Tafazzin knockdown interrupts cell cycle progression in cultured neonatal ventricular fibroblasts

Quan He, Miao Wang, Nicole Harris, and Xianlin Han

1Diabetes and Obesity Research Center, Sanford-Burnham Medical Research Institute, Orlando, Florida; and 2Hypertension and Vascular Research Division, Department of Internal Medicine, Henry Ford Hospital, Detroit, Michigan

Submitted 30 January 2013; accepted in final form 21 August 2013

He Q, Wang M, Harris N, Han X. Tafazzin knockdown interrupts cell cycle progression in cultured neonatal ventricular fibroblasts. Am J Physiol Heart Circ Physiol 305: H1332–H1343, 2013. First published August 30, 2013; doi:10.1152/ajpheart.00084.2013.—Mutation of the mitochondrial protein tafazzin causes dilated cardiomyopathy in Barth syndrome. Previous studies have shown that tafazzin knockdown promotes hypertrophy of neonatal cardiac myocytes. The current investigation was designed to show whether tafazzin knockdown affects cardiac fibroblast proliferation and collagen secretion, which contribute to fibrosis in dilated cardiomyopathy. In primary cultures of neonatal ventricular fibroblasts (NVFs) transduced with a tafazzin short hairpin RNA adenovirus, tafazzin knockdown increased production of reactive oxygen species and activation of mitogen-activated protein kinases and induced protein and DNA synthesis via cell cycle regulators. It also reduced intracellular ATP, activated AMPK, and caused multinucleation, hypertrophy, and enhanced collagen secretion. We concluded that tafazzin knockdown interrupts the NVF cell cycle and this in turn may contribute to fibrosis and dilated cardiomyopathy in Barth syndrome.

TAFAZZIN IS A MITOCHONDRIAL protein that plays an important role in cardiolipin synthesis and remodeling (15, 73). Cardiolipin is predominantly present in the inner membrane of mitochondria and is essential for their optimal function (13, 36). Studies have shown that knockdown of tafazzin results in cardiac and skeletal muscle defects and prenatal cardiomyopathy in mice (1, 58), retarded cardiac development and brady-cardia in zebrafish (40), and impaired motor activity in Droso-phila (72). Tafazzin mutation reduces cardiolipin, impairs mitochondrial function (1, 15, 16, 28, 72), and causes dilated cardiomyopathy (DCM) in Barth syndrome, a rare and often fatal x-linked genetic disorder accompanied by aciduria, neu-tropenia, and myocardial noncompaction (7, 64).

Fibrosis is a hallmark of cardiac dysfunction in cardiomyopathy and heart failure. It is characterized by proliferation of cardiac fibroblasts, excessive extracellular matrix (ECM) deposition, and transformation of fibroblasts into myofibroblasts. Fibroblasts constitute over 90% of nonmyocyte cells in the heart (2), yet occupy only one third of cardiac tissue (9). Because of their overwhelming number, proliferation, and the proteins they secrete, fibroblasts play fundamental roles in the heart (3, 10, 43), including 1) structural support; 2) regulation of ECM deposition and turnover; 3) secretion of paracrine factors including cytokines, chemokines, and growth factors; 4) electro-mechanical connections among myocytes, fibroblasts, and the ECM; and 5) sensing environmental changes. During the development of DCM, both cardiac myocytes and fibroblasts undergo remodeling. In contrast with eccentric hypertropy (elongation) of myocytes (60), fibroblasts proliferate and become myofibroblasts, a process that enhances ECM secretion and deposition (51, 57). Interrupted fibroblast and ECM networks directly contribute to the fibrosis and cardiac dysfunction seen in DCM.

Cell proliferation undergoes four steps from the quiescent G1 phase to S (DNA synthesis), G2 (DNA duplication), and M (mitosis). There is a restriction point between the G1 and S phases that must be overcome by mitogenic promoters to have the cell progress to S phase. Studies have shown that cell proliferation is induced by 1) activation of MAPKs including p42/44 and p38 (65), 2) induction of transcription and translation factors [e.g., early growth response protein (Egr-1) (5) and p70 s6 kinase (25)], and 3) induction of other cell cycle regulatory proteins [e.g., cyclins (66), proliferating cell nuclear antigen (PCNA) (50), and phosphorylation of retinoblastoma protein (Rb) (76)]. However, it is unknown whether tafazzin knockdown affects cardiac fibroblast proliferation and thereby contributes to the fibrosis and DCM manifest in Barth syndrome.

