MicroRNAs in the Human Heart
A Clue to Fetal Gene Reprogramming in Heart Failure

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Background—Chronic heart failure is characterized by left ventricular remodeling and reactivation of a fetal gene program; the underlying mechanisms are only partly understood. Here we provide evidence that cardiac microRNAs, recently discovered key regulators of gene expression, contribute to the transcriptional changes observed in heart failure.

Methods and Results—Cardiac transcriptome analyses revealed striking similarities between fetal and failing human heart tissue. Using microRNA arrays, we discovered profound alterations of microRNA expression in failing hearts. These changes closely mimicked the microRNA expression pattern observed in failing cardiac tissue. Bioinformatic analysis demonstrated a striking concordance between regulated messenger RNA expression in heart failure and the presence of microRNA binding sites in the respective 3′ untranslated regions. Messenger RNAs upregulated in the failing heart contained preferentially binding sites for downregulated microRNAs and vice versa. Mechanistically, transfection of cardiomyocytes with a set of fetal microRNAs induced cellular hypertrophy as well as changes in gene expression comparable to the failing heart.

Conclusions—Our data support a novel mode of regulation for the transcriptional changes in cardiac failure. Reactivation of a fetal microRNA program substantially contributes to alterations of gene expression in the failing human heart. (Circulation. 2007;116:258-267.)

Key Words: cardiomyopathy ■ heart failure ■ fetal heart ■ microRNAs

Systolic heart failure, a leading cause of hospital admission and mortality in industrialized nations, is characterized by left ventricular remodeling and dilatation associated with activation of a fetal gene program triggering pathological changes in the myocardium associated with progressive dysfunction.

MicroRNAs (miRNAs) are recently discovered regulatory molecules consisting of ~22 noncoding nucleotides that regulate gene expression by hybridization to messenger RNAs (mRNAs) with the consequence of mRNA degradation or translational inhibition of targeted transcripts. Initially, the importance of miRNAs has been demonstrated in plant biology, cancer (Esquela-Kerscher and Slack), viral diseases, and developmental processes.

ES,21,22 However, recent evidence suggests that miRNAs also participate in control of cardiac hypertrophy and development; in the fruit fly, miRNA1 (miR-1) targets transcripts encoding the Notch ligand delta, thus regulating the expansion of cardiac and muscle progenitor cells. Accumulation of miR-1 in the heart impairs the pool of proliferating ventricular myocytes, whereas targeted deletion of miR-1 results in dysregulation of cardiogenesis. MiR-133 enhances skeletal myoblast proliferation by repressing serum response factor. Thus far, >350 human miRNAs have been identified (see http://microrna.sanger.ac.uk), each targeting numerous different RNA transcripts with the consequence of

Clinical Perspective p 267

The online-only Data Supplement, consisting of tables, is available with this article at http://circ.ahajournals.org DOI: 10.1161/CIRCULATIONAHA.107.687947/DC1.
Interesting Regulated miRNAs in Failing and Fetal Human Hearts

