Mesenchymal stem cells preserve neonatal right ventricular function in a porcine model of pressure overload

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Wehman B, Sharma S, Pietris N, Mishra R, Siddiqui OT, Bigham G, Li T, Aiello E, Murthi S, Pittenger M, Griffith B, Kaushal S. Mesenchymal stem cells preserve neonatal right ventricular function in a porcine model of pressure overload. Am J Physiol Heart Circ Physiol 310: H1816–H1826, 2016. First published April 22, 2016; doi:10.1152/ajpheart.00955.2015.—Limited therapies exist for patients with congenital heart disease (CHD) who develop right ventricular (RV) dysfunction. Bone marrow-derived mesenchymal stem cells (MSCs) have not been evaluated in a preclinical model of pressure overload, which simulates the pathophysiology relevant to many forms of CHD. A neonatal swine model of RV pressure overload was utilized to test the hypothesis that MSCs preserve RV function and attenuate ventricular remodeling. Immunosuppressed Yorkshire swine underwent pulmonary artery banding to induce RV dysfunction. After 30 min, human MSCs (1 million cells, n = 5) or placebo (n = 5) were injected intramyocardially into the RV free wall. Serial transthoracic echocardiography monitored RV functional indices including 2D myocardial strain analysis. Four weeks postinjection, the MSC-treated myocardium had a smaller increase in RV end-diastolic area, end-systolic area, and tricuspid vena contracta width (P < 0.01), increased RV fractional area of change, and improved myocardial strain mechanics relative to placebo (P < 0.01). The MSC-treated myocardium demonstrated enhanced neovessel formation (P < 0.0001), superior recruitment of endogenous c-kit+ cardiac stem cells to the RV (P < 0.0001) and increased proliferation of cardiomyocytes (P = 0.0009) and endothelial cells (P < 0.0001). Hypertrophic changes in the RV were more pronounced in the placebo group, as evidenced by greater wall thickness by echocardiography (P = 0.008), increased cardiomyocyte cross-sectional area (P = 0.001), and increased expression of hypertrophy-related genes, including brain natriuretic peptide, β-myosin heavy chain and myosin light chain. Additionally, MSC-treated myocardium demonstrated increased expression of the antihypertrophy secreted factor, growth differentiation factor 15 (GDF15), and its downstream effector, SMAD 2/3, in cultured neonatal rat cardiomyocytes and in the porcine RV myocardium. This is the first report of the use of MSCs as a therapeutic strategy to preserve RV function and attenuate remodeling in the setting of pressure overload. Mechanistically, transplanted MSCs possibly stimulated GDF15 and its downstream SMAD proteins to antagonize the hypertrophy response of pressure overload. These encouraging results have implications in congenital cardiac pressure overload lesions.

stem cell therapy; congenital heart disease; right ventricle; pressure overload

NEW & NOTEWORTHY

We demonstrate for the first time that intramyocardial injection of mesenchymal stem cells preserves right ventricular function

CONGENITAL HEART DISEASE (CHD) is the leading cause of morbidity and mortality in children with birth defects. While surgical interventions have dramatically improved outcomes and longevity in patients with CHD, many patients still progress to heart failure, a population that has grown significantly over the last decade (30). In contrast to adult patients, in whom ischemic heart disease is the predominant etiology of heart failure, children with CHD are frequently exposed to acute or chronic ventricular pressure and volume overload, which if untreated can progress to ventricular dysfunction and ultimately to heart failure. Further, patients with CHD who develop right ventricular (RV) dysfunction have the poorest outcomes (23, 26), particularly in those patients with univentricular heart disease in whom the RV is the systemic pumping chamber (22, 24, 28). In the presence of such abnormal loading conditions, the RV undergoes a maladaptive remodeling process characterized by chamber dilatation, hypertrophy, myocardial fibrosis, and ultimately failure (18). Existing medical therapies have been extrapolated from experience in adult patients and have not been shown to halt the ventricular remodeling process or promote systolic and diastolic functional recovery (31). In the event of progression to overt RV failure, heart transplantation remains the only definitive treatment. Novel, paradigm-shifting therapies are urgently needed to address the unmet needs of pediatric heart failure (4).

Stem cell therapy has shown promise over the last decade as a safe and feasible intervention for adults with acute or chronic myocardial infarction. A well-studied type of stem cells consists of bone marrow-derived mesenchymal stem cells (MSCs), an undifferentiated population of cells capable of multipotent differentiation. Transplanted MSCs functionally recover acute and chronic ischemic myocardium in preclinical models of myocardial infarction, despite their limited engraftment in the injured myocardium (33). The precise mechanism of action has yet to be defined, but it may involve secretion of paracrine factors and stimulation of endogenous mechanisms of myocardial repair (3). As a therapeutic for adults, MSCs have recently emerged as a leading stem cell candidate based on a series of early phase clinical trials that have shown a favorable safety profile, feasibility as an allogeneic cell therapy product, and preliminary efficacy to improve contractility, quality of life,
and reduce myocardial scar formation (11, 12, 20). Additionally, MSCs have shown the capacity to recruit and activate resident c-kit+ cardiac stem cells (CSCs), a myocardial compartment of undifferentiated progenitor cells, to areas of myocardial injury (13). This has implications for the use of MSCs in pediatric patients, who are anticipated to have a robust response to MSC treatment. This expected response is based on our previous study, which demonstrated an increased number and enhanced regenerative capacity of resident CSCs in pediatric patients compared with adult patients (34). Should MSC therapy provide a similar benefit to RV performance, as has been observed for LV models of myocardial infarction, it would represent a breakthrough in the treatment of children with RV dysfunction. Also, this type of therapy may potentially be applied to adults who suffer from RV dysfunction secondary to pulmonary hypertension.