Based on our previous study showing that tafazzin knockdown causes hypertrophy of neonatal cardiac myocytes (34), we hypothesized that it interrupts cardiac fibroblast cell cycle progression. Transduction of rat neonatal ventricular fibroblasts (NVFs) with a tafazzin short hairpin (sh)RNA adenovirus knocked down tafazzin mRNA and protein, reduced cardiolipin, enhanced reactive oxygen species (ROS) production from mitochondria, activated p42/44 and p38 MAPKs, and induced transcriptional and translational factors and other cell cycle regulators (e.g., cyclin E, PCNA, and Rb phosphorylation). It also reduced intracellular ATP, activated AMPK, and halted proliferation. Tafazzin knockdown ultimately resulted in multinucleation, hypertrophy, and enhanced collagen secretion in NVFs.

MATERIALS AND METHODS

Animal protocols were approved by the Institutional Animal Care and Use Committees of the Sanford-Burnham Medical Research Institute, Orlando Diabetes and Obesity Research Center, and Henry Ford Hospital.

Supplies and chemicals. Phosphatase and proteinase inhibitor cocktail tablets (PhosSTOP and Complete Mini) were obtained from Roche Applied Science (Indianapolis, IN). Primary antibodies against phospho-p38 (Thr180/Tyr182), phospho-p42/44 (Thr202/Tyr204), phospho-SAPK/JNK (Thr183/Tyr185), phospho-Rb (Ser795), phospho-p70 s6 kinase (Thr389), phospho-AMPKα (Thr172), PCNA, Egr-1, β-actin, and GAPDH, and a horseradish peroxidase-conjugated secondary antibody against rabbit IgG were purchased from Cell
Signaling Technology (Boston, MA). Antibodies against cyclin E and tafazzin were from Santa Cruz (Santa Cruz, CA). The antibody against H9251-smooth muscle actin was obtained from Abcam (Cambridge, MA). Coomassie protein assay kit, SuperSignal West Pico chemiluminescent substrate kit, and Restore Plus Western blot stripping buffer were purchased from Thermo Scientific (Rockford, IL). DMEM and cell culture supplements, precast tris-glycine polyacrylamide gels, polyvinylidene fluoride membranes, SYBR Green PCR Master Mix, MitoTracker Green, the mitochondrial superoxide indicator MitoSOX Red, and Texas Red- and FITC-conjugated anti-rabbit IgG were obtained from Life Technologies (San Diego, CA). 3H-Thymidine and 3H-leucine were purchased from PerkinElmer (Waltham, MA).

Table 1.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin A1</td>
<td>5’ AGCAACGCGATTGTGTTCAAG 3’</td>
<td>5’ CAACTCGGAATTGGCTGCTC 3’</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>5’ GCCATCTGGACACAAATCT 3’</td>
<td>5’ GGCAGAGTACATTCCAACAAG 3’</td>
</tr>
<tr>
<td>Cyclin E1</td>
<td>5’ ATGTCCAAGTGGCCTACGTC 3’</td>
<td>5’ TCTGCATCAACTCCAACGAG 3’</td>
</tr>
<tr>
<td>Cyclin B1</td>
<td>5’ CTGCTGCAGGAGACCATGTA 3’</td>
<td>5’ CTACGGAGGAAGTGCAGAGG 3’</td>
</tr>
<tr>
<td>H9251-Acgon</td>
<td>5’ ACCAGACCGTGACACAAAGG 3’</td>
<td>5’ ACCAGACCGTGACACAAAGG 3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’ ATTCAACGGCACAGTCAAGG 3’</td>
<td>5’ TGGATGCAGGGATGATGTTC 3’</td>
</tr>
<tr>
<td>Tafazzin</td>
<td>5’ TGGGAAGCCCTTCAGTACAC 3’</td>
<td>5’ TGATTGTGGAGCTGTTCTGC 3’</td>
</tr>
</tbody>
</table>

For vector construction

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tafazzin</td>
<td>5’ ATGCCCCTCCATGTGAAG 3’</td>
<td>5’ TAGTGGACATGCTGGAAAT 3’</td>
</tr>
<tr>
<td>Tafazzin with linker</td>
<td>5’ ATGAATTCATGCCCCTCCATGTGAAG 3’</td>
<td>5’ CTATCTTCCAGGCTGGAAAT 3’</td>
</tr>
</tbody>
</table>

Fig. 1. Tafazzin short hairpin (sh)RNA adenovirus knocked down tafazzin expression leading to cardiolipin reduction. Neonatal ventricular fibroblasts (NVFs) were transfected with the tafazzin (Taz) shRNA adenovirus for 48 h and tested for tafazzin expression and cardiolipin.

