Molecular Signature of a Right Heart Failure Program in Chronic Severe Pulmonary Hypertension

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Right heart failure is the cause of death of most patients with severe pulmonary arterial hypertensive (PAH) disorders, yet little is known about the cellular and molecular causes of right ventricular failure (RVF). We first showed a differential gene expression pattern between normal rat right and left ventricles, and postulated the existence of a molecular right heart failure program that distinguishes RVF from adaptive right ventricular hypertrophy (RVH), and that may differ in some respects from a left heart failure program. By means of microarrays and transcriptional sequencing strategies, we used two models of adaptive RVH to characterize a gene expression pattern reflective of growth and the maintenance of myocardial structure. Moreover, two models of RVF were associated with fibrosis, capillary rarefaction, the decreased expression of genes encoding the angiogenesis factors vascular endothelial growth factor, insulin-like growth factor 1, apelin, and angiotensin-1, and the increased expression of genes encoding a set of glycolytic enzymes. The treatment of established RVF with a β -adrenergic receptor blocker reversed RVF, and partly reversed the molecular RVF program. We conclude that normal right and left ventricles demonstrate clearly discernable differences in the expression of mRNA and microRNA, and that RVH and RVF are characterized by distinct patterns of gene expression that relate to cell growth, angiogenesis, and energy metabolism.

Keywords: pulmonary hypertension; right heart failure; gene expression

Chronic right heart failure is usually a pump failure and a consequence of the loss or impairment of functional contractile elements or inadequate adaptation to a variety of congenital or acquired cardiac abnormalities (1, 2). Because considerable experimental knowledge exists regarding different forms of chronic left ventricular failure (LVF), our concepts of mechanisms of right ventricular failure (RVF) have been largely shaped by investigations of models of LVF. An extensive review of the literature revealed that mechanisms of acute RVF were examined in great detail in animal models of acute right ventricular afterload increase (3), but that no comparable knowledge base exists regarding differences

between RV hypertrophy and chronic RVF in the setting of the remodeled lung circulation in chronic severe pulmonary hypertension (PH). Yet RVF is increasingly recognized as an important clinical problem (4), and most patients with severe PH die from RVF. Equally important, RVF is the worst prognostic indicator in patients with LVF (5, 6). The two principal barriers responsible for this knowledge gap include a paucity of data based on experimental chronic RVF, and the notion that RVF is irreversible.

Because the embryological development of the left ventricle (LV) and right ventricle (RV) are different and the normal RV is thin-walled compared with the LV, we propose that the means by which the RV adapts to pressure overload and some of the cellular and molecular events that lead to RV hypertrophy and failure may be different from those means and events in the left chamber of the heart. During cardiac development, the primary heart field gives rise to the LV, whereas cells of the secondary heart field develop into the RV (7, 8). We now understand that chamber-specific gene expression with different transcriptional control leads to the formation of the RV and LV (9–11). For example, the transcription factor paired-like homeodomain 2 (Pitx2) is responsible during the primitive heart tube stage for the right/left specification of the chambers and outflow tracts, and GATA binding protein 4 is required for the expression of heart and neural crest derivatives expressed 2 and the formation of the RV (12–14). The homeodomain transcription factor Pitx2 is coexpressed with Tbx1 in secondary heart field cells, and the activation of the Pitx2 enhancer by T-box 1 (Tbx1) affects asymmetric cardiac morphogenesis (15).

Of importance in considering potential differences between right-sided and left-sided heart adaptation and failure, the α 1-adrenergic agonists increase the contractile force of the LV, whereas they cause a reduction of force in the corresponding normal RV (16). Likewise, the long-term infusion of norepinephrine led to LV hypertrophy, whereas the RV did not respond with hypertrophy (17).

Finally, the irreversibility of RVF is of widespread concern. In fact, RV function fully recovers within weeks after lung transplantation and RV unloading in patients with endstage PH (18). How the failing RV returns to normal function after lung transplantation remains unknown.

Because a temporal, structural, and molecular transition from compensated RV hypertrophy to chronic RVF is likely, we designed experiments to contrast RV hypertrophy and RVF after we had applied two converging strategies to demonstrate differences in molecular pattern between the normal RV and normal LV. We compared the global mRNA expression pattern between RV and LV tissue, using microarray technology, and we then sequenced the RV and the LV transcriptome to identify differences in microRNA (miRNA) expression between the two ventricles, which have not been investigated to the best of our knowledge. We included miRNA analyses in our studies because of the increasing recognition of the role of miRNA in the control of cardiac growth and function.