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Fold Change, Diseased Versus Healthy</th>
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<th>Potential Cardiovascular Important Target Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa_miR_212</td>
<td>8.1</td>
<td>2.1</td>
<td>…</td>
<td>...</td>
<td>Titin (TTN; −1.51; <em>P</em>&lt;0.01) Calcium/calmodulin-dependent protein kinase kinase 1-α (CAMKK1; −1.32; <em>P</em>=0.12) Dynein light chain 2 (Dlc2; −1.34; <em>P</em>&lt;0.05) Bone morphogenetic protein 5 (BMP5; −1.80; <em>P</em>&lt;0.05)</td>
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<td>10.9</td>
<td>↑ Hepatocellular carcinoma</td>
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<td>Tumor-associated calcium signal transducer 1 (TACSTD1; −2.59; <em>P</em>&lt;0.001) Jagged 1 (JAG1; −1.22; <em>P</em>&lt;0.17)</td>
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<td>hsa_miR_29b</td>
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<td>1.2</td>
<td>Expressed in astrocytes</td>
<td>13</td>
<td>Fibronectin type III domain containing 5 (FNDC5; −2.19; <em>P</em>&lt;0.008)</td>
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<tr>
<td>hsa_miR_29a</td>
<td>3.3</td>
<td>−5.3</td>
<td>Expressed in astrocytes</td>
<td>13</td>
<td>Epiregulin (EREG; −1.98; <em>P</em>&lt;0.07) Cell division cycle and apoptosis regulator 1; −1.61; <em>P</em>&lt;0.01 Prominin 1 (PROM1; −1.59; <em>P</em>&lt;0.18) Nuclear factor-κB repressing factor (NFKB; −1.49; <em>P</em>&lt;0.01) Angiopoietin-like 7 (ANGPTL7; −1.49; <em>P</em>&lt;0.05) Tubulin-β (TUBB; −1.32, <em>P</em>&lt;0.05) Phospholamban (PLN; −1.46; <em>P</em>&lt;0.05) Neuregulin 1 (NRG1; −1.32; <em>P</em>&lt;0.05) Amphiregulin (AREG; −2.59; <em>P</em>&lt;0.05) Potassium channel, subfamily K, member 1 (KCNK1; −2.29; <em>P</em>&lt;0.01) Potassium voltage-gated channel, subfamily G, member 1 (KCNN1; −1.57; <em>P</em>&lt;0.05) Cell division cycle associated 7-like (CDCA7L; −1.55; <em>P</em>&lt;0.01)</td>
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<td>mmu_miR_17_3p</td>
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<td>1.8</td>
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<td>Phosphatase and actin regulator 1 (PHACTR1; −7.16; <em>P</em>&lt;0.001)</td>
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<td>hsa_miR_210</td>
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<td>4.3</td>
<td>Regulation of oogenesis</td>
<td>14</td>
<td>Inositol hexaphosphate kinase 3 (IPHK3; −2.45; <em>P</em>&lt;0.001) Potassium intermediate calcium-activated channel, member 2 (KCNN2; −1.89; <em>P</em>&lt;0.001) Immunoglobulin superfamily, member 11 (IGSF11; −1.65; <em>P</em>&lt;0.05) SCL2-like 12 (BCL2L12; −1.59; <em>P</em>&lt;0.01) Protein kinase C, α-binding protein (PRKCBP; −1.57; <em>P</em>&lt;0.05)</td>
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<td>hsa_miR_211</td>
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<td>3.1</td>
<td>↑ Cell growth</td>
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<td>Myocilin, trabecular meshwork inducible glucocorticoid response (MYOC; −1.86; <em>P</em>&lt;0.01) Peroxisome proliferative activated receptor-γ, coactivator 1 (PPARGC1A; −1.44; <em>P</em>&lt;0.05) Growth differentiation factor 9 (GDF9; −1.33; <em>P</em>&lt;0.03) GLI-Kruppel family member (HHR3; −2.72; <em>P</em>&lt;0.001) Proline dehydrogenase (oxidase) 1 (PRODH; −1.50; <em>P</em>&lt;0.01) P450 (cytochrome) oxidoreductase (POR; −1.40; <em>P</em>&lt;0.01) Succinate dehydrogenase complex, subunit A, flavoprotein-like 2 (SDHCL2; −2.79; <em>P</em>&lt;0.001) Spen homolog, transcriptional regulator (Drosophila) (SPEH; −2.15; <em>P</em>&lt;0.01) Thrombomodulin (THBD; −1.58; <em>P</em>&lt;0.05) Phosphofructokinase, muscle (PFK; −1.44; <em>P</em>&lt;0.01) Latent transforming growth factor-β binding protein 1 (LTBP1; −1.38; <em>P</em>&lt;0.05) Calpain 5 (CAPN5; −1.31; <em>P</em>&lt;0.05)</td>
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gene silencing. Databases and computational approaches have been developed to predict target genes of miRNAs, but thus far only few have been validated.30,31

In the present study we tested our hypothesis that alterations of specific miRNAs contribute to reactivation of fetal gene programs in human heart failure. Using microarray and miRNA stem loop real-time polymerase chain reaction (RT-PCR) technology, we investigated miRNA profiles as well as the cardiac miRNA transcriptome in left ventricular tissue from patients with end-stage heart failure in comparison with healthy adult and fetal human hearts. Potential target genes of regulated miRNAs were identified and validated by database searches and simultaneous transcriptome and quantitative RT-PCR analyses. We finally tested whether the reexpression of fetal miRNAs in cultured neonatal or adult cardiomyocytes would initiate gene programs and morphological changes as observed during heart failure.