Random primers and Omniscript reverse transcriptase were obtained from Qiagen (Valencia, CA). The OxiSelect intracellular ROS assay kit was obtained from Cell Biolabs (San Diego, CA). Custom primers were synthesized by TIB MolBiT (Adelphia, NJ). The shRNA adenovirus (pSilencer adeno 1.0-CMV) (34) was expanded and purified by the Gene Therapy Center Virus Core Facility of the University of North Carolina at Chapel Hill. The AAVCMV expression vector was obtained from the Penn Vector Core of the University of Pennsylvania (Philadelphia, PA). An ATP assay kit, antimycin A, oligomycin, picric acid, Direct Red 80, and the collagen standard were purchased from Sigma (St. Louis, MO). Mitochondria-targeted antioxidant mito-TEMPO, p38 MAPK inhibitor SB203580, MEK inhibitor U0126, and FCCP (carbonyl cyanide 4-trifluoromethoxyphenylhydrazone) were obtained from ENZO Life Sciences (Farmingdale, NY). Clone vector pGEM-T Easy, a PCR amplification kit, restriction endonucleases EcoR I and Kpn I, RNase inhibitor RNasin, FuGENE 6 transfection reagent, and oligo(dT) primer were obtained from Promega (Madison, WI), XF cell culture microplates, XF Calibrant, and XF96 extracellular flux assay kits were purchased from Seahorse Bioscience (North Billerica, MA). Other routine supplies and chemicals were purchased from Fisher and Sigma.

Cell culture. Cardiac fibroblasts were generated from Sprague-Dawley rat pups (Charles River Laboratories) at the preplating stage of the cardiac myocyte preparation (34). Cells were cultured in DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% fetal bovine serum in a humidified CO2 incubator at 37°C. Cells were passed with 0.05% trypsin/EDTA when they reached confluence. Passage 2 cells were used for all experiments.

Construction of tafazzin adeno-associated viral expression vector. Tafazzin cDNA was amplified by PCR with tafazzin-specific primers (Table 1) using oligo(dT) reverse transcripts as a template, which was generated by reverse transcription with total RNA from male c57 mice. The PCR product of tafazzin cDNA was cloned into pGEM-T Easy, and the tafazzin-coding region was re-amplified by PCR with primers containing Ecor I and Kpn I recognition sites (Table 1). The PCR product was digested with Ecor I and Kpn I and inserted into an AAVCMV plasmid, which was digested with the same restriction endonucleases. The coding region of tafazzin in AAVCMV under the CMV promoter was confirmed by sequencing at the Analytical Genomics Core Facility of Sanford Burnham Medical Research Institute.

Adenoviral transduction of NVF. Cells at 80% confluence were transduced with the tafazzin shRNA adenovirus or its control (6–10 pfu/cell) and maintained under serum-free conditions with DMEM supplemented with (in mg/l) 5 insulin, 5 transferrin, and 2.5 selenium. Cells were harvested for assay after 48 h incubation.

Transfection of NVF with plasmid DNA. NVFs were transfected with 1 μg plasmid DNA per well of a 6-well plate using FuGENE 6 transfection reagent in 1 ml DMEM supplemented with insulin, transferrin, and selenium following the manufacturer’s instructions. NVFs were incubated with DNA and transfection reagent for at least 40 h before assay.

Shotgun lipidomics. Cells were scraped into PBS and the pellets kept at −80°C until just before processing. Lipids were extracted and cardiolipin and the polyunsaturated fatty acid oxidation product 4-hydroxy-2-(E)-nonenal analyzed by shotgun lipidomics (31, 70, 74).

ROS. Cells were incubated with 1 μM DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate) for 1 h, lysed, and assayed for ROS using an OxiSelect kit from Cell Biolabs following the manufacturer’s instructions. ROS were represented by green fluorescence intensity, which was corrected for protein concentration and expressed as relative fluorescence units per milligram of protein.

To determine mitochondrial ROS, cells were incubated with 300 nM MitoTracker Green in serum-free medium at 37°C for 45 min. The cells were washed with HBSS (Hanks’ balanced salt solution) and incubated

Fig. 2. Tafazzin knockdown increased reactive oxygen species (ROS) production. ROS were determined after NVFs were transected with the tafazzin shRNA adenovirus. A: cells were transduced with the tafazzin shRNA adenovirus for 24 h, and ROS were measured with a green fluorescence kit and expressed as relative units per milligram of protein (RU/mg). Data represent means ± SD of 3 separate experiments. *P < 0.01 vs. SCR. B: 4-Hydroxy-2-(E)-nonenal (HNE) was analyzed by shotgun lipidomics (31, 70, 74). C: cells were incubated with 1 μM MitoTracker Green in serum-free medium at 37°C for 45 min. The cells were washed with HBSS (Hanks’ balanced salt solution) and incubated

AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00084.2013 • www.ajpheart.org
with 5 μM MitoSOX Red at 37°C for 5 min in HBSS. The cells were kept in HBSS after wash. Images were captured and analyzed in a similar way as described under Immunocytochemical staining.

