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Review

The current status and future of cardiac stem/progenitor cell therapy for congenital heart defects from diabetic pregnancy

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Pregestational maternal diabetes induces congenital heart defects (CHDs). Cardiac dysfunction after palliative surgical procedures contributes to the high mortality of CHD patients. Autologous or allogeneic stem cell therapies are effective for improving cardiac function in animal models and clinical trials. c-kit$^+$ cardiac progenitor cells (CPCs), the most recognized CPCs, have the following basic properties of stem cells: self-renewal, multicellular clone formation, and differentiation into multiple cardiac lineages. However, there is ongoing debate regarding whether c-kit$^+$ CPCs can give rise to sufficient cardiomyocytes. A new hypothesis to address the beneficial effect of c-kit$^+$ CPCs is that these cells stimulate endogenous cardiac cells through a paracrine function in producing a robust secretome and exosomes. The values of other cardiac CPCs, including Sca1$^+$ CPCs and cardiosphere-derived cells, are beginning to be revealed. These cells may be better choices than c-kit$^+$ CPCs for generating cardiomyocytes. Adult mesenchymal stem cells are considered immune-incompetent and effective for improving cardiac function. Autologous CPC therapy may be limited by the observation that maternal diabetes adversely affects the biological function of embryonic stem cells and CPCs. Future studies should focus on determining the mechanistic action of these cells, identifying new CPC markers, selecting highly effective CPCs, and engineering cell-free products.

MATERNAL DIABETES-INDUCED CONGENITAL HEART DEFECTS

Congenital heart defect (CHD) is the most common birth anomaly, accounting for 28% of all major birth defects (1). Pregestational maternal diabetes is well known to be a risk factor for birth defects affecting both fetal and neonatal outcomes, including CHD and central nervous system defects (2–19). The risk of CHD is five-fold higher in the infants of diabetic mothers than in those of non-diabetic mothers with tetralogy of Fallot, dextrotransposition of the great arteries, ventricular septal defect, total anomalous pulmonary venous return, aortic stenosis, left ventricular outflow tract obstruction associations, right ventricular outflow tract obstruction associations, perimembranous ventricular septal defect, atrial septal defect secundum, atrial septal defect not otherwise specified, and ventricular septal defect with atrial septal defect (20,21).

Recent analysis of age-specific mortality caused by CHD has revealed that the rate is highest during the first year of life (22). However, the CHD-related mortality among adults aged >65 years has increased significantly since 1985 (22). Although survival after bypass surgery in CHD has dramatically improved because of surgical advances, an increase in the CHD prevalence in children and adults because of the expanding population and long-term survivors contributes to the high mortality of CHD patients after palliative surgical procedures (23,24). As a result, the increased incidence of CHD requires repeated hospitalization due to the significant morbidity and increased risk of mortality (25).

MECHANISMS OF MATERNAL DIABETES-INDUCED CHD

The pathophysiology of maternal diabetes-induced CHD is complex and not entirely understood; however, animal studies from our laboratory have shown that it is associated with decreased cell proliferation and increased cell apoptosis from high oxidative stress (13). Maternal type 1 diabetes increases the production of reactive oxygen species that oxidize Trx and dissociate Trx from apoptosis signal-regulating kinase 1 (ASK1), activating ASK1 by autophosphorylation. Next, activated-ASK1 induces the proapoptotic endoplasmic reticulum stress–JNK1/2 pathway, which may contribute to cardiac cell apoptosis. Meanwhile, ASK1 represses cyclin D expression and bone morphogenetic protein 4 signaling as well as upregulates cell cycle inhibitors p21 and p27 to suppress heart cell proliferation. In addition, ASK1 impedes the cardiogenesis pathway, which is possibly through modulation of hypoxia-inducible factor-1α activities (10). Maternal type 1 diabetes-induced oxidative stress also impairs Wnt signaling in the developing heart. Briefly, oxidative stress induced by diabetes inhibits the canonical Wnt signaling pathway through increasing its antagonist expression and the activity of its negative regulator as well as the noncanonical Wnt signaling pathway via downregulating Wnt5a (9).

