Intersectin-1s Regulates the Mitochondrial Apoptotic Pathway in Endothelial Cells*

Sanda A. Predescu 1, Dan N. Predescu, Ivana Knezevic, Irene K. Klein, and Asrar B. Malik

From the Department of Pharmacology, University of Illinois College of Medicine, Chicago, Illinois 60612

Intersectins (ITSNs) are multidomain adaptor proteins implicated in endocytosis, regulation of actin polymerization, and Ras/MAPK signaling. We have previously shown that ITSN-1s is required for caveolae fission and internalization in endothelial cells (ECs). In the present study, using small interfering RNA to knock down ITSN-1s protein expression, we demonstrate a novel role of ITSN-1s as a key antiapoptotic protein. Knockdown of ITSN-1s in ECs activated the mitochondrial pathway of apoptosis as determined by genomic DNA fragmentation, extensive mitochondrial fission, activation of the proapoptotic proteins BAK and BAX, and cytochrome c efflux from mitochondria. ITSN-1 knockdown acts as a proapoptotic signal that causes mitochondrial outer membrane permeabilization, dissipation of the mitochondrial membrane potential, and generation of reactive oxygen species. These effects were secondary to decreased activation of Erk1/2 and its direct activator MEK. Bcl-X<sub>L</sub> overexpression prevented BAX activation and the apoptotic ECs death induced by suppression of ITSN-1s. Our findings demonstrate a novel role of ITSN-1s as a negative regulator of the mitochondrial pathway-dependent apoptosis secondary to activation of the Erk1/2 survival signaling pathway.

ITSN-1 is a multidomain adaptor protein, which binds to and scaffolds the endocytic machinery of clathrin- and caveolae-mediated endocytic pathways (1–3). Two major ITSN-1 transcripts have been described in mammals, the ubiquitously expressed ITSN-1s and the neuron specific long isoform, ITSN-1L (1, 4, 5). A highly similar human gene, ITSN-2, generates by alternative splicing two ITSN-2 isoforms widely expressed in human tissues (lung endothelium included) and showing a high degree of similarity to ITSN-1 proteins (2, 6). ITSN-1s, the only ITSN-1 isoform present in the ECs lining the blood vessels wall (3), comprises two Eps15 homology domains, a central coiled-coil domain, and five consecutive SH3<sup>2</sup> domains (SH3A to -E) (1, 7). Although ITSN-1 is best known for its role in endocytosis, recent studies also implicate ITSNs as mediators of the MAPK signaling pathway (8). ITSNs interact via the SH3A domain with the mammalian Son-of-sevenless, a guanine-nucleotide exchange factor for Ras (9). The association of ITSN with mammalian Son-of-sevenless leads to increased levels of RasGTP with functional consequences in signaling Erk1/2 activation (9). Activated Ras initiates the phosphorylation cascade, leading to phosphorylation and activation of p42/p44 MAPKs (Erk1 and Erk2). In both neurons and nonneuronal cell lines, overexpression of the SH3A to -E, presumed to function in a dominant negative manner (11), attenuated the activation of EGF-mediated Ras/MAPK pathway (8). The concept of ITSNs as mediators of MAPK signaling is further supported by studies showing that full-length ITSN overexpression in HEK cells generated increased Ras-GTP levels associated with cytoplasmic vesicles, resulting in the c-Jun NH2-terminal kinase (JNK) activation (12). More recent work (13) in NIH 3T3 fibroblasts demonstrated that the long ITSN isoform mediates JNK activation, and this effect is due to the capability of ITSN-L to activate both Ras and Cdc42. Together, these findings suggest that ITSNs regulate the MAPK pathways important for essential cellular functions, such as cell survival, proliferation, migration, and differentiation.

In the present study, we used the siRNA to specifically knock down ITSN-1s protein expression and to analyze its effects on ECs. Biochemical and morphological analyses of cells lacking ITSN-1s showed morphological changes characteristic to apoptosis, such as genomic DNA fragmentation, extensive mitochondrial fission, blebbing of the mitochondrial membrane, widening of the narrow tubular cristae, and swollen and unstacked Golgi apparatus. Moreover, the proapoptotic proteins of the Bcl-2 family, BAX and BAK, were activated, resulting in the release of cytochrome c from the mitochondrial intermembrane space (IMS) to the cytosol and mitochondrial dysfunction. These effects were secondary to decreased MEK/Erk1/2 activation. Overexpression of the antiapoptotic protein Bcl-X<sub>L</sub> prevented BAX activation and inhibited apoptotic cell death caused by ITSN-1s down-regulation. Thus, we conclude that ITSN-1s is a key link between the regulatory signaling mechanisms of the mitochondrial death and upstream survival signaling pathways.

