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Fibrosis-related Gene Expression in Single Ventricle Heart Disease

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Abstract

Objective—To evaluate fibrosis and fibrosis-related gene expression in the myocardium of pediatric single ventricle (SV) subjects with right ventricular (RV) failure.

Study design—Real-time quantitative polymerase chain reaction was performed on explanted RV myocardium of pediatric SV subjects and non-failing controls. Subjects were divided into 3 groups: SV failing (SV-F, RV failure prior to or following Stage I palliation), SV non-failing (SV-NF, infants listed for primary transplant with normal RV function), and Stage III (Fontan or RV failure following Stage III). In order to evaluate subjects of similar age and RV volume loading, SV-F was compared with SV-NF and Stage III was compared with NF RV. Histologic fibrosis was assessed in all hearts. Mann-Whitney tests were performed to identify differences in gene expression.

Results—Collagen (Col1 α , Col3) expression is decreased in SV-F compared with SV-NF ($P = .019$ and $p = 0.035$, respectively), and is equivalent in Stage III compared with NF RV. Tissue inhibitors of metalloproteinase (TIMPs1, 3 and 4) are down-regulated in Stage III compared with NF RV ($p = 0.0047$, $p = 0.013$ and $p = 0.013$, respectively). Matrix metalloproteinases (MMP2, MMP9) are similar between SV-NF and SV-F, and between Stage III and NF RV. There is no

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difference in the prevalence of RV fibrosis by histology in SV subjects with RV failure (18%) compared with those with normal RV function (38%).

Conclusions—Fibrosis is not a primary contributor to RV failure in infants and young children with SV. Additional studies are required to understand whether anti-fibrotic therapies are beneficial in this population.

Keywords

Hypoplastic left heart syndrome (HLHS); congenital heart disease; pediatric heart failure; collagen; matrix metalloproteinases (MMPs); tissue inhibitors of metalloproteinases (TIMPs)

Hypoplastic left heart syndrome (HLHS) and other single ventricle congenital heart diseases (SV) characterized by severe underdevelopment of the left ventricle (LV), result in a single functional pumping chamber of right ventricular (RV) morphology. The single RV in these patients must function in relation to the increased afterload of the systemic circulation. As a result, heart failure (HF) can result in death or the need for heart transplantation in children with SV^{1, 2}. However, the exact mechanisms underlying SV HF are poorly understood, limiting the ability to identify effective medical therapies.

Cardiac fibrosis is associated with the progression of HF in adults, and the severity of fibrosis is independently associated with cardiovascular mortality^{3, 4}. Diffuse, reactive fibrosis of the LV is present by histopathologic evaluation in the majority of explanted hearts from adults with end-stage HF⁵. Chronic activation of the renin angiotensin aldosterone system (RAAS) in adult HF is one mechanism contributing to pathologic cardiac fibrosis. Increased angiotensin II and aldosterone promote the maladaptive accumulation of extracellular matrix (ECM) proteins through an imbalance of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), in part via elevated transforming growth factor beta (TGF β) signaling, resulting in increased collagen synthesis and decreased collagen breakdown. This imbalance in ECM homeostasis is a hallmark of myocardial fibrosis, and its physiological consequences include increased mechanical stiffness, decreased compliance, and ultimately contractile dysfunction^{3, 4, 6–10}.

Pharmacologic therapies targeting multiple components of the RAAS pathway have significantly decreased morbidity and mortality in adult LV HF patients; aldosterone antagonists, for example, are used in the setting of RAAS activation to minimize adverse cardiac remodeling including progression of fibrosis^{11–14}. However activation of the RAAS pathway has not been demonstrated specifically in pediatric SV patients and a double-blind placebo-controlled trial of the angiotensin converting enzyme inhibitor (ACEi) enalapril in infants with SV physiology demonstrated that enalapril did not prevent the development of ventricular dysfunction or HF¹⁵. The purpose of this study was to profile fibrosis-related gene expression and histologic fibrosis in the myocardium of pediatric SV patients with RV failure.