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Received 20 June 2017; accepted 3 October 2017; advance online publication 15 November 2017. doi:10.1038/pr.2017.259

Similar to the observations in type 1 diabetic embryopathy, maternal type 2 diabetes causes heart defects in the developing embryo that are manifested as oxidative stress, endoplasmic reticulum stress, and excessive apoptosis in cardiac cells (11).

In addition, our recent research has indicated that high glucose suppresses embryonic stem cell cardiogenesis (26). When glucose-responsive E14 mouse embryonic stem cells (GR-E14 cells) are cultured under high glucose conditions, high glucose prevents the differentiation of GR-E14 cells into contracting cardiomyocytes. Further study has revealed that high glucose represses the expression of essential genes for cardiogenesis, inhibiting the maturation of differentiated cardiomyocytes from GR-E14 cells and reducing potassium channel proteins that are important for cardiomyocyte contraction (26). Similarly, a recent study revealed that pregestational maternal diabetes suppresses the differentiation of murine embryonic D3 stem cells into cardiomyocytes (27). Another recent study from our laboratory has demonstrated that both maternal diabetes *in vivo* and high glucose *in vitro* affect cardiac progenitor cells (CPCs) (28). $Sca1^+$ progenitor is a cardiac cell population expressing marker stem cell antigen-1 (Sca1), and it has significant cardiac regeneration ability to differentiate into cardiomyocytes and other cells in the heart (28). Maternal diabetes and high glucose induce $Sca1^+$ progenitor cell apoptosis, which may be related to oxidative stress. Pro-apoptotic transcription factor Forkhead O 3a (FoxO3a) is involved in $Sca1^+$ progenitor cell death because hyperglycemia activates FoxO3a via dephosphorylating its threonine 32 residue (28). FoxO3a deletion *in vivo* prevents diabetes-induced $Sca1^+$ progenitor cell apoptosis, and FoxO3a-negative mutant *in vitro* reverses high glucose-triggered $Sca1^+$ progenitor cell death (28). Collectively, these results suggest that maternal diabetes and high glucose induce $Sca1^+$ progenitor cell apoptosis via activating FoxO3a to limit its regenerative potential (28). The above studies may serve as an optimized guide, providing information on autologous cell therapy with cardiac stem/progenitor cells in CHD from diabetic pregnancy.

In conclusion, high oxidative stress induced by maternal diabetes triggers cardiac cell apoptosis, inhibits cell proliferation, and constantly suppresses the regenerative capacity of CPCs to recover damaged cardiomyocytes, which finally leads to CHD.

HEART TRANSPLANTATION AND MEDICAL AND DEVICE THERAPIES FOR CHD

Animal studies have indicated that maternal diabetes-induced heart failure (HF) can be characterized by a gradual loss of cardiomyocytes, and experimental inhibition of apoptosis is responsible for improving cardiac function (26,28). The current standard therapy for HF caused by cardiomyocyte loss is heart transplantation (HT) or mechanical circulatory support (29,30). HT remains the final end-stage therapeutic option to treat patients with CHD. However, there is a high risk of mortality after implantation because of CHD (31). The survival at 3 months post-HT was dramatically worse in CHD patients than in those without CHD (32). On the other hand, the number of infants who have undergone HT has decreased owing to donor limitations (33).

Neonatal HT is uncommon; however, significant progress has been made in the treatment of CHD, including new medical and device therapies during the past 20 years (34). Clinical studies focus on two major pharmacological agents, beta-blockers and angiotensin-converting enzyme inhibitors, to treat CHD patients (35–37). However, randomized-controlled trials have shown that β-blockers do not significantly improve the clinical HF outcomes in patients with CHD (38). Similarly, pharmacological blockade of angiotensin-converting enzyme in infants with CHD does not improve cardiac function or HF status (39,40). In addition, animal studies have indicated that *N*-acetylcysteine alleviates CHD induced by pregestational diabetes (41); however, the safety and feasibility of *N*-acetylcysteine need to be investigated by more clinical trials.