The potential efficacy of MSCs has not previously been evaluated in a preclinical model relevant to the pathophysiologic of RV dysfunction. We therefore undertook the following experiments to test the hypothesis that human MSCs preserve RV function and attenuate ventricular remodeling compared with placebo, using a porcine model of RV pressure overload. Mechanistically, we tested whether MSC treatment resulted in the activation of the antihypertrophy factor GDF15, a secreted member of the transforming growth factor (TGF)-β superfamily because GDF15 functionally protects the myocardium and has antihypertrophic properties in a rodent model of left ventricle cardiac pressure overload (40, 41).

METHODS

Study design. This study was performed using protocols that were reviewed and approved by our Institutional Animal Care and Use Committee and following the 1996 Guide for the Care and Use of Laboratory Animals. Immunosuppressed Yorkshire swine (6–9 kg, 14–21 days of life) underwent pulmonary artery (PA) banding followed by injection of MSCs (n = 5) or placebo (n = 5) at 30 min postbanding. Conventional and speckle-tracking echocardiography was performed to assess structural and functional changes at baseline, postbanding (<72 h from banding), and at 4 wk.

Culture and flow cytometric analysis of MSCs. Fresh bone marrow aspirates were obtained from the iliac crest of two human donors to obtain MSCs (Lonza, Walkersville, MD). MSCs were isolated from other bone marrow cells by Ficoll density centrifugation and plastic adherence, as previously described (29, 42). MSCs at passage 3 were evaluated by flow cytometry with a Becton-Dickinson FACS Caliber (San Jose, CA) with 10,000 events collected. Cells were incubated with fluorochrome-conjugated primary antibodies against c-kit+1, the hematopoietic lineage surface marker CD34, the mesenchymal stromal lineage markers, CD90, CD44, CD105, CD106, and CD140b, and the endothelial cell surface marker CD31 (BD Pharmingen, San Jose, CA, and R&D Systems, Minneapolis, MN). Isotype controls were run for each immunosubtype.

Induction of RV pressure overload. Swine were anesthetized with ketamine 15–20 mg/kg im) and xylazine (1–2 mg/kg im). Animals were intubated and ventilated with 100% oxygen. Anesthesia was maintained with 1–3% inhaled isoflurane. Fentanyl was started as a continuous infusion (30–50 μg kg−1 h−1 iv). A left anterior thoracotomy was performed in the fifth intercostal space to expose the RV, main PA, and aorta. The pericardium was opened longitudinally with electrocautery. The left lung was gently retracted with wet gauze to expose the descending thoracic aorta. The pleural reflection was cleared from the descending aorta, and a 16 French catheter was secured for continuous monitoring of intra-arterial systemic blood pressure. The PA was dissected from the ascending aorta. A 5-mm wide GoreTex band was encircled around the PA, 10 mm away from the pulmonary valve. The band was progressively tightened around the PA with medium-sized hemoclips to induce an RV/systemic/systolic pressure ratio of ~75%. In the event this ratio exceeded 75% or there was hemodynamic compromise, one or more hemoclips were removed. When hemodynamically stable at the desired pressure ratio, the band was sutured to the PA adventitia with a 5-0 polypropylene suture to prevent band migration. All swines were alive for 4 wk.

Intramyocardial injection. On the morning of stem cell injection, MSCs at passage 3 were harvested with TrypLE Express (Gibco, Life Technologies, Grand Island, NY) and resuspended in PBS. Prior to injection, swines were treated with amiodarone (2 mg/kg iv bolus followed by 0.5 mg kg−1 h−1 infusion) (25). After 30 min postbanding, swines underwent stem cell injection (n = 5, 5 × 106 MSCs) or placebo (n = 5, PBS) injection to the RV myocardium via six separate 200-μl aliquots (total volume of 1.2 ml) with a 29-gauge needle at a 30-degree angle to the epicardium. We used a weight-based dose of ~125,000 MSCs/kg, which was an intermediate dose extrapolated from a previous dose-escalation study using doses of 20,000 to 200,000 MSCs/kg in a phase II human clinical trial (11).