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†To whom correspondence should be addressed: Dept. of Pharmacology, University of Illinois College of Medicine, 835 S. Wolcott Ave., Chicago, IL 60612. Tel.: 312-996-1412; E-mail: sandap@uic.edu.

The abbreviations used are: SH3, Src homology 3; ITSN, intersectin; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; IMS, intermembrane space; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; EC, endothelial cell; MEF, mouse embryonic fibroblast; BMH, bis-maleimido-hexane; EM, electron microscopy; Ab, antibody; pAb, polyclonal antibody; mAb, monoclonal antibody; siRNA, small interfering RNA; RT, reverse transcription; GST, glutathione S-transferase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; DAPI, 4′,6-diamidino-2-phenylindole; MTP, mitochondrial permeability transition pore; HBSS, Hanks’ balanced salt solution; TMRE, tetramethylrhodamine ethyl ester perchlorate; ROS, reactive oxygen species; Puro, puromycin.
**EXPERIMENTAL PROCEDURES**

**Reagents and Cells**

Human lung microvascular endothelial cells (ECs) were obtained from Clonetics (San Diego, CA), and wild-type mouse embryonic fibroblasts (MEFs) were from ATCC (Manassas, VA). The retroviral packaging PT67 cell line was from Clontech (Mountain View, CA). siRNA SMART池 reagents for ITSN-1, siCONTROL™ functional nontargeting RNA, GloCytochelin B siRNA, and the corresponding transfection reagent were from Dharmacon (Lafayette, CO). Human Bcl-XL, cyclophilin B siRNA, and the corresponding transfection reagent were from Calbiochem; anti-BAX mAb from Chemicon International (Temecula, CA); anti-Bcl-XL mAb, phospho-Erk1/2, phospho-p38, and phospho-JNK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-phospho-MEK1/2 (Sigma). Anti-ITSN pAb was generated as in Refs. 7 and 14. Horseradish peroxidase-conjugated reporters IgGs were from Cappel, Organon Teknika (Durham, NC), and Alexa 594- and Alexa 488-conjugated reporters were from Molecular Probes.

**Cell Culture and siRNA**

ECs were grown as described (14). For silencing ITSN-1 gene expression, we used Dharmacon SMART池 reagents. Preliminary experiments were carried out to establish optimized transfection and cell culture conditions. RNAse-free conditions and reagents were used throughout the silencing experiments. The individual siGENOME duplex most efficient in knocking down ITSN-1s protein expression (ITSN-1s RNAi), GGA-CAGUACAACUAUGUCCUU (sense sequence) and 5'-UUUCAGUACACAUUGCCUU (antisense), was delivered in Prolong Antifade Kit (Molecular Probes, Inc., Eugene, OR); bis-maleimidohexane (BMH) cross-linker (Pierce). All electron microscopy (EM) grade reagents were from EM Science (Forth Washington, PA). All other reagents were from Sigma if not otherwise specified. Specific antibodies (Abs) were obtained from the following sources: anti-cytochrome c mAbs, Bcl-2 family antibody sampler kit, anti-BAK, and anti-BAX pAbs from Calbiochem; anti-BAX mAb from Chemicon International (Temecula, CA); anti-Bcl-XL mAb, phospho-Erk1/2, phospho-p38, and phospho-JNK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-phospho-MEK1/2 (Sigma). Anti-ITSN pAb was generated as in Refs. 7 and 14. Horseradish peroxidase-conjugated reporters IgGs were from Cappel, Organon Teknika (Durham, NC), and Alexa 594- and Alexa 488-conjugated reporters were from Molecular Probes.