Protein extraction and Western blot. Protein was isolated from NVFs with a lysis buffer containing proteinase and phosphatase inhibitors and subjected to Western blot (34).

ATP assay. ATP assay was performed as described previously (34).

Determination of oxygen consumption rate. NVFs (1 × 10^5/ well) were plated in the Seahorse 96 well culture plate overnight and transduced with shRNA adenovirus for 48 h after serum starved 24 h. Before loading the plate in an XF96 analyzer and measurement of oxygen consumption rate, OCR, cells were washed with PBS (warmed at 37°C) and incubated in Seahorse assay medium at 37°C for 1 h. OCR was determined following Seahorse Bioscience procedure.

Real-time RT-PCR. Total RNA isolation from NVFs and real-time RT-PCR were performed using the primers listed in Table 1 in an Eppendorf RealPlex2 as described previously (34). Target gene mRNA levels were determined using the ΔΔCt method (71) and expressed as relative to control (NVFs infected with a scrambled adenovirus).

3H-thymidine and 3H-leucine incorporation. Rates of DNA and protein synthesis by NVFs were estimated by 3H-thymidine and 3H-leucine incorporation, respectively. NVFs were plated onto 6-well plates at a density of 5 × 10^4 cells per well and incubated in DMEM containing 10% FBS. The medium was changed to serum-free DMEM when they reached 80% confluence. Cells were transduced with 5 × 10^8 pfu shRNA adenovirus or transfected with plasmid DNA, and 1 μCi 3H-thymidine or 3H-leucine was added to each well 6 h later. After 40 h, the cells were harvested for trichloroacetic acid precipitates, which were counted for 3H activity (in counts per minute 3H incorporation). Tritium activity of NVFs represents DNA or protein synthesis expressed as a percentage of control (e.g., NVFs transfected with a scrambled adenovirus or control DNA).

Immunocytochemical staining. Cells plated onto Lab-Tek slides were washed with PBS, immunostained with a β-actin antibody, and visualized with a Texas Red-conjugated secondary antibody. The nuclei were counterstained by 4',6-diamidino-2-phenylindole. Five images of each sample were acquired under an Olympus IX71 inverted microscope with fluorescence filters for Texas Red and 4',6-diamidino-2-phenylindole. The two images from the same field were merged with SPOT software. The nuclei were counted and cell surface area analyzed with ImageJ.

Collagen assay. Cells were transduced with a shRNA adenovirus in serum-free medium for 48 h. The culture medium was collected and kept at −80°C after spinning out debris, while the cells were trypsinized and counted. For the collagen assay, duplicate samples were incubated with picrosirius red (0.1% in saturated aqueous picric acid) for 30 min at room temperature. The picrosirius red-bounded collagen precipitates were spun out and washed with 0.1 M NaOH and optical density read at 540 nm. Collagen content was based on a standard curve of type I collagen run with samples in parallel and expressed as micrograms per million cells.

Statistical analysis. Data were expressed as means ± SE or SD for the low replicate experiments. Differences in mean values were analyzed using a two-tailed t-test, where *P < 0.05.

RESULTS

Tafazzin knockdown decreased cardiolipin. To see if tafazzin shRNA would knock down tafazzin expression, NVFs were

Fig. 3. Tafazzin knockdown activated MAPKs and induced transcriptional and translational factors. NVFs were transected with the tafazzin shRNA adenovirus, and activated MAPKs, transcriptional factor early growth response (Egr)-1, and phosphorylated (active form) translation factor p70 s6 kinase determined by Western blot with specific antibodies, using GAPDH as a loading control. Levels of both factors were expressed as the ratio of the factor to GAPDH. A: phospho-p38 (Thr180/Tyr182). Data represent means ± SD of 3 separate experiments. #P < 0.05 vs. SCR. B: phospho-p42/44 (Thr202/Tyr204). Data represent means ± SE of 4 separate experiments. *P < 0.05 vs. SCR. C: EGR-1. Data represent means ± SD of 3 separate experiments. *P < 0.05 vs. SCR. D: phospho-p70 s6 kinase (Thr389). Data represent means ± SD of 3 separate experiments. *P < 0.05 vs. SCR.
transduced with the tafazzin shRNA adenovirus for 48 h and its expression was measured by real-time RT-PCR and Western blot. We found that the tafazzin shRNA adenovirus significantly knocked down both tafazzin mRNA and protein compared with the scrambled adenovirus (Fig. 1, A and B). Because tafazzin plays an important role in cardiolipin synthesis and mutation of tafazzin decreases cardiolipin (1, 15, 28, 68, 72), we measured cardiolipin by shotgun lipidomics and found that tafazzin knockdown significantly reduced cardiolipin in NVFs (Fig. 1C). Cardiolipin species data indicated that tafazzin knockdown mainly decreased unsaturated species (Fig. 1D). Monolysocardiolipin and cardiolipin ratio is a more sensitive parameter of cardiolipin effects of tafazzin knockdown (44), but we found that monolysocardiolipin present in NVFs at very low level, which could not be quantified accurately.