A variety of assist devices, such as left ventricle assist devices, and a total artificial heart have been used to decrease the mortality and morbidity in patients awaiting HT (34). Although many assistive devices have been developed as replacement therapy for critically ill patients with CHD to support their failing heart, limitations, including significant mortality and morbidity after implantation of assist devices, device reliability, cardiac arrhythmia, risk of infection, and anticoagulation, should be considered (34,42).

CARDIAC STEM/PROGENITOR CELL THERAPY FOR CHD

Beyond HT and medical care, new discoveries on the regenerative potential of cardiac stem cells and progenitor cells have emerged as a novel therapeutic strategy for treating and preventing CHD (29,43,44). *In utero* intracardiac delivery of CPCs through ultrasound-guided microinjection in mice has been demonstrated that engrafted CPCs incorporate to the heart and differentiated into the cardiomyocytes (45). Thus prenatal transplantation of CPCs rescues the cardiac dysfunctions (45). Previous studies from our laboratory have indicated that maternal diabetes and high glucose affect stem/progenitor cells in cardiogenesis by suppressing differentiation or inducing cell death (26,28). Therefore, cardiac stem/progenitor cell therapy for maternal diabetes-induced CHD is a possible solution for surgical and medical care challenges. Recent studies have indicated that preexisting cardiomyocytes in humans could be replaced at early childhood (46) and dedifferentiate after injury in adulthood (47). However, the capacity for human cardiac regeneration is insufficient to self-repair HF, and strategies for repopulating the damaged myocardium in CHD are being explored (48–51). Many different cell types are considered candidates for cardiac cell therapy, including skeletal myoblasts, bone marrow–derived cells, embryonic stem cells, and endogenous cardiac stem/progenitor cells, which might give rise to cardiomyocytes (29,44).

c-KIT–expressing (c-kit$^+$) CPCs are the most recognized CPCs in heart disease for several reasons, including their natural location, their function in the heart to maintain homeostasis during aging, and their support of cardiac function for potential myocardial-lineage differentiation after transplantation (52). Several studies have shown that these cells are clonogenic, self-renewing, and are able to differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells *in vitro*, contributing to the formation of myocardium and vessels after transplantation *in vivo* (53,54). In one study, transplanted fetal c-kit$^+$ CPCs after expansion *in vitro* promoted heart repair (55). However, there is ongoing debate regarding whether c-kit$^+$ CPCs can give rise to sufficient cardiomyocytes. Recent studies with lineage-tracing approaches have shown that c-kit$^+$ CPCs isolated from the adult mouse hearts have no regenerative potential to differentiate into cardiomyocytes *in vitro* or for transplantation into hearts after injury (56,57). Further studies suggest that resident c-kit$^+$ CPCs may not be CPCs because they generate cardiomyocytes within the heart at a functionally insignificant level. By contrast, c-kit$^+$ CPCs contribute to endothelial cell production instead of myocyte generation in the infarcted hearts (58,59).

Other CPCs may be better choices than c-kit$^+$ CPCs for generating cardiomyocytes. Sca1$^+$ CPCs are closest to cardiomyocytes (60), and they have significant cardiac regeneration ability to differentiate into cardiomyocytes and other cardiac cells (61–63). Although Sca1$^+$ CPCs rarely give rise to cardiomyocytes during normal conditions (64), they home to the injury site in the mouse model of myocardial ischemia and improve cardiac repair and function by forming new cardiomyocytes (65). In contrast, deletion of *Sca1* leads to decreased cardiac function and hypertrophic response (66). Moreover, integrin-linked kinase-overexpressed Sca1$^+$ CPCs improve cardiac function in myocardial infarction after transplantation (67). However, the functional advantage of Sca1$^+$ CPCs in cell therapies for maternal diabetes-induced CHD remains elusive.