### Table: Potential Cardiovascular Important Target Genes

<table>
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<tr>
<th>miRNAs</th>
<th>Fold Change, Diseased Versus Healthy</th>
<th>Fold Change, Fetal Versus Healthy</th>
<th>Known Expression/Function</th>
<th>Reference</th>
<th>Potential Cardiovascular Important Target Genes</th>
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<tr>
<td>hasa_let_7c</td>
<td>2.2</td>
<td>2.9</td>
<td>↑ Embryogenesis</td>
<td>15</td>
<td>Membrane protein, palmitoylated 3 (MP3; −6.54; (P&lt;0.001)) Succinate dehydrogenase complex, subunit A, flavoprotein-like 2 (SDHAD; −2.79; (P&lt;0.001)) Neurturin (NRT; −1.82; (P&lt;0.05)) Carbonic anhydrase IV (CA4; −1.47; (P&lt;0.05)) Arginase, type II (ARG2; −1.46; (P&lt;0.09)) Neuregulin 1 (NRG1; −1.32; (P&lt;0.05)) Succinate dehydrogenase complex, subunit A, flavoprotein-like 2 (SDHAD; −2.79; (P&lt;0.001)) CASP8 and FADD-like apoptosis regulator (CFLAR; −2.08; (P&lt;0.01))</td>
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<td>hasa_miR_372</td>
<td>2.3</td>
<td>1.1</td>
<td>↑ Proliferation, tumorigenesis</td>
<td>16</td>
<td>E2F transcription factor 5 (E2F5; −1.43; (P&lt;0.05)) Occludin (CLUD; −1.42; (P&lt;0.06))</td>
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<td>↓ Drosophila metamorphosis</td>
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<td>Tumor-associated calcium signal transducer 1 (TACSTD1; −2.59; (P&lt;0.001))</td>
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<td>miRNAsdownregulated</td>
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<td></td>
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<td>Fibroblast activation protein, alpha (FAP; 2.56; (P&lt;0.01)) Cold shock domain protein A (CSDA; 2.06; (P&lt;0.01)) Catalase (CAT; 1.56; (P&lt;0.01)) Interferon-γ (IFN; 1.55; (P&lt;0.01)) Angiopoietin-like 1 (ANGPT1; 2.82; (P&lt;0.0001)) Actinin-α (ACTN1; 1.84; (P&lt;0.01)) Reticnoic acid receptor-γ (RAR; 2.46; (P&lt;0.001)) Immediate early response 3 (IER3; 2.01; (P&lt;0.05)) Fas ligand (tumor necrosis factor superfamily, member 6) (FASL; 1.63; (P&lt;0.05))</td>
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<td>...</td>
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<td>Tumor necrosis factor receptor superfamily, member 10 (TNFRSF10A; 1.48; (P&lt;0.05))</td>
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<td>hasa_miR_182</td>
<td>−2.8</td>
<td>−1.3</td>
<td>↓ Apoptosis</td>
<td>17</td>
<td>Carbonic anhydrase III, muscle specific (CA3; 4.00; (P&lt;0.01)) AE binding protein 1 (AEBP1; 3.22; (P&lt;0.01)) Phosphodiesterase 1A, calmodulin-dependent (PDEA1A; 2.39; (P&lt;0.05)) T-cell immunomodulatory protein (CD80B; 1.85; (P&lt;0.001)) Sarcoglycan-ε (SGCE; 1.47; (P&lt;0.01)) Inteukin 8 (IL8; 1.38; (P=0.05)) Sarcoplasm (SLN; 3.34; (P&lt;0.0001)) Folate receptor 2 (fetal) (FOLR2; 2.68; (P&lt;0.001)) Cathespin K (CTSK; 2.23; (P&lt;0.01)) Peptidase D (PEPD; 1.66; (P&lt;0.01)) Glutathione peroxidise 1 (GPX1; 1.53; (P&lt;0.05))</td>
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<td>hasa_miR_107</td>
<td>−1.6</td>
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<td>↓ Cell growth</td>
<td>12</td>
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</table>

