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Reciprocal Regulation of MicroRNA-1 and Insulin-Like Growth Factor-1 Signal Transduction Cascade in Cardiac and Skeletal Muscle in Physiological and Pathological Conditions

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Background—MicroRNAs (miRNAs/miRs) are small conserved RNA molecules of 22 nucleotides that negatively modulate gene expression primarily through base paring to the 3′ untranslated region of target messenger RNAs. The muscle-specific miR-1 has been implicated in cardiac hypertrophy, heart development, cardiac stem cell differentiation, and arrhythmias through targeting of regulatory proteins. In this study, we investigated the molecular mechanisms through which miR-1 intervenes in regulation of muscle cell growth and differentiation.

Methods and Results—On the basis of bioinformatics tools, biochemical assays, and in vivo models, we demonstrate that (1) insulin-like growth factor-1 (IGF-1) and IGF-1 receptor are targets of miR-1; (2) miR-1 and IGF-1 protein levels are correlated inversely in models of cardiac hypertrophy and failure as well as in the C2C12 skeletal muscle cell model of differentiation; (3) the activation state of the IGF-1 signal transduction cascade reciprocally regulates miR-1 expression through the Foxo3a transcription factor; and (4) miR-1 expression correlates inversely with cardiac mass and thickness in myocardial biopsies of acromegalic patients, in which IGF-1 is overproduced after aberrant synthesis of growth hormone.

Conclusions—Our results reveal a critical role of miR-1 in mediating the effects of the IGF-1 pathway and demonstrate a feedback loop between miR-1 expression and the IGF-1 signal transduction cascade. (***Circulation***. 2009;120:2377-2385.)

Key Words: heart failure ■ hypertrophy ■ IGF-1 ■ microRNA ■ signal transduction

MicroRNAs (miRNAs/miRs) are small conserved RNA molecules of ≈22 nucleotides that negatively modulate gene expression in eukaryotic organisms. The base pairing of a specific miR to the 3′ untranslated region (UTR) of its messenger RNA (mRNA) targets leads to mRNA cleavage and/or translation repression.[1] Bioinformatic analysis predicts that each miR may regulate hundreds of targets, indicating their important role in most biological processes such as cell proliferation, apoptosis, and stress responses.[2–5] Recently, these small RNA molecules have also been demonstrated to be involved in myocardial and cardiac stem cell development,[6] cardiac hypertrophy and remodeling, and arrhythmias,[7–11] as is the case of miR-1,[6,12–14] the expression of which inversely correlates with cardiac hypertrophy.[11,15] Notably, mice harboring deletion of miR-1 showed cardiac defects, including misregulation of cardiac morphogenesis, electric conduction, and cell proliferation.[10,14] Among putative miR-1 targets, proteins such as growth factors (Toll-like), transcription factors, and signal transduction kinases have been validated as true targets.[12,13]

Clinical Perspective on p 2385

Insulin-like growth factor-1 (IGF-1) is a key regulator of growth, survival, and differentiation in most cell types,[16] and its importance is demonstrated by the conservation throughout the evolutionary scale.[16] In myocardial biology, IGF-1 and its signal transduction cascade are involved in the control of virtually every critical biological process, including development, cardiomyocyte size and survival, action potential, and excitation-contraction coupling.[17,18] In this report, we

Figure 1. IGF-1 regulation by miR-1. A, IGF-1 seed sequence alignment in different species. B, Northern blot analysis for miR-1 overexpressed by adenovirus (Ad-miR-1) in neonatal cardiomyocytes. C and D, Western blot analysis and real-time polymerase chain reaction for IGF-1 on neonatal cardiomyocytes infected with adenovirus expressing miR-1, miR-133, and empty vector (mean±SE, minimum of 3 experiments per group). GAPDH was used as internal control. E, Luciferase reporter assay (mean±SE, minimum of 3 experiments per group) on 293T cells, performed by cotransfection of miR-1 oligonucleotide (20 nmol/L) with a luciferase reporter gene linked to wildtype (wt) or mutated (mt) 3′ UTR of IGF-1 (different doses). *$P<0.05$ vs control (wt 3′ UTR using miR-143).

demonstrate not only that IGF-1 is a target of miR-1[19] but, remarkably, that miR-1 expression per se depends on the activation state of the IGF-1 signal transduction cascade. Indeed, IGF-1 upregulation correlates with miR-1 depression and vice versa. Together, these findings unravel a unique reciprocal molecular circuit between miR-1 and IGF-1.

Methods

Animals

All procedures involving animals were performed in accordance with institutional guidelines for the care and use of laboratory animals.

Transverse aortic constriction (TAC) experiments were performed on male C57/Bl6 mice ranging in age from 10 to 12 weeks, with 6 animals per group. AKT transgenic mice with cardiac-specific overexpression of constitutively active AKT were male mice aged 10 to 12 weeks.

Cell Cultures and Adenoviral Infection

Mouse neonatal cardiomyocytes were prepared as described previously.[11] Mouse C2C12 and 293T cells were obtained from ATCC (Manassas, Va) and maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, Calif) supplemented with 4.5 g/L glucose, 4 mmol/L L-glutamine, 10% fetal bovine serum, and penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere. C2C12 cells were differentiated in Dulbecco's modified Eagle's medium
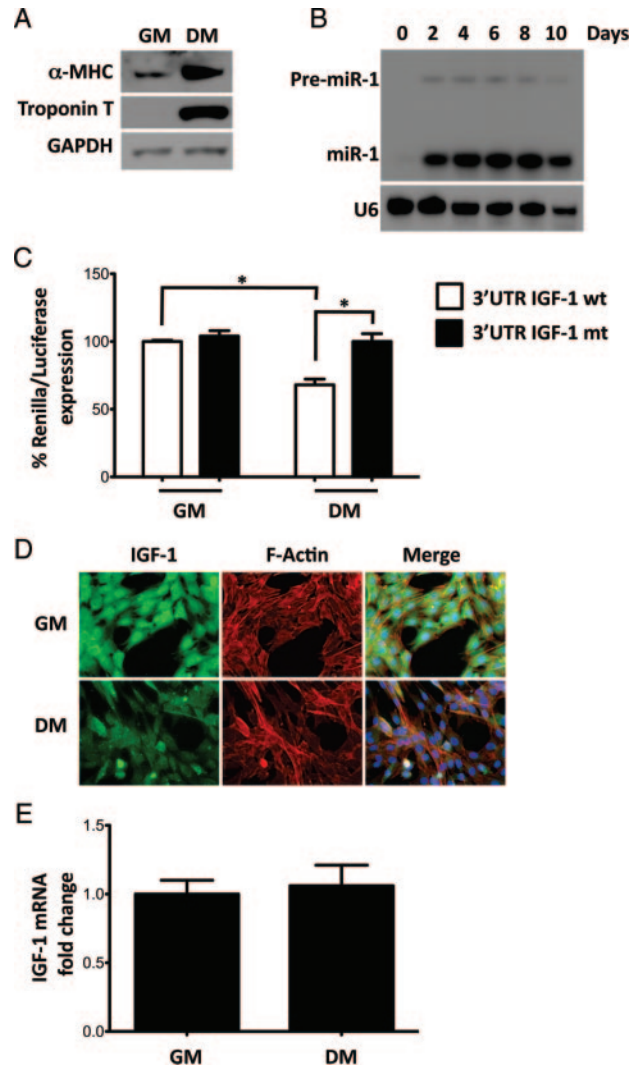