Another population with CPCs that has begun to be revealed in preclinical and clinical studies includes cardiosphere-derived cells. Human cardiosphere-derived cells and their derivatives have the capacity to give rise to cardiomyocytes both *in vitro* and *in vivo* (68,69). More importantly, when injected into animal models of myocardial infarction, they contributed to the functionally meaningful renewal and repair by paracrine effector secretion (70–72). In addition, cardiosphere-derived cells are currently undergoing phase I clinical evaluation to test the feasibility and the procedural safety (73).

Mesenchymal stem cells (MSCs) are mesoderm-derived stem cells with potential of self-renewal, differentiation, and distribution in various organs, such as bone marrow, umbilical cord blood, adipose tissue, and heart (74). Human MSCs were used as a therapeutic strategy to preserve neonatal right ventricular function in a porcine model by antagonizing the hypertrophy response of pressure overload (75). Furthermore, intramyocardial delivery of human MSCs has been shown to preserve global and regional cardiac functions, attenuate remodeling, and stimulate endogenous progenitor cell proliferation to improve neovascular formation and myocyte cycling. Moreover, right ventricular hypertrophy could be reversed via growth differentiation factor 15 signaling, which is directly secreted through transplanted MSCs (30).

Both CPCs and MSCs have similar features that they may secrete paracrine factors to enhance cardiac regeneration. However, CPCs possess the potential of cardiomyocyte differentiation (76,77). Transplanted MSCs have a tendency to differentiate into osteoblasts rather than cardiomyocytes (78).

Encouraging results in animal studies have elucidated the therapeutic potential of these four types of stem/progenitor cells (67,79–85). However, more efforts should be made to apply cardiac CPCs therapy for CHD from diabetic pregnancy.

MECHANISMS OF CARDIAC STEM/PROGENITOR CELL-BASED THERAPY FOR CHD

Thus far, there are many uncertainties related to stem/progenitor cells therapies for cardiac repair in CHD patients. It was originally thought that transplanted stem/progenitor cells could restore lost myocardium and cardiac function by regenerating cardiomyocytes, endothelial cells, and smooth muscle cells to promote the formation of new cardiac tissue (86–88). However, subsequent animal studies have revealed that transplanted stem/progenitor cells have poor survivability and are unable to truly regenerate myocardium or differentiate into cardiomyocytes *in vivo* (89–91). For example endothelial progenitor cells have not been found to differentiate into cardiomyocytes *in vivo*, but they promote angiogenesis (92,93). In addition, transplanted MSCs have been shown to differentiate into bone-forming osteoblasts instead of cardiomyocytes in mice (78). The results suggest that it is lack of efficacy to differentiate sufficient cardiomyocytes for cardiac repair in transplanted hearts by stem/progenitor cell therapies.

Instead of creating new cardiomyocytes, transplanted cells may secret paracrine factors to stimulate resident stem/progenitor cell activation to promote vascular growth and give rise to new myocardium for cardiac repair (94,95). Recent preclinical studies have indicated that angiogenetic factors secreted by transplanted MSCs can suppress inflammation-associated overproliferation of pulmonary artery smooth muscle cells in a model of pulmonary hypertension (96,97). These factors involve specific cytokines, angiogenic growth factors, and proper factors for stem cell mobilization or recruitment (98). Further investigations have revealed that the paracrine functions of stem/progenitor cells are potentially mediated by extracellular vesicles, such as exosomes (99–101). Exosomes are lipid bilayer nanovesicles that are released on fusion of multivesicular endosomes with the plasma membrane (100). Increasing studies have indicated that exosomes

are important mediators of cell–cell communication (102,103). Injection of c-KIT-expressing neonatal CPC (nCPC)-derived exosomes has been shown to protect against injury and promote regeneration in a myocardial infarction murine model (101,104). Accumulating evidence has indicated that CPC-derived exosomes and packed microRNA (miRNA) may be critical mediators involved in the communication between transplanted cells and endogenous stem/progenitor cells (101,105).