### Methods

Approval was obtained from the ethical committees of the Medical School of Hannover (Germany) and the University of Utrecht (Netherlands). We examined cardiac tissue from patients undergoing heart transplantation because of end-stage heart failure due to dilative cardiomyopathy and, for comparison, healthy adult and fetal human heart samples (gestation age, 12 to 14 weeks). Immediately after explantation, tissue pieces were removed from the left ventricles, and excised tissue was shock-frozen in liquid nitrogen and stored at −80°C until analysis.

**miRNA Expression and Transcriptome Analyses**

Frozen heart tissue was minced under liquid nitrogen and subjected to total RNA isolation including small RNAs (mirVana miRNA Isolation Kit; Ambion). We separately isolated total RNA and purified miRNAs using the flashPAGE Fractionator system (Ambion). Capillary electrophoresis (Bioanalyzer 2100, Agilent) was used to assess quality of total RNA and purity of isolated miRNA. miRNA obtained from 10 μg total RNA was labeled with the dye...
Cy3 (Molecular Probes, Carlsbad, Calif) with the use of the mirVana miRNA Labeling Kit (Ambion) according to the manufacturer’s recommendations. Each target was hybridized to a separate array (n = 6 failing left ventricles; n = 4 normal adult left ventricles; n = 6 fetal hearts). Microarrays with a set of 384 miRNAs (mirVana miRNA Probe Set, Ambion) were spotted in-house on SCHOTT Nexterion Slide E microarray slides in quadruplicate. The oligonucleotide probes were 42 to 46 nucleotides long and consisted of an 18- to 24-nucleotide segment that targeted a specific known human, mouse, or rat miRNA. A complete listing of the probes in the probe set can be found at www.ambion.com/techlib/resources/miRNA_array/index.html. Slide processing, miRNA purification and enrichment, and labeling were performed according to the Ambion mirVana manuals (www.ambion.com/techlib/prot/). Data acquisition was done with the use of ScanAlyze Software (M. Eisen, LBNL). Regulated miRNAs were validated by specific miRNA stem loop primer reverse transcription followed by TaqMan RT-PCR analyses (see below).

For transcriptome analyses, reverse transcription, second-strand synthesis, and cleanup of double-stranded cDNA were performed according to the Affymetrix protocols (One-Cycle cDNA synthesis Kit, Affymetrix) starting from 2 μg of total RNA (n = 4 failing left ventricles; n = 4 normal adult left ventricles; n = 6 fetal hearts). Synthesis of biotin-labeled cRNA was performed with the use of the IVT Labeling Kit (Affymetrix). cRNA concentration was determined and the distribution of cRNA fragment sizes was checked by gel electrophoresis. For hybridization of the human genome U133 Plus 2.0 GeneChip, 15 μg of fragmented cRNA was used.

**Array Data Analyses**

For both oligonucleotide arrays and spotted miRNA arrays, data analysis was done with the use of R packages from the Bioconductor project (www.bioconductor.org). Resulting signal intensities were normalized by variance stabilization. Quality of all data sets was tested, and statistical analysis to select differentially expressed genes was performed with the use of the limma (Linear Models for Microarray Analysis) package. The core of the limma package is an implementation of the empirical Bayes linear modeling approach of Smyth and can be used for stable analysis even for smaller sample sizes.

For data analysis, a special principal correspondence analysis (supervised classification method termed between-group analysis) was used to ordinate groups rather than individual samples as described. Classic Venn diagram was used to show numbers of miRNAs that were regulated in failing and fetal human hearts.