Methods

Explanted myocardium from children (10 years of age or younger) of all races, sex, and ethnic backgrounds were available for this study through the Institutional Review Board-approved University of Colorado Pediatric Cardiac Tissue Bank. All heart tissue was dissected and rapidly flash frozen in the operating room immediately after removal from the subject. SV subjects were defined as those patients with ductal-dependent single ventricle physiology. Only those patients with a systemic RV and a hypoplastic LV (n=19) were included; SV subjects of LV morphology were excluded. All SV hearts were obtained from patients at the time of transplant. SV patients were divided into 3 groups: the SV non-failing (SV-NF) group included infants listed for primary transplant without evidence of RV systolic dysfunction on echocardiography, the SV failing (SV-F) group included infants either without any prior surgical intervention or infants status post Stage I surgical palliation with decreased RV systolic function on echocardiogram and RV failure as the main indication for transplant, and the Stage III group included children with end-stage Fontan failure (RV failure and/or plastic bronchitis or protein losing enteropathy). Non-failing RV (NF RV, n=8) myocardium was obtained from biventricular hearts without congenital heart disease, acquired from donor children whose hearts could not be placed for technical reasons. In order to evaluate subjects of similar age and RV volume loading, SV-F was compared with SV-NF (both primarily comprised of infants) and Stage III was compared with the NF RV.

At the time of explant, the basal aspect of the ventricles is sent to the clinical pathology laboratory at the Children's Hospital Colorado for routine examination. Hematoxylin and eosin (H&E) staining was performed on all SV samples and trichrome staining was performed on select specimens at the discretion of the clinical pathologist. For the purposes of this study, a single pediatric pathologist reviewed all available slides. Qualitative assessment of fibrosis was descriptive, noting the presence or absence of fibrosis as well as location, consistent with routine clinical pathologic evaluation.

Total RNA from each frozen tissue sample was extracted using MirVanaRNA Isolation Kit (Ambion #AM1561) as previously described²⁰. Reverse transcription of mRNA was performed using the iScript™ cDNA Synthesis Kit (BioRad), and reverse transcription of microRNA (miRNA) was performed using the miScript Reverse Transcription Kit (Qiagen) as described⁷. Relative expression of each gene target was quantified using real-time polymerase chain reaction (RT-PCR). mRNA levels of 18S were used for normalization of all mRNA targets, whereas hsa-miR-361 was used as an internal control for miRNA¹⁶. The average of at least two technical replicates was used for each independent experiment; primer sequences are listed in Table I (available at www.jpeds.com).

All statistical procedures were performed with GraphPad Prism software (GraphPad Software, Inc). Mann-Whitney tests were performed to compare non-parametric RT-PCR data between 2 groups. Statistical significance was set a priori at $p < 0.05$ and all data are presented as mean $\pm$ standard error of the mean (SEM). Linear regression was performed to evaluate correlation between continuous variables.

Results

SV demographic information, indication for transplant, medications, and histology results are included in Table 2. The SV-NF group had 7 subjects with a median age of 0.19 years (range 0.05 – 0.38) and was 86% male. The SV-F group had 6 subjects with a median age of 0.66 years (range 0.41 – 1.27) and was 67% male. The NF RV group had 8 subjects with a median age of 7.2 years (range 1.3 – 9.5) and was 63% male. The Stage III group had 6 subjects with a median age of 5.5 years (range 3.3 – 10.1) and was 83% male. 6 of the SV-NF subjects (86%), 3 of the SV-F subjects (50%), and 3 of the Stage III subjects (50%) were on ACEi therapy. Representative histologic sections of SV myocardium are illustrated in Figure 1, A–C (available at www.jpeds.com), demonstrating the variability with which fibrosis is detected in this population. The presence of RV fibrosis by histology in young SV subjects is inconsistent and is not more prevalent in SV subjects with end-stage RV failure: 3/8 (38%) of subjects with normal RV function had evidence of RV fibrosis while only 2/11 (18%) subjects with RV failure had histologic evidence of RV fibrosis (Figure 1; available at www.jpeds.com).