ADVANTAGE OF NEONATAL HEART-DERIVED STEM/PROGENITOR CELLS

A recent study has elucidated the phenotypic characteristics of human nCPCs and adult CPCs (101). CPCs are derived from the heart specimens that are routinely discarded in cardiopulmonary bypass surgery or HT (68,70,101,106,107). After cell dissociation, CPCs are isolated with microbeads (101). Thus the derived CPCs bypass the contentious ethical issue. The results indicated that the proliferative capacity of neonatal c-kit$^+$ CPCs is superior to that of adult c-kit$^+$ CPCs. In addition, the growth properties and functional activity of adult c-kit$^+$ CPCs are affected by age; they are characterized by loss of c-kit expression, reduced cell division, telomere length shortening, and induction of senescence. nCPCs have also been revealed to have a greater potential for myocardial regeneration to improve cardiac function in a myocardial infarction model (101). Moreover, transplanted CPCs may secret paracrine factors that are essential for stem function and cardioprotection through exosomes to repair injured hearts, and nCPCs secrete more paracrine factors than adult CPCs (101). Taken together, these results suggest that nCPCs may be more suitable for use in CPC therapy to treat patients with maternal diabetes-induced CHD.

STRATEGIES FOR CELL DELIVERY IN CPC THERAPY

For CPC therapy used to treat patients with maternal diabetes-induced CHD, two major cell delivery strategies may be used: direct cell injection and cardiac patch implantation. The most common cell replacement therapy used in patients with CHD is direct injection of stem/progenitor cells through the coronary arteries or using catheters. Direct cell injection has been validated to significantly improve cardiac function. This beneficial effect may be attributed to the delivery of multiple cell types, paracrine factors secreted by injected cells, and relative immaturity of the transplanted cells. However, the engraftment efficiency of a direct cell injection trial is $<10\%$ because of rapid wash out, ejection, and death. Cardiac patch implantation may be an alternative to cell injection. Cell engraftment and survival have been improved by cardiac patch technologies. Engineered cardiac tissues by scaffolds are designed to facilitate cell assembly into the host myocardium and have enabled release of bioactive peptides or paracrine factors in a targeted and controlled manner (108). Furthermore, the majority of three-dimensional engineered cardiac tissues have been demonstrated beneficial and three-dimensional printed scaffolds have been used *in vivo* (109). However, numerous challenges need to be addressed, such as an appropriate cell dose for injection and the choice of implantation location.

CASE REPORTS AND TRIALS OF CPC THERAPY IN CHD

Several encouraging case reports in the end stage of pediatric HF, including dilated cardiomyopathy (110), double outlet right ventricle (111), pulmonary atresia with ventricular septal defect (111), and hypoplastic left heart syndrome (112), have evaluated the safety and preliminary efficacy of stem/progenitor cell transplantation. However, further studies have suggested that cell therapy fails to treat some patients with CHD, leading to death or subsequent HT (111,113). Therefore, clinical trials are urgently needed to verify the benefit and risk of stem/progenitor cell transplantation therapy in CHD.