Immune suppression. All swines were treated with cyclosporine and methylprednisone to attenuate the immune response to the injected human cell xenografts (17). Two days prior to cell or placebo injection, animals were started on oral cyclosporine 10 mg/kg in two divided doses daily. Cyclosporine doses were titrated to target a serum level of 125 to 225 ng/ml (39). Methylprednisone (6 mg/kg im) was administered the morning of intramyocardial injection and tapered to 3 mg/kg im daily over the first 2 wk after transplantation, and they were continued on that dose for the duration of the study.

Transthoracic echocardiography. Transthoracic echocardiograms were performed with a General Electric Vivid Q Ultrasound and a 3S probe (General Electric, New York, NY). Echocardiographic images were obtained with the subject sedated. Parasternal short axis and apical four-chamber views were captured at baseline, postbanding, and 4 wk. Two-dimensional images were acquired in addition to measurements made by pulsed and continuous wave Doppler to assess the tricuspid and pulmonary valve parameters. For assessment of RV function and size were selected from current recommendations (32) for 2D assessment in the bi-ventricular heart which included RV fractional area change (FAC), defined as [(end-diastolic area − end-systolic area)/end-diastolic area] × 100. Tricuspid valve vena contracta width was assessed as a marker for severity of tricuspid regurgitation, and measurements were made with color Doppler at the narrowest point of the regurgitant jet (35). All echocardiographic images were reviewed and analyzed by a single, blinded investigator.

Analysis of myocardial strain. Measurements of strain and strain rate were determined as previously described (5). Speckle-tracking measures of deformation from the apical four-chamber view included peak global longitudinal strain and strain rate, and were calculated from the combined deformation of the myocardial segments in each imaging plane. Myocardial motion was tracked through the cardiac cycle calculating myocardial deformation. The speckle-tracking algorithm then divided the myocardium into six segments in the four-chamber view. Measurements were performed in triplicate, and tracking was visually assessed. A single, blinded observer performed offline analysis of images in Digital Imaging and Communications in Medicine format using vendor-independent software (2D Cardiac Performance Analysis, TomTec Imaging Systems, Munich, Germany). Deformation curves were not accepted if greater than two segments demonstrated inadequate tracking.

Myocardial histology. After necropsy was performed, six different sites in the RV, two sites in the septum, and four different sites in the LV were biopsied. Primary antibodies used were c-kit+ (AbD Serotec, Raleigh, NC), Ki67 (EMD Millipore, Billerica, MA), N11CD (Abcam (36)), GATA-4 (Santa Cruz, Dallas, TX), smooth muscle actin (Sigma, St. Louis, MO), α sarcomeric actin (Sigma), phallolidin...
H1818 MESENCHYMAL STEM CELLS PRESERVE NEONATAL RV FUNCTION

(Sigma), CD31 (Abcam, Cambridge, MA), isoelectin B4 (Life Technologies, Green Island, NY), trypsin (Abcam), human antimyocardial antibody (EMD Millipore), and wheat germ agglutinin (Abcam). For secondary antibodies, conjugated AlexaFluor fluorochromes were used (Jackson ImmunoResearch Laboratories, West Grove, PA).

A Nikon Eclipse microscope system (Melville, NY) visualized and captured images of antibody-mediated immunofluorescence. Random fields were selected while viewing the slides, and the mean number of positive cells or neovessels identified in five views was extrapolated for a count per square millimeter or cubic centimeter. To quantify cardiomyocyte cross-sectional area, wheat germ agglutinin staining of cardiomyocyte plasma membranes was used to trace five distinct green outlines from five random fields in Image Pro Plus software (Media Cybernetics, Rockville, MD). The cross-sectional area within the traced border was calculated to generate an average value for each subject.

RNA isolation and RT-PCR analysis. Total RNA was isolated from biopsy specimens of explanted hearts with RNeasy Kit (Qiagen, Germantown, MD), according to the manufacturer’s instructions, and quantified using a Nanodrop 2000 (Thermo Fisher Scientific, Rockville, MD). Five hundred nanograms of RNA were reverse-transcribed with a cDNA synthesis kit, according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). Each reaction was performed on a StepOnePlus (Applied Biosystems) polymerase chain reaction (PCR) machine. Primer/probe sets were obtained from Life Technologies: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), myosin light chain-2v (MLC-2v), β-myosin heavy chain 7 (β-MHC), and brain natriuretic peptide (BNP). Quantitative PCR reactions were performed in quadruplicate. The cycle threshold (CT) values of the housekeeping gene (GAPDH) were subtracted from the corresponding gene of interest (ΔCT). Relative RNA abundance was calculated using the following equation: 2ΔΔCT. The final values were averaged and results represented as relative expression to GAPDH with the standard error of the mean.

In vitro hypertrophy assays. Primary neonatal rat cardiomyocytes (NRCMs) were purchased from Lonza and cultured according to manufacturer’s instructions. NRCMs were preconditioned with MSCs for 24 h and were then serum free for 24 h before exposure to a hypertrophic agonist (25 μM) phenylephrine (PE) and 1 μM angiotensin II (ANGII) (40, 41). NRCMs with or without MSC preconditioning were exposed to PE/ANGII for 48 h before being fixed in formalin for staining with phallolidin or undergoing immunoblot analysis for growth differentiation factor 15 (GDF15) and SMAD 2/3 expression. Calculation of cross-sectional area of NRCMs was performed by tracing five distinct cardiomyocytes from five random fields in Image Pro Plus software and averaged for each group.

