Mitofusin 2 Triggers Vascular Smooth Muscle Cell Apoptosis via Mitochondrial Death Pathway

Xiaomei Guo,* Kuang-Hueih Chen,* Yanhong Guo, Hua Liao, Jian Tang, Rui-Ping Xiao

Abstract—Previous studies have shown that mitofusin 2 (Mfn-2) (or hyperplasia suppressor gene [HSG]) inhibits vascular smooth muscle cell (VSMC) proliferation. Here, we demonstrate that Mfn-2 is a primary determinant of VSMC apoptosis. First, oxidative stress with H2O2, inhibition of protein kinase C with staurosporine, activation of protein kinase A with forskolin, and serum deprivation concurrently elevate Mfn-2 expression and induce VSMC apoptosis. Second, overexpression of Mfn-2 also triggers apoptosis of VSMCs in culture and in balloon-injured rat carotid arteries, thus contributing to Mfn-2–mediated prevention of neointima formation after angioplasty. Third, Mfn-2 silencing protects VSMCs against H2O2 or Mfn-2 overexpression–induced apoptosis, indicating that upregulation of Mfn-2 is necessary and sufficient for oxidative stress–mediated VSMC apoptosis. The Mfn-2 proapoptotic effect is independent of its role in mitochondrial fusion but mainly mediated by inhibition of Akt signaling and the resultant activation of the mitochondrial apoptotic pathway, as manifested by decreased Akt phosphorylation, increased mitochondrial Bax/Bcl-2 ratio, cytochrome c release, and activation of caspases-9 and caspase-3. Furthermore, Mfn-2–induced apoptosis was blocked by overexpression of an active phosphoinositide 3-kinase mutant or Bcl-xL or inhibition of caspase-9 but not caspases-8. Thus, in addition to its antiproliferative effects, Mfn-2 constitutes a primary determinant of VSMC apoptosis. (Circ Res. 2007;101:1113-1122.)

Key Words: PI3K-Akt ■ apoptosis ■ HSG ■ Mfn-2 ■ vascular smooth muscle cells

Apoptosis is a programmed cell death that is essential for embryonic development and for tissue homeostasis, remodeling, and immune responses. There are 2 major apoptotic signaling cascades: the first is the death receptor (Fas or tumor necrosis factor receptor)–mediated pathway involving activation of caspase-8 and its downstream executioner caspases; the other is the mitochondrial pathway activated by cellular deprivation or stress, and it involves sequentially the release of cytochrome c, the recruitment of apoptotic protease activating factor-1 (Apaf-1), and the activation of caspase-9 and downstream executioner caspases.1-3 Defects (inhibition or exacerbation) of either pathway trigger proliferative or degenerative disorders, including atherosclerosis, restenosis, myocardial infarction, cancers, neurodegenerative diseases, and AIDS.3-7

Ras, a small GTPase, plays a central role in the regulation of many fundamental biological processes, such as cell proliferation, differentiation, senescence, survival, and growth via activation of a wide array of downstream signaling pathways. Among them, the Ras-Raf-MEK-ERK/mitogen-activated protein kinase (MAPK) pathway and the Ras-PI3K-Akt (also known as protein kinase [PK]B) pathway are vital for cell proliferation and cell survival.8-11 Whereas the Ras-MAPK pathway drives cell cycle progression, the activation of the Ras-PI3K-Akt signaling blocks apoptotic cell death.11 In particular, Akt-mediated phosphorylation of proapoptotic members of the Bcl-2 family, including Bad and Bax, prevents translocation of those proapoptotic molecules from the cytoplasm to the mitochondria, thereby inhibiting the mitochondrial apoptotic pathway and preventing cells from apoptosis.12,13

Although Ras was originally identified as a viral oncogene, over the past decade, increasing evidence has placed Ras signaling at the center of pathways for a wide array of cardiovascular diseases, such as hypertensive vascular proliferation, injury-associated arterial restenosis, cardiac hypertrophy and failure, angiogenesis, and endothelial dysfunction.14-16 Thus, the identification and characterization of novel control points in the Ras pathways have been a focus of cardiovascular biology and medicine.14 In this regard, our recent studies have demonstrated that mitofusin 2 (Mfn-2) (also named hyperplasia suppressor gene [HSG]) acts as an endogenous Ras inhibitor and that deregulation of Mfn-2 expression leads to vascular proliferative disorders in the

Materials and Methods

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich. An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Primary VSMC Culture and Adenoviral Infection

VSMCs were isolated, cultured, and infected as described previously.17 See the online data supplement for further details.