Collagen (Col) gene expression, association with age, and expression of myocardial microRNA 29b (miR-29b, a regulator of collagen levels) in SV are shown in Figure 2. Both Col1 α and Col3 are higher in the SV-NF group in comparison with the SV-F group (Figure 2, A and B $p=0.019$ and $p=0.035$, respectively). There is a trend toward an increase in the ratio of Col1 α /Col3 in SV-NF compared with SV-F (Figure 2, C; $p=0.051$), however this trend did not meet statistical significance. Myocardial miR-29b is upregulated in SV-F in comparison with SV-NF (Figure 2, D; $p=0.014$). Collagen expression, including Col1 α /Col3, and miR-29b expression are similar between SV Stage III and NF RV (Figure 2, E; $p=0.41$; Figure 2, F; $p=0.85$; Figure 2, G; $p>0.99$; Figure 2, H; $p=0.41$). Linear regression of Col1 α and Col3 versus age at explant in SV-F and SV-NF subjects reveals moderate inverse correlation, with higher collagen expression in younger subjects (Figure 2, I; $R^2=0.54$ and $p=0.004$; Figure 2, J; $R^2=0.34$ and $p=0.036$). Linear regression of the Col1 α /Col3 ratio versus age at explant in SV subjects does not demonstrate significant correlation (Figure 2, K; $R^2=0.22$ and $p=0.11$). Additionally, there is no correlation between Col1 α and Col3 versus age at explant in Stage III and NF RV subjects (Col1 α $R^2=0.16$ and $p=0.16$, Col3 $R^2=0.20$ and $p=0.10$, Col1 α /Col3 $R^2=0.08$ and $p=0.34$).

Expression of other genes involved in ECM regulation are shown in Figure 3. MMP2 expression is similar between SV-NF and SV-F (Figure 3, A; $p=0.37$), and there is a trend toward increased MMP9 expression in the SV-F group (Figure 3, B; $p=0.07$). TGF β 1 and TGF β 2 expression is similar between SV-NF and SV-F groups (Figure 3, C $p=0.95$ and Figure 3, D $p=0.73$, respectively). In the failing SV post stage III palliation, MMP2 and MMP9 expression is similar to NF RV (Figure 3, E $p=0.85$ and Figure 2, F $p=0.14$, respectively). There are trends towards lower TGF β 1 and higher TGF β 2 expression in Stage III compared with NF RV, although neither reached statistical significance (Figure 3, G $p=0.081$ and Figure 3, H $p=0.081$, respectively). Myocardial gene expression of TIMPs 1–4 are similar between SV-NF and SV-F groups (Figure 3, I $p=0.95$; Figure 3, J $p=0.95$; Figure 3, K $p=0.45$; Figure 3, L $p=0.73$). Although TIMP2 was equivalent between Stage III and

NF RV (Figure 3, N, $p=0.75$), TIMPs 1, 3 and 4 are all significantly down-regulated in Stage III (Figure 3, M $p=0.0047$; Figure 3, O $p=0.013$; Figure 3, P $p=0.013$).

Myocardial gene expression of biomarkers associated with fibrosis are demonstrated in Figure 4. Corin (Figure 4, A; $p=0.53$), suppression of tumorigenicity 2 ligand (ST2L; Figure 4, B; $p>0.99$), and galectin-3 (Gal3; Figure 3C, $p=0.70$) are similar between SV-NF and SV-F groups. Myocardial Corin (Figure 4, D; $p=0.14$) and ST2L (Figure 4, E; $p=0.14$) expression is similar between Stage III and NF RV, whereas Gal3 is lower in the Stage III group (Figure 4, F; $p=0.043$).

Discussion

Our current findings demonstrate that neither histologic fibrosis nor a pro-fibrotic gene expression profile is a consistent hallmark of RV failure in young patients with single ventricle. Given known developmental upregulation and accumulation of collagen during the neonatal period^{17, 18}, infant patients with single ventricle. were analyzed separately from the Stage III pediatric subjects, which also served to minimize changes that may arise solely from differences in RV volume load. Because the primary emphasis of our analysis was to study fibrosis and changes in fibrotic gene expression in response to RV failure, SV infants with RV failure (SV-F) were compared with infants with single ventricle. with normal RV function who underwent elective heart transplantation (SV-NF) and pediatric subjects with single ventricle with RV failure post-stage III (Stage III) were compared with RV from age-matched, non-failing, biventricular subjects (NF RV). Results from both infant and young subjects with single ventricle suggest that pathologic RV fibrosis may not be a primary contributor to RV dysfunction in pediatric patients with single ventricle and the putative benefit of anti-fibrotic agents may be limited in this population^{15, 19, 20}.