Immunoblot analysis. RV myocardial biopsy specimens were harvested and homogenized in T-PER buffer (Thermo Fisher Scientific) containing a complete protease inhibitor cocktail (Roche Applied Science). Tissue homogenate was further sonicated on ice with 15 s of sonication cycles for three times before the samples were centrifuged to pellet cell or tissue debris. Protein concentration was measured by Bradford assay, containing a complete protease inhibitor cocktail (Roche Applied Science). Tissue homogenate was further sonicated on ice with 15 s of sonication cycles for three times before the samples were centrifuged to pellet cell or tissue debris. Protein concentration was measured by Bradford assay. Tissue homogenate was further sonicated on ice with 15 s of sonication cycles for three times before the samples were centrifuged to pellet cell or tissue debris. Protein concentration was measured by Bradford assay. Tissue homogenate was further sonicated on ice with 15 s of sonication cycles for three times before the samples were centrifuged to pellet cell or tissue debris. Protein concentration was measured by Bradford assay. Tissue homogenate was further sonicated on ice with 15 s of sonication cycles for three times before the samples were centrifuged to pellet cell or tissue debris. Protein concentration was measured by Bradford assay. Tissue homogenate was further sonicated on ice with 15 s of sonication cycles for three times before the samples were centrifuged to pellet cell or tissue debris. Protein concentration was measured by Bradford assay. Tissue homogenate was further sonicated on ice with 15 s of sonication cycles for three times before the samples were centrifuged to pellet cell or tissue debris. Protein concentration was measured by Bradford assay. Tissue homogenate was further sonicated on ice with 15 s of sonication cycles for three times before the samples were centrifuged to pellet cell or tissue debris. Protein concentration was measured by Bradford assay. Tissue homogenate was further sonicated on ice with 15 s of sonication cycles for three times before the samples were centrifuged to pellet cell or tissue debris. Protein concentration was measured by Bradford assay.

Statistics. Continuous data are reported as means ± SE. The two groups were compared at 4 wk with an unpaired Student’s t-test. When more than two groups were present, comparisons were made by a one-way analysis of variance with a Tukey’s posttest to separately compare values between each group. Within-group comparisons were made by a paired Student’s t-test or repeated measures analysis of variance with a Tukey’s posttest, where appropriate. Statistical analysis was performed with Graphpad Prism software (Ver. 5.0c, La Jolla, CA).

RESULTS

Characterization of MSCs. Human MSCs were isolated and expanded from bone marrow aspirates. MSCs were examined for their expression of mesenchymal stromal lineage markers by fluorescence-activated cell sorting analysis. Expanded MSCs demonstrated expression for mesenchymal markers CD90, CD44, CD105, CD106, and CD146 (≥80%) and were negative or expressed very low levels for hematopoietic markers CD45, CD34, or CD31. These results confirmed the biochemical characteristics of the expanded MSCs.

RV pressure overload and immune suppression. Pressure overload in the RV was induced in neonatal swine with a PA band, which was physiologically calibrated to acutely increase the RV to systemic blood pressure ratio from 0.35 ± 0.02 to 0.75 ± 0.02 in the placebo group and 0.36 ± 0.01 to 0.77 ± 0.03 in the MSC group, indicating an equivalent and highly consistent rise in RV afterload across both groups. Thirty minutes postbanding, swines underwent injection of MSCs (1 × 10^6 MSCs, six locations) or placebo (phosphate buffered saline). Both groups received immune suppression with cyclosporine and methylprednisolone to reduce xenotransplants rejection. Cyclosporine trough levels remained within the therapeutic window for both groups throughout the study (144.8 ± 47.0 vs. 141.3 ± 49.4, placebo vs. MSCs, respectively, P = 0.96). There were no opportunistic infections, signs of end-organ dysfunction, or other adverse sequelae seen in the model. All swines survived the duration of the protocol. At 4 wk, the luminal diameter at the level of the PA band was equivalent in both groups with minimal variation, indicating no PA band loosening or migration during the study period (9.22 ± 0.9 mm vs. 9.09 ± 0.7 mm, placebo vs. MSCs, respectively, P = 0.91). Necropsy revealed that the PA band was secured in its original PA position in both groups.