Gene Silencing Through RNA Interference

Cultured VSMCs were transfected with a pool of 4 short interfering (si)RNAs (100 nmol/L) targeted specifically to rMfn-2 using a Basic Nucleofector kit for primary smooth muscle cells (Amaxa Inc) according to the protocol provided by the manufacturer. See the online data supplement for further details.

Balloon Injury and Morphometric Analysis of Intimal Thickening

Balloon denudation and morphometric analysis of intimal thickening were performed as described previously.17 See the online data supplement for further details.

Figure 1. Overexpression of rMfn-2 induces apoptotic death of cultured rat VSMCs. A, Overexpression of rMfn-2 by adenoviral gene transfer, shown by Western blot with an anti–rMfn-2 antibody (n=5; *P<0.01 vs uninfected or Adv-GFP–infected group). B, DNA laddering to detect fragmented DNA in cells infected with Adv-rMfn-2-GFP or Adv-GFP for a variable period of time, as indicated. Similar results were obtained in 5 independent experiments. C, Time course of rMfn-2–mediated apoptosis, assayed by Cell Death ELISA, in VSMCs infected with either Adv-rMfn-2-GFP or Adv-GFP (75 mois) for the indicated time (from 24 to 72 hours) (n=5; *P<0.01 vs uninfected or Adv-GFP–infected group). D, Titer dependence of rMfn-2–induced apoptosis detected by Cell Death ELISA. Cells were infected with 50, 75, and 100 mois of Adv-rMfn-2-GFP or Adv-GFP for 60 hours (n=5; *P<0.01 vs Adv-GFP–infected group). E, Representative TUNEL staining in VSMCs in the presence or absence of Adv-rMfn-2-GFP or Adv-GFP infection for 72 hours. Magnification, ×200. F, Quantitative analysis of TUNEL-positive cells. The number of TUNEL-positive cells was measured by counting 1000 cells from random fields. The data are shown as the means±SE (n=5; *P<0.01 vs uninfected or Adv-GFP–infected group).
Statistical Analysis
All data were expressed as means±SE or proportion. Two-tailed *t*-tests and ANOVA, followed by the Bonferroni procedure, were used to test the differences in continuous variables. χ² test was used to test the differences in categorical variables. *P*<0.05 was considered statistically significant.

Results
Overexpression of rMfn-2 Triggers Apoptosis in Cultured VSMCs
To explore the potential role of rMfn-2 in regulating VSMC survival, primary cultured rat VSMCs were infected with adenoviral vectors expressing either rMfn-2 (Adv-rMfn-2-GFP) or green fluorescent protein (GFP) (Adv-GFP as a control virus) at 75 multiplicities of infection (mois). Figure 1A shows that 68-kDa rMfn-2 protein abundance was increased by 3.7-fold in cells infected with Adv-rMfn-2-GFP for 24 hours relative to the Adv-GFP–infected group.