**Tafazzin knockdown increased ROS production and activated MAPKs.** Because cardiolipin is essential for optimal mitochondrial function and mitochondrial malfunction enhances ROS production (18), we next tested the effect of tafazzin knockdown on ROS production and found that it significantly increased ROS levels compared with the scrambled virus treatment (Fig. 2A). As an indicator of enhanced ROS production, 4-hydroxy-2-((E))-nonenal (an oxidized product of unsaturated fatty acids) was significantly increased by tafazzin knockdown (Fig. 2B). To access the ROS levels in the mitochondria, cells were stained with mitochondrial superoxide indicator, MitoSOX Red and MitoTracker Green. As showed in Fig. 2C, tafazzin knockdown obviously increased MitoSOX Red intensity. The stains of MitoSOX Red and MitoTracker Green were perfectly overlapped, indicating its mitochondria specificity. Quantitative analysis showed that tafazzin knockdown significantly increased mitochondrial ROS level (Fig. 2D).

Because it is well established that ROS activate MAPKs (63), we examined the effect of tafazzin knockdown on MAPK activation and found that it potently increased p38 phosphorylation compared with the scrambled adenovirus (Fig. 3A); moreover, phosphorylated p42/44 MAPK was significantly elevated as well (Fig. 3B). Activated MAPKs may induce downstream transcriptional and translational factors leading to cell proliferation.

**Tafazzin knockdown induced transcriptional and translational factors.** When we questioned whether tafazzin knockdown stimulates transcriptional and translational factors, we found that it increased the early growth response transcriptional factor Egr-1 (Fig. 3C). It also potently activated p70 s6 kinase, which phosphorylates the 40s subunit of the ribosome and thus heightens translation of the proteins involved in cell cycle regulation and cell proliferation (25, 39) (Fig. 3D). Both increased expression of Egr-1 and activation of p70 s6 kinase may influence regulation of the cell cycle.

**Tafazzin knockdown caused induction of cell cycle regulator.** We questioned whether tafazzin knockdown induces expression of cyclins (a family of major cell cycle regulatory proteins) and found that it more than doubled both cyclin E mRNA (Fig. 4A) and protein levels (Fig. 4B) compared with the scrambled adenovirus. Tafazzin knockdown increased PCNA, a marker of cell proliferation (21), over 10-fold compared with the scrambled adenovirus treatment (Fig. 4C). Other studies have shown that PCNA interacting with cyclin E activates cyclin-activated kinase (CDK), which promotes cell cycle progression (50). In turn, CDK phosphorylates tumor suppressor protein Rb and...
stimulates the genes that regulate the cell cycle (77). We found that tafazzin knockdown potently increased phosphorylation of Rb (Fig. 4D), which could promote cell cycle progression by favoring interaction with the transcription factor E2F, which activates transcription of the genes involved in cell cycle progression (76). Induction of cell cycle regulators (e.g., cyclin E, PCNA, and Rb) is a sign that tafazzin knockdown promotes cell cycle progression.

**Tafazzin knockdown induced DNA and protein synthesis.**

We next tested whether induction of cell cycle regulators would increase DNA and protein synthesis. We found that tafazzin knockdown significantly increased DNA synthesis as indicated by heightened $^3$H-thymidine incorporation compared with the scrambled adeno virus (Fig. 5A). To exclude the effects of adenoviral proteins, we performed transfection experiments with a plasmid containing tafazzin shRNA (pshTaz) and found that NVFs transfected with pshTaz exhibited increased $^3$H-thymidine incorporation compared with cells transfected with a plasmid containing scrambled shRNA (pshSCR) (Fig. 5B). Transfection experiments with tafazzin overexpression constructs (AAVCMVTaz) confirmed that $^3$H-thymidine incorporation was reduced in NVFs transfected with AAVCMVTaz compared with an AAVCMV empty vector and labeled with $^3$H-thymidine. Data represent means ± SE of 6 separate experiments. $^*P < 0.05$ vs. AAVCMV. Both knockdown and overexpression experiments confirmed the DNA synthetic effects of tafazzin indicating that tafazzin shRNA is highly specific to tafazzin. The offsite effects of tafazzin shRNA may be minor since we did not find any effects of tafazzin shRNA on GAPDH protein levels (Figs. 3 and 4).