Preservation of RV size and function. Both groups had equivalent baseline (prebanding) values for RV FAC, a comparable measure to ejection fraction, and a similarly modest decline postbanding (Fig. 1A, supplemental movies S1 and S2, respectively; Supplemental Material for this article is available online at the Journal website). At 4 wk postbanding, placebo-treated swines showed significant deterioration in RV FAC from baseline and postbanding values (P = 0.001), whereas MSC-treated swines demonstrated near baseline levels of FAC. Compared with the placebo group, MSC-treated swines had a significantly greater FAC (P = 0.016, Fig. 1A, supplemental movies S3 and S4). Placebo-treated swines also demonstrated marked dilatation of the RV at 4 wk postbanding, evidenced by increased end-diastolic area (EDA) and end-systolic area (ESA) compared with baseline and postbanding values (P = 0.006 and P = 0.004, respectively), whereas EDA and ESA values in MSC-treated swines were relatively preserved (P = 0.13 and P = 0.29, respectively, Fig. 1, B and C). The severity of tricuspid regurgitation was assessed by measuring the width of the vena contracta. Placebo-treated swines demonstrated a regurgitant jet across the tricuspid valve with a significantly greater vena contracta width compared with MSC-treated swines, indicating a more severe degree of tricuspid regurgitation at 4 wk postbanding (P = 0.019, Fig. 1, H–J). Tricuspid
valve papillary muscle rupture, leaflet perforation, and endocarditis were not observed.

To corroborate the echocardiographic findings with an operator-independent imaging modality, parameters of RV strain using speckle-tracking echocardiography were utilized. Measures of longitudinal strain and strain rate were equivalent between the two groups at baseline and postbanding (Fig. 2, C and D). At 4 wk postbanding, however, strain parameters in the placebo-treated swines were significantly reduced compared with the MSC-treated swines, indicative of diminished contractility in the RV of placebo-treated swines ($P = 0.002$ and $P = 0.004$, respectively).

Fig. 1. Echocardiographic assessment of neonatal Yorkshire swine following pulmonary arterial banding and RV injection with placebo or MSCs. A–C: RV FAC, EDA, and ESA are equivalent pre- and postbanding. At 4 wk, the RV FAC in MSC-treated swines was preserved relative to baseline and postbanding values, whereas the FAC of placebo-treated swines was significantly reduced (placebo, $n = 5$, 29.9 ± 4.7%, vs. MSCs, $n = 5$, 47.7 ± 3.4%; $P = 0.016$). The ESA and EDA in the placebo group were significantly enlarged at 4 wk relative to the MSC-treated swines, which had relatively preserved size (EDA: placebo, $n = 5$, 16.9 ± 3.7 cm, vs. MSCs, $n = 5$, 7.2 ± 1.2 cm, $P = 0.036$; ESA: placebo, $n = 5$, 12.1 ± 2.8 cm, vs. MSCs, $n = 5$, 3.9 ± 0.8 cm, $P = 0.023$). D–G: representative echocardiographic images of the RV immediately and at 4 wk postbanding in the placebo-treated and MSC-treated swines. H–J: tricuspid valve (TV) vena contracta width was significantly greater at 4 wk in the placebo-treated vs. MSC-treated swines (placebo, $n = 5$, 4.4 ± 0.4 mm, vs. MSCs, $n = 5$, 3.0 ± 0.2 mm, $P = 0.019$). Data are presented as means ± SE. EDA, end-diastolic area; ESA, end-systolic area; FAC, fractional area of change; MSCs, human mesenchymal stem cells; RV, right ventricle; TV, tricuspid valve. $^*P < 0.05$ and $^{**}P < 0.01$, unpaired $t$-test between groups at 4 wk postbanding; $^1P < 0.05$ and $^2P < 0.01$, within-group repeated measures analysis of variance.
Fig. 2. A–D: analysis of myocardial strain in neonatal Yorkshire swines following pulmonary arterial banding and RV injection with placebo or MSCs. A: individual segments are reviewed frame by frame to ensure adequate tracking is present throughout the cardiac cycle. B: graphical representation of longitudinal strain of individual wall segments. C: while strain parameters were equivalent at baseline and postbanding, RV peak GLS was greater in the MSC-treated swines at 4 wk after cell injection compared with placebo (placebo, n = 5, −13.05 ± 0.6%, vs. MSCs, n = 5, −16.14 ± 0.6%, P = 0.002). D: strain rate was significantly greater in the MSC-treated swines compared with the placebo-treated swines at 4 wk (placebo, n = 5, 0.97 ± 0.09/s, vs. MSCs, n = 5, 1.39 ± 0.08/s, P = 0.004). E and F: analysis of myocardial fibrosis following placebo or MSC treatment. E: MSC-treated swines exhibited reduced myocardial fibrosis in RV specimens as evidenced by Mason’s trichrome staining (placebo, n = 5, 8.8 ± 2.5%, vs. MSCs, n = 5, 1.4 ± 0.28%, P = 0.009), whereas no difference in fibrosis was observed between groups in the septum or LV (septum: placebo, n = 3, 3.5 ± 1.6%, vs. MSC, n = 3, 0.48 ± 0.23%, P = 0.14; LV: placebo, n = 3, 9.1 ± 5.1, vs. MSCs, n = 3, 0.47 ± 0.18%, P = 0.17). F: MSC-treated swines had reduced expression of collagen type I and increased expression of the antifibrotic protein BMP-2, whereas no difference TGF-β expression was seen (fold change relative to GAPDH listed below bands). Data are presented as means ± SE. GLS, global longitudinal strain; MSC, mesenchymal stem cell; RV, right ventricle; BMP-2, bone morphogenetic protein 2; TGF-β, transforming growth factor β. **P < 0.01, unpaired t-test between groups at 4 wk postbanding; †P < 0.05 and ‡P < 0.01, within-group repeated measures analysis of variance.
Stimulation of endogenous myocardial repair. The relative percentage of myocardial fibrosis from MSC-treated vs. placebo-treated swines at 4 wk was significantly reduced as determined by Masson’s trichrome staining \((P = 0.009, \text{Fig. 2E})\). There was no difference in the percent fibrosis in the septum or LV between the groups \((P = 0.14 \text{ and } P = 0.17, \text{respectively})\).