There was a robust time-dependent increase in VSMC apoptosis, as evidenced by DNA fragmentation assayed by DNA laddering (Figure 1B) and Cell Death ELISA (Figure 1C), when cells were infected with Adv-rMfn-2-GFP at a titer of 75 mois for ≈60 to 72 hours. Figure 1D illustrates the titer-dependent apoptosis induced by Adv-rMfn-2-GFP. Apoptotic cells were characterized by 2 common morphological features: cellular shrinkage and rounding with DNA condensation and fragmentation, as revealed by TUNEL staining. There were no obvious morphological changes or TUNEL-positive nuclei in uninfected VSMCs or those infected with Adv-GFP (Figure 1E). Notably, the percentage of TUNEL-positive cells was 3.8±0.5% and 84.3±2.5% (n=5, *P*<0.0001) in cells infected with Adv-GFP and Adv-rMfn-2-GFP for 72 hours, respectively (Figure 1F).

rMfn-2–Mediated Apoptosis Is Independent of Mitochondrial Fusion
We and others have demonstrated previously that the transmembrane domain of rMfn-2 protein is essential for its role in mitochondrial fusion by targeting the protein to the outer membrane of mitochondria.17,18,19 Disruption of rMfn-2 mitochondrial targeting leads to a rather uniform cytotoxic distribution of rMfn-2, while retaining its antiproliferative effect.17 To determine whether enhanced mitochondrial fusion is required for rMfn-2–mediated apoptosis, we infected VSMCs with an adenovirus expressing the rMfn-2 transmembrane domain deletion mutant [Adv-rMfn-2-TMD(Δ)]. Figure 2A shows that forced expression of the rMfn-2 mutant is more potent than overexpression of wild-type rMfn-2 in promoting VSMC apoptosis, indexed by Cell Death ELISA.

The robust proapoptotic effect of the rMfn-2 mutant was more potent than overexpression of wild-type rMfn-2 in promoting VSMC apoptosis, indexed by Cell Death ELISA. The robust proapoptotic effect of the rMfn-2 mutant was further manifested by a marked increase in TUNEL-positive staining cells (Figure 2B). Thus, rMfn-2 proapoptotic competency is independent of its known functional role in mitochondrial fusion.

rMfn-2 Expression Is Elevated in Response to Oxidative Stress and Other Apoptotic Stimuli
To evaluate the potential physiological and pathological relevance of rMfn-2–mediated apoptosis, we first determined the expression of endogenous rMfn-2 in response to proapoptotic oxidative stress with H2O2 in cultured VSMCs.20 We found that treating VSMCs with 25 to 100 μmol/L H2O2 for 24 hours markedly induced VSMC apoptosis in a concentration-dependent manner, as manifested by activation of caspase-9 and caspase-3 (Figure 3A). Interestingly, H2O2 treatment also concomitantly elevated rMfn-2 protein abundance in VSMCs in a similar concentration-dependent fashion, with a maximal increase of 3.2-fold over baseline (Figure 3A and 3B). Moreover, other death-inducing stimuli, including inhibition of PKC signaling with staurosporine,21 activation of PKA signaling by forskolin,22 and serum deprivation,23 also markedly increase Mfn-2 protein expression in VSMCs (Figure 3C), suggesting that Mfn-2 is a primary determinant of VSMC apoptosis.

Mfn-2 Is Necessary for H2O2-Induced VSMC Apoptosis
To further explore the possible role of rMfn-2 in VSMC apoptosis, we used RNA interference to knockdown rMfn-2 expression in VSMCs. Four rMfn-2 siRNAs were transfected into VSMCs. Western blot analysis revealed a marked decrease (~95%) in rMfn-2 protein abundance in cells treated with 2.5 μg of rMfn-2 siRNA (siMfn-2) as compared with either untreated or control siRNA-treated cells (Figure 4A, top and middle), indicating that the rMfn-2 siRNA pool can effectively and specifically reduce rMfn-2 protein abundance in growth-arrested VSMCs.
Next, we sought to determine whether rMfn-2 is necessary for oxidative stress or adenoviral gene transfer of rMfn-2–mediated apoptosis using rMfn-2 gene silencing. Figure 4A and 4B illustrates that rMfn-2 silencing not only significantly reduced basal rMfn-2 protein abundance but also prevented H₂O₂-induced and adenovirus-mediated upregulation of rMfn-2. Most importantly, silencing of rMfn-2 protected VSMCs from overexpression of rMfn-2– or H₂O₂-mediated apoptosis, as manifested by Cell Death ELISA and attenuation of cleaved caspase-9 (Figure 4A and 4B, top and bottom). These results indicate that Mfn-2 upregulation is obligatory to H₂O₂ or overexpression of rMfn-2–induced VSMC apoptosis.