**miRNA Target Prediction Methods**

The miRNA database miRBase (http://microrna.sanger.ac.uk/) was used to identify potential miRNA targets. The miRanda algorithm was used to scan all available miRNA sequences for a given genome against 3’ UTR sequences of that genome. The algorithm uses dynamic programming to search for maximal local complementa-
tary alignments, which correspond to a double-stranded antiparallel duplex. A positive score is given for complementary base pairing, and a negative score is given to mismatches, gap opening, and gap extension. Importantly, scores derived from the 5' end of the miRNA were multiplied by a scaling factor to reflect the apparent importance of perfect Watson-crick base pairing, which has been observed experimentally. Subsequently, Karlin-Altschul normalization was performed (miRBase Targets version 3.0; http://microrna.sanger.ac.uk/targets/v3/). In an alternative approach, we searched for highly conserved species for which a certain miRNA site was found. The predicted miRNA targets obtained by these 2 methods were then compared with our gene expression data (Affymetrix Genechip System). Additionally, all >4-fold regulated genes in end-stage human heart failure were screened for potential miRNA binding sites of regulated miRNAs with the use of the miRanda algorithm.

MicroRNA Stem Loop RT-PCR

A variety of regulated miRNAs were validated by quantitative miRNA stem loop RT-PCR technology (TaqMan MicroRNA Assays, Applied Biosystems, Foster City, Calif). All miRNA samples were derived from isolations having the same total RNA concentration. We used highly target-specific stem loop structure and reverse transcription primer, and after reverse transcription used specific TaqMan hybridization probes for miRNA amplification. This allows high specificity for only the mature miRNA target and formation of a reverse transcription primer/mature miRNA chimera, extending the 5' end of the miRNA. Semiquan-

titative RT-PCR of selected target genes in transfected rat cardiomyocytes was done as described. More than 95% of cultured cardiomyocytes stained positive for actinin, demonstrating high purity of cell cultures. Cy3-labeled scrambled-miR, -miR-21, -miR-129, and -miR-212 (Cy3-pre-miR miRNAs; Ambion; 100 nmol/L, 48 hours) were transfected separately or in combination with neonatal cardiomyocytes by a liposomal-based method (Lipofectamine, Invitrogen). Forty-eight hours after transfection, cells were harvested, and cell lysates were prepared as described. Adult cardiomyocytes were isolated and cultivated as described. Cardiomyocytes were identified by typical rod-shaped morphology, and cultures were of high purity. Adult cardiomyocytes were transfected with scrambled Cy3-labeled miRNA or a set of fetal miRNAs (miR-21, miR-129, and miR-212; 72 hours, 25 nmol/L) by a liposomal-based approach (Lipofectamine, Invitrogen). Transfection efficacy was monitored by the use of Cy3-labeled miRNAs and by quantitative miRNA stem loop RT-PCR measurement of miR-21, miR-129, and miR-212 (TaqMan MicroRNA Assays, Applied Biosystems). As a marker for cell size, surface area of neonatal (48 hours after transfection) and adult (72 hours after transfection) cardiomyocytes was calculated with the use of

Figure 2. Altered miRNAs in failing and fetal heart tissue compared with controls. Comparison of induced (top) or repressed (bottom) miRNAs in failing heart tissue with that of fetal human heart tissue is shown. A very close relationship of miRNA expression between fetal and failing hearts is shown, eg, 86.6% of induced miRNAs (>1.5-fold) and 83.7% of repressed miRNAs (>1.5-fold) were regulated in the same direction in fetal and failing heart tissue compared with healthy adult control left ventricles. The exact values are shown in Table III in the online-only Data Supplement.