In the predominantly infant aged cohort that either did not undergo any surgical palliation or underwent Stage I palliation, collagen expression is significantly less in subjects that were transplanted for RV failure compared with those subjects with single ventricle that underwent elective heart transplantation. This finding is contrary to the hypothesis that myocardial fibrosis is contributory to RV failure in this population. Moreover, this decreased collagen expression in SV-F may represent adverse remodeling that predisposes to ventricular dilation and dysfunction, akin to the adverse ECM remodeling process acutely post-myocardial infarction where failure of ECM support has been associated with ventricular wall thinning and slippage of myocyte fascicles²¹. In addition, the trend toward increased MMP9 and unchanged TIMP expression may favor ECM degradation and loss of ECM organization in the SV-F group. Independent of the involvement of MMP9 in ECM degradation, MMP9 is known to compromise contractile function of cardiomyocytes through the proteinase-activated receptor-1 (PAR-1) signaling cascade²², and may contribute to RV dysfunction in SV-F. Additionally, the miR-29 family targets and down-regulates many fibrosis-related mRNAs, including multiple collagen types, fibrillins, and elastin²³. Elevated miR-29b in SV-F may be consistent with ongoing collagen degradation and the general anti-fibrotic milieu in this group, which is in stark contrast to the downregulation of miR-29 in the region of fibrotic scar after myocardial infarction²⁴.

A caveat to the argument that the lower collagen content in SV-F is detrimental arises from the significant age difference between the SV-NF and SV-F groups. Although all subjects in the SV-NF and SV-F groups were < 1.5 years of age, subjects in the SV-NF group are younger than those in the SV-F group ($p=0.0012$). Given the significant inverse correlation between Col1 α and Col3 expression and age in this cohort (Figure 2), higher Col1 α and Col3 gene expression in the SV-NF group may be a physiological process that contributes to a developmental accumulation of functional collagen similar to what has been previously described in animal models^{17, 18}. Lastly, an attenuated fibrotic response has been demonstrated specifically in young rats²⁵ and in pediatric patients with dilated cardiomyopathy²⁶, suggesting that age and duration of disease also influence fibrosis development. Although we cannot determine whether collagen downregulation is pathologic in SV based on our current associative data, our results do support the conclusion that RV failure can occur independently from histologic fibrosis and pro-fibrotic gene expression, the latter of which also varies with age in young SV patients.

With regard to patients with single ventricle who required heart transplant following Stage III surgical palliation, there is also absence of a pro-fibrotic collagen expression profile. In particular, prior studies have demonstrated that an elevated Col1 α /Col3 ratio is associated with a detrimental increase in myocardial stiffness, decreased ejection fraction, and increase left ventricular end diastolic pressure^{27–30}. Interestingly, in our SV cohort, there is a trend toward increased Col1 α /Col3 ratio in subjects with histologic evidence of RV fibrosis, illustrating the association between gene expression and the histologic presence of fibrosis ($p=0.084$, Figure 5; available at www.jpeds.com). Notably, the Col1 α /Col3 ratio in Stage III subjects was equivalent to the NF RV group. Additionally, Gal3, a soluble beta-galactoside-binding lectin released by activated cardiac macrophages has been shown to induce cardiac fibroblast proliferation, collagen deposition, and ventricular dysfunction in animal models³¹, with inhibition of Gal3 resulting in attenuated cardiac fibrosis³². Low Gal3 expression in Stage III when compared with NF RV supports a possible down-regulation of Gal3-related fibrotic pathways.