Overexpression of rMfn-2 Induces VSMC Apoptosis In Vivo
To further explore the pathological significance of rMfn-2 proapoptotic effect in vivo, rat carotid arteries were subjected to balloon injury and simultaneously infected with either Adv-rMfn-2-GFP or Adv-GFP as described previously. The efficiency of in vivo adenoviral gene transfer of rMfn-2 was tested by immunohistochemical staining with the anti-rMfn-2 antibody. Five days after the surgery, the rMfn-2 protein level was significantly increased in arteries infected with Adv-rMfn-2-GFP compared with those from the sham and PBS control groups and those infected with Adv-GFP (Figure 5A). Whereas there was no detectable TUNEL-positive cell in the sham-operated group, balloon injury led to 7.8 ± 1.5% and 9.4 ± 1.7% (n = 10, P > 0.05) TUNEL-positive cells in the absence or presence of Adv-GFP infection, respectively. More importantly, the percentage of TUNEL-positive VSMCs was further augmented by ~2-fold in arteries infected with Adv-rMfn-2-GFP (17.9 ± 2.1%) relative to the control groups (n = 10, P < 0.01) (Figure 5B and 5C).

At day 21 after balloon angioplasty, rat arteries from different experimental groups were harvested for histological analysis. Figure 5D shows representative examples of hematoxylin/eosin-stained cross-sections of the vessels. The balloon injury–induced increase in the ratio of intima to media area was markedly attenuated by overexpression of rMfn-2 (Figure 5D and 5E). These in vivo observations demonstrate that overexpression of rMfn-2 by adenoviral gene delivery greatly promotes medial VSMC apoptosis, thus contributing, at least in part, to rMfn-2–mediated inhibition of neointima formation after balloon injury.

Overexpression of rMfn-2 Activates Mitochondrial Apoptotic Pathway
To discriminate which apoptotic pathway was responsible for rMfn-2–induced apoptosis, we examined the activation status of caspase-8 and caspase-9 in response to rMfn-2 overexpression. VSMCs infected with Adv-rMfn-2-GFP displayed increased cleavage of procaspase-9 without altering the cleavage of procaspase-8 (Figure 6A and 6B). Activation of caspase-9 was detectable 42 hours after infection and further elevated in a time-dependent manner (Figure 6A). Likewise, caspase-3 was activated by overexpression of rMfn-2 with a similar temporal profile (Figure 6A). Furthermore, inhibition of caspase-9 with Z-LEHD-FMK (15 μmol/L) largely prevented rMfn-2–elicited activation of caspase-3 (Figure 6C). Thus, rMfn-2 promotes VSMC apoptosis by activation of caspase-9 and
caspase-9 (Figure 7E). This protective effect was also confirmed by Cell Death ELISA (Figure 7F). In contrast, infection of cells with the control virus, Adv-LacZ, had no such effect (Figure 7E and 7F). This further substantiates our conclusion that rMfn-2 promotes VSMC apoptosis via the mitochondrial apoptotic signaling pathway.