To examine whether a molecular RVF program exists, both the expression of miRNA and mRNA in normal hearts and in RV and

(Received in original form October 19, 2010 and in final form June 28, 2011)

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This work was supported by the Victoria Johnson Center for Obstructive Lung Disease Research. B.C. was supported by the 2009 Step-Up Undergraduate Summer Research Program of the National Institutes of Health. J.I.D. is a Graduate Student in the Integrative Life Sciences Program of Virginia Commonwealth University. H.J.B. was supported by a stipend from the Netherlands Heart Foundation.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 45, pp 1239–1247, 2011

Originally Published in Press as DOI: 10.1165/rcmb.2010-0412OC on June 30, 2011

Internet address: www.atsjournals.org

LV tissue from two different models of RV stress without RVF and two models of endstage pulmonary arterial hypertension (PAH) and RVF were compared. Finally we attempted to rescue the failing RV, to begin to assess the reversible components of a molecular RV failure program.

MATERIALS AND METHODS

Models of Compensated RV Hypertrophy and RV Failure

Using different modes of hemodynamic stress and dietary or pharmaceutical interventions, we created models of either compensated RV hypertrophy (pulmonary artery banding [PAB] and chronic hypoxia) or RV failure (Sugen5416 [SU5416]/hypoxia or PAB with a Cu^{2+} -depleted diet). This distinction was made on the basis of a combination of findings: (1) a clearly reduced cardiac output, as measured by thermodilution; (2) changes consistent with RV dysfunction, imaged by cardiac ultrasound (i.e., a significant decrease in tricuspid annular plane systolic excursion [TAPSE] compared with control rats, paradox movements of the interventricular septum, or pericardial fluid); (3) a loss of myocardial capillaries, as indicated by immunohistochemistry or confocal microscopy; and (4) myocardial fibrosis, as demonstrated by immunohistochemistry (19).

Additional Research

PAB, chronic hypoxia, angioproliferative PH, and treatment with carvedilol were investigated as described previously (19).

Additional details regarding the isolation of RNA, microarray analysis, quantitative RT-PCR, miRNA sequencing, and Western blot analysis are available in the online supplement. The decision-making process that guided the experimental strategy and analysis of data is presented in Figure 1.

RESULTS

Screening of Microarray Gene Expression in Right Ventricles from Animals with and without RVF

Before identifying differences in gene expression patterns in the models of RV hypertrophy without failure (chronic hypoxia) and of RVF (SU5416/hypoxia), we sought to identify differences between the normal RV and normal LV, using microarray analysis (Figure E1 in the online supplement) and miRNA sequencing (Figure E2). This analysis yielded a small but significant set of

differences in gene expression between the two ventricles (Table E1). These data served as a reference for the subsequent comparison of expression data derived from rats with RV hypertrophy and RVF.

We next extracted total RNA from the free walls of RVs in rats injected with the vascular endothelial growth factor (VEGF) receptor antagonist SU5416, rats exposed to chronic hypoxia for 4 weeks, and rats receiving SU5416 in combination with chronic exposure to hypoxia. The administration of SU5416 alone caused emphysema and a mild degree of PH in rats (20). Chronic exposure to hypoxia for 4 weeks generated moderate PH and RV hypertrophy, but not RVF (21). Treatment with SU5416 in combination with chronic hypoxia was characterized by severe PH and RVF (Figure E3) (19, 22).

The heat-map display (Figure 2a) generated from microarray hybridization and detailed analysis illustrates that the RVF gene expression pattern was indeed distinct from the expression pattern observed in RV hypertrophy triggered by chronic hypoxia. The gene expression pattern of the failing RV (SU5416/chronic hypoxia) was also distinct from the expression pattern observed in the RV of normal control animals and animals treated with SU5416 alone. Table E2 lists the biological processes associated with the differential expression between the normal and failing RV. Finally, the gene expression pattern in the failing RV was also different from that in the nonfailing companion LV (Figure E4).