A striking difference between Stage III and NF RV emerges when evaluating TIMP expression: TIMPs 1, 3 and 4 are markedly down-regulated in the Stage III group. Interestingly, the down-regulation of TIMPs 1, 3 and 4 in Stage III is similar to what has previously been described in ischemic cardiomyopathy³³ as well as adult idiopathic dilated cardiomyopathy²⁶ although increases in TIMPs 1–4 have also been described in adult dilated cardiomyopathy²⁹. TIMPs are potent reversible inhibitors of activated MMPs and may have additional MMP-independent pro-fibrotic activity³⁴. Alterations in TIMP expression appear to be variable among disease etiologies and heart failure stages³⁴, suggesting that the regulation of TIMPs are not solely determined by MMP levels even though an interplay between MMPs and TIMPs is required to maintain ECM homeostasis. It is postulated that this low expression of TIMPs in Stage III would favor ECM degradation and turnover. Alternatively, this pattern of TIMP downregulation may represent a common, end-stage phenotype.

Assessment of TGF β 1 and TGF β 2 expression did not demonstrate any statistically significant differences between Stage III subjects and NF RV. Both TGF β 1 and TGF β 2 are

pro-fibrotic^{35, 36}, with trends towards increased TGF β expression in adult dilated cardiomyopathy³⁰. Given the known upregulation of TGF β 1 by angiotensin II³⁷ it is worth noting that 50% of the Stage III subjects were on ACEi at the time of explant; however among our small cohort of Stage III subjects (n=6), TGF β 1 expression was similar between those with and without ACEi treatment (p>0.99). The role of TGF β 2 in cardiac fibrosis is not well-defined, although in a model of hypertrophic cardiomyopathy, there is suggestion that a decrease in myocardial TGF β 2 is associated with less fibrosis³⁶. The trend toward increased TGF β 2 in Stage III could represent a propensity for fibrosis, however this is difficult to conclude particularly in the context of equivocal or anti-fibrotic regulation of other fibrosis-related genes. Certainly, TGF β s are involved in a wide variety of cell functions aside from promoting ECM deposition.

The current study has several limitations. First, histologic results were obtained from routine clinical pathologic evaluation of the explanted myocardium by H&E and occasional trichrome staining; thus, it is possible that more specific histologic evaluation of the myocardium could reveal a higher incidence of RV fibrosis. However, the minimal histologic evidence of fibrosis in SV is consistent with the lack of upregulation of fibrosis-related gene expression in the SV myocardium. Second, gene expression assessment of MMPs and TIMPs at the mRNA level may not completely reflect their physiologic level of activity. Although all SV-F samples are considered to have end-stage RV failure, assessment of MMP and TIMP levels at one point in time may not necessarily reflect the dynamic nature of this proteolytic system²¹. Nevertheless, alterations in fibrosis-related genes are present prior to overt phenotypic changes³⁸; thus, the lack of a prominent fibrotic gene expression pattern may suggest that, excluding new cardiovascular insults, future fibrosis would be minimal. Third, in this study we chose to investigate select genes of interest implicated in promoting fibrosis in humans and animal models of HF^{29, 39–42}. There are likely other mechanisms contributing to RV dysfunction in SV that were not evaluated, such as enhanced myocardial collagen cross-linking⁴³ or increased myofibril-mediated stiffness via titin hypophosphorylation²⁸. Fourth, it is important to recognize that the SV explants in our cohort are infants and children with a maximum age of 10 years (with only 3 patients older than 5 years). There is a population of older SV patients that experience RV failure as adolescents and young adults and given the significant influence of age, it is unlikely that our results are translatable to an older SV HF population. Fifth, as noted above, it is possible that the use of ACEi (86% of SV-NF, 60% of SV-F, 50% of Stage III) may have decreased fibrosis development. Further statistical investigations were limited secondary to the relatively small sample size, however 80% of SV subjects with histologic evidence of fibrosis were on ACEi therapy. Importantly, possible prevention of fibrosis with ACEi did not preclude development of RV failure. Finally, our cohort is small, which is a limitation inherent to single center studies in the SV population and makes it challenging to draw definitive conclusions based on statistical significance.

In conclusion, fibrosis is not a primary contributor to RV failure in infants and young children with SV. This attenuated fibrotic response may be secondary to young age and short duration of disease (characteristic of this disease process) and possibly differences between RV and LV adaptation. Thus, pharmacologic interventions targeting the development of

fibrosis may have limited benefit and require additional investigations specifically in the young SV population.