rMfn-2 Induces VSMC Apoptosis by Inhibiting Akt Activation

Ras coordinates cell proliferation and cell survival via activating an array of downstream signaling cascades, particularly the Ras-Raf-MEK-ERK/MAPK and the PI3K-Akt signaling pathways.8,11,14 We have previously demonstrated that rMfn-2 binds to Ras and inhibits the Ras-activated extracellular signal regulated kinase (ERK)/MAPK pathway, resulting in VSMC growth arrest.17 Next, we sought to delineate the relative importance of the 2 aforementioned Ras-elicited pathways in rMfn-2–mediated VSMC apoptosis by examining the phosphorylation status of Akt and ERK1/2. Profoundly, rMfn-2 overexpression fully abolished endothelin (ET)–1–induced Akt activation, as evidenced by suppression of Akt phosphorylation at Ser473, in a titration- and time-dependent manner in cells infected by Adv-rMfn-2-GFP but not Adv-GFP (Figure 8A through 8C). In addition, infection of VSMCs with Adv-rMfn-2-GFP also decreased ET-1–mediated activation of ERK1/2. However, under the same experimental conditions, rMfn-2–induced inhibition of Akt activation was much greater and occurred earlier than the inhibition of ERK1/2 activation (Figure 8C and 8D). It is noteworthy that the overexpression of rMfn-2 also markedly reduced basal Akt phosphorylation (Figure 8E). Furthermore, we coexpressed rMfn-2 with a constitutively active phospho-
inositide 3-kinase (PI3K) mutant (CA-PI3K), an upstream kinase of Akt, via adenoviral gene transfer (Adv-CA-PI3K at 75 mois). Indeed, cotransfection of cells with Adv-CA-PI3K, but not with the control Adv-LacZ, not only abolished rMfn-2–mediated inhibition of Akt phosphorylation (Figure 8E) but also prevented caspase-9 activation (Figure 8F) and VSMC apoptosis, as assayed by Cell Death ELISA (Figure 8G).

**Discussion**

The major finding of the present study is that upregulation of rMfn-2 is both necessary and sufficient for inducing VSMC apoptosis. This conclusion is based on several lines of evidence. First, in primary cultured rat VSMCs, oxidative stress with H2O2 treatment concurrently elevates the expression of endogenous rMfn-2 and apoptotic cell death. Second, other apoptotic-inducing stimuli, including inhibition of PKC signaling with staurosporine,21 activation of PKA signaling by forskolin,22 and serum deprivation,21,23 can markedly elevate Mfn-2 protein expression (Figure 3C), which is associated with increased VSMC apoptosis (data not shown). Third, adenovirus-mediated overexpression of rMfn-2 is sufficient to trigger robust apoptosis of VSMCs in culture and in vivo (Figures 1 through 8) and in multiple cancer cell lines (Figure I in the online data supplement). Finally and importantly, RNA interference–mediated gene knockdown of rMfn-2 protects VSMCs from either overexpression of rMfn-2 or oxidative stress–mediated apoptosis. These findings mark rMfn-2 as an important determinant of cell apoptosis in physiological and pathological contexts and suggest that upregulation of this well-conserved gene might open a novel therapeutic avenue for a variety of proliferative disorders such as cardiovascular proliferative diseases and certain types of tumors.
Molecular Mechanism Underlying rMfn-2–Mediated Apoptosis

Our previous studies have shown that overexpression of rMfn-2 suppresses cell proliferation via inhibiting the Ras-Raf-ERK/MAPK signaling pathway. Although the possible involvement of inhibition of ERK1/2 activation in rMfn-2–mediated apoptosis cannot be completely excluded, the present results indicate that the apoptotic effect of rMfn-2 appears to be mainly attributable to rMfn-2–mediated suppression of the PI3K-Akt cell survival signaling pathway. This conclusion is corroborated by the following lines of evidence: (1) overexpression of rMfn-2 markedly inhibits both basal and mitogenic stimulus–induced Akt activation; (2) under the same experimental conditions, the rMfn-2–mediated inhibition of Akt activation is much greater and occurs earlier than the inhibition of ERK1/2 activation; (3) forced expression of the constitutively active PI3K, an upstream kinase of Akt, fully blocks rMfn-2–induced cleavage of caspase-9 and VSMC apoptosis (Figure 8F and 8G).