Predictability of the RVF Signature

We questioned whether the gene expression pattern could distinguish between a normal RV, an RV undergoing adaptive hypertrophy, and a failing RV. We thus performed a prediction analysis, applying a leave-one-out cross-validation (LOOCV). The dataset consisted of expression data from six biological replicates per class ($n = 6$ from each class: normal RV, hypoxia RV, and SU5416/hypoxia RV). The LOOCV method takes into account the sample size, and is suitable for small sample numbers (23–25). Averaging the technical replicate data in each class, four prediction algorithms were used: diagonal linear discriminant analysis, the one-nearest neighbor, the three-nearest neighbors, and nearest centroid. Performing 2,000 permutations, which resulted in $P < 0.0005$ for all prediction algorithms,

Figure 1. Experimental strategy. Schematic representation of the approach taken to identify the molecular signature that can discriminate between right ventricular (RV) hypertrophy and RV failure. miRNA, microRNA; qRT-PCR, quantitative, real-time PCR.

Figure 2. Comparison of differentially expressed genes between normal RV, hypertrophied RV, and failing RV. (a) Six biological replicates per condition were hybridized to Agilent Whole Rat Genome microarrays (Agilent Technologies, Santa Clara, CA). We identified 3,399 genes as differentially expressed between normal RV and failing RV, using the significance analysis of microarrays (SAM) algorithm at a Δ value of 0.54 (false discovery rate = 4.85%). This list was further narrowed down by using the SAM algorithm. A two-class unpaired comparison between hypoxia (Hx) RV and SU5416/hypoxia (SuHx) RV yielded 1,175 differentially expressed genes at a Δ value of 0.71 (false discovery rate = 4.93%). The average expression values of normal RV, hypertrophied RV, and failing RV relative to the average of normal RV expression for these significant genes were clustered using Cluster 3.0 (55), and visualized using Java Tree-view (56). *Green* indicates lower expression under a given condition than in normal RV, and *red* indicates higher expression under a given condition than in normal RV. *Black* indicates that the expression under the given condition is equal to the expression in normal RV. (b) Above, quantitative, real-time PCR analysis of insulin-like growth factor 1 (IGF-1) (a, $P < 0.01$ versus control; b, $P < 0.005$ versus control; c, $P < 0.05$ versus Hx; d, $P < 0.025$ versus pulmonary artery banding [PAB]) mRNA in normal (Control), hypoxic (Hx), PAB, SuHx, and pulmonary arteries banded with low Cu^{2+} (PAB/C) rat RVs. Data were normalized to the expression of 18S rRNA, and are shown as means $\pm$ SEM ($n = 6$). Below, representative Western blots for the expression of IGF-1, phospho-Akt (p-Akt), and total Akt from these groups. Densitometry was performed using Quantity One 1-D Analysis software (Bio-Rad, Hercules, CA) for normalized IGF-1 (a, $P < 0.001$ versus Hx; b, $P < 0.025$ versus PAB) and p-Akt (a, $P < 0.005$ versus control; b, $P < 0.005$ versus Hx; c, $P < 0.005$ versus PAB). (c) Quantitative, real-time PCR analysis of angiotensin-1 (a, $P < 0.001$ versus control [CTRL]; b, $P < 0.001$ versus Hx; c, $P < 0.005$ versus PAB), apelin (a, $P < 0.001$ versus control; b, $P < 0.005$ versus control; c, $P < 0.001$ versus Hx; d, $P < 0.005$ versus PAB), and vascular endothelial growth factor (VEGF) (a, $P < 0.001$ versus control; b, $P < 0.001$ versus Hx; c, $P < 0.05$ versus PAB) mRNA in normal (Control), Hx, PAB, SuHx, and PAB with low Cu^{2+} (PAB-Cu) rat RVs. Data were normalized to the expression of 18S rRNA, and are shown as means $\pm$ SEM ($n = 6$). Representative Western blots for the expression of angiotensin-1, apelin, and α -actinin from these groups are also provided. Densitometry was performed using Quantity One 1-D Analysis software (Bio-Rad) for normalized Ang1 (no significant difference) and apelin (a, $P < 0.005$ versus control; b, $P < 0.001$ versus Hx; c, $P < 0.05$ versus control; d, $P < 0.005$ versus PAB).

we found 499 probes that showed a prediction accuracy of 100% for all four algorithms. Because 49 of 499 probes failed when applying the LOOCV method, these 49 probes were eliminated from the final prediction dataset (Figure E5). However, the remaining 450 probes could be used to predict in which class a new sample belonged. Using this strategy, we identified the gene expression pattern that predictably differentiates RV hypertrophy from RVF (Figure 1).

Confirmation of Microarray Gene Expression Data and Analysis of Candidate Genes Associated with RVF in Two Additional Models

We next focused on genes that demonstrated, according to our microarray analysis, a fold-change difference in gene expression between RV hypertrophy and RVF (Figure 1), and we selected a number of genes and proteins for quantitative RT-PCR and Western blot confirmation (Figures 2b and 2c). Furthermore, we examined RV tissue from two additional models: one of RV hypertrophy (i.e., PAB) and one of RVF (PAB plus Cu²⁺-depleted diet). We focused on three categories of genes already identified by our microarray data analysis: genes promoting cell growth, genes encoding proteins involved in angiogenesis, and genes encoding glycolytic enzymes.

Molecular RVF Program: Loss of Genes Promoting Cell Growth

We analyzed RV and LV tissue from rats 6 weeks after chronic hypoxia, PAB, and SU5416/hypoxia, and from PAB animals receiving a Cu²⁺-depleted diet (a model of RVF) (19). As expected, in the models of hypertrophy and RVF, re-expression of the fetal genes myosin heavy chain β (MHC β) (19) and atrial natriuretic peptide (ANP) occurred (Figure E6), whereas the expression of these genes did not distinguish between RVH and RVF, in contrast to the two models of RV hypertrophy (either chronic hypoxia or PAB), which were associated with a 3- to 4-fold increase in the expression of RV tissue insulin-like growth factor 1 (IGF-1) mRNA. RV tissue from the failing RV (SU5416/hypoxia and PAB/low Cu²⁺) failed to induce this gene above the expression level of the control RV (Figure 2b). Correspondingly, the expression of the IGF-1 protein was drastically reduced in the failing RV when compared with the nonfailing, hypertrophied RV (Figure 2b). Of note, the downstream signaling protein phospho-Akt (p-Akt) was similarly affected in the RVs in the models of failure (Figure 2b), and of further interest, the baseline expression of IGF-1 mRNA was greater in the normal RV compared with the normal LV (Figure E1b). Because Kruppel-like factor 5 (Klf5) was recently described as a factor that transactivates IGF-1 in cardiac fibroblasts, which in turn causes the growth of cardiomyocytes (26) and controls cell differentiation and apoptosis (27), we compared the expression of Klf5 in the RV hypertrophy and RVF models, and found that Klf5 was overexpressed in the hypertrophied RV but not in the failing RV from SU5416/hypoxia animals (Figure E7).

Normal HIF-1 α Expression but Impairment of Angiogenic Capillary Maintenance in the Failing RV

Hypoxia inducible factor 1 α (HIF-1 α) was associated with the development of cardiac hypertrophy (28). Although the expression of HIF-1 α mRNA was no different between RV hypertrophy and RVF samples (data not shown), signaling downstream from HIF-1 α via VEGF and Akt was affected in the failing RV compared with the nonfailing RV (Figures 2b and 2c).

We previously showed that the failing RV can be characterized by capillary rarefaction (19) (Figure E3), and here we show that the expression of Ang-1, apelin, and VEGF mRNA was reduced in the failing RV tissue compared with the RV tissues of hypoxic and PAB animals (Figure 2c). Although the reduced expression of the gene encoding VEGF signaling may also reflect a loss of myocardial vessels, the nearly abolished expression of genes encoding the angiogenesis factors Ang-1 and apelin, and also of the apelin protein expression (29) (Figure 2c), points toward a global impairment of the process of capillary growth and maintenance in the failing RV. Again, this pattern was not present in the nonfailing companion LV (Figure E4).

Elevated Expression of Genes Encoding Glycolytic Enzymes

To begin to understand some of the pathways involved in the different responses to hypertrophy and failure in the RV, we used the Ingenuity Pathway Analysis program (<http://www.ingenuity.com>) and further explored the microarray gene expression dataset. The expression pattern of the glycolysis/gluconeogenesis canonical pathway was revealed to have a greater association between the genes in the microarray dataset than expected by chance alone ($P = 0.009$), and we found that seven different genes encoding enzymes involved in glycolysis were differentially expressed (Figure E8). Most notably, hexokinase-1 gene expression was increased fivefold in the failing (SU5416/hypoxia) RV compared with the hypertrophied (chronic hypoxia) RV (Table 1), whereas the gene encoding phosphofructokinase was threefold up-regulated in expression. We also found that the mitochondrial uncoupling protein 2 (Ucp2) was significantly increased in expression in the failing compared with the nonfailing RV (Table 1). In contrast, the alcohol dehydrogenase 7 gene was decreased twofold in the failing compared with the hypertrophied RV (Table 1). Importantly, these differences were reflected in the dataset derived from the prediction analysis (Figure E5).

Expression of miRNA in Failing RV

In addition to the changes in mRNA expression pattern in tissue with RVF (in the models of both SU5416/hypoxia and the PAB/Cu²⁺ depletion), changes in miRNA expression were also identified (Figure E9a). Chronic hypoxia decreased the RV expression of miRNA-21 and 34c*, whereas RVF suppressed the expression of miRNA 133a (30–32), 139_3p (which we had found to be expressed in equal abundance in the normal RV and LV; Figure E2b), 21, and 34c* (Figure E9b). In contrast, miRNA 27a and 29a were not changed by hypoxia, PAB, or conditions leading to RVF (Figure E9b).

TABLE 1. ELEVATED EXPRESSION OF GENES ENCODING GLYCOLYTIC ENZYMES

Genes	Fold Change		
	Hypertrophy	Failure	Failure with Carvedilol
ADH7	3.45	1.59	1.09
HK1	−3.73	1.33	1.01
PFKM	−1.48	2.31	1.15
Ucp2	−1.25	1.86	1.06

Definition of abbreviations: ADH7, alcohol dehydrogenase 7; HK1, hexokinase 1; Ucp2, mitochondrial uncoupling protein 2; PFKM, phosphofructokinase; RV, right ventricle.

HK1 mRNA is clearly overexpressed in the failing RV, compared with the hypertrophied RV of chronically hypoxic rats. Likewise, PFKM is overexpressed in the failing RV. Fold change is presented relative to the normal RV.

RVF Molecular Signature

Guided by our algorithm (Figure 1), we distinguished between quantitative and categorical (defined as the opposite direction of gene expression) differences characterizing RV hypertrophy and RVF. We thus arrived at the validated gene expression pattern (Table 2) that clearly separates RV hypertrophy from RVF. This pattern was termed the RVF signature.

Reversal of Established RV failure

Finally, we examined the changes in RV tissue gene expression associated with the significant reversal of established (SU5416/hypoxia) RVF in rats treated with the adrenergic receptor blocker carvedilol (33). The partial reversal of RVF was characterized by a reduction in RV hypertrophy and dilatation and myocardial fibrosis and restored RV capillarization, as recently reported (Figure 3) (33). This functional improvement was accompanied by a return of VEGF gene expression toward normal, and a small but significant increase in the expression of the angiopoietin 1 (Ang1) and apelin genes, whereas no effect of carvedilol treatment was evident in RV myocardial IGF-1 expression (Figure 4). However, we found a doubling of the expression of the gene encoding the transcription factor Klf5 when compared with that of the RV from SU5416/hypoxia rats (Figure E7). We also assessed the impact of carvedilol treatment on the expression of genes encoding glycolytic enzymes (Table 1), and we show the change in the gene expression pattern of SU5416/hypoxia carvedilol-treated RVs (Figure E10).

DISCUSSION

One objective of our investigation concerned the exploration of differences in the normal pattern of gene expression between the two chambers of the heart, and here we show clearly discernable differences in the expression of both mRNA and miRNA in the normal RV and LV. In the case of the transcription factor Irx2, the difference was categorical (i.e., no expression in the normal RV) (Figure E1b), whereas in the case of a larger number of genes, as exemplified by nuclear receptor subfamily 2, group F, member 2 and IGF-1 and as delineated in Table E1, the differences in expression were a matter of degree. This was also the case for miRNA, where a higher level of expression was generally found in the normal LV (Figure E2a), as validated by quantitative PCR for miRNA 27b and 34c* (Figure E2b). These differences in miRNA and mRNA expression may be attributable to the different embryological development of the two chambers, or to the fact that the RV is a low-pressure chamber and the LV a high-pressure chamber. The lack of Irx2 expression in the normal RV may account for the absence of ANP expression in the normal RV (34, 35). The normal pattern of RV gene expression served as the reference when comparing the expressed gene patterns between RV hypertrophy and RVF.

Another objective involved the characterization of differences in patterns of gene expression between compensated RV hypertrophy and RVF. In Figure 1, we illustrate the decision-making process and the rationale for the selection of genes of interest, which guided the path from expression analysis to the analysis of the RVF signature. We induced right heart failure in rats by combining chronic VEGF receptor blockade and chronic hypoxia (19, 22, 33). Whereas the RV hypertrophy phenotype was characterized by normal cardiac output, RVF was characterized echocardiographically by RV chamber dilatation, hemodynamically by a reduction in cardiac output, and histologically by myocardial fibrosis and capillary rarefaction (Figure E3) (19). We analyzed the patterns of mRNA and miRNA expression in search of mRNA and miRNA patterns that would differentiate RV hypertrophy from RVF.

The pattern of molecular failure (Figure 2a) was analyzed and categorized by a number of biological processes (Table E2). A predication analysis (Figure E5) revealed a set of 450 probes that can distinguish RV hypertrophy from RVF. We found markers of RVF expression and, as expected, directionally similar changes in expression that were shared by RV hypertrophy and RVF. Examples include the expression of ANP, brain natriuretic peptide (BNP), connective tissue growth factor (CTGF), and regulator of calcineurin 1 (Rcan1) mRNA, which were all increased in both hypertrophied and failing RVs (Figure E6). Based on microarray gene expression screening (Figure 2a and Table E2), the expression of selected genes was confirmed using quantitative, real-time PCR (Figure 2). In search of categorical markers that separate RV hypertrophy from RVF, we identified one miRNA and five mRNA signals (Table 2) that were also contained within the prediction dataset. The combination of these six differentiating signals was termed the “RVF molecular signature.”

The factors and mechanisms that ultimately determine the switch from compensated RV hypertrophy to RVF remain unclear, and longitudinal studies will be necessary to examine this transition. Although our experiments were not designed to address the events that govern the transition from RV hypertrophy to RVF, we explored changes in expression that relate to cell growth, angiogenesis, and aspects of cardiac energy metabolism. We assume, but do not know, that the angiogenesis and cardiac growth factor IGF-1 had been increased in expression during the early phase of RV hypertrophy in SU5416/hypoxia-treated animals before the onset of RVF, comparable to the increased expression in the nonfailing PAB RV. Based on this reasoning, we postulate that the low expression of IGF-1 in the failing RV of the SU5416/hypoxia animals and PAB/low Cu²⁺ animals is part of a failure program, or an inhibition of a cell growth and angiogenesis program (Figure 2c) that may also include the expression of the transcription factor Klf5 (26). Interestingly, the expression of IGF-1 mRNA was reported to increase in endstage cardiomyopathy (36) and in myocardial biopsies from

TABLE 2. GENE EXPRESSION PATTERN THAT SEPARATES RV HYPERTROPHY FROM RVF

	RV Hypertrophy	RVF	RVF with Carvedilol Treatment
MicroRNA 133a	Normal	Decreased	N/A
Apelin	Increased	Severely reduced	Increased
Insulin-like growth factor 1	Increased	No increase (compared to normal RV)	No change
VEGF	Increased	Reduced	Increased
HK1	Decreased	Increased	Decreased
ADH7	Normal	Decreased	N/A

Definition of abbreviations: ADH7, alcohol dehydrogenase 7; HK1, hexokinase 1; N/A, not available; RV, right ventricle; RVF, right ventricular failure; VEGF, vascular endothelial growth factor.

Comparisons were performed between normal and hypertrophied RVs, between hypertrophied and failing RVs, and between failing RVs treated with carvedilol and failing RVs with no treatment.

Figure 3. Maladaptive remodeling in rat models of right heart failure. (a) Echocardiography of the failing SuHx RV shows dilatation, paradoxical movements of the interventricular septum, and free wall hypertrophy. Immunohistochemistry with anti-CD31 antibodies (c) shows capillary rarefaction, whereas Masson's trichrome stain (e) shows myocardial fibrosis. Four weeks of carvedilol treatment (15 mg/kg) are associated with decreased RV dilatation and hypertrophy (b), normalization of the shape and movements of the interventricular septum (b), the reversal of capillary rarefaction (d), and fibrosis (f). Arrows point to the RV, dashed lines indicate the position of the septum.

failing hearts (37). Based on our data, chronic hypoxic PAH apparently induces apelin, IGF-1, Klf5, and VEGF mRNA in the hypertrophied RV (Table 1). Apelin, induced by Ang-1, enhances endothelial cell proliferation in the presence of VEGF and controls blood vessel diameter during angiogenesis (29). The hypoxia-induced expression of apelin was linked to endothelial cell

proliferation and regenerative angiogenesis (38, 39). Ang-1 also promotes cardiac myocyte survival (40) and endothelial cell survival via phosphoinositide 3-kinase–Akt signaling (41).

It is tempting to speculate that the progression from RV hypertrophy to RVF involves the failure of expression (or active repression) of IGF-1, apelin, and angiopoietin, leading to both

Figure 4. Comparison of differentially expressed genes between the normal RV, failing RV, and failing RV treated with carvedilol (Carv). Quantitative, real-time PCR analysis was performed on angiopoietin-1 (a, $P < 0.001$ versus control; b, $P < 0.025$ versus SuHx), apelin (a, $P < 0.001$ versus control; b, $P < 0.05$ versus SuHx), IGF-1 (a, $P < 0.025$ versus control; b, $P < 0.01$ versus SuHx), and brain natriuretic peptide (BNP) (a, $P < 0.001$ versus control; b, $P < 0.05$ versus control; c, $P < 0.05$ versus SuHx) mRNA in normal (Control), SuHx, and SU5416/hypoxia + carvedilol (SuHx-Carv) rats RVs, administered for 4 weeks at a dose of 15 mg/kg. Data were normalized to the expression of 18S rRNA, and are presented as means $\pm$ SEM ($n = 3$).

myocardiocyte and endothelial cell death, whereas the expression of genes encoding ANP and BNP (considered biomarkers of heart failure) is not impaired. Although we show, as an example, that the expressions of apelin and IGF-1 protein and genes are matched, whereas the expressions of the Ang-1 gene and protein are not, a more systematic exploration of the changes in protein expression is beyond the scope of this study.

A switch from fatty-acid utilization to glycolysis and lipid accumulation, as reported in the setting of LVF (42), may also occur during the progression from RV hypertrophy to RVF (43). Endothelial cell fatty-acid uptake was reported to be controlled by VEGF (44), and the expression of VEGF was found to be decreased in the failing SU5416/hypoxia RV (Figure 2c), whereas VEGF mRNA was increased in the corresponding SU5416/hypoxia LV (Figure E4). Apparently, HIF-1 α -dependent transactivation of VEGF is impaired in failing SU5416/hypoxia RV tissue, whereas the transactivation of glucose transporter 1 is not (data not shown) (19). The importance of cardiac VEGF signaling for the maintenance of proper cardiac function cannot be overstated, in light of the findings by May and colleagues (45), who showed in genetically modified mice that the inhibition of VEGF receptor signaling caused capillary rarefaction and induced a state of myocardial hibernation, which were both reversible upon the release of blocked VEGF signaling. Moreover, VEGF was shown to induce the expression of Rcan1, which acts as an antihypertrophy signal (33).

The increased expression of genes encoding multiple enzymes of the glycolytic pathway (Figure E8) and the increased expression of the uncoupling protein Ucp2 (46) in the failing RV is consistent with the energy limitation and impaired utilization described in LVF (47). The increased gene expression in both the natriuretic peptide and glycolytic pathway enzyme (48, 49) may perhaps be seen as “attempts” of the failing RV to survive and maintain some myocontractility.

