels after 20 years of age.[95] The density of CPCs in the myocardium also decreases with age as discussed previously.[38] These findings suggest that the myocardium is most plastic in the early years of life—a notion that has been reinforced by relatively impressive improvements after clinical transplantation of MSCs, UCB-derived MSCs, and CDCs in pediatric populations,[96,97] with greater benefit observed in younger patients.[83]

Dosing and Method of Administration

The extension of stem cell therapy to the pediatric population uncovers a set of issues related to dosing and route of administration that are particular to the children. One approach for intracoronary administration of stem cells is the stop-flow technique. Commonly used in adults, this technique relies on an angioplasty balloon to occlude the targeted coronary artery while cells are infused distal to the balloon. Occlusion may be maintained ≤2 minutes before reperfusion. Both the TICAP and PERSEUS trials utilized this technique, using amiodarone loading to reduce the risk of significant arrhythmia. Intracoronary delivery was extremely safe in these trials, despite the challenges and risks associated with engaging the coronary ostia in young patients. The only adverse event reported was the transient periprocedural troponin elevation with no clinical evidence of MI. Another important consideration when performing intracoronary stem cell administration in children is the size of the injected cells, which, if too large, can occlude the myocardial capillary beds themselves or form occlusive cell aggregates. The TICAP and PERSEUS demonstrated the safety of CDC injection using this technique, but other stem cells may warrant a different route of administration because of their larger dimensions. MSCs, for example, typically range in size from 20 to 50

μm,[98] and microvascular occlusion after MSC administration has been demonstrated both hemodynamically and histologically using the stop-flow technique in animal models.[99,100] Direct intramyocardial injection is an alternative delivery technique and is particularly convenient for CHD patients when performed in the context of another open cardiac surgical procedure. With this method, the total cell dose is divided into multiple small aliquots that are injected directly into the free wall of the RV. Preclinical studies have validated this methodology, and it is now being practiced in 2 enrolling HLHS clinical trials (Table). Intramyocardial stem cell administration adds negligible risk to the single ventricle palliative operations and eliminates the small but real risks of selective coronary catheterization and intracoronary cell delivery. Should repeated cell delivery be demonstrated to be beneficial, it is likely that a combination of intracoronary and intramyocardial delivery will be used.

Novel Stem Cell-Based Therapeutics

Because there is an absence of significant engraftment or retention by transplanted stem cells, the secretome is now considered the functional unit of these cells, and its various components have been successfully used to recover the injured myocardium. To further reinforce this paradigm, we have reported that injecting a single dose of the total conditioned media from neonatal CPCs into a rodent MI model outperformed the transplantation of live neonatal CPCs with respect to functional recovery.[41] The exosomal fraction of the total conditioned media was then purified and injected into the same model, resulting in functional recovery comparable with that achieved using live cells. It may, therefore, be possible to design future stem cell-based therapeutic agents around these secreted products rather than the cells themselves. As the understanding of the molecular mechanisms underlying the benefits of stem cell therapy becomes more sophisticated, it may also be possible to modify cells to optimize their elaboration of the most critical secreted factors.[80,101] Novel, customized, secretome-derived agents would likely be superior to live cells from immunologic and logistic standpoints, and pending functional evaluation may represent the next generation of stem cell therapeutics.

Conclusions

There is accumulating evidence to support the therapeutic potential of stem cell administration in children with CHD, who, indeed, may be the best responders to this type of therapy. Preclinical studies have verified the benefits of various stem cell types in animal models relevant to CHD and have identified the secretome as the primary means by which these cells exert their favorable effects. Although a small but growing number of clinical trials in CHD patients has shown stem cell-dependent improvements in ventricular function, further investigation will define the ideal timing, dosing, and stem cell type or types for the treatment of CHD. As the field continues to evolve, we expect that stem cell-based therapeutics will offer otherwise unattainable myocardial protection and regeneration in the setting of previously untreatable congenital cardiac conditions, offering hope for better longevity and quality of life to patients with CHD.

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Disclosures

None.

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