To evaluate the impact of stem/progenitor cell therapy for patients with CHD, several cell therapy trials have been performed. A TICAP (transcoronary infusion of CPCs in patients with single ventricle physiology) phase 1 trial was the first complete clinical investigation for hypoplastic left heart syndrome patients. This trial aimed to evaluate the feasibility and safety of CPC infusion after staged palliation as well as the effects on heart dysfunction followed by the infusion of CPCs. No serious adverse effects were found in the TICAP trial. CPC infusion improved the HF status and somatic growth through increasing ventricular function and reducing the tricuspid valve diameter, ventricular volume echocardiography, and ventriculogram. Long-term observation of the TICAP study confirmed these beneficial effects and the lack of ectopic tumor formation (114). Following the TICAP phase 1 trial, a PERSEUS (CPC infusion to treat univentricular heart disease) phase 2 trial was undertaken to validate the therapeutic efficacy of CPCs. Similar to the TICAP phase 1 study, CPCs favorably improved the cardiac function in CPC transplantation. Additionally, these beneficial effects were not related to the palliation treatment received before CPC infusion. Patients with CHD have a better quality of life after these therapy trials (115). In addition to these two complete cell therapy trials, several successive clinical studies are ongoing, such as the APOLLON (cardiac stem/progenitor cell infusion in univentricular physiology) phase 3 trial (116). These trials also promote the improvement of stem/progenitor cell therapy to treat CHD patients from diabetic pregnancy.

FUTURE DIRECTIONS

As described above, maternal diabetes causes adverse effects on the biological function of embryonic stem cells and CPCs (26,28). Another recent study has shown that human umbilical cord mesenchymal stromal cells isolated from women with gestational diabetes display premature aging, poorer cell growth, and mitochondrial dysfunction, reducing the therapeutic potential of stem cells (117). This is a barrier for direct cardiac stem/progenitor cell therapy to treat CHD from diabetic pregnancy. Furthermore, the biological function

of CPC remains elusive. Future studies should focus on the mechanistic action of these cells to diminish the adverse effects caused by maternal diabetes.

CPCs are a heterogeneous group of cells distributed throughout the heart that have been subclassified by a variety of cell markers or transcription factors, including c-Kit$^+$ CPCs, cardiosphere-derived cells, and Sca-1$^+$ CPCs (76,118). However, unlike other adult cell types for which surface markers have been extensively characterized, endogenous CPCs have mixed and overlapping expression of stem cell markers (76). Highly specific markers for CPCs need to be identified in follow-up studies.

Previous studies have shown that maternal diabetes affects the expression of miRNAs in the developing mouse embryonic neural tube and heart, and alterations of several miRNAs contribute to the formation of neural tube defect (119–122), suggesting that maternal diabetes-altered miRNAs may also be involved in CHD formation. miRNAs have been shown to negatively regulate gene expression and signaling pathways in various tissues (123). Whole-genome miRNA screening has been performed to identify that a subset of miRNAs promotes endogenous cardiomyocytes proliferation and induces myocardial regeneration (124). In addition, studies in animal model have indicated that suppression of miRNA-208a and miRNA-21 may be involved in reduction of cardiac fibrosis, which prevents myocardial remodeling (125–127). More recently, several studies have shown some novel mechanisms by which tissue noncoding RNA expression contributes to regulate myocyte and vessel growth or myocardial fibrosis (128–130). Furthermore, miRNAs have been implicated in the clinical pathogenesis of several types of CHD and circulating miRNAs may be promising clinical biomarkers for disease diagnosis in future (131,132). Although the therapeutic potential of miRNAs in CHD has not yet been revealed, analysis between miRNAs and CPC-derived exosomes has indicated that signaling pathway regulated by miRNAs may be age specific and can provide essential information for novel therapeutic strategies in CHD (104), especially in cases induced by maternal diabetes.

Currently, the practice of cardiac stem/progenitor cell therapy for CHDs through neonatal cardiac surgery is confined to a few institutions that have well-established multidisciplinary team with a pediatric cardiac surgeon, cardiologist, and fetal echocardiography specialists. In addition, precision diagnosis of fetal CHDs is critical for the prenatal *in utero* stem cell delivery to treat CHDs.

STATEMENT OF FINANCIAL SUPPORT

This work is supported by the NIH grants NIH R01DK083243, R01DK101972, R01HL131737, R01HL134368, R01HL139060, and R01DK103024.

Disclosure: The authors declare no conflict of interest.

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