Cardiomyocytes from neonatal rats were isolated and cultivated as described. More than 95% of cultured cardiomyocytes stained positive for actinin, demonstrating high purity of cell cultures. Cy3-labeled scrambled-miR, -miR-21, -miR-129, and -miR-212 (Cy3-pre-miR miRNAs; Ambion; 100 nmol/L, 48 hours) were transfected separately or in combination with neonatal cardiomyocytes by a liposomal-based method (Lipofectamine, Invitrogen). Forty-eight hours after transfection, cells were harvested, and cell lysates were prepared as described. Adult cardiomyocytes were isolated and cultivated as described. Cardiomyocytes were identified by typical rod-shaped morphology, and cultures were of high purity. Adult cardiomyocytes were transfected with scrambled Cy3-labeled miRNA or a set of fetal miRNAs (miR-21, miR-129, and miR-212; 72 hours, 25 nmol/L) by a liposomal-based approach (Lipofectamine, Invitrogen). Transfection efficacy was monitored by the use of Cy3-labeled miRNAs and by quantitative miRNA stem loop RT-PCR measurement of miR-21, miR-129, and miR-212 (TaqMan MicroRNA Assays, Applied Biosystems). As a marker for cell size, surface area of neonatal (48 hours after transfection) and adult (72 hours after transfection) cardiomyocytes was calculated with the use of
the AxioVison Rel 4.4 package (Carl Zeiss GmbH). Data are expressed as mean±SEM. Statistical analysis was performed by 1-way ANOVA followed by multiple comparisons by the Fisher protected least-significant difference test. Statistical analysis was performed with the use of the StatView 5.0 statistical program (Abacus Concepts, Berkley, Calif). Statistical significance was assumed at \( P < 0.05 \).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Cardiac Transcriptome and miRNA Expression: Striking Similarities Between Fetal and Failing Human Heart Tissue

miRNA and total RNA isolated simultaneously from human left ventricular tissue were subjected to miRNA microarray and transcriptome analysis, respectively. This allowed comparisons of both miRNA expression and transcript levels of potential target genes within the same tissue.

Transcriptome (Affymetrix Genechip System) analysis of >50 000 transcripts revealed 461 genes to be upregulated >2-fold (\( P < 0.05 \)) and 569 to be downregulated >2-fold (\( P < 0.05 \)) in left ventricular tissue from patients with end-stage heart failure compared with healthy controls (Table I in the online-only Data Supplement). Hierarchical cluster analysis of >4-fold regulated genes revealed extensive activation of gene programs known to be induced in cardiac hypertrophy or failure, such as 16- and 57-fold increases in the expression of atrial or brain natriuretic peptide (ANP, BNP), whereas genes coding for the voltage-dependent Ca\(^{2+}\) channel (CACNB2) or the sodium-calcium exchanger 1 (Slc8a1) were highly repressed, as recently described in end-stage heart failure.\(^4\) Reactivation of single fetal genes such as ANP, β-myosin heavy chain (MHC), or α-skeletal actin in heart
failure has been described; however, comprehensive identification of the fetal gene program by combined transcriptome and miRNA analyses in left ventricular tissue from failing and fetal human hearts has never been performed.

We show a closely related gene expression profile of regulated genes in fetal human hearts (gestation age, 12 to 14 weeks) compared with failing hearts (Figure 1). Correspondence analysis demonstrates that gene expression profiles between failing and fetal human heart tissue converge with increasing levels of regulation (Figure 1B). This demonstrates the close relationship of gene expression programs in fetal and failing human hearts.

Because miRNAs have superordinate regulatory functions during cardiac development, we analyzed miRNA expression profiles in fetal and failing hearts and developed a strategy to relate alterations of gene expression to the number of binding sites for fetal miRNAs in heart failure. miRNA microarray analysis revealed 67 miRNAs upregulated >1.5-fold in failing left ventricles versus normal hearts, whereas 43 miRNAs were downregulated >1.5-fold (Table and Table II in the online-only Data Supplement). Ten regulated miRNAs were also analyzed with the use of miRNA stem loop primer RT-PCR. The findings with RT-PCR were in good agreement with the microarray data (Table III in the online-only Data Supplement). Alterations in miRNA expression in heart failure displayed a pattern strikingly similar to that observed in the fetal heart (Figure 2, Table, and Table II in the in the online-only Data Supplement). Indeed, 86.6% of induced miRNAs (>1.5-fold) and 83.7% of repressed miRNAs (<1.5-fold) were regulated in the same direction in fetal and failing heart tissue compared with healthy adult control left ventricle (Figure 2). There were some minor exceptions, in
which miRNAs were repressed in fetal human hearts and upregulated in failing hearts and vice versa; future studies must show whether these discordant regulated miRNAs may be of importance in heart failure.