A wide array of external signals evokes apoptosis through 2 major signaling pathways: the death receptor–mediated pathway and the mitochondrial pathway.2–3 The Bcl-2 family, including both antiapoptotic (eg, Bcl-2 and Bcl-xL) and proapoptotic members such as Bax, plays a pivotal role in the mitochondrial apoptotic pathway. Bax insertion into the mitochondrial membrane causes release of cytochrome c, resulting in cellular apoptosis,3 whereas Bcl-2 blocks mitochondrial cytochrome c release and thus prevents subsequent activation of caspase-9 and caspase-3.24,25 Here, we have shown that the rMfn-2 apoptotic effect is mediated by the mitochondrial apoptotic pathway, because rMfn-2 profoundly decreases the level of mitochondrial antiapoptotic protein Bcl-2, while increasing Bax mitochondrial accumulation, resulting in mitochondrial cytochrome c release and activation of caspase-9 and caspase-3 but not caspase-8. Moreover, inhibition of caspase-9 or overexpression of the mitochondrial antiapoptotic protein Bcl-xL is able to abolish the apoptotic effect of rMfn-2. In determining the link between upregulation of rMfn-2 and activation of the mitochondrial death pathway, we found that overexpression of rMfn-2 profoundly suppresses both basal and mitogenic stimulus–induced activation of Akt. Furthermore, enforced expression of a constitutively active PI3K mutant fully protects cells against adenoviral gene transfer of rMfn-2–induced apoptosis. These findings provide the first documentation that rMfn-2–induced VSMC apoptosis is dependent on inhibition of Akt signaling pathway, which, in turn, leads to activation of the mitochondrial apoptotic signaling cascade.

Potential Pathological and Therapeutic Implications of rMfn-2–Mediated Apoptosis

The present study may have important pathological and therapeutic implications because overgrowth of VSMCs is a pivotal etiologic factor in the development of atherosclerosis and restenosis after angioplasty.26–28 To date, inhibiting VSMC proliferation is among the most effective strategies for preventing their overgrowth and controlling neointimal thickening.14 Previous studies have shown that targeting Ras with negative regulators or blocking the Ras downstream pathways is able to effectively attenuate restenosis from balloon catheterization.14,15,29–33 Our recent studies have demonstrated that rMfn-2 is a powerful endogenous Ras inhibitor and that somatic gene transfer of rMfn-2 profoundly inhibits VSMC proliferation and balloon injury–induced neointima thickening in vivo by inhibiting the Ras-Raf-MEK-ERK/MAPK signaling pathway.17

In addition to inhibition of cell proliferation, growing evidence has indicated that apoptosis also plays an essential role in the control of neointimal thickening. VSMC apoptosis in both the intima and the media can limit neointimal formation at a defined time point and is inversely correlated with restenosis.34–38 It is noteworthy that upregulation of rMfn-2 by gene transfer markedly triggers apoptosis in cultured VSMCs via inhibition of the Ras-PI3K-Akt cell survival pathway and subsequent activation of the mitochondrial death pathway. Moreover, adenoviral gene transfer of rMfn-2 in balloon-injured rat
carotid arteries increases VSMC apoptosis and attenuates angioplasty-induced neointima thickening (Figure 5). Together with its antiproliferative effect, the proapoptotic effect marks rMfn-2 as a potential therapeutic target in treating cardiovascular proliferative disorders.

Ras mutations and the subsequent constitutive activation of MAPK and PI3K-Akt signaling pathways have been implicated in tumorigenesis. In this regard, our preliminary studies have shown that overexpression of human Mfn-2 profoundly inhibits proliferation and induces apoptosis in most of the tested human cancer cell lines and largely blocks the growth of tumors in null mice (K.-H.C., X.-Y.Q., X.G., Z.-X.L., Y.K., J.T., 2005, unpublished data).

In summary, we have demonstrated, for the first time, that rMfn-2 displays a profound proapoptotic effect in VSMCs and that this effect is mediated by suppressing an Akt-dependent cell survival signaling pathway, thus leading to activation of the mitochondrial apoptotic signaling cascade. Importantly, RNA interference–mediated gene silencing protects cells against oxidative stress with H2O2 and adenoviral gene transfer of rMfn-2–induced apoptosis, implying that the well-conserved gene rMfn-2 constitutes a crucial determinant of cell fate. Furthermore, overexpression of rMfn-2 substantially triggers apoptotic death in multiple cancer cell lines and in VSMCs in vivo and markedly attenuates balloon injury–induced neointima formation in rat carotid arteries, suggest-