**Tafazzin knockdown induced protein synthesis.**

A: NVFs were transfected with an adenovirus containing sham RNA and metabolically labeled with $^3$H-leucine. Data represent means ± SD of 3 separate experiments. $^*P < 0.05$ vs. SCR. B: NVFs were transfected with an adenovirus containing sham RNA and metabolically labeled with $^3$H-leucine. Data represent means ± SE of 6 separate experiments. $^*P < 0.05$ vs. SCR.
Tafazzin knockdown also increased protein synthesis as indicated by increased 
\(^3\)H-leucine incorporation (Fig. 6A). However, whereas we expected to see heightened cell proliferation due to increased DNA and protein synthesis, cell number did not increase (data not shown). Consistent with the increased 
\(^3\)H-leucine incorporation, the protein content of the NVFs subjected to tafazzin knockdown was significantly increased compared with cells transfected with the scrambled virus (Fig. 6B).

**Tafazzin knockdown decreased intracellular ATP and activated AMPK.** Tafazzin knockdown decreased cardiolipin, which is essential for optimal mitochondrial function (13). As shown in Fig. 7A, tafazzin knockdown significantly decreased maximal and basal OCR compared with scrambled virus control. We questioned whether tafazzin knockdown might affect intracellular ATP and found that in fact it significantly decreased intracellular ATP compared with the control (NVFs transfected with the scrambled virus) (Fig. 7B). This decline in ATP resulted in increased phosphorylation of the energy sensor AMPK\(\alpha\) (Fig. 7C). As a complementary response of the decreased ATP and activation of AMPK, mitochondrial density was increased as indicated by the elevated mitochondrial DNA concentration (Fig. 7D). The increased mitochondrial density was also evidenced by the increased MitoTracker Green fluorescence intensity in Fig. 2C (top).

To confirm involvement of the above-identified signaling molecules in tafazzin knockdown induced signaling events, we used pharmacological reagents to block mitochondrial ROS, MAPK, and AMPK individually. We found that mitochondria-targeted antioxidant mito-TEMPO abrogated tafazzin knockdown induced elevation of EGR-1, phospho-p70 s6 kinase, and phospho-Rb (Fig. 8A). MEK inhibitor U0126 inhibited tafazzin knockdown induced EGR-1, PCNA, and phospho-Rb (Fig. 8B). However, p38 MAPK inhibitor SB203580 (10 \(\mu\)M), which blocked p38 activation in cultured cardiac fibroblasts (17, 54), had no effect on tafazzin knockdown induced signaling molecules (Fig. 8C). AMPK inhibitor compound C enhanced tafazzin knockdown induced phospho-Rb and phospho-p70 s6 kinase but had no effect on cyclin E (Fig. 8D). These results suggested that tafazzin knockdown-induced signaling was originated from mitochondrial ROS and mediated by MAPKs, gene expression factors, and cell cycle regulators. This signaling cascade was negatively regulated by AMPK.

**Tafazzin knockdown resulted in multinucleation and hypertrophy.** AMPK has been postulated as a metabolic checkpoint in the cell cycle (29). When cellular energy is depleted (e.g., reduction of intracellular ATP and activation of AMPK), proliferation decreases. This notion was supported by our results showing that increased DNA and protein synthesis did not result in proliferation of NVFs subjected to tafazzin knockdown. In addition, we found that tafazzin knockdown resulted in multinucleation (Fig. 9, A and B) and hypertrophy as indicated by increased protein synthesis (Fig. 6) and cell surface area (Fig. 9C).

**DISCUSSION**

Tafazzin knockdown causes hypertrophy of neonatal cardiac myocytes (34), and mutation of the tafazzin gene causes dilated
cardiomyopathy in Barth syndrome (64). Our work with neonatal cardiac fibroblasts (NVFs) showed that tafazzin knockdown increased ROS production, activated MAPKs including p42/44 and p38, stimulated transcriptional and translational factors, which in turn activated cell cycle regulators, and increased DNA and protein synthesis. On the other hand, tafazzin knockdown also decreased intracellular ATP, activated AMPK, and halted the energy-consuming process (i.e.,

![Fig. 8. Confirmation of tafazzin knockdown induced signaling molecules. NVFs were transduced with the shRNA adenovirus for 24 h and treated with 25 μM mitochondria-targeted antioxidant mito-TEMPO (mito-TP; A), 10 μM MEK inhibitor U0126 (B), 10 μM p38 MAPK inhibitor SB203580 (C), or 10 μM AMPK inhibitor compound C (ComC; D) for 24 h. The elevated signaling proteins were determined by Western blot with specific antibodies. GAPDH was used as a loading control (Cont). Data represent 3 separate experiments.]

![Fig. 9. Tafazzin knockdown induced multinucleation and hypertrophy. NVFs were transduced with an shRNA adenovirus and fixed with formaldehyde, then immunostained with Texas red-conjugated antibody and the nuclei counterstained with 4',6-diamidino-2-phenylindole (DAPI; A). Pictures represent 20 images from 4 separate experiments. Multinucleated cells were counted and expressed as a percentage of total cells (B). Data represent means ± SE of 4 separate experiments. *P < 0.01 vs. SCR. Cell surface area was measured using ImageJ software and expressed as a percentage of SCR (C). Data represent means ± SE of 4 separate experiments. *P < 0.01 vs. SCR.]

AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00084.2013 • www.ajpheart.org
cell proliferation), ultimately resulting in multinucleation, hypertrophy, and enhanced collagen secretion.

Tafazzin plays an important role in de novo cardiolipin synthesis and remodeling in the mitochondria. Tafazzin knockdown leads to reduced cardiolipin, which is consistent with previous studies involving yeast (28), Drosophila (72), and human skin fibroblasts (68). To be consistent with what is reported in mice (1) and human skin fibroblasts (69), knockdown tafazzin mainly decreased unsaturated cardiolipin species including tetralinoleoyl cardiolipin. In contrast with adult heart, tetralinoleoyl cardiolipin is not the major species in NVFs. It is not a predominant cardiolipin species in neonatal mouse heart either (41). This may be the reason, at least in part, that the level of cardiolipin reduced by tafazzin knockdown was not dramatic as expected. It is worth noting that the decreased tetralinoleoyl cardiolipin due to tafazzin knockdown is not the primary mechanism of cell cycle interruption in NVFs. Tafazzin knockdown impaired mitochondrial function more profoundly than the reduction of cardiolipin, suggesting that NVFs are more sensitive to cardiolipin alteration since cardiolipin contents are dramatically increased during postnatal development (41). Cardiolipin is essential for proper function of the mitochondrial respiration chain, and malfunction of the chain leads to increased production of ROS (23). ROS play important roles in the pathophysiology of cardiac remodeling (67). Our data showing that tafazzin knockdown increased ROS production are consistent with this notion. Our previous study showed that tafazzin knockdown induced cardiomyocyte hypertrophy (34), which is most likely mediated by ROS. However, responses to ROS differ between cardiac myocytes and fibroblasts; for example, myocytes undergo hypertrophy, contractile dysfunction, and death, whereas fibroblasts undergo proliferation and transformation into myofibroblasts and enhanced extracellular matrix secretion (45). Our preliminary data showed that tafazzin knockdown increased ROS in neonatal cardiac myocytes as measured by CM-H2DCFDA staining (He et al., unpublished data), whereas Chen et al. (12) reported that tafazzin mutation increased ROS in yeasts. Thus our data suggest that cardiolipin deficiency due to tafazzin dysfunction could result in enhanced ROS production.

It is well established that ROS activate MAPKs, including p42/44 and p38 (18), and that this contributes to proliferation of several types of cells including vascular smooth muscle cells (61), endothelial cells (39), lung fibroblasts (30, 53), tumor cells (47), and cardiac fibroblasts (22). Downstream mediators of MAPKs include transcriptional and translational factors, which increase expression of the genes involved in cell cycle regulation. Our data showed that tafazzin knockdown induced transcriptional factor Egr-1, a ubiquitous early growth response protein whose induction by p42/44 or p38 promotes cell growth (75). It also stimulated phosphorylation of p70 s6 kinase, which in turn increased phosphorylation of the ribosome s40 subunit and led to enhanced translation of the genes involved in cell cycle regulation (39). Other studies have demonstrated crosstalk between MAPKs and p70 s6 kinase (48). Taken together, induction of Egr-1 and activation of p70 s6 kinase induced by tafazzin knockdown contribute to expression of the genes that regulate the cell cycle.

Previous studies have shown that activation of p70 s6 kinase mediates cyclin E expression, which promotes cell cycle progression, whereas rapamycin-induced G1 arrest is associated with reduced expression of cyclin E (14, 20). Cyclin E is a member of the cyclin protein family that controls cell cycle progression through activation of CDK. Cyclin E binds to CDK and forms an activated complex, which is required for transition from G1 to S phase (42). Cyclin E-activated CDK phosphorylates Rb and increases expression of the genes that regulate the cell cycle (77). Elevated expression of cyclin E has been found in tumors characterized by enhanced cell prolifer-
Knocking down cyclin E with a siRNA inhibits growth of breast cancer cells (46). Selective repression of cyclin E, CDK2, and E2F has been found in replicating senescent human lung fibroblasts (38). PCNA, another cell cycle regulator, is also involved in the effects of tafazzin knockdown on cell cycle progression. It is a marker of proliferating cells that has a number of related functions, including DNA replication and repair and cell cycle control. PCNA is shaped like a ring and works as a sliding clamp for DNA polymerases and a docking station for other proteins including CDKs, cyclins, and other DNA replication factors (55). The correlation between elevated PCNA and cell cycle progression is well established even though its functions are not fully understood (50); induction of PCNA has been reported in superoxide-mediated NVF proliferation (59).