Close Relationship Between miRNAs and the Cardiac Transcriptome in the Failing Heart

Single miRNAs often target multiple genes, and, conversely, single genes are regulated by several miRNAs. We therefore searched for miRNA binding sites in highly regulated genes (>4-fold) to link the observed changes in both miRNA expression and the cardiac transcriptome. A total of 543 binding sites for the miRNAs represented on the microarray were identified within the sequences of these regulated genes (Table IV in the online-only Data Supplement). To analyze a potential relation between number of miRNA binding sites and changes in gene expression, we determined the percentage of binding sites for upregulated or downregulated miRNAs (>1.2-fold) for each of the regulated genes (Figure 3 and Table IV in the online-only Data Supplement). More binding sites for upregulated miRNAs were found in repressed genes than in induced genes in heart failure (48.1% versus 39.3%), although this was of borderline statistical significance ($P=0.07$). Upregulated miRNAs had significantly more binding sites for miRNAs repressed in heart failure than downregulated genes (30.2% versus 14.3%; $P=0.0002$). Although induction of fetal miRNAs in failing heart tissue likely results in repression of various target genes, the presence of more binding sites for repressed miRNAs appears to be involved in the upregulation of miRNAs in heart failure (Figure 3).

Overexpression of a Distinct Set of Fetal miRNAs in Cultured Cardiomyocytes Initiates Fetal Gene Programs and Morphological Changes Comparable to Those in Failing Hearts

Computational prediction identified ≈1000 potential target genes with at least 1 binding site for highly upregulated miRNAs during heart failure (eg, miR-21, miR-29b, miR-129, miR-210, miR-212; Table). However, in the transcriptome analysis conducted in parallel, more theoretically predicted target genes were upregulated, demonstrating no general preponderance of gene repression. Thus, the presence of 1 binding site for a specific miRNA does not always confer strong regulatory action, but several binding sites for upregulated and downregulated miRNAs work in concert to regulate gene expression (Figure 3).

To finally prove the importance of miRNAs for the initiation of fetal gene programs, we mimicked a fetal miRNA environment in cultured cardiomyocytes by overexpressing 3 fetal miRNAs upregulated in the failing heart (miR-21, miR-129, and miR-212; Figure 4). Transfection efficiency was monitored by using Cy3-labeled pre-miRNA and was >85% in both neonatal and adult cardiomyocyte cultures (Figures 4A and 5A). Only viable cardiomyocytes were transfectable (Figure 5B). Although the overexpression of a single miRNA had only minor effects, the simultaneous upregulation of all 3 miRNAs resulted in morphological changes of neonatal cardiomyocytes similar to that observed in the failing heart, eg, hypertrophy (Figure 4A and 4B).

Therefore, we investigated changes in gene expression in these transfected cardiomyocytes. Strikingly, a number of both established (ANP, BNP, β-MHC, α-skeletal actin) and newly identified fetal genes (villin2, cspg2, phlda1, hsp90, RASA1, MEF2a, cradd, dtna) were reactivated or silenced by this modulation of the miRNA environment (Figures 3 and 4C). Likewise, transfection of isolated adult rat cardiomyocytes with a set of fetal miRNAs (miR-21, miR-129, and miR-212) resulted in cellular hypertrophy and activation of a fetal gene program (Figure 5C to 5F). We cannot completely rule out off-target effects after miRNA transfection. However, this seems to be unlikely because overexpression of a scrambled miRNA did not significantly alter cellular size or gene expression. In addition, miRNA levels in transfected adult cardiomyocytes were lower than in neonatal cells, but comparable changes in hypertrophy and activation of fetal genes were still observed.

Discussion

Comparison With Other Studies in the Field

Published studies thus far focused on the initial phases of cardiac hypertrophy in mice, whereas our report is based on fetal and failing human heart tissue. Olson’s group also investigated expression of a limited amount of 6 different miRNAs in human failing heart tissue, whereas we investigated >350 different miRNAs. The reported data of the Olson laboratory on miRNA expression in failing human hearts are based on Northern blots, whereas our results were obtained by miRNA microarray and stem loop miRNA RT-PCR experiments. However, with 1 exception (miR-195), our miRNA data are in good agreement with the 6 miRNAs found to be regulated in human heart failure reported by Olson’s group. The data obtained after aortic banding or with transgenic mice overexpressing activated calcineurin A leading to cardiac hypertrophy are not comparable to a situation of end-stage human heart failure because during initiation and progression of cardiac hypertrophy, different signaling pathways and subsequent gene networks are activated compared with the failing heart (see Reference 1 for review). Thus, different miRNA expression patterns between heart tissue of patients with end-stage heart failure and a model of cardiac hypertrophy in mice may have been expected. Further differences between the studies may depend on the different miRNA isolation processes.