Figure 7. rMfn-2–mediated VSMC apoptosis is via the mitochondrial apoptotic signaling pathway. A, Overexpression of rMfn-2 increases cytochrome c release from mitochondria to cytosol. A representative Western blot with a cytochrome c antibody to assay cytochrome c content in the cytosol and mitochondria from cultured VSMCs infected with Adv-GFP or Adv-rMfn-2-GFP at 75 mos for 60 hours (n=5) is shown. Cytochrome c oxidase subunit IV (COX4) was used as a mitochondrial marker. All blots were reprobed with an anti–α-actin antibody for loading normalization. B, The ratio of cytochrome c abundance in cytosol to that in mitochondria is increased in cells overexpressing rMfn-2. C, The protein level of Bcl-2 was decreased and the content of Bax was increased in the mitochondrial fraction of Adv-rMfn-2-GFP–infected cells, as examined by Western blot with an anti–Bcl-2 or anti–Bax antibody. D, Quantitative analysis of Bcl-2 and Bax abundance in the mitochondria fraction of cells uninfected or infected with adenovirus (n=5; *P<0.01 vs the control group). E, Bcl-xL overexpression rescues VSMCs from rMfn-2–induced apoptosis. VSMCs were transfected with the indicated combinations of adenoviral vectors for 48 hours. The whole cell extracts were analyzed by Western blot with an anti–Bcl-xL antibody or an anti–cleaved caspase-9 antibody. The blot was stripped and reprobed for α-actin. Similar results were obtained in 5 independent experiments. F, Apoptosis in VSMCs infected with the indicated combinations of adenoviral vectors was assayed by Cell Death ELISA. The data are shown as the means±SE (n=5).
ing that rMfn-2 might be a clinically important therapeutic target in diverse cellular proliferative diseases.

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**Disclosures**

None.
References


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Expanded Materials and Methods

**Reagents and antibodies**

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich. The following monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) were used: anti-rMfn-2 pAb and anti-α-actin pAb (Sigma-Aldrich); anti-phospho-Akt pAb (Ser 473), anti-cleaved caspase-3 pAb (Asp175), and anti-cleaved caspase-9 pAb (Asp353, rat specific) (Cell Signaling); anti-Bax (N-20) pAb, anti-caspase-8 p20 mAb, and anti-Bcl-2 (C-2) mAb (Santa Cruz); peroxidase-labeled anti-mouse and anti-rabbit secondary antibodies (Amersham, GE); anti-cytochrome c pAb and anti-COX4 mAb (BD Biosciences). The PhosphoPlus p44/42 MAP kinase (Thr202/Tyr204) antibody kit was from Cell Signaling. The ApoAlert Cell Fractionation kit and caspase-9 inhibitor Z-LEHD-FMK were from BD Biosciences.

**Primary VSMC culture and adenoviral infection**

VSMCs were isolated from male Wistar Kyoto (WKY) rats by collagenase digestion of the aortic media, as described previously. VSMCs were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS, Invitrogen). VSMC synchronization was achieved by culturing cells in DMEM with 0.5% FBS for 24 h.

The construction and preparation of Adv-LacZ, Adv-GFP, Adv-rMfn-2-GFP or Adv-rMfn-2-TMD(Δ) have been previously described. The recombinant adenoviral vectors Adv-CA-PI3K and Adv-Bcl-xL were also described previously. For infection
with adenoviral vectors, an accurate cell count was performed before infection and cells were infected with the indicated MOI of adenovirus in DMEM containing 0.5 % FBS.

**TUNEL assay**

Cultured rat VSMCs were fixed in 10% formalin, washed twice with PBS, labeled with DeadEnd Colorimetric TUNEL System (Promega) following manufactory’s instruction, and counterstained with hematoxylin. The paraffin-embedded sections obtained from balloon-injured rat carotid arteries 5 days after operation were stained by the same kit, following the recommended procedure. VSMCs or sections treated with 0.1 mg/ml DNase I (Sigma-Aldrich) were used as positive controls. The amount of apoptotic cells was assessed by counting a total of 1000 cells from random fields.