Figure 2. IGF-1 regulation by miR-1 on skeletal muscle C2C12 cells. A, Western blot analysis for the C2C12 differentiation markers α-myosin heavy chain (α-MHC) and troponin T. B, miR-1 expression in C2C12 cells after differentiation into myotubes. Cells were switched to differentiation medium (DM) at day 0. C, Luciferase reporter assay (mean±SE, minimum of 3 experiments per group) on C2C12 cells cultured in growth medium (GM) and differentiation medium with a luciferase reporter gene linked to wild-type (wt) or mutated (mt) 3′ UTR of IGF-1. *$P<0.05$ vs controls (wt 3′ UTR in growth medium or mt 3′ UTR in differentiation medium). D, Immunofluorescence staining for IGF-1 and F-actin of C2C12 cells cultured in growth medium and differentiation medium for 2 days. E, Quantitative polymerase chain reaction for IGF-1 on C2C12 cells cultured in growth medium and differentiation medium. GAPDH was used as internal control.

supplemented with 4% horse serum. MiR-1 and miR-133 adenoviral vectors (Ad-miR-1 and Ad-miR-133, respectively) as well as the Ad-Empty control have been described previously.[11]

Bioinformatics

MiR-1 target prediction was performed with the use of the following algorithms: miRanda (http://microrna.sanger.ac.uk), TargetScan (http://www.targetscan.org), and PicTar (http://pic-tar.bio.nyu.edu). The 3′ UTR ΔG was calculated with the use of the software mFOLD (http://frontend.bioinfo.rpi.edu/applications/mfold/). MiR-1 promoter analysis was performed with the use of the software MatInspector (http://www.genomatix.de/products/MatInspector).
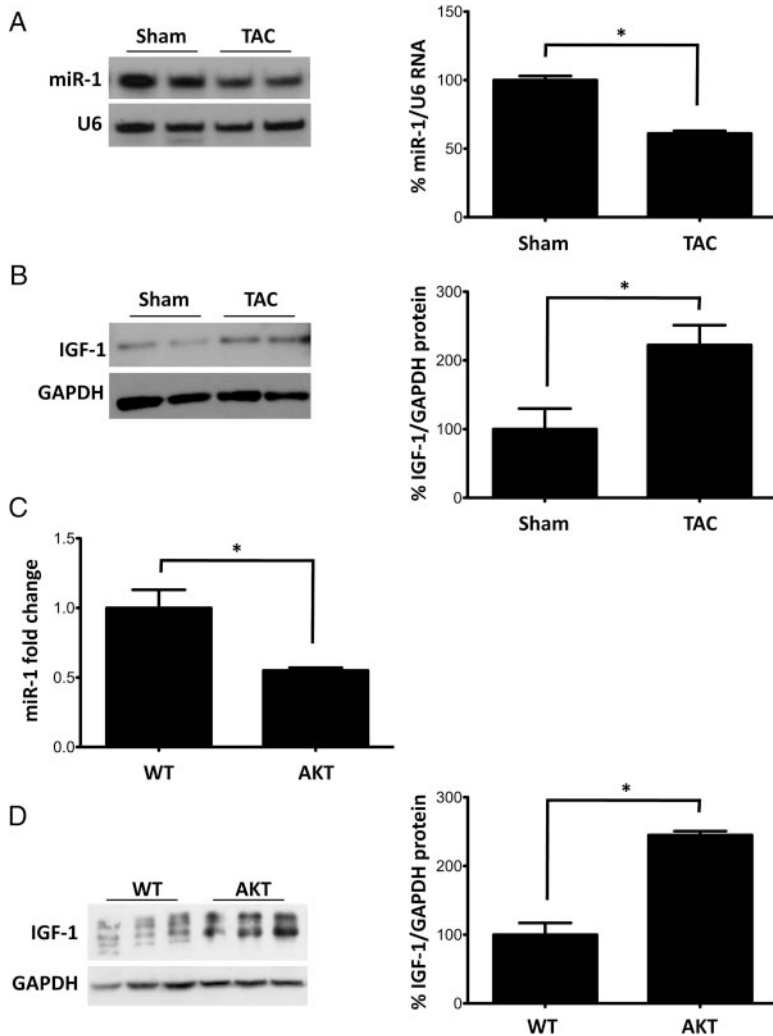
Figure 3. Reciprocal regulation of miR-1 and IGF-1 expression in TAC and AKT overexpression models of cardiac hypertrophy. A, Northern blot analysis and relative expression values of miR-1 in sham-operated and TAC mice (representative results, mean±SE of a minimum of n=5 mice per group). B, Western blot analysis for IGF-1 in left ventricles from sham- and TAC-operated mice (representative results, mean±SE of a minimum of n=5 mice per group). Band intensities were quantified with the use of ImageJ software version 1.34 (http://rsb.info.nih.gov/ij/) and normalized to GAPDH. C, miR-1 quantitative real-time polymerase chain reaction on AKT and wild-type (WT) mice. D, Western blot analysis for IGF-1 expression on AKT and wild-type mice (representative results, mean±SE of a minimum of n=5 mice per group). *$P<0.05$ vs controls (sham or WT).