Induction of cyclin E and cyclin-dependent kinases leads to hyperphosphorylation of Rb and promotes cell cycle progression (27). Rb was found to mute retinoblastoma (32) and serves as a conduit between the cell cycle machinery and transcriptional factors (32, 76). CDKs are not active in quiescent cells (early G1 phase), whereas Rb is hypophosphorylated and interacts with E2F. When CDKs are activated during the G1-S transition, Rb is hyperphosphorylated and releases its inhibition to E2F, thereby activating transcription of the genes involved in the cell cycle (27). Consistent with the present study showing that tafazzin knockdown increased phosphorylation of p42/44 MAPK and Rb, Garnovskaya et al. (24) reported that activation of p42/44 MAPK increased Rb phosphorylation in vascular smooth muscle cells. Tafazzin knockdown stimulates cyclin E, which in turn may activate cyclin-dependent kinases and increase Rb phosphorylation, and also potently enhanced elevation of DNA replication and protein synthesis required for cell proliferation (62).

Cell proliferation is an energy-consuming process. Mitochondrial oxidative phosphorylation is the major intracellular ATP contributor although aerobic glycolysis is enhanced in the proliferating cells. The enhanced glycolysis mostly meets the metabolic requirements (49). In our tafazzin knockdown NVF model, intracellular ATP was decreased, and this limited cell mitosis although the DNA was duplicated. Tafazzin knockdown increased DNA synthesis, which is consistent with Hauff’s (33) study showing that lymphoblasts from patients with Barth syndrome tended to have increased 3H-thymidine incorporation under serum-free conditions. Abnormal cell proliferation is also supported by studies from Phoon et al. (58) showing that tafazzin knockdown decreased cellular proliferation during embryogenesis in mice. Activation of AMPK due to decreased ATP also halts cell proliferation and has been postulated as a cell cycle metabolic checkpoint (29). AMPK activation reportedly suppressed proliferation of vascular smooth muscle cells (35) and tumor cells (26) and also phosphorylated several proteins involved in cell cycle regulation (4). Multinucleation induced by tafazzin knockdown is most likely due to decreased ATP and activation of AMPK. AMPK reduces p70 s6 kinase phosphorylation in cardiac myocyte (11), and p70 s6 kinase phosphorylates α2AMPK at serine491 and results in inactivation in mouse hypophalumus (19). However, the precise mechanism of NVF cell cycle interruption and cardiac fibrosis needs to be investigated further in vivo.

Our data showed that tafazzin knockdown increased collagen secretion. This was not due to transformation of cardiac fibroblasts into myofibroblasts, since NVFs subjected to tafazzin knockdown did not show an increase in α-smooth muscle actin, a marker of myofibroblasts (data not shown). Rather, it was most likely due to the increased cell size. Phoon et al. (58) reported that tafazzin knockdown in mice leads to diastolic dysfunction without fibrosis. As pointed out in the article, the effects of tafazzin knockdown at a different embryonic developmental stage are variable. It is also possible that knocking down tafazzin in a different model system displays different phenotypes since there is no genotype-phenotype correlation in Barth syndrome (6). Ninety percent of Barth syndrome patients had diagnosed cardiomyopathy (typically dilated cardiomyopathy) (64). During the development of dilated cardiomyopathy, cardiac myocytes undergo remodeling including elongation and death, whereas cardiac fibroblasts are activated characterized by increased proliferation and enhanced collagen secretion. Myocardial fibrosis has been reported from Barth syndrome patients (37, 52, 56).

Motivated by our previous finding that tafazzin knockdown causes cardiac myocyte hypertrophy (34), we have now established that tafazzin knockdown caused mitochondrial dysfunction, increases ROS and decreased ATP production, activated MAPKs and AMPK, and ultimately resulted in cardiac fibroblast multinucleation, hypertrophy, and enhanced collagen secretion (Fig. 11). These results may be helpful in explaining how tafazzin mutation and cardiolipin deficiency contribute to dilated cardiomyopathy in Barth syndrome and other diseases.

ACKNOWLEDGMENTS

We thank Jacina Redden for her technical assistance with the lipid analyses.

GRANTS

This work was supported by grants from the Barth Syndrome Foundation and the American Heart Association.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Q.H. conception and design of research; Q.H., M.W., and N.H. performed experiments; Q.H., M.W., N.H., and X.H. analyzed data; Q.H., M.W., and N.H. interpreted results of experiments; Q.H. prepared figures; Q.H. drafted manuscript; Q.H. and X.H. edited and revised manuscript; Q.H. approved final version of manuscript.

REFERENCES