By immunoblot analysis, there was significantly reduced expression of collagen I protein levels in MSC-treated RV myocardium relative to placebo as well as enhanced expression of the antifibrotic protein, BMP-2 \((\text{Fig. 2F})\). There was no difference between the two groups, however, in protein expression of the profibrotic marker, TGF-β.

At 4 wk postbanding, we observed very few MSCs retained in the RV myocardium \((0.34 \pm 0.1 \text{ cells/mm}^2)\). A significantly greater number of neovessels and arterioles were identified in the RV myocardium of MSC-treated swines compared with placebo \((P = 0.0005 \text{ and } P = 0.026, \text{respectively}, \text{Fig. 3, A and B})\). The number of endogenous c-kit⁺ CSCs were identified in the myocardium by their expression for c-kit⁺ as well as a cardiac lineage marker, GATA-4, and the absence of a mast cell marker, tryptase. Significantly higher numbers of c-kit⁺/GATA-4⁺/tryptase⁻ CSCs were present in the RV free wall myocardium in the MSC-treated swines compared with the placebo-treated swines \((P = 0.014, \text{Fig. 3C})\). There was no difference, however, in the number of c-kit⁺ CSCs present in the LV myocardium \((P = 0.54)\).

The number of proliferating cardiomyocytes and endothelial cells, as determined by coexpression of the surface marker Ki67, was also more abundant in the MSC-treated swines, suggesting enhanced cellular proliferation and more active myocardial repair \((P = 0.0009 \text{ and } P < 0.0001, \text{respectively}, \text{Fig. 3, D and E})\). Proliferating cardiomyocytes and endothelial cells failed to coexpress the active fragment of the Notch 1 receptor, N1ICD \((\text{Fig. 3, F and G})\), suggesting the proliferating cardiomyocytes were not derived from recruited c-kit⁺ CSCs, which occur in the maturing neonatal rodent heart \(36\).

Attenuation of the hypertrophic response to pressure overload. To evaluate whether MSC therapy may attenuate hypertrophic RV myocardial changes during pressure overload, an RV myocardial evaluation using three different modalities was performed, including echocardiography, histology, and biochemical data. The RV free wall thickness in the placebo-treated swine was much greater at 4 wk compared with postbanding values, as anticipated. In contrast, the RV free wall thickness in the MSC-treated swine underwent no significant change in wall thickness during the 4-wk study period \((P = 0.0004 \text{ and } P = 0.51, \text{respectively}, \text{Fig. 4A})\).

Additionally, myocardial sections stained with wheat germ agglutinin revealed the cross-sectional area of cardiomyocytes was significantly larger in the placebo-treated swines than in the MSC-treated swines \((P = 0.001, \text{Fig. 4B})\). The RV myocardium from placebo-treated swines exhibited significantly higher expression of BNP as well as other markers for cardiomyocyte hypertrophy \((2, 37)\), including MLC-2v and β-myosin heavy chain, compared with that of MSC-treated swines \((P = 0.05, \text{P} = 0.02, \text{and } P = 0.02, \text{respectively}, \text{Fig. 4, C-E})\).

Since previous studies have shown that elevated GDF15 levels functionally protect the myocardium through an antihypertrophy response during left ventricle pressure overload, the protein levels of GDF15 and its known downstream effector, SMAD 2/3, were determined in RV myocardium \((40, 41)\).

Relative to placebo, MSC treatment of myocardium increased protein expression of both GDF15 and phosphorylated SMAD 2/3 based on immunoblot staining and densitometry analysis \((\text{Fig. 4F})\).

The potential for MSC treatment to abrogate the cardiomyocyte hypertrophy response was further validated in vitro \((40)\). Primary neonatal rat cardiomyocytes \((\text{NRCM})\) exposed to the hypertrophy agonist combination PE/ANGII demonstrated a dose-dependent rise in cardiomyocyte cross-sectional area \((\text{Fig. 5A})\).