In the failing RV of SU5416/hypoxia animals, we found a global increase in the expression of miRNA (Figure E9a), but a decrease of the expression of miRNA 133a (Figure E9b), and this decrease also occurred in the other models of RVF induced by PAB/Cu²⁺ depletion. These results were similar to the data in left heart failure induced by transverse aortic constriction, insofar as van Rooij and colleagues also reported the decreased expression of miRNA 133a in the failing LV (50, 51). Consistent with a postulated role of miRNA 133a as a negative regulator of cardiac fibrosis (31, 52), the failing RV (SU5416/hypoxia and PAB/Cu²⁺ depletion) with the reduced expression of miRNA 133a also showed a drastic up-regulation of expressed CTGF mRNA, which was reported to occur during LV fibrosis (Figure E6). In addition, we identified an increased expression of genes encoding fibrosis-controlling proteins: TGF- β 1, TGF- β 2, Smad4, mitogen-activated protein kinase 3, and forkhead box A2 (data not shown). In contrast, whereas miRNA 21 and 34c* were reported to increase during LVF, a decrease in these miRNA species occurred in both models of RVF (Figure E9b). Using three different target prediction approaches, namely, PicTar (<http://pictar.mdc-berlin.de/>), miRanda (<http://www.microrna.org>), and TargetScan (<http://www.targetscan.org>), we identified Rcan1 to be regulated by miRNA 21. A decreased expression of miRNA 21 in the failing RV would predict the increased expression of Rcan1, which indeed was found (Figure E6). The expression of miRNA 92a (an endogenous repressor of an endothelial cell angiogenic program) (53) was not affected in the failing RV.

To test whether the partial reversal of RVF in the SU5416/hypoxia model by the drug carvedilol, as previously reported (33), was also associated with a change in the pattern of gene expression associated with RVF, we compared the pattern of gene expression in RVF with that in SU5416/hypoxia animals, in whom RVF had been reversed by treatment with carvedilol (33). Indeed,

the expression of the six genes listed in Table 2 changed as a consequence of treatment with carvedilol. The expression of Ang-1 and apelin mRNA was significantly affected, but not normalized, after the treatment of SU5416/hypoxia rats with carvedilol, whereas the expression of VEGF mRNA returned to normal control levels (Figure 4), and the expression of the genes encoding Klf5 and hexokinase-2 doubled when compared with SU5416/hypoxia RV tissue (Figure E7). The directional changes in expression of the genes encoding glycolytic enzymes in the carvedilol-treated SU5416/hypoxia animals (Figure E10) may be consistent with some degree of myocardial metabolic recovery.

Consistent with our previous characterization of the RVF phenotype in the SU5416/hypoxia model by cardiac output, TAPSE, myocardial capillary density, and fibrosis (19, 22, 33), the partial reversal of the gene expression pattern in the failing RV by carvedilol is related to functional improvement in the RV. Whether the alterations in gene expression pattern are responsible for the functional improvement, or merely accompany the improvement, remains unclear. A rigorous, mechanistic assessment awaits experiments based on gene knockout strategies in pulmonary hypertensive rats.

Most likely, our short list of miRNA and mRNA representing RVF failure (in contrast to RV hypertrophy) is incomplete, and an extended analysis of the prediction dataset (Figure E5) and expressed miRNA (Figure E9) will lead to the discovery of additional RVF-associated and repair-associated genes. It remains to be explored whether interventions designed to prevent the down-regulation of genes encoding apelin, VEGF (54), and Ang-1 would prevent the development of RVF.

In conclusion, we describe a gene expression pattern that distinguishes between adaptive RV hypertrophy and RVF, and we provide for the first time, to the best of our knowledge, a set of expression data that can predict experimental RVF. Although the clinical importance of this RV dysfunction-associated gene expression signature is unclear and awaits confirmation after analyzing human hearts, we find it intriguing that the set of genes (Table 2) that discriminates between RV hypertrophy and RVF includes miRNA133a, three growth and angiogenesis factors, and two genes encoding enzyme proteins involved in energy metabolism. We thus interpret our data to propose that RVF is a condition characterized, on the level of gene expression, by a pattern reflecting impaired cell growth, impaired angiogenesis, and a shift in energy metabolism (Figure E8).

The prevention and reversal of RVF have become goals of treatment, and therapeutic strategies may include agents and nonpharmaceutical measures that preserve microvessel density and function and the myocardial energy metabolism of the afterload-stressed RV. At this stage, we do not claim that our results are representative of data derived from other models of RVF or RV strain or reflect changes of gene expression in failing human RVs. However, our data may provide a basis and a reference for comparison in other animal species and models. Efforts to translate the findings and concepts presented here will depend on the availability and exploration of heart-tissue samples from patients with PAH and on mechanistically informative biomarkers of RVF, which have yet to be identified.

Author Disclosure: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Donatas Kraskauskas for outstanding technical assistance with the rat experiments.

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