Limitations

Mammalian miRNAs exert their function by both transcript degradation and translational inhibition, resulting in changes in gene and/or protein expression. This study sought to investigate a potential underlying role of miRNAs in the observed changes in gene expression in end-stage heart failure. Therefore, we did not investigate potential changes in protein expression. However, this is certainly of great importance and will be an ambitious task for various laboratories in the world for many years.

Conclusions and Perspective

We discovered for the first time a highly concordant microRNA expression pattern in fetal and failing human cardiac
tissue. Reexpression of fetal miRNAs during heart failure provides a basis to modulate a substantial fraction of the cardiac transcriptome, including the activation of a fetal gene program. Future identification of miRNA actions and functions will substantially improve our understanding of cardiovascular biology. Development of drugs and molecules that specifically regulate cardiac miRNAs with subsequent normalization of altered target expression may lead to novel treatments for heart failure.

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Disclosures
Drs Thum and Bauersachs have filed a patent regarding the diagnostic and therapeutic use of microRNAs in cardiovascular disease. The remaining authors report no conflicts.

References
Thus, targeting miRNAs may finally open novel mechanistic treatment concepts for heart failure prevention and therapy. Systemically or locally applied precursor or inhibitory miRNA may provide new therapeutic strategies for the treatment of heart failure. Further, the development of endogenous miRNAs for the treatment of heart failure and other cardiac conditions is not only conceivable but a realistic goal. Identifying endogenous miRNAs that are specifically present in cardiomyocytes or endothelial cells and that can evoke cardiac hypertrophy and heart failure will help to develop and validate new therapeutic approaches for heart failure prevention and therapy.

**CLINICAL PERSPECTIVE**

Chronic systolic heart failure is characterized by progressive left ventricular remodeling associated with the reactivation of a fetal gene program. MicroRNAs (miRNAs), recently discovered small regulatory molecules that posttranscriptionally regulate the expression of several target genes, play a critical role during cardiac development and potentially in cardiac disease. The present study demonstrates highly congruent messenger RNA and miRNA expression patterns in fetal and failing human cardiac tissue. The striking concordance between messenger RNA expression and the presence of miRNA binding sites in regulated genes in failing heart tissue suggests that reexpression of fetal miRNAs during heart failure modulates a substantial fraction of the cardiac transcriptome, including the activation of a fetal gene program. Indeed, overexpression of specific miRNAs in cultured cardiomyocytes modified a set of fetal cardiac genes and led to development of cellular hypertrophy. Identification of cardiac miRNA actions and functions will substantially improve our understanding of heart failure development and progression. Systemically or locally applied precursor or inhibitory miRNA molecules that specifically influence cardiac miRNAs are expected to beneficially modulate gene networks in the heart. Thus, targeting miRNAs may finally open novel mechanistic treatment concepts for heart failure prevention and therapy.
MicroRNAs in the Human Heart: A Clue to Fetal Gene Reprogramming in Heart Failure
Thomas Thum, Paolo Galuppo, Christian Wolf, Jan Fiedler, Susanne Kneitz, Linda W. van Laake, Pieter A. Doevendans, Christine L. Mummery, Jürgen Borlak, Axel Haverich, Carina Gross, Stefan Engelhardt, Georg Ertl and Johann Bauersachs

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In the article, “MicroRNAs in the Human Heart: A Clue to Fetal Gene Reprogramming in Heart Failure,” by Thum et al (Circulation. 2007;116:258-267), 2 errors appeared:

(1) In Figure 1B, right panel, in the y-axis (“BGA axis2”), “2.0” should read “0.2” and “0.15” should read “0.05.”

(2) In the legend for Figure 2, “The exact values are shown in Table III...” should have read “The exact values are shown in Table II...”

The authors regret these errors.

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