Reporter Assays

The miR-1 promoter, which contains 2 potential binding sites for Foxo3a, was amplified from mouse chromosome 2 with the use of KOD Taq polymerase (Novagen, San Diego, Calif) and the following primers: forward, 5′-CTCTCAGTATCCTAATCCTC-3′; and reverse, 5′-GTAGGCACTCCTGCGCCGGC-3′. The promoter was cloned into the reporter plasmid, pGL3.basic (Promega, Madison, Wis). For the promoter experiment, 293T cells were seeded in 24-well plates (Nunc, Rochester, NY) and infected with adenoviruses (Ad) carrying a dominant negative (DN) or dominant active (DA) form of AKT[20,21] or Foxo3a[22] (kindly provided by Dr Domenico Accili, Columbia University) and subsequently cotransfected with the miR-1 promoter-luciferase reporter (100 ng) construct and the Renilla luciferase plasmid (10 ng) with the use of Lipofectamine 2000 (Invitrogen). For IGF-1 and IGF-1 receptor (IGF-1R) 3′ UTR reporter assays, experiments were performed on human 293T and murine C2C12 cell lines. The 3′ UTR segments of IGF-1 and IGF-1R 3′UTR mRNAs were subcloned by standard procedures into psiCHECK-2 (Promega) immediately downstream of the stop codon of the luciferase gene. MiR-1 seed mutagenesis was performed as described by the manufacturer (Stratagene, La Jolla, Calif). 293T cells were transfected with the reporter plasmid (10, 50, and 100 ng) and miR mimics (20 nmol/L) (Dharmacon, Lafayette, Colo). The IGF-1 reporter plasmid (50 ng) was transfected into C2C12 cells with Lipofectamine following a modified manufacturer's protocol.[23] Cells were lysed and assayed for luciferase activity either 24 or 48 hours after transfection. All luciferase assays were performed with the use of the Dual Luciferase kit (Promega), as described previously.[11,12]

Western Blot Analyses

Neonatal cardiomyocytes, C2C12, and 293T cells were collected at different time points (24, 48 hours) and lysated with the use of RIPA buffer. The following antibodies were used: IGF-1 (Laboratory Vision Corporation, Fremont, Calif), α-myosin heavy chain (ABcam, Cambridge, Mass), troponin T (Thermo Scientific, Waltham, Calif), GAPDH (mouse anti-rabbit GAPDH, Cell Signaling, Danvers, Mass), total AKT (Cell Signaling), Ser473 P-AKT (Cell Signaling), total Foxo3a (Cell Signaling), and Tyr32 P-Foxo3a (Cell Signaling). Densitometry analyses were performed with the use of ImageJ (National Institute of Health).

RNA Quantification

For IGF-1 RNA quantification, total RNA was extracted with the use of the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Complementary DNA was prepared with the use of SuperScript Reverse Transcriptase cDNA Kit (Invitrogen). Sybr green quantitative polymerase chain reaction was performed with the use of the following primers: IGF-1-5′ (5-CACCTCAGACAGGCATTGTG-3); IGF-1-3′ (5-TCTGA-GTCTTGGGCATGTCA-3); GAPDH-5′ (5-GACGGCCGCATC-TTCTTGT-3), GAPDH-3′ (5-CACACCGACCTTCACCATTTT-3). For miRNA quantitative reverse transcription–polymerase chain reaction, total RNA was extracted with the use of TRIzol; primers and probes specific for human/mouse miR-1 and the internal controls Sno202 RNA and U6 were purchased from Applied Biosystems. Amplification and detection were performed with the use of the 7300 Sequence Detection System (ABI), with 40 cycles of denaturation at
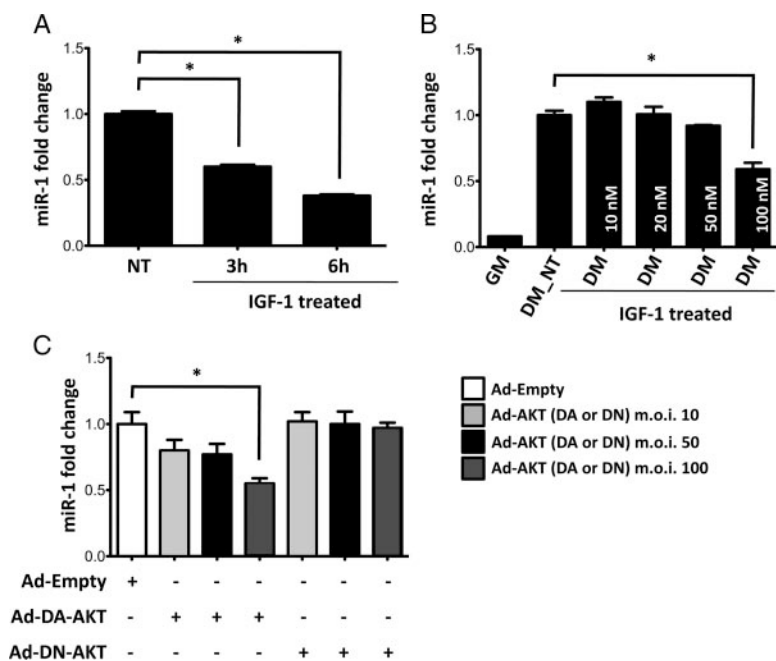
Figure 4. Regulation of miR-1 by the IGF-1 pathway. A, miR-1 quantitative real-time polymerase chain reaction on neonatal cardiomyocytes treated with 10 nmol/L IGF-1 at different time points. NT indicates neonatal cardiomyocytes not treated. Sno202 RNA was utilized as internal control. B, miR-1 quantitative real-time polymerase chain reaction on differentiated C2C12 cells treated with various doses of IGF-1 for 2 days. GM indicates C2C12 kept in growth medium and not treated; DM NT, C2C12 differentiated not treated; and DM, C2C12 differentiated. Sno202 RNA was utilized as internal control. C, miR-1 quantitative real-time polymerase chain reaction on neonatal cardiomyocytes infected with Ad-DA-AKT and Ad-DN-AKT at different multiplicity of infection (m.o.i) values (Ad-Empty m.o.i 100; Ad-Empty m.o.i 90+Ad-AKT [DA or DN] m.o.i. 10; Ad-Empty m.o.i 50+Ad-AKT [DA or DN] m.o.i. 50; Ad-AKT [DA or DN] m.o.i. 100). Sno202 RNA was utilized as internal control. *P<0.05 vs controls (neonatal cardiomyocytes NT, C2C12 DM NT, and Ad-Empty). Data are expressed as the mean±SE of 3 independent experiments.

95°C (15 seconds) and annealing/extension at 60°C (60 seconds). For the miRNA quantification, this was preceded by reverse transcription at 42°C for 30 minutes and denaturation at 85°C for 5 minutes.

Northern blotting was performed to confirm the expression levels of miR-1. Probes and anti-sense oligonucleotides against mature miR-1 and U6 were locked nucleic acid based (Exiqon, Vedbaek, Denmark). Densitometry analyses were performed with the use of ImageJ (National Institutes of Health).

Immunofluorescence
C2C12 myoblasts were plated in Laboratory-Tek 2-well chamber slides (2.10^5 cells per well). IGF-1 staining was performed with the use of primary antibody (Laboratory Vision Corporation) together with Alexa Fluor 594 phalloidin (Molecular Probes, Carlsbad, Calif), diluted in 5% BSA/0.1% Triton X-100/PBS. Goat anti-rabbit antibody conjugated to Alexa Fluor 488 (Molecular Probes) was used as secondary antibody.

Myocardial Biopsies
Eight patients (6 male, 2 female; mean age, 46.8±13.6 years) with acromegaly were evaluated with noninvasive (ECG, 2-dimensional echocardiography) and invasive cardiac studies (cardiac catheterization, coronary and left ventricular angiography, and left ventricular endomyocardial biopsy) before neurosurgery and/or somatostatin analogue therapy. All invasive studies were performed after informed consent was obtained and with the approval of the ethics committee of the Department of Cardiovascular and Respiratory Science, University La Sapienza, Rome, Italy. Endomyocardial biopsies were drawn, stored, and processed as described previously.[24]

Statistical Analysis
Statistical and frequency distribution analysis was performed by GraphPad Prism 4.0 (GraphPad Software, Inc, La Jolla, Calif). Data in Figure 1 were analyzed with the use of a 2-way ANOVA adjusted pairwise test. Data in Figures 2 and 3 were analyzed with 2-way ANOVA adjusted for multiple comparisons and 1-way ANOVA, respectively. Data in Figures 4 and 5 were analyzed with 2-way ANOVA adjusted for multiple comparisons. Data in Figure 6 and Figure I in the online-only Data Supplement were analyzed with 1-way ANOVA and 2-way ANOVA adjusted for multiple comparisons, respectively. The linear correlation on acromegaly patient parameters was calculated with the Pearson product moment correlation coefficient. A value of $P<0.05$ was considered to be statistically significant.

Results

Bioinformatics
The identification of true miR target genes is a great challenge, and computational algorithms have been the major driving force in predicting miR targets to date.[25–27] In the present study, we used the following target prediction algorithms: miRanda (http://microrna.sanger.ac.uk),[28] TargetScan (http://www.targetscan.org),[29] and PicTar (http://pic-tar.bio.nyu.edu).[5,30] These approaches are based on the identification of elements in the 3′ UTR of target genes complementary to the seed sequence of the miR of interest, calculation of thermodynamic properties of 3′ UTRs, and phylogenetic conservation of complementary sequences in the 3′ UTRs of orthologous genes. To reduce the number of false-positive targets, we also analyzed mRNA secondary structure flanking the seed sequence. In fact, several lines of evidence have shown that complex mRNA tertiary structures might prevent miR/mRNA interactions that limit the number of single-stranded regions accessible for binding to miRs.[10,13] Using this approach, we identified several putative targets of cardiac relevance, including, as shown recently, IGF-1.[19] Its 3′ UTR contains only 1 seed sequence, which has a ΔG energy value of −4.5 and −11.9 kcal/mol for mRNA sequences 5′ and 3′ of the seed sequence, respectively. Interestingly, this sequence is evolutionarily conserved between species (Figure 1A). Another putative target included IGF-1R, which is involved in the IGF-1 pathway (Figure IA in the online-only Data Supplement). IGF-1R belongs to the large class of tyrosine kinase transmembrane receptors and is directly activated by IGF-1 as well as the related growth factor IGF-2.[31] The ΔG energy values of the 5′ and 3′ RNA sequences around their seed sequences are −18 and −14 kcal/mol for IGF-1R.
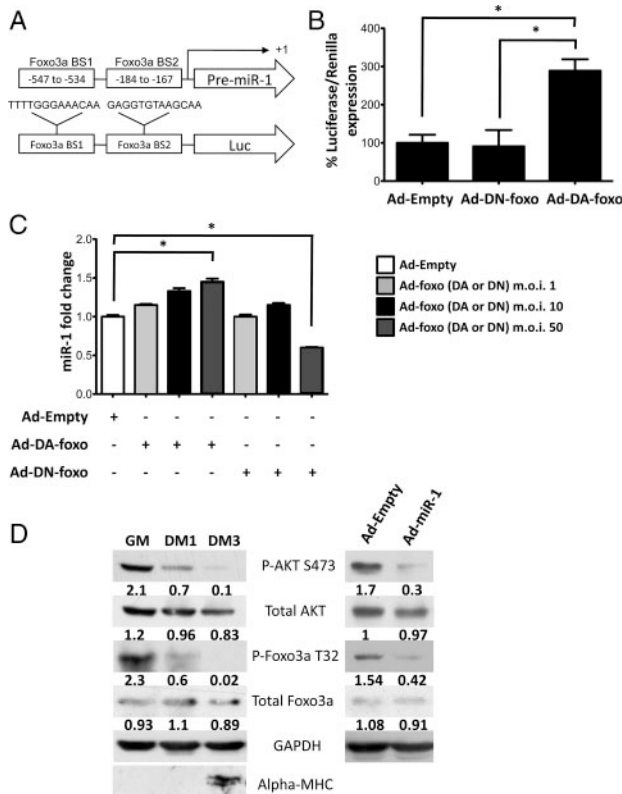
Figure 5. Foxo3a regulates miR-1 expression. A, The 2 potential Foxo3a binding sites on miR-1 promoter are indicated as BS1 and BS2. Both fragments of the miR-1 promoter were synthesized and linked to the luciferase (Luc) reporter gene. B, 293T cells were infected with Ad-Empty or Ad-DA-foxo and Ad-DN-foxo at a multiplicity of infection (m.o.i.) of 50. Twenty-four hours after infection, cells were transfected with empty vector (pGL3.basic) or miR-1 promoter constructs, respectively. Firefly luciferase activities were normalized to Renilla luciferase activities. C, Neonatal cardiomyocytes were infected with Ad-DA-foxo and DN-foxo at different m.o.i (Ad-Empty m.o.i 50; Ad-Empty m.o.i 49+Ad-foxo [DA or DN] m.o.i. 1; Ad-Empty m.o.i 40+Ad-foxo [DA or DN] m.o.i. 10; Ad-foxo [DA or DN] m.o.i. 50). Cells were harvested 48 hours after infection and analyzed for miR-1 levels by quantitative real-time polymerase chain reaction. Sno202 RNA was utilized as internal control. D, Western blot on C2C12 (DM1 and DM3 indicate differentiation medium at days 1 and 3, respectively) (left) and neonatal cardiomyocytes infected with Ad-miR-1 (m.o.i. 100) (right) for total AKT, P-AKT, total Foxo3a, and P-Foxo3a. Band intensities were quantified (bottom of each panel) as described in Figure 3. MHC indicates myosin heavy chain. *$P<0.05$ vs controls (Ad-Empty, Ad-DN-foxo). Data are expressed as the mean±SE of 3 independent experiments.

IGF-1 Is a Target of miR-1 in Cardiac Myocytes

In neonatal cardiomyocytes, overexpression of miR-1 but not its physiologically coexpressed miR-133 resulted in a significant decrease in IGF-1 protein levels (Figure 1B and 1C) without affecting its mRNA levels (Figure 1D). To confirm IGF-1 as a real miR-1 target, the wild-type 3′ UTR of IGF-1 was cloned downstream of the luciferase gene and assayed in 293T cells, which do not express endogenous miR-1 (data not shown). When cotransfected with miR-1 mimics, the assay resulted in a significant decrease (>45%) in luciferase activity (Figure 1E). In contrast, no effect was observed when an unrelated miR (miR-143) with no complementarity to any seed sequence in the 3′ UTR of IGF-1 was used (Figure 1E).

Consistent with these data, cotransfection of miR-1 with constructs containing mutated 3′ UTR seed sequences did not result in any decrease in luciferase activity (Figure 1E). Similar results were obtained for luciferase constructs containing 3′ UTRs of IGF-1R, in which cotransfection with miR-1 mimics resulted in a significant decrease in luciferase activity (Figure IB in the online-only Data Supplement). Consistent downregulation of IGF-1R protein level was found in differentiated C2C12 cells (Figure IC in the online-only Data Supplement).

Reciprocal Regulation of miR-1 and IGF-1 During C2C12 Skeletal Muscle Cell Differentiation

It is well established that IGF-1 regulates skeletal muscle cell differentiation[32] and that the miR-1 level changes during development in skeletal muscle cells.[12] In the present study, we used C2C12 cells as an in vitro cell model that recapitulates skeletal muscle differentiation. On serum withdrawal, C2C12 myoblasts stop proliferating, irreversibly exit from the cell cycle, differentiate, and fuse into multinucleated skeletal myotubes after the concomitant coexpression of muscle differentiation markers.[33] Consistent with that, upregulation of cell differentiation markers 2 days after serum withdrawal confirmed C2C12 differentiation (Figure 2A). Precursor and mature miR-1 levels, although absent at the myoblast stage, were remarkably increased at day 2 of differentiation (Figure 2B), confirming previous observations.[12] The expression of miR-1 increased significantly after the switch to differentiation conditions and remained at a high level up to day 10, when myotube formation was completed and contractile activity could be observed (data not shown).

To monitor changes in the miR-1 level on C2C12 differentiation, we performed a luciferase assay by transfecting the IGF-1 luciferase construct in proliferating and differentiating C2C12 cells. Results show a significant reduction (>35%) in luciferase expression in differentiated cells compared with mutant IGF-1 3′ UTR vector and with myoblast state (Figure 2C), indicating that inhibition of the reporter gene was a consequence of the increased endogenous level of miR-1. No changes were obtained when a luciferase reporter not carrying any 3′ UTR was transfected (data not shown). In addition, immunofluorescence staining of C2C12 cells kept in differentiation medium demonstrated a striking decrease in the IGF-1 protein level starting from day 2 of differentiation (Figure 2D). In contrast, the IGF-1 mRNA level was unaltered (Figure 2E).

Reciprocal Regulation of miR-1 and IGF-1 Expression in the TAC and AKT Overexpression Mouse Models of Cardiac Hypertrophy

We[11] and others[15] have previously shown an inverse correlation between miR-1 expression and cardiac hypertrophy. We thus hypothesized that repression of miR-1 in the TAC model (Table I in the online-only Data Supplement) would be accompanied by an increase in the IGF-1 protein level. In agreement with this, Western blot analysis demonstrated an increased IGF-1 protein level after TAC, together with a decreased miR-1 level (Figure 3A and 3B). Similarly, a reduction in miR-1 expression was found in another mouse
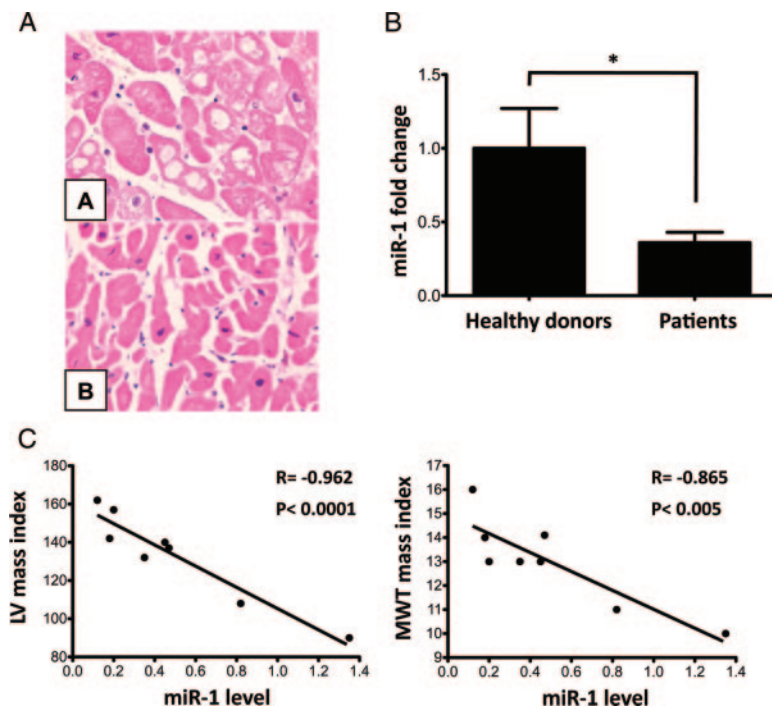
Figure 6. miR-1 expression in acromegalic patients. A, Left ventricular biopsy (A) from a patient with acromegalic cardiomyopathy showing remarkable cardiomyocyte hypertrophy compared with normal control (B) (hematoxylin-eosin staining; magnification ×250). B, miR-1 quantitative real-time polymerase chain reaction on heart biopsies from acromegaly patients compared with healthy donors. U6 RNA was utilized as internal control. C, Pearson product moment linear correlation between the left ventricular (LV) mass index, maximal wall thickness (MWT), and level of miR-1 in acromegaly patients. *$P<0.05$ vs control (healthy donors). Data are expressed as the mean±SE of 3 independent experiments.

model of cardiac hypertrophy, in which the downstream target of IGF-1, AKT, is constitutively overexpressed.[20] Consistent with our observations, decreased miR-1 expression was accompanied by an increased IGF-1 protein level (Figure 3C and 3D).

The IGF-1 Pathway Controls miR-1 Levels Through Foxo3a Transcriptional Regulation

Molecular circuits in which miRs control the level of a factor that in turn regulates the same miRs have been described. For instance, Fontana and al[34] described a circuitry involving sequentially miR-17-5p-20a-106a and AML1, in which miR-17-5p-20a-106a functions as a master gene complex interlinked with AML1 in a mutual negative feedback loop. We hypothesized that a circuit between miR-1 and IGF-1 might exist in striated muscle. To test this assumption, we measured miR-1 levels in different in vitro cell models in which IGF-1 activity was either diminished or increased. In neonatal cardiomyocytes treated with IGF-1, miR-1 expression was significantly decreased starting from 3 hours after treatment and remained depressed until 6 hours after treatment (Figure 4A). Notably, in C2C12 differentiated cells in which miR-1 is highly expressed, treatment with increasing IGF-1 doses induced a concomitant decrease in miR-1 expression with a significant reduction at a dose of 100 nmol/L IGF-1 (Figure 4B). A similar downregulation in miR-1 levels was observed when neonatal cardiomyocytes were infected with an adenovirus carrying a dominant active form of AKT (DA-AKT)[20] but not a dominant negative mutant (DN-AKT) (Figure 4C).

To study miR-transcriptional regulation, we analyzed the promoter region of miR-1 and identified 2 potential binding sites for Foxo3a, a transcription factor with cellular localization and transcriptional activity depending on its phosphorylation status, which in turn is regulated by AKT (Figure 5A). The miR-1 level was measured in neonatal cardiomyocytes

infected with adenovirus carrying a dominant active (DA-foxo) or dominant negative form (DN-foxo) of Foxo3a. In DA-foxo, all 3 potential AKT phosphorylation sites have been replaced by nonphosphorylatable amino acids, preventing it from being excluded from the nucleus in response to insulin, making it constitutively active.[22] In DN-foxo, the transactivation domain (Δ256), which is important for coactivator recruitment, has been deleted. Our results demonstrate that although DA-foxo could upregulate miR-1 levels, the DN-foxo mutant could not (Figure 5C). These data strongly suggest that Foxo3a is able to influence miR-1 expression levels. Moreover, to test whether Foxo3a can regulate miR-1 promoter activity, we cloned a fragment containing the 2 potential binding sites mapped on mouse chromosome 2 into the luciferase vector. As shown in Figure 5B, DA-foxo, but not DN-foxo, induced a marked elevation of miR-1 promoter activity.

miR-1 overexpression should induce a decrease in the phosphorylation state of AKT and Foxo3 because of the decreased levels of IGF-1 and IGF-1R and thus a generalized decrease of IGF-1 signal transduction pathway. We therefore determined phospho-Foxo3a and phospho-AKT in differentiated C2C12 and neonatal cardiomyocytes infected with Ad-miR-1; data show a significant downregulation of both phospho-Foxo3a and phospho-AKT (Figure 5D). Taken together, these data suggest that Foxo3a can transcriptionally regulate miR-1 expression.

Repression of miR-1 Levels and Correlation With Cardiac Hypertrophy in Acromegalic Patients

Acromegaly is a syndrome that results from overproduction of growth hormone, which in turn increases IGF-1 synthesis in peripheral tissues and leads to a significant elevation in the IGF-1 plasma level.[35] Cardiac hypertrophy is a dangerous consequence of growth hormone overproduction, and echo-

Table. Clinical and Echocardiographic Characteristics of Acromegalic Patients

	Patients							
Variables	1	2	3	4	5	6	7	8
Age, y	37	30	49	31	69	55	57	47
Sex	M	M	M	F	F	M	M	M
Body surface area, g/m^2	1.95	1.99	2.03	2.27	1.68	2.11	2.10	2.11
Presumed disease duration, y	7	2	4	4	10	1	2	4
IGF-1 at biopsy, ng/mL*	1241	1205	634	952	657	938	938	787
LV ejection fraction, %	54	58	56	60	50	50	52	55
Maximal wall thickness, mm	14	13	10	11	13	14	16	13
LV mass index, g/m^2†	151	132	88	103	143	132	160	136

LV indicates left ventricular.
*Normal range=80-330 ng/mL.
†LV mass index calculated according to Devereux and Reichek[36]; normal values=91±20 g/m^2.

cardiographic analysis of cardiac function in patients with acromegaly consistently showed significantly increased left ventricular mass as well as end-diastolic left ventricular wall and septum thickness (Table).[36] Furthermore, histological examination of myocardial biopsies showed hypertrophied cardiomyocytes with interstitial, perivascular, and focal replacement fibrosis (Figure 6A). Because blood levels of IGF-1 are elevated in patients with acromegaly, we measured miR-1 levels in myocardial biopsies by quantitative reverse transcription–polymerase chain reaction (Figure 6B). Interestingly, all patients showed a significant reduction in cardiac miR-1 levels compared with healthy donors, and there was a linear inverse correlation between miR-1 levels and left ventricular mass and end-diastolic left ventricular wall thickness (Figure 6C).

Discussion

MiR-mediated posttranscriptional gene regulation is now considered a fundamental player of many genetic programs. However, despite our ability to identify miRs, few regulatory targets have been established for vertebrate miRs. In this study, we demonstrated the potential feedback loop in which miR-1 and its target IGF-1 are reciprocally regulated.

Binding of IGF-1 to its receptor activates its intrinsic receptor tyrosine kinase, which phosphorylates several intracellular substrates such as the insulin receptor substrate-1 and Shc,[37,38] leading to the activation of signaling pathways, including phosphatidylinositol 3-kinase/AKT pathways.[39] Active AKT in turn phosphorylates and inhibits the winged-helix family of transcription factors, Foxo3a. In the present study, we demonstrated not only through IGF-1 stimulation but also with transfection of AKT or its downstream transcription factor Foxo3a that the IGF-1 pathway controls miR-1 expression (Figure 7). In fact, overexpression of AKT in neonatal cardiomyocytes reduces miR-1 expression, whereas overexpression of Foxo3a increases the miR level. These data are further supported by the identification of IGF-1R[31] as a miR-1 target.

Several miR-1 cardiac targets involved in the control of hypertrophy, cell cycle, excitation-contraction coupling, and membrane excitability have been identified, including HDAC4,[12] Hand2,[13] the K$^+$ channel subunit, Kir2.1 (KCNJ2),

connexin 43 (GJA1),[14] Ras GTPase–activating protein (Ras-GAP), cyclin-dependent kinase 9 (Cdk9), fibronectin, Ras homolog enriched in brain (Rheb),[15] and, as recently shown, IGF-1.[19] This indicates that miR-1 is involved in many different biological processes.

The importance of the IGF-1 pathway in cardiac function is well established.[40] Indeed, the effects of IGF-1 on adult muscle are pleiotropic, ranging from antiapoptotic,[18,41–43] hypertrophic, and regenerative effects on cardiac muscle.[31,44,45] Thus, both miR-1 and IGF-1 are involved in most biological processes controlling cardiac function. Notably, our results strongly indicate miR-1 as a modifier and a regulator of the multiple effects of IGF-1 in cardiac muscle. The possibility that IGF-1 is a target of miR-1 has been suggested previously.[19,46] However, we show here the biological relevance of this functional interaction and demonstrate that it is part of the autoregulatory circuit in cardiac and skeletal muscle (Figure 7). Our results also indicate that miR-1 may simultaneously downregulate IGF-1R together with IGF-1 protein, leading to a significant downmodulation of the whole downstream signal transduction cascade. The importance of our observation is strengthened by data from human samples, which strongly indicate the involvement of the miR-1 and IGF-1 regulatory loop in controlling human
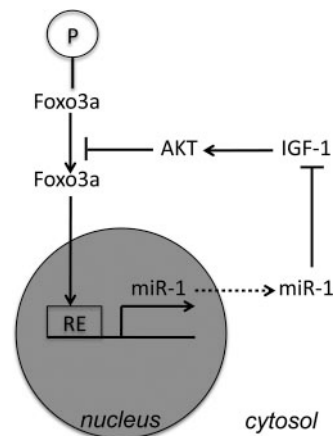
Figure 7. Proposed model of the miR-1/IGF-1 regulatory loop. RE indicates responsive element.

cardiac hypertrophy. Indeed, the striking inverse correlation between miR-1 levels and the degree of cardiac hypertrophy indicates that miR-1 is a key player in this process in humans. The interplay between IGF-I, AKT, Foxo3a, and miR-1 may provide a new paradigm to understand the manner in which IGF-1 modulates cardiac and skeletal muscle structure and function.[18,47]

Disclosures

None.

References

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116:281–297.
2. Cheng AM, Byrom MW, Shelton J, Ford LP. Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res*. 2005;33:1290–1297.
3. Felli N, Fontana L, Pelosi E, Botta R, Bonci D, Facchiano F, Liuzzi F, Lulli V, Morsilli O, Santoro S, Valtieri M, Calin GA, Liu CG, Sorrentino A, Croce CM, Peschle C. MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. *Proc Natl Acad Sci U S A*. 2005;102:18081–18086.
4. Xu P, Guo M, Hay BA. MicroRNAs and the regulation of cell death. *Trends Genet*. 2004;20:617–624.
5. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N. Combinatorial microRNA target predictions. *Nat Genet*. 2005;37:495–500.
6. Ivey KN, Muth A, Arnold J, King FW, Yeh RF, Fish JE, Hsiao EC, Schwartz RJ, Conklin BR, Bernstein HS, Srivastava D. MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. *Cell Stem Cell*. 2008;2:219–229.
7. van Rooij E, Sutherland LB, Liu N, Williams AH, McAnally J, Gerard RD, Richardson JA, Olson EN. A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc Natl Acad Sci U S A*. 2006;103:18255–18260.
8. van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J, Olson EN. Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science*. 2007;316:575–579.
9. Kwon C, Han Z, Olson EN, Srivastava D. MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling. *Proc Natl Acad Sci U S A*. 2005;102:18986–18991.
10. Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, Tsuchihashi T, McManus MT, Schwartz RJ, Srivastava D. Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell*. 2007;129:303–317.
11. Care A, Catalucci D, Felicetti F, Bonci D, Addario A, Gallo P, Bang ML, Segnalini P, Gu Y, Dalton ND, Elia L, Latronico MV, Hoydal M, Autore C, Russo MA, Dorn GW II, Ellingsen O, Ruiz-Lozano P, Peterson KL, Croce CM, Peschle C, Condorelli G. MicroRNA-133 controls cardiac hypertrophy. *Nat Med*. 2007;13:613–618.
12. Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL, Wang DZ. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet*. 2006;38:228–233.
13. Zhao Y, Samal E, Srivastava D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature*. 2005;436:214–220.
14. Yang B, Lin H, Xiao J, Lu Y, Luo X, Li B, Zhang Y, Xu C, Bai Y, Wang H, Chen G, Wang Z. The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. *Nat Med*. 2007;13:486–491.
15. Sayed D, Hong C, Chen IY, Lypowy J, Abdellatif M. MicroRNAs play an essential role in the development of cardiac hypertrophy. *Circ Res*. 2007;100:416–424.
16. Humbel RE. Insulin-like growth factors I and II. *Eur J Biochem*. 1990; 190:445–462.
17. Latronico MV, Costinean S, Lavitrano ML, Peschle C, Condorelli G. Regulation of cell size and contractile function by AKT in cardiomyocytes. *Ann N Y Acad Sci*. 2004;1015:250–260.
18. Santini MP, Tsao L, Monassier L, Theodoropoulos C, Carter J, Lara-Pezzi E, Slonimsky E, Salimova E, Delafontaine P, Song YH, Bergmann M, Freund C, Suzuki K, Rosenthal N. Enhancing repair of the mammalian heart. *Circ Res*. 2007;100:1732–1740.
19. Shan ZX, Lin QX, Fu YH, Deng CY, Zhou ZL, Zhu JN, Liu XY, Zhang YY, Li Y, Lin SG, Yu XY. Upregulated expression of miR-1/miR-206 in a rat model of myocardial infarction. *Biochem Biophys Res Commun*. 2009;281:597–601.
20. Condorelli G, Drusco A, Stassi G, Bellacosa A, Roncarati R, Iaccarino G, Russo MA, Gu Y, Dalton N, Chung C, Latronico MV, Napoli C, Sadoshima J, Croce CM, Ross J Jr. Akt induces enhanced myocardial contractility and cell size in vivo in transgenic mice. *Proc Natl Acad Sci U S A*. 2002;99:12333–12338.
21. Bellacosa A, Chan TO, Ahmed NN, Datta K, Malstrom S, Stokoe D, McCormick F, Feng J, Tsichlis P. Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene*. 1998;17: 313–325.
22. Nakae J, Kitamura T, Silver DL, Accili D. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J Clin Invest*. 2001;108:1359–1367.
23. Mercer SE, Ewton DZ, Deng X, Lim S, Mazur TR, Friedman E. Mirk/Dyrk1B mediates survival during the differentiation of C2C12 myoblasts. *J Biol Chem*. 2005;280:25788–25801.
24. Frustaci A, Chimenti C, Calabrese F, Pieroni M, Thiene G, Maseri A. Immunosuppressive therapy for active lymphocytic myocarditis: virological and immunologic profile of responders versus nonresponders. *Circulation*. 2003;107:857–863.
25. Bentwich I. Prediction and validation of microRNAs and their targets. *FEBS Lett*. 2005;579:5904–5910.
26. Rajewsky N. microRNA target predictions in animals. *Nat Genet*. 2006; 38(suppl):S8–S13.
27. Maziere P, Enright AJ. Prediction of microRNA targets. *Drug Discov Today*. 2007;12:452–458.
28. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res*. 2006;34:D140–D144.
29. Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell*. 2007;27:91–105.
30. Chen K, Rajewsky N. Natural selection on human microRNA binding sites inferred from SNP data. *Nat Genet*. 2006;38:1452–1456.
31. Reiss K, Cheng W, Pierzchalski P, Kodali S, Li B, Wang S, Liu Y, Anversa P. Insulin-like growth factor-1 receptor and its ligand regulate the reentry of adult ventricular myocytes into the cell cycle. *Exp Cell Res*. 1997;235:198–209.
32. Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD, Glass DJ. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol*. 2001;3:1009–1013.
33. Gu W, Schneider JW, Condorelli G, Kaushal S, Mahdavi V, Nadal-Ginard B. Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell*. 1993;72: 309–324.
34. Fontana L, Pelosi E, Greco P, Racanicchi S, Testa U, Liuzzi F, Croce CM, Brunetti E, Grignani F, Peschle C. MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. *Nat Cell Biol*. 2007;9:775–787.
35. Ren J, Samson WK, Sowers JR. Insulin-like growth factor I as a cardiac hormone: physiological and pathophysiological implications in heart disease. *J Mol Cell Cardiol*. 1999;31:2049–2061.
36. Devereux RB, Reichek N. Echocardiographic determination of left ventricular mass in man: anatomic validation of the method. *Circulation*. 1977;55:613–618.
37. Backer JM, Myers MG Jr, Sun XJ, Chin DJ, Shoelson SE, Miralpeix M, White MF. Association of IRS-1 with the insulin receptor and the phosphatidylinositol 3′-kinase: formation of binary and ternary signaling complexes in intact cells. *J Biol Chem*. 1993;268:8204–8212.
38. Sasaoka T, Rose DW, Jhun BH, Saltiel AR, Draznin B, Olefsky JM. Evidence for a functional role of Shc proteins in mitogenic signaling induced by insulin, insulin-like growth factor-1, and epidermal growth factor. *J Biol Chem*. 1994;269:13689–13694.

39. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*. 1999;96:857–868.

40. Saetrum Opgaard O, Wang PH. IGF-I is a matter of heart. *Growth Horm IGF Res*. 2005;15:89–94.

41. Muta K, Krantz SB. Apoptosis of human erythroid colony-forming cells is decreased by stem cell factor and insulin-like growth factor I as well as erythropoietin. *J Cell Physiol*. 1993;156:264–271.

42. Matthews CC, Feldman EL. Insulin-like growth factor I rescues SH-SY5Y human neuroblastoma cells from hyperosmotic induced programmed cell death. *J Cell Physiol*. 1996;166:323–331.

43. Jung Y, Miura M, Yuan J. Suppression of interleukin-1 beta-converting enzyme-mediated cell death by insulin-like growth factor. *J Biol Chem*. 1996;271:5112–5117.

44. Torella D, Rota M, Nurzynska D, Musso E, Monsen A, Shiraishi I, Zias E, Walsh K, Rosenzweig A, Sussman MA, Urbanek K, Nadal-Ginard B, Kajstura J, Anversa P, Leri A. Cardiac stem cell and myocyte aging, heart failure, and insulin-like growth factor-1 overexpression. *Circ Res*. 2004; 94:514–524.

45. Lembo G, Rockman HA, Hunter JJ, Steinmetz H, Koch WJ, Ma L, Prinz MP, Ross J Jr, Chien KR, Powell-Braxton L. Elevated blood pressure and enhanced myocardial contractility in mice with severe IGF-1 deficiency. *J Clin Invest*. 1996;98:2648–2655.

46. McCarthy JJ, Esser KA. MicroRNA-1 and microRNA-133a expression are decreased during skeletal muscle hypertrophy. *J Appl Physiol*. 2007; 102:306–313.

47. Lara-Pezzi E, Winn N, Paul A, McCullagh K, Slominsky E, Santini MP, Mourkioti F, Sarathchandra P, Fukushima S, Suzuki K, Rosenthal N. A naturally occurring calcineurin variant inhibits FoxO activity and enhances skeletal muscle regeneration. *J Cell Biol*. 2007;179:1205–1218.

CLINICAL PERSPECTIVE

MicroRNAs (miRNAs/miRs) are small conserved RNA molecules of 22 nucleotides that negatively modulate gene expression primarily through base paring to the 3′ untranslated region of target messenger RNAs. The muscle-specific miR-1 has been implicated in cardiac hypertrophy, heart development, cardiac stem cell differentiation, and arrhythmias through targeting of regulatory proteins. In this study, we investigated the molecular mechanisms through which miR-1 intervenes in regulation of cardiac and skeletal muscle cell growth and differentiation. We demonstrate that miR-1 controls the expression of insulin-like growth factor-1 (IGF-1) and IGF-1 receptor protein levels by translation. The IGF-1 pathway is critically involved in many aspects of cardiac development and cardiac function. We found that in conditions in which miR-1 is decreased, such as cardiac hypertrophy, IGF-1 is increased; the regulation is reciprocal because IGF-1 stimulation leads to downregulation of miR-1 levels. This effect is dependent on IGF-1 inhibition of the Foxo3 transcription factor, which regulates miR-1 promoter activity. The clinical relevance of these observations is demonstrated by analyzing the expression of miR-1 in cardiac biopsies of patients with acromegaly, a condition in which growth hormone and IGF-1 are overproduced and cardiac myocyte size is dramatically increased; miR-1 levels inversely correlate with echocardiographic parameters of cardiac mass in these patients. Our results add information in regard to the manner in which the IGF-1 pathway regulates cardiac function, demonstrating the role of miR-1 in response to cardiac stress and hypertrophy.