Preconditioning of NRCM with MSCs, however, suppressed this response, as evidenced by a significant reduction in cross-sectional area compared with control with no MSC treatment \((P < 0.0001, \text{Fig. 5B})\). Consistent with our findings in vivo, NRCM preconditioned with MSCs prior to induction of hypertrophy demonstrated increased GDF15 expression as well as phosphorylated SMAD 2/3 compared with control without MSC pretreatment \((\text{Fig. 5C})\).

DISCUSSION

The potential for a cell-based therapy that is safe, effective, and readily available may be an attractive option for patients with CHD who develop RV dysfunction and for whom effective therapies are currently lacking. The present study demonstrates for the first time that direct injection of human MSCs in a porcine model of RV pressure overload preserves ventricular function, attenuates ventricular remodeling, and reduces myocardial fibrosis through enhanced neovessel formation, proliferation of cardiomyocyte and endothelial cells, and recruitment of endogenous c-kit⁺ CSCs. We also present evidence that shows that MSC activation of GDF15 and its associated downstream SMAD 2/3 protein suppresses the hypertrophy response in vitro as well as in the neonatal swine model for RV pressure overload. Finally, these results are promising in support of the use of MSCs in pediatric patients who develop RV dysfunction.

Functional benefit of MSC injection. Our findings of preserved ventricular size and function of the RV with MSC therapy are consistent with previous animal models of myocardial infarction in the LV. Further, our findings demonstrate the universal potential of MSC treatment for both RV and LV dysfunction despite the biochemical, genetic, and physiological differences between the two ventricular chambers \((10)\).

Amado et al. \((1)\) showed in a porcine model of myocardial infarction that intramyocardial MSC injection led to an improvement in LV ejection fraction from 25.3 ± 1.6% postinfarction to 41.9 ± 0.7% at 8 wk of follow-up, whereas the placebo group showed no improvement. Similarly, Zhao et al. \((42)\) demonstrated that myocardial contractility in the adjacent zone of infarction recovered to near preinfarction levels in sheep treated with MSCs, whereas myocardial contractility continued to deteriorate in the control group. These studies also showed a significant decrease in myocardial scar formation in the cell-treated group. Although no previous animal model has looked specifically at the effect of MSC therapy recovering RV dysfunction, a similar study in neonatal sheep showed that direct injection of umbilical cord blood stem cells to the RV after PA banding led to significant improvement in load-independent indices of systolic and diastolic function in the RV, as assessed by pressure-volume loop analysis \((7)\). However, this study did not evaluate treatment-associated histolog-
A and B: neovessel formation was enhanced in the MSC-treated swines (placebo, \(n = 5\), 13.2 ± 0.9 vessels/mm\(^2\), vs. MSCs, \(n = 5\), 29.9 ± 2.0 vessels/mm\(^2\), \(P < 0.0001\)) as was the number of arterioles (placebo, \(n = 5\), 4.8 ± 1.1 arterioles/mm\(^2\), vs. MSCs, \(n = 5\), 9.2 ± 1.2 arterioles/mm\(^2\), \(P = 0.026\)).

C: MSC-treated swines exhibited enhanced mobilization of endogenous cardiac stem cells to the RV free wall compared with placebo (placebo, \(n = 5\), 333.3 ± 333.3 cells/cm\(^3\), vs. MSCs, \(n = 5\), 2,667 ± 667 cells/cm\(^3\), \(P = 0.014\)). This effect was not observed in the LV (placebo, \(n = 5\), 357.1 ± 357.1 cells/cm\(^3\), vs. MSCs, \(n = 5\), 714.3 ± 437.4 cells/cm\(^3\), \(P = 0.54\)).

D and E: MSC-treated myocardium also demonstrated increased stimulation of cardiomyocyte and endothelial cell proliferation, indicated by the presence of the proliferative cell surface marker Ki67 (Ki67+ cardiomyocytes: placebo, \(n = 5\), 0.89 ± 0.6 cells/mm\(^2\), vs. MSCs, \(n = 5\), 5.5 ± 0.6 cells/mm\(^2\), \(P = 0.0009\); Ki67+ endothelial cells: placebo, \(n = 5\), 0.07 ± 0.03 cells/mm\(^2\), vs. MSCs, \(n = 5\), 0.55 ± 0.03 cells/mm\(^2\), \(P < 0.0001\)).

F and G: expression of the Notch 1 receptor fragment, N1ICD, was not present on proliferating cardiomyocytes and endothelial cells within MSC-treated myocardium. All images were taken at \(\times 20\) with insets at \(\times 60\) magnification. Data are presented as means ± SE. LV, left ventricle; MSCs, mesenchymal stem cells; RV, right ventricle. *\(P < 0.05\); ***\(P < 0.0001\), unpaired \(t\)-test.
ical and biochemical changes occurring in the RV myocardium or reveal a mechanism for this recovery.

**Mechanism for attenuating cardiac hypertrophy.** We observed both in vitro and in vivo histological and biochemical evidence to suggest that MSC therapy prevents cardiomyocyte hypertrophy. These findings are in line with previous evidence in a sheep model of myocardial infarction that showed a reduction in global ventricular and individual cardiomyocyte hypertrophy, as well as a decrease in expression of hypertrophy-related genes compared with MSC-treated swines, which included BNP, β-MHC, and MLC-2v. Future studies are needed to determine how MSCs activate the GDF15 pathway and how GDF15 elicits cardioprotection in the pressure-overloaded RV myocardium.