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Abbreviations

HLHS	Hypoplastic left heart syndrome
SV	single ventricle congenital heart disease
RV	right ventricle
HF	heart failure
RAAS	renin angiotensin aldosterone system
ECM	extracellular matrix
MMPs	matrix metalloproteinases
TGFβ	transforming growth factor beta
TIMPs	tissue inhibitors of metalloproteinases
NF	non-failing
Col	collagen
ST2L	suppression of tumorigenicity 2 ligand
Gal3	galectin-3
miR-29b	microRNA-29b

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Figure 1. Collagen gene expression profile in SV hearts

A. *Col1 α* gene expression is lower in the SV-F group compared with the SV-NF group ($p=0.019$). B. *Col3* gene expression is lower in the SV-F group compared with the SV-NF group ($p=0.035$). C. *Col1 α /Col3* ratio is similar between SV-F and SV-NF ($p=0.051$). D. Myocardial miR-29b is higher in SV-F compared with SV-NF ($p=0.014$). E–G. *Col1 α* , *Col3* and *Col1 α /Col3* are similar between Stage III and NF RV ($p=0.41$, $p=0.85$, $p>0.99$). H. Myocardial miR-29b is similar between Stage III and NF RV ($p=0.41$). I. Linear regression of *Col1 α* versus age in SV-NF and SV-F subjects demonstrates a moderate inverse correlation ($R^2=0.54$, $p=0.004$). J. Linear regression of *Col3* versus age in SV-NF and SV-F subjects demonstrates a mild inverse correlation ($R^2=0.34$, $p=0.036$). K. Linear regression of *Col1 α /Col3* ratio versus age in SV-NF and SV-F subjects does not demonstrate a significant correlation ($R^2=0.22$, $p=0.11$).

Figure 2. Gene expression of extracellular matrix regulators in SV myocardium

A–B. MMP2 and MMP9 expression is similar between SV-F and SV-NF ($p=0.37$, $p=0.07$).
 C–D. TGFβ1 and TGFβ2 expression is similar between SV-F and SV-NF ($p=0.95$, $p=0.73$).
 E–F. MMP2 and MMP9 expression is similar between Stage III and NF RV ($p=0.85$, $p=0.14$).
 G–H. TGFβ1 and TGFβ2 expression is similar between Stage III and NF RV ($p=0.081$, $p=0.081$).
 I–L. TIMP1–4 expression is similar between SV-F and SV-NF ($p=0.95$, $p=0.95$, $p=0.45$, $p=0.73$).
 MP. TIMP1, 3 and 4 are lower in Stage III compared with NF RV ($p=0.0047$, $p=0.013$, $p=0.013$).
 TIMP2 expression is similar between the two groups ($p=0.75$).

Figure 3. Myocardial expression of fibrosis-related biomarkers in SV

A–C. Myocardial Corin, ST2L, and Gal3 expression is similar between SV-F and SV-NF ($p=0.53$, $p>0.99$, $p=0.70$). D–F. Myocardial Corin and ST2L expression is similar between Stage III and NF RV ($p=0.14$, $p=0.14$). Gal3 expression is decreased in Stage III compared with NF RV ($p=0.043$).

Figure 4.

Figure 5.

Table 1**Primer Sequences for RT-PCR**

Listed are the forward (F) and reverse (R) primer sequences used for each target gene. All primers are presented in a 5'-3' orientation.

Primer	Sequence
Corin F	GCT GGC AGG AGA TAT TGA GTC
Corin R	TGT AAA GTG GTC CCA TTG AGG
Gal-3 F	GAG CCA GCC AAC GAG CGG AA
Gal-3 R	CTT GAG GGT TTG GGT TTC CAG A
ST2L F	CCT ACC ATT GAC CTC TAC AAC TG
ST2L R	TAA TCA CCT GCG TCC TCA G
TGFβ1 F	GCC TTT CCT GCT TCT CAT GG
TGFβ1 R	TCC TTG CGG AAG TCA ATG TAC
TGFβ2 F	CCA AAG GGT ACA ATG CCA AC
TGFβ2 R	TCT AAA TCT TGG GAC ACG CAG
TIMP-1 F	GGT CCCAGA TAG CCT GAA TC
TIMP-1 R	GAA AGA TGG GAG TGG GAA CAG
TIMP-2 F	CCC TCT GTGACT TCA TGG TG
TIMP-2 R	GAG ATG TAG CAC GGG ATC ATG
TIMP-3 F	CCT TCT GCA ACT CCG ACA TC
TIMP-3 R	GCC TCG GTA CAT CTT CAT CTG
TIMP-4 F	CTA CCA TCT GAA CTG TGG CTG
TIMP-4 R	CTA CCA TCT GAA CTG TGG CTG
MMP-2 F	GAC GAT GAG CTA TGG ACC TTG
MMP-2 R	GTT GTA CTC CTT GCC ATT GAA
MMP-9 F	CTG GAG GTT CGA CGT GAA G
MMP-9 R	TCC TGG CAG AAA TAG GCT TTC
miR-29b	GGG TAG CAC CAT TTG AAA TCA GTG TT
Collagen1α F	CCCTGGAAAGAATGGAGATG
Collagen1α R	TCCAAACCACTGAAACCTCTG
Collagen3 F	AAG TCA AGG AGA AAG TGG TGG
Collagen3 R	CTC GTT CTC CAT TCT TAC GAC G
U6	CGG CAG CAC ATA TAC TAA AAT
18S F	GCC GCT AGA GGT ATT CTT G
18S R	CTT TCG CTC TGG TCC GTC GTC TT

Table 2

SV demographics and histology

All SV subjects are listed, including age at heart explant, sex, most recent surgical intervention prior to explant, time from last surgical palliation, indication for heart transplantation, and medications at the time of explant. RV fibrosis type for each subject is listed, obtained from histologic evaluation of the explanted myocardium by clinical pediatric pathologists. SV-NF = single ventricle, non-failing. SV-F = single ventricle, failing. F = female. M = male. NS = no surgery. N/A = not applicable. PB = protein losing enteropathy. PGE = prostaglandin E1. ACEi = angiotensin converting enzyme inhibitor. PDE3i = phosphodiesterase 3 inhibitor. PDE5i = phosphodiesterase 5 inhibitor. EFE = endocardial fibroelastosis.

Group	Age (years)	Sex	Surgical Intervention	Time from last palliation (years)	Indication for Transplant	Medications	RV Fibrosis
SV-NF	0.05	F	NS	N/A	Elective	Diuretic, PGE	None
SV-NF	0.09	M	NS	N/A	Elective	ACEi, Diuretic, PGE	None
SV-NF	0.18	M	NS	N/A	Elective	ACEi, Diuretic	None
SV-NF	0.19	M	NS	N/A	Elective	ACEi	None
SV-NF	0.23	M	NS	N/A	Elective	ACEi, Diuretic	Interstitial and EFE
SV-NF	0.34	M	NS	N/A	Elective	ACEi, PGE	None
SV-NF	0.38	M	NS	N/A	Elective	ACEi, Diuretic	EFE
SV-F	0.41	M	NS	N/A	RV failure	Diuretic, PDE3i	None
SV-F	0.44	M	Stage I	0.42	RV failure	ACEi, Diuretic, PDE3i	Subendocardial
SV-F	0.58	M	Stage I	0.53	RV failure	Unknown	None
SV-F	0.73	F	Stage I	0.48	RV failure	Digoxin, Diuretic	None
SV-F	0.99	F	Stage I	0.92	RV failure	Digoxin, ACEi, Diuretic	None
SV-F	1.27	M	Stage I	1.3	RV failure	ACEi, Diuretic	None
Stage III	3.28	M	Stage III	0.58	RV failure	Digoxin, ACEi, Diuretic, PDE3i	None
Stage III	4.76	M	Stage III	1.8	RV failure, PB	ACEi, Diuretic, PDE3i, PDE5i	Interstitial and subendocardial
Stage III	4.96	M	Stage III	2.0	RV failure	Digoxin, ACEi, Diuretic, PDE3i	None
Stage III	6.05	F	Stage III	1.9	RV failure, PLE	Diuretic, PDE3i, PDE5i	None
Stage III	7.46	M	Stage III	4.8	RV failure	Unknown	None
Stage III	10.06	M	Stage III	6.2	PLE	Diuretic, PDE3i, PDE5i	Focal myocardial, EFE