**Cell Death ELISA & DNA laddering**

Cellular DNA fragmentation was quantified by the Cell Death ELISA (Roche Applied Science), which measures cytoplasmic DNA-histone complexes generated during apoptotic DNA fragmentation, following the manufacture’s instruction. 3 x 10⁴ control or infected VSMCs (with Adv-GFP, Adv-rMfn-2-GFP, or Adv-rMfn-2-TMD(Δ)) were used for each test.

DNA fragmentation was also assayed by DNA laddering as described. Briefly, 1.5 µg of DNA was loaded in each lane, size-fractioned on a 1.5% agarose gel in Tris-Acetate-EDTA buffer, and then stained with ethidium bromide (Invitrogen).

**Gene silencing through RNA interference**

Cultured VSMCs were transfected with a pool of four short interfering RNAs (siRNAs; 100 nmol/L) targeted specifically to rMfn-2 using Basic Nucleofector kit for
primary smooth muscle cells (Amixa Inc.) according to the protocol provided by the company. siRNA duplexes were designed and synthesized by Dharmacon Inc. to target the rat Mfn-2: (a) 5’-GCUCCUGGCUCAAGACUAUUU-3’, (b) 5’-GCACCGCCAUAUAGAGGAAUU-3’, (c) 5’-GGAUAAGGCUUGGCUGGUUUU-3’, (d) 5’-CGGCUGAGAUUCAUGGACAUU-3’. As a negative control, cells were transfected with Silencer Negative Control #1 siRNA (Ambion Inc.). Experiments were performed between 48 to 72 hours after transfection.

**Western blot analysis**

Western blot analysis was performed as previously described.¹ For protein quantification, the data image from each western blot was opened using ImageQuaNT software (Molecular Dynamics, Inc.). The appropriate bands were identified and the integrated pixel intensity of each band was recorded as the raw intensity value for the band. Final band intensity values were obtained by subtracting the background intensity values from the raw intensity value of each band.

**Cytochrome c release**

To assay cytochrome c release, mitochondrial and cytosolic fractions were separated using the ApoAlert Cell Fractionation kit (BD Biosciences). These cellular fractions were analyzed for the presence of cytochrome c by immunoblotting with an anti-cytochrome c antibody. The purity of each cytosol and mitochondrial fraction was examined by monitoring the level of cytochrome C oxidase 4 (COX4), a mitochondrial marker.

*Balloon injury and morphometric analysis of intimal thickening*
Balloon denudation of the left common carotid arteries of male WKY rats and morphometric analysis of intimal thickening were performed as previously described\textsuperscript{1}. All procedures involving experimental animals were performed in accordance with protocols approved by the committee for animal research of Tongji Hospital, Huazhong University of Science and Technology. Immediately following injury, PBS or 2x10\textsuperscript{9} MOI of Adv-rMfn-2-GFP or Adv-GFP was delivered into vessel walls. The arteries were collected on day 5 and day 21 after operation and embedded in paraffin. Ten animals were used in each experimental group.

References


Supplemental Figure Legend

Supplemental Figure 1. Proapoptotic effect of Mfn-2 on cancer cell lines. A, lung cancer cell line A549. A549 cells were infected with 75 MOI of Adv-rMfn-2-GFP or Adv-GFP for indicated time (from 24 to 48 h). B, hepatic cancer cell line Bel 7402. Bel 7402 cells were infected with 100, 200, and 300 MOI of Adv-rMfn-2-GFP or Adv-GFP for 24 h. Apoptosis was assayed by Cell Death ELISA. Data are shown as the mean ± SE (n=5).
Supplemental Figure 1

(A) Cell Death ELISA (OD405 nm) over Infection Time (hr) for control, Adv-GFP, and Adv-rMfn-2-GFP.

(B) Cell Death ELISA (OD405 nm) over Titer (MOI / cell) for Adv-GFP and Adv-rMfn-2-GFP.