**Remodeling of the myocardium.** Our results demonstrated that injected MSCs remodeled the pressure-induced hypertrophy of RV myocardium by promoting endogenous myocardial repair pathways. These pathways include increasing the number of c-kit+ CSCs, decreasing myocardial fibrosis, stimulating neovascularization (14, 15), and stimulating cardiomyocyte and endothelial proliferation (19). Other studies have demonstrated that injected MSCs activated and recruited resident c-kit+ CSCs to areas of LV ischemic myocardium. Hatzistergos et al. (13) first demonstrated that MSCs stimulated c-kit+ CSC proliferation into areas of LV infarction in a swine model. At 2 wk postinjection, the MSCs recruited ~9,000...
c-kit+/GATA-4+ CSCs per cm³ into the infarcted myocardium compared with a scarce number of these cells in the control. While the absolute number of c-kit+ CSCs observed to be recruited in our study is less (~3,000 CSCs per cm³), this may be due to two factors: our analysis was performed at a greater interval following the delivery of cells (4 wk rather than 2 wk postinjection), and we used a different model of myocardial injury, which was one of global RV stress compared with their focal region of ischemic infarcted left ventricle. Despite these differences, our observations are consistent with Hatzistergos et al. in that they suggest trophic actions produced by MSCs facilitate endogenous myocardial repair through activation and mobilization of c-kit+ CSCs. Interestingly, we found no relative increase in c-kit+ CSCs in the LV of MSC-treated swines, which provides further evidence for preferential recruitment of CSCs to areas of MSC injection. In addition, our study showed that MSCs stimulated the remodeling of the RV myocardium by stimulating endogenous cardiomyocyte and endothelial cell to proliferate which occurs by an unknown paracrine response elicited by the transplanted MSCs. MSC remodeling of the myocardium by secreted paracrine factors is supported by our data and others (39), which showed that very few transplanted MSCs were present at 4 wk postinjection (8). Our results show that the mechanism by which transplanted MSCs promote myocardial remodeling remains to be fully elucidated, and it is critical to determine the role of GDF15 in this myocardial remodeling process.

**Study limitations.** Our study was limited by a small sample size and short duration of follow-up. We described the benefits of MSC therapy at 4 wk postinjection, yet the durability of these improvements over a longer period of follow-up is unknown. On the basis of previous studies in models of myocardial infarction carried out for 12 wk or more, it seems possible that the positive effects of the MSCs would persist, but long-term studies using a model of chronic RV dysfunction will be required to address this important question. Additionally, the present model does not capture all of the salient features of complex CHD, which often includes a combination of pressure and volume overload, ischemia, chronic/acute conditions, and dysrhythmias. Although this study captures the acute conditions of RV pressure overload seen in CHD with very consistent physiological and biochemical findings at 4 wk, an alternative chronic model of pressure and volume overload might provide additional data for the use of MSC treatment in complex CHD patients.

**Implications for patients with congenital heart disease.** The findings from our study reinforce the hypothesis that cell-based therapies can provide a functional benefit to the nonischemic
MESENCHYMAL STEM CELLS PRESERVE NEONATAL RV FUNCTION

H1825

RV dysfunction seen in CHD patients. One of the most complex CHD conditions is hypoplastic left heart syndrome (HLHS), a condition where the LV is underdeveloped and the RV supports the systemic circulation through a series of staged palliative operations. The mortality rate for HLHS patients is high at 25–30% at 1 yr of life (27), which is due in part to RV dysfunction (24) and lack of adequate treatment options. Consequently, HLHS patients represent a CHD patient population that may benefit from rigorous stem cell trials. One such trial will be conducted at our own institution, delivering allogeneic MSCs to the RV of HLHS patients during stage II palliative, bidirectional cavopulmonary connection operations (38).

MSCs have been shown in animal studies, as well as in a recent randomized clinical trial in infected myocardial adult patients, to be safely administered as an allogeneic therapy for several reasons: the reduced expression of major histocompatibility complex I and the lack of expression of major histocompatibility complex II and costimulatory molecules CD80, CD86 and CD40, obviating the need for immune suppression (11, 21). Should we find similar benefits to RV function with MSC therapy, it would represent a potentially paradigm-shifting therapeutic alternative for patients with HLHS and other forms of CHD where the RV is under the stress of abnormal loading conditions (18).

In conclusion, we have demonstrated for the first time that direct injection of MSCs preserves RV function in an acute porcine model of pressure overload and attenuates ventricular remodeling. Further, therapy with MSCs increased the expression of GDF15, which has known antipathy and protective activites. These results establish the benefit of MSC treatment in this porcine model of RV dysfunction, a relevant model for studying stem cell biology for patients with CHD.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES


