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the function of AKT by which it inhibits apoptosis through miR- 
21-dependent suppression of FasL.

Materials and Methods

DNA Constructs Cloned into Recombinant Adenovirus—A 
320-bp sequence encompassing the stem-loop of miR-21 was 
amplified from mouse genomic DNA by PCR using the follow-
ing primers: 5′-CTGGCTTGACCACCTCGTG-3′ and 5′- 
GACTGTGACGACTACCCCAA-3′ and cloned into recombi-
nant adenovirus downstream of a cytomegalovirus (CMV) 
promoter (23). For a control, a scrambled sequence (5′-GAA-
CCAGCCACCAGCGAC-3′) replaced the mature miRNA se-
quenue within its stem-loop structure (23). This was 
as a control in all experiments. The miR-21 eraser was synthe-
sized in the form of a tandem repeat antisense sequence of 
mature miR-21 terminating in (T)6. This construct was cloned 
to recombinant adenovirus downstream of a U6 promoter. 
Human PTEN cDNA (accession number NM_000314), pur-
chased from Oriogene, and short hairpin RNA targeting PTEN, 
synthesized in the form of a hairpin-forming oligonucleotide

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corresponding to bases 1471–1491 of *Mus musculus* PTEN (accession number NM_008960), were cloned into recombinant adenovirus downstream of a CMV and U6 promoter, respectively. Akt1/PKβa cDNA (activated) and dominant negative, kinase-defective, *dnAkt1* cDNA in pUSEamp from Upstate (Millipore), were cloned into recombinant adenovirus downstream of a CMV promoter.

**Construction of the miR-21 Transgenic Mouse**—The miR-21 transgene was constructed using the miR-21 DNA described above, cloned downstream of the α-myosin heavy chain promoter and upstream of an SV40 polyadenylation signal. The transgenic mouse was generated at the Transgenic Core Service, University of Medicine and Dentistry of New Jersey by Dr. Gassan Yehia. All required animal protocols were approved by the Institutional Animal Care and Use Committee at the New Jersey Medical School.

**Culturing Cardiac Myocyte and Treatments**—Cardiac myocytes were prepared as previously described (24). Briefly, hearts were isolated from 1- to 2-day-old Sprague-Dawley rats. After dissociation the cardiac myocytes were differentially separated by Percoll gradient centrifugation followed by a differential plating step for further separation of non-cardiac cells. Myocytes were then plated in Dulbecco’s modified essential medium/Ham’s F-12 (1:1) supplemented with 10% fetal bovine serum. Twenty-four hours after plating, the medium was changed to serum-free, and the cells are infected with recombinant adenoviruses at a multiplicity of infection of 10–20 particles/cell. Myofibroblast that were separated from the cardiac myocytes were isolated from the same Percoll gradient and cultured in Dulbecco’s modified essential medium/Ham’s F-12 (1:1) supplemented with 10% fetal bovine serum.

SW-480 colon cancer cell line was purchased from ATCC (catalogue number CCL-228) and propagated in Leibovitz’s L-15 medium supplemented with 2.0 mM L-glutamine and 10% fetal bovine serum, in a CO₂-free incubator. The medium was changed to Dulbecco’s modified essential medium/Ham’s F-12 (1:1) just before transfer to a hypoxic chamber. An MCF-7 breast adenocarcinoma cell line was purchased for ATCC (catalogue number HTB-22) and propagated in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum and 0.1 mg/ml bovine insulin, as recommended by the manufacturer.

**Construction of Adenoviruses**—Recombinant adenoviruses were constructed, propagated, and titered, as previously described by Dr. Frank Graham (25). Briefly, pHGHloxΔE1,3Cre (Microbix), including the ΔE1 adenoviral genome, was co-transfected with the pDC shuttle vector containing the gene of interest, into 293 cells using Lipofectamine (Invitrogen). Through homologous recombination, the test genes integrate into the E1-deleted adenoviral genome. The viruses were propagated on 293 cells and purified using CsCl₂ banding followed by dialysis against 20 mM Tris-buffered saline with 2% glycerol. Titering was performed on 293 cells overlaid with Dulbecco’s modified Eagle’s medium plus 5% equine serum and 0.5% agarose.

**Northern Blotting**—This was carried out as described previously (23).
Malated to that of U6 (n = 4). The numbers were averaged and plotted as relative values to the control adjusted to 1. Error bars represent ± S.E. and * p < 0.05 versus normoxia (0 time point). b, 16-week-old C57Bl/6 mice were subjected to coronary artery occlusion (CAO) for the intervals indicated. For each time point cardiac tissue from the ischemic and remote regions, and a sham-operated heart, were dissected, and total RNA was extracted and used for Northern blot analysis. The autoradiogram signals for miR-21 were quantified and normalized to that of U6 (n = 4). The numbers were averaged and plotted as relative values to the sham adjusted to 1. Error bars represent ± S.E. and *, p < 0.05 versus sham. c, myofibroblasts, MCF-7, and SW480 cells were exposed to hypoxia for the indicated periods. RNA was extracted and analyzed by Northern blotting. The numbers below the autoradiogram are the relative levels of miR-21/U6 signal (n = 2).

and a knot was tied on top of the tubing to occlude the left anterior descending coronary artery. At this point, if permanent occlusion is the objective, the chest cage is then closed in layers and the pneumothorax reduced. On the other hand, for ischemia/reperfusion, the left anterior descending coronary artery was occluded for 45 min only, after which the knot was released for a 16-h reperfusion period. The loosened ligature was left in place for subsequent occlusion prior to infusion of Evans blue dye, which was used to assess the area at risk at the conclusion of the reperfusion period. The coronary occlusion and reperfusion were verified by visual inspection and by electrocardiograph monitoring. Throughout the procedure, a 36.8–37 °C rectal temperature was maintained.

**Triphenyltetrazolium Chloride Staining**—At the conclusion of the ischemia/reperfusion protocol, the animals were anesthetized, the chest cage was opened, and the coronary artery was re-occluded at the same site, using the ligature that was loosened in situ. Evans Blue dye (0.5%) was perfused for a few minutes into the coronary artery above the site of ligation before the hearts were excised and sliced into a 1-mm-thick cross-section. The sections were fixed with formaldehyde and stained with triphenyltetrazolium chloride (1%) solution for 15 min at 37 °C. Both sides of each slice were photographed using a camera-mounted dissecting microscope. ImageJ software was then used to assess the percent infarct area relative to the area at risk.

**Echocardiography**—Mice were anesthetized with 2.5% avertin (0.010–0.015 ml/g body weight) administered by intraperitoneal injection. Transthoracic echocardiography (Sequoia C256, Acuson, Mountain View, CA) was performed using a 13-MHz linear ultrasound transducer. The chest was shaved. Mice were placed on a warm saline bag in a shallow left lateral position, and warm coupling gel was applied to the chest. Electrocardiographic leads were attached to each limb using needle electrodes. Two-dimensional images and M-mode tracings (sweep speed = 100–200 mm/s) were recorded from the parasternal short-axis view at the mid papillary muscle level. Care was taken not to apply too much pressure to the chest wall. The images were recorded on videotape, and freeze frames were printed on a Sony color printer, scanned into a PC using Photoshop (Adobe). The images were then analyzed by using the NIH Imagej program. M-mode measurements of LV internal diameter (LVID) and wall thickness were made from three consecutive beats and averaged using the leading edge-to-leading edge convention adopted by the American Society of Echocardiography. End-diastolic measurements are taken at the time of the apparent maximal LV diastolic dimension. End-systolic measurements are made at the time of the most anterior systolic excursion of the posterior wall. LVEF is calculated by the cubed methods as follows: LVEF = LVIDd − LVID LVId, where d indicates diastolic and s indicates systolic. LV percent fractional shortening (LVFS) is calculated as LVFS% = [(LVEDD − LVESD)/(LVEDD)]/100. Heart rate is calculated from the period between two consecutive electrocardiogram tracings.

**Hemodynamic Measurements**—Mice were anesthetized as described above, and a 1.4-French (Millar Instruments) catheter tip micromanometer catheter was inserted through the right carotid artery into the aorta and then into the LV where pressures, dp/dt, and −dp/dt were recorded.

**Statistics**—For comparing two experimental groups, an unpaired, two-tailed, Student t test (Excel software) was used for calculating the probability values. p < 0.05 was considered significant.

**RESULTS**

**Hypoxia Induces Down-regulation of miR-21**—Because miR-21 has been reported to target a plethora of antiapoptotic genes, we decided to examine its expression levels in cardiac myocytes exposed to hypoxia and in the ischemic myocardium. Fig. 1a

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**FIGURE 1.** Down-regulation of miR-21 in cardiac myocytes exposed to hypoxia. a, myocytes were exposed to hypoxia for increasing intervals, as indicated. The treatments were staggered to allow synchronized extraction of total RNA and Northern blot analysis. The autoradiogram signals for miR-21 were quantified and normalized to that of U6 (n = 4). The numbers were averaged and plotted as relative values to the control adjusted to 1. Error bars represent ± S.E. and *, p < 0.01 versus normoxia (0 time point). b, 16-week-old C57Bl/6 mice were subjected to coronary artery occlusion (CAO) for the intervals indicated. For each time point cardiac tissue from the ischemic and remote regions, and a sham-operated heart, were dissected, and total RNA was extracted and used for Northern blot analysis. The autoradiogram signals for miR-21 were quantified and normalized to that of U6 (n = 4). The numbers were averaged and plotted as relative values to the sham adjusted to 1. Error bars represent ± S.E. and *, p < 0.05 versus sham. c, myofibroblasts, MCF-7, and SW480 cells were exposed to hypoxia for the indicated periods. RNA was extracted and analyzed by Northern blotting. The numbers below the autoradiogram are the relative levels of miR-21/U6 signal (n = 2).
miR-21 Targets FasL and PTEN during Hypoxia—AKT is an established anti-apoptotic gene that has been implicated in the negative regulation of FasL (29, 30). Therefore, we questioned whether AKT regulates miR-21 or its targets in cardiac myocytes. To address this, we overexpressed constitutively active AKT (caAKT) in the myocytes before exposure to hypoxia. Similar to overexpression of miR-21, this resulted in inhibition of basal levels of either PTEN or FasL in cardiac myocytes but did completely suppress their up-regulation after exposure to hypoxia (Fig. 2c). In contrast, knockdown of miR-21 by an antisense construct (miR-21-eraser) induced up-regulation of both genes. This suggests that miR-21 is saturating during normoxia but limiting/reduced during hypoxia, relative to its target mRNAs. This is in contrast to the results observed with the luciferase reporter assay in Fig. 2b, where the cells were supplemented with both an exogenous target (luciferase reporter) and miR-21.

There have been contradictory reports regarding the localization of Fasl to cardiac myocytes (26–28). Also, because primary myocyte cultures usually contain <10% of non-myocytes, the results of Western blot analysis could be ambiguous. Thus, to address this issue we co-immunostained myocytes for FasL and anti-αMHC (red), and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, blue) (n = 3). The asterisk marks non-cardiac, αMHC-negative cells. Arrowheads mark FasL-positive vesicles.

AKT Regulates miR-21 Expression

miR-21 Targets FasL and PTEN during Hypoxia—PTEN is a validated miR-21 target (2, 4, 5). Using TargetScan v5.1 and Microcosm target prediction software, we identified Fasl as another miR-21-predicted target, which has not been previously experimentally validated. Fig. 2a shows the alignment between miR-21 and FasL 3'-UTR of both the mouse and human genes. Inclusion of this sequence within the 3'-UTR of a luciferase gene rendered it responsive to hypoxia and to overexpression of miR-21, which induced an increase or a decrease in its expression, respectively, as demonstrated in Fig. 2b. However, mutations within the seed sequence’s binding site abrogated these responses. This suggests that miR-21 directly targets and inhibits FasL.

We also tested the effect of miR-21 on the expression of the endogenous protein. Interestingly, overexpression of miR-21 did not inhibit basal levels of either PTEN or FasL in cardiac myocytes but did completely suppress their up-regulation after exposure to hypoxia (Fig. 2c). In contrast, knockdown of miR-21 by an antisense construct (miR-21-eraser) induced up-regulation of both genes. This suggests that miR-21 is saturating during normoxia but limiting/reduced during hypoxia, relative to its target mRNAs. This is in contrast to the results observed with the luciferase reporter assay in Fig. 2b, where the cells were supplemented with both an exogenous target (luciferase reporter) and miR-21.

shows that miR-21 declined by 40 ± 7% within 1 h and by 75 ± 8% within 12–24 h of exposure to hypoxia. Meanwhile, there was no significant change in miR-1 levels (Fig. 1a). Similarly, within 6 h of coronary artery occlusion in mice, miR-21 was reduced by 40% in the ischemic region (Fig. 1b). In contrast, though, it increased by 1.8-fold in the perifarzone, as expected in myocytes undergoing hypotrophy. At this early stage of ischemia there was no significant inflammatory cell infiltration or myofibroblast proliferation detected in the ischemic region. We also examined the response of other cell types to hypoxia. Interestingly, colon cancer (SW480) and breast cancer (MCF-7) cell lines exhibited a slight increase in miR-21 after 24 h of hypoxia (Fig. 1c). However, fibroblasts isolated from the neonatal rat heart (myofibroblasts) exhibited a reduction in miR-21 within the same time frame. These differences between cell types may reflect a contrast in their tolerance to hypoxia, cancer cells being the most resilient. The results also suggest that miR-21-targeted genes may play a role in a normal cell’s response to hypoxia.

miR-21 Targets FasL and PTEN during Hypoxia—PTEN is a validated miR-21 target (2, 4, 5). Using TargetScan v5.1 and Microcosm target prediction software, we identified Fasl as another miR-21-predicted target, which has not been previously experim
In consensus, knockdown of PTEN (Ad.siPTEN) resulted in up-regulation of phospho-AKT (Fig. 3c) and inhibited hypoxia-induced FasL (Fig. 3a). To determine whether the effect of PTEN was mediated through inhibition of AKT, we delivered it to the cells in the presence of caAKT. The results show that the effect of caAKT was dominant and counteracted that of PTEN (Fig. 3a and b). Surprisingly, the results also revealed that caAKT inhibited the expression of the exogenously delivered PTEN (Fig. 3b). This suggested that AKT inhibition of both FasL and PTEN might be mediated through up-regulation of miR-21. Indeed, knockdown of miR-21 resulted in reversal of caAKT inhibition of FasL expression (Fig. 3a). Moreover, Northern blot analysis confirmed that caAKT induced up-regulation of miR-21 and rescued its down-regulation during hypoxia, which was abrogated upon pretreatment of the cells with Ad.miR-21-eraser (antisense miR-21) (Fig. 3, d and f). This suggests that caAKT suppresses FasL and PTEN during hypoxia through an miR-21-dependent mechanism.

The AKT pathway is commonly regarded in terms of its activation but rarely its inhibition. However, we speculated that if activated AKT inhibits hypoxia-induced FasL and PTEN and increases miR-21, it is likely that inhibition of endogenous AKT would be responsible for the reverse effects seen during hypoxia. Although determining the effect of knockdown of endogenous AKT by short silencing RNA on miR-21 and its targets was complicated by the rapid ensue of cell death, its inhibition by overexpression of a dominant negative mutant (Ad.dnAKT), PTEN, or treatment of the cells with wortmannin...
induced up-regulation of junctional FasL (Fig. 3a) and down-regulation of miR-21 (Fig. 3, e and f). This indicated that inhibition of AKT is sufficient for inducing down-regulation of miR-21 and may, thus, be the mediating signal during hypoxia. Fig. 3g shows that exposure of myocytes to hypoxia results in a biphasic effect on phospho-AKT and miR-21 levels. During short intervals (15 min) of hypoxia, AKT phosphorylation is enhanced and is associated with an increase in PTEN phosphorylation that deactivates PTEN and an increase in miR-21. However, longer periods of hypoxia induced a reduction in phospho-AKT, associated with an increase in total PTEN and a decrease in its phosphorylation, and reduced miR-21 levels. The data suggest that long term hypoxia induces inhibition of AKT, which mediates the down-regulation of miR-21 and up-regulation of FasL and PTEN. This may be initiated by dephosphorylation and activation of PTEN, which in turn deactivates the AKT pathway. The data also suggest that down-regulation of miR-21 during hypoxia is mediated through inhibition of AKT.

miR-21 Enhances AKT Phosphorylation—Because miR-21 suppresses the expression of PTEN it would, accordingly, be expected to enhance AKT phosphorylation. Thus, to confirm its inhibition of PTEN and also determine if this effect has any functional significance, we tested the consequence of overexpressing miR-21 on AKT phosphorylation. Under quiescent conditions, miR-21 had no influence on the phosphorylation levels of AKT, which reconciles with its lack of inhibition of PTEN under the same conditions (see Fig. 2c). In contrast, when it was added before stimulating the cells with an activator of the AKT pathway, such as insulin, we observed a 5-fold increase in phospho-threonine 308 and a 20-fold increase in phospho-serine 473 following 1 h of insulin treatment (Fig. 4, a and d). In contrast, the miR-21 eraser inhibited AKT phosphorylation by 40% (Fig. 4, b and d). Insulin was selected here for two reasons: one, it is a robust activator of AKT and, second, insulin resistance has been attributed to an increase in PTEN (31–36). As seen in Fig. 4a, insulin stimulation was accompanied by a 1.9-fold increase in PTEN that was completely suppressed by overexpression of miR-21. To determine if the effect of miR-21 was indeed mediated through suppression of PTEN, we supplied the cells with miR-21 in the presence of exogenous PTEN. This treatment resulted in complete abrogation of miR-21-enhanced AKT phosphorylation (Fig. 4, c and d). Thus, by suppressing PTEN, miR-21 enhances AKT activity, which, in turn, induces up-regulation of miR-21 (Fig. 3c), creating a positive feedback loop.

miR-21 Is Necessary for AKT-mediated Inhibition of Apoptosis—Because miR-21 is required for AKT-mediated inhibition of FasL during hypoxia, we questioned whether it is necessary or sufficient for mediating the antiapoptotic effects of this kinase. To address this we treated myocytes with caAKT in the presence or absence of miR-21-eraser, or independently with miR-21, before exposing them to hypoxia. After 24 h, live cells were loaded with JC-1 dye and immediately imaged. Fig. 5a shows that the JC-1 dye predominantly aggregates in the healthy mitochondria and emits a red florescence, whereas the excess monomeric form remains in the cytosol, emitting green florescence (Fig. 5a). During hypoxia the mitochondrial outer membrane is damaged, and this is reflected by a loss in the red JC-1 aggregates. Both overexpression of caAKT and miR-21 prevented mitochondrial damage by 80 ± 20% and 50 ± 13%, respectively, whereas knockdown of miR-21 partly reversed the effect of caAKT (35 ± 15%, Fig. 5, a and b). Interestingly, though, neither miR-21-eraser nor dnAKT were sufficient for inducing mitochondrial damage when applied during normoxia, despite the fact that they were both sufficient for inducing up-regulation of FasL.

Caspase-8 is a direct downstream mediator of the Fas-induced death signal, thus, to confirm the antiapoptotic effects of miR-21 and its contribution to AKT function, we measured the activity of this caspase. Interestingly, unlike its effect on mitochondrial damage, miR-21 completely inhibited hypoxia-induced activity of caspase-8, whereas the miR-21-eraser completely abolished AKT inhibition (Fig. 5c). This indicated that probably both the intrinsic and extrinsic apoptosis pathways are involved in mitochondrial injury during hypoxia, where miR-21 has a major role in inhibiting the latter only through suppression of FasL. On the other hand, AKT, through suppression of FasL, as well as BAD and Bax, has the potential to inhibit both pathways. Hence, miR-21 partly mediates the antiapoptotic effects of AKT.
Overexpression of miR-21 Retards Ischemic Damage and Heart Failure—To investigate the role of miR-21 in the heart in vivo, we generated a cardiac-specific transgenic mouse model overexpressing miR-21 (miR-21-TG). Two lines were obtained with 10- and 20-fold overexpression of the transgene relative to the wild-type littermates (Fig. 6a). These animals show no overt phenotype, and all physical and functional parameters of the heart are comparable to the wild-type mice before or after short term transverse aortic constriction. To examine whether miR-21 could exert its antiapoptotic effect in vivo in the adult myocytes, we subjected the mice to 45 min of ischemia followed by 16 h of reperfusion (I/R). Fig. 6b shows the results of Evans blue and triphenyltetrazolium chloride staining in cardiac sections from the miR-21-Tg and wild hearts after I/R. The infarct zone/area at risk was significantly smaller in the transgenic model (Fig. 6c). This is consistent with suppression of junc- tional FasL and, thus, cell death by the overexpressed miR-21.

We also investigated the effect of the transgene on long term ischemic injury in the heart. After 4 weeks of left coronary artery occlusion, cardiac function and physical parameters were assessed by echocardiography and hemodynamic measurements. Heart sections revealed a less dilated left ventricular chamber in the miR-21-Tg versus the wild-type mice (Fig. 6d) that was confirmed by echocardiography measurements of the left ventricular end diastolic and end systolic dimensions (Fig. 6f, LVEDD and LVESD). This, in addition to lower left ventricular end diastolic and systolic pressure (Fig. 6f, LVEDP and LVSP), and higher ejection fraction (EF(%)) in the miR-21-TG, demonstrates reduced signs of cardiac failure. In agreement, there was no detectable lung congestion or increase in lung weight in the transgenic mice (Fig. 6e). Moreover, there was less collagen deposition, which is a reflection of reduced myocyte death, inflammatory cell infiltration, and myofibroblast proliferation (Fig. 6g), in addition to inhibition of ischemia-induced up-regulation of PTEN, FasL, and caspase-6, and down-regulation of phospho-AKT, in the left ventricle of the miR-21-TG (Fig. 6h). We also confirmed that FasL was localized to the intercalated discs in the ischemic heart, which was undetectable in the transgenic model (Fig. 6i). Thus, the results demonstrate that the miR-21 transgene effectively inhibits PTEN and...

FIGURE 5. miR-21 inhibits hypoxia-induced apoptosis. a, myocytes plated on fibronectin-coated glass chamber slides were treated with Ad.miR-21, Ad.miR-21-eraser, Ad.dnAKT, Ad.caAKT, or a control virus, separately or in combination, for 24 h, as indicated, before exposing them to 24 h of hypoxia. Cells were then loaded with JC-1 dye and imaged live (n = 8). b, the red mitochondrial signals were quantified and graphed as relative values to control normoxia, adjusted to 1. Error bars represent ± S.E. and *, p < 0.05 versus normoxia. **, p < 0.05 versus control hypoxia. #, p < 0.05 versus caAKT with hypoxia. c, myocytes were treated as in a. Protein was extracted and analyzed for caspase-8 activity (n = 6). Results were averaged and plotted as relative activity/µg of protein. Error bars represent ± S.E. and *, p < 0.01 versus control normoxia. **, p < 0.05 versus caAKT plus hypoxia.
**AKT Regulates miR-21 Expression**

FasL during myocardial ischemia and ameliorates signs of cardiac failure.

**DISCUSSION**

miR-21 is one of the most commonly and highly up-regulated microRNA in cancer and cardiovascular diseases and, thus, one of the most studied. A preponderance of reports shows that it targets anti-apoptotic and tumor suppressor genes and is involved in promoting cardiac myocyte and cancer cell survival. Its antiapoptotic effect has been predominantly attributed to inhibition of PTEN and PDCD4 expression (2, 4–7). Whereas up-regulation of miR-21 in various pathological conditions has been the prevailing finding, we discovered herein that miR-21 is down-regulated in the ischemic heart, consistent with the results of a recent report (16). We observed a similar down-regulation in isolated cardiac myocytes and myofibroblasts after prolonged hypoxia, albeit at different rates. On the other hand, brief periods of hypoxia (15 min) induced up-regulation of miR-21 in myocytes, in concordance with its increase seen during ischemia preconditioning (37). In contrast, MCF-7 and SW-480 cancer cell lines exhibited a slight increase in miR-21 that did not decline for up to 48 h of hypoxia. This indicated that the pathway that induced down-regulation of miR-21 in myocytes is refractory to hypoxia in cancer cells. We found this to be an AKT-dependent pathway, whose inhibition during hypoxia may be initiated by dephosphorylation and activation of PTEN. This explains why cancer cells that are frequently deficient in PTEN might be insensitive to hypoxia-induced inhibition of AKT.

Thus, in this report we have identified AKT as a positive upstream regulator of miR-21, experimentally validated that FasL is a direct target of miR-21, and showed that AKT regulates PTEN and FasL expression in cardiac myocytes. We also show that this pathway is deactivated during ischemia or hypoxia, which is required for down-regula-
AKT Regulates miR-21 Expression

![Diagram of the hypoxia-regulated AKT-miR-21 pathway](image)

**FIGURE 7.** A diagram of the hypoxia-regulated AKT-miR-21 pathway. Long term hypoxia induces dephosphorylation and deactivation of AKT. This results in down-regulation of miR-21 and, consequently, up-regulation of its targets that include PTEN and FasL. FasL activates Fas and its downstream caspase cascade, whereas PTEN inhibits AKT, creating a positive feedback loop. This pathway can be counteracted by constitutively active AKT or overexpression of miR-21.

Survival function unto the cells. More significantly, AKT has been implicated in the negative regulation of FasL in smooth muscle (30) and cancer cells (38), and reduction of PDCD4 expression in cancer cells (39, 40), both of which are now validated targets of miR-21. Thus, AKT-induced up-regulation of miR-21 provides a mechanism whereby AKT inhibits these molecules. As evidence, we show that AKT-mediated suppression of FasL during hypoxia in cardiac myocytes was reversed upon knockdown of miR-21 (Fig. 3a), as was its effect on caspase-8 activity (Fig. 5c). However, its effect on mitochondrial integrity was only partly reversed (Fig. 5a). This suggests that up-regulation of miR-21 and suppression of FasL only partly contribute to the antianti apoptotic effects of AKT. Our recently published data support this conclusion, because it shows that, in addition to up-regulation of miR-21, AKT induces down-regulation of miR-199a and up-regulation of hypoxia-inducible factor-1alpha (Hif-1α) and Sirt-1 (41). It is important to note that during short term hypoxia Hif-1α exerts protective effects, whereas during prolonged hypoxia it is involved in a proapoptotic function that involves stabilization of nuclear p53, which in turn induces an increase in FasL mRNA (42). We have also previously shown that this could be suppressed by overexpression of miR-199a, which inhibits the expression of Hif-1α and, thus, p53 (43).

PTEN is an established negative regulator of AKT, but a reciprocal effect has not been shown. Upon overexpression of caAKT we found that it not only inhibited FasL but also suppressed PTEN. This is consistent with AKT inducing up-regulation of miR-21, which in turn inhibits PTEN. Conversely, PTEN may inhibit miR-21, via inhibiting AKT. This reciprocal relation between a miRNA and its target has been previously described for several miRNA target pairs, including miR-200 family and ZEB1 (44), miR-9 and REST (45), miR-145 and OCT4 (46), and let-7 and lin-28 (47), where the miRNA target is itself a negative regulator of the miRNA, creating a double negative feedback loop that could potentially augment their effects.

In our transgenic model, overexpression of miR-21 had no impact on physical or functional parameters of the heart during normal conditions. Similarly, overexpression of miR-21 in cultured myocytes had no effect on basal levels of its targets, PTEN or FasL, during resting conditions. This indicated that its levels are saturating relative to its targets under these settings. On the other hand, its function was uncovered following its down-regulation by an antisense construct or during ischemia, which proved necessary for up-regulation of its targets (Figs. 2c and 6(b-f)). Thus, the question remains regarding its role during cardiac hypertrophy or in cancer, in which it is highly up-regulated. One explanation is that it is an adaptive mechanism against the gradual development of ischemia as mass outgrows vascular supply during enhanced tissue growth. Alternatively, the increase in miR-21 parallels the increase in its target mRNAs, as a consequence of enhanced global transcription associated with induction of growth. Another possibility is that miR-21 might be targeting other genes under those conditions. Indeed, Thum et al. reported that miR-21 is predominantly up-regulated in the myofibroblast during cardiac hypertrophy or failure, where it promotes cell survival and fibrosis through inhibition of sprouty1 (13). Consequently, knockdown of miR-21 induced myofibroblast apoptosis and reduced fibrosis during cardiac failure. On the other hand, our data show that overexpression of miR-21 could reduce fibrosis during ischemic heart disease or failure by decreasing myocyte cell death and, thus, inflammatory cell infiltration, and fibroblast proliferation.

In conclusion, we have outlined a unique aspect of the AKT survival pathway: one in which it regulates the extrinsic apoptotic pathway in cardiac myocytes via miR-21-dependent sup-

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**FIGURE 6.** miR-21 protects the heart against ischemic injury. a, a transgenic mouse model was generated with a 320-bp sequence encompassing the stem-loop of mouse miR-21 downstream of the α-myosin heavy chain promoter (αMHC). Two lines were obtained and diagnosed by Northern blot analysis for miR-21 expression in the hearts of 10-week-old mice. b, miR-21-Tg and wild-type littermate mice were subjected to 45-min ischemia followed by 16-h reperfusion. Hearts were then perfused with Evans blue dye, fixed, sectioned, and stained with triphenyltetrazolium chloride. Shown are images from both sides of a representative section from each of the mice. c, the % area of risk (right) and % infarct zone/area at risk (left) were measured, averaged, and plotted (n = 6). Error bars represent ± S.E. and *, p < 0.05 versus wild type. d, 20-week-old transgenic and wild-type littermates were subjected to complete left coronary artery occlusion (CAO) or a sham operation for 4 weeks. The hearts were then isolated and sectioned to reveal the extent of left ventricular chamber dilatation (n = 6, each). e, lung weight and heart weight/tibial length (HW/TL) were calculated and graphed. Error bars represent ± S.D. and *, p < 0.01 versus sham, f, before sacrifice, cardiac wall and chamber dimensions, and functions, were assessed by echocardiography and hemodynamic measurements. *, p < 0.05 versus sham of matching genotype. f, p < 0.05 versus WT-CAO. g, hearts were fixed in formaldehyde, sectioned, and stained with Sirius Red for detection of collagen (red). The collagen was quantified (n = 3, 3 sections each) and graphed as fold increase in CAO hearts versus sham-operated ones. Error bars represent ± S.D. and *, p < 0.001 versus sham, and **, p < 0.001 versus wild type-CAO. h, total protein was extracted from similarly treated mice groups and analyzed by Western blotting for the molecules listed on the left of each panel. i, 20-week-old miR-21-Tg and wild-type littermates were subjected to CAO for 16 h. The hearts were isolated and fixed in formaldehyde and immunostained for FasL (purple).
pression of FasL. Thus, in general, the discovery of miRNA, and their functions, is introducing a new dimension to our existing knowledge of signaling molecules and pathways that remains to be explored and exploited for more precise therapeutically targeting.

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