**RT-PCR**

RNA was isolated from cultured cells using Qiagen RNeasy Mini RNA isolation kit as per the manufacturer’s instructions. cDNA was generated by the first step of the Invitrogen Thermoscript 2 Step reverse transcription (RT)-PCR kit as described in the manufacturer’s protocol. Amplifications were carried out using the following primers: ITSN-1s (5'-TTTGTGTTCCCAAGGTTTACG-3'), 5'-AGGCTTATCTGGCGGTTTCTC-3'), ITSN-1L (5'-GTGTGTGTTGAAACGTTGTTG-3', 5'-CCATCCCCATATCCGGTGAC-3'), ITSN-2s (5'-CTGTCACATCCCTTCGACCATT-3'), ITSN-2L (5'-CAGCTGATTTTCTCCTGGTGCGT-3'), 5'-ACATCTCCCGACGCGGTAA-3'). An equal volume of cDNA from the first step was used as template for the PCR containing standard conditions as per the manufacturer’s instructions. After 30 cycles, products were run in 1.5% agarose gel (Invitrogen) and visualized using ethidium bromide. Relative amounts of RNA were assessed by amount of PCR product in comparison with PCR product for glyceraldehyde-3-phosphate dehydrogenase.

**Retroviral Transfection**

Retrovirus was produced by transfection into the retroviral packaging PT67 cell line. Briefly, vectors (10 μg) were transfected into PT67 cells using TransIT-LT1 transfection reagent (Mirus, Madison, WI). Viral supernatants, collected 48 h post-transfection, were used to infect cultured ECs for 4 h in the presence of 4 μg/ml Polybrene. Subsequently, the cells were selected in Dulbecco’s modified Eagle’s medium containing 1 mg/ml puromycin (Clontech, Mountain View, CA) and then transfected with a 100 nM final concentration of ITSN-1s RNAi. 48 h post-siRNA transfection EC monolayers were used for biochemical and morphological analyses.

**Immunofluorescence Microscopy**

EC monolayers grown on glass coverslips were stained with MitoTracker Red CMXRos according to the manufacturer’s instructions. After MitoTracker staining, ECs were washed in PBS, fixed in 3.7% paraformaldehyde for 15 min at room temperature, and permeabilized in PBS containing 0.2% Triton X-100, for 5 min at room temperature. After quenching in PBS containing 1% bovine serum albumin for 1 h at room temperature, the fixed and permeabilized ECs were incubated with anti-cytochrome c or anti-Bax mAbs diluted in 0.1% bovine serum albumin in PBS, for 1 h at room temperature. The cells were washed (5 × 5 min) and then were incubated with an anti-mouse IgG-AlexaFluor 488 conjugated for an additional 1 h at room temperature. After washing as above, the cells were mounted on glass slides using the Prolong Antifade kit.

**Electron Microscopy and Morphometric Analysis**

Control and RNAi ITSN-1s transfected cells were subjected to standard EM procedure. Briefly, EC monolayers were fixed in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, containing 5% sucrose for 30 min at room temperature. After washing in the same buffer (3 × 10 min), the cells were post-fixed in Palade’s 1% osmium tetroxide for 1 h on ice, stained
Intersectin-1s Promotes Cell Survival

with Kellenberger buffer, dehydrated in graded ethanol, and embedded in Epon at 60 °C. For analyzing the morphological changes induced by ITSN-1 RNAi transfection, 15–20 sections/grid and 3–5 grids/block from six blocks chosen at random were used. The mitochondrial profiles were recorded on 100 micrographs (×64,000 magnification) for each experimental condition, and their number was normalized per 100 μm² of EC surface.

Generation of Anti-ITSN Polyclonal Ab

A polyclonal anti-ITSN Ab was raised in New Zealand rabbits against GST fusion protein GST-human ITSN-1-s (residues 1–440) as in Ref. 7. The cDNA of human ITSN-1 was kindly provided by Dr. Suzana de la Luna (Molecular Genetics Center IRO, Barcelona, Spain). The Ab was affinity purified on immobilized GST-human ITSN-(1–440) and characterized by Western blotting using human cultured EC 1% Triton X-100 extracts.

Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) Assay

The TUNEL assay was carried out for detection of apoptosis induced by ITSN-1 siRNA knockdown. Control and siRNA-treated cells (ECs or MEFs), 48 h after transfection, were washed in PBS and then permeabilized by methanol fixation for 5 min, at −20 °C. The TUNEL reaction mixture (label and enzyme solutions), prepared as indicated by the manufacturer, was applied on cell monolayers for 60 min, at 37 °C in a humidified atmosphere in the dark. EC monolayers reacted with the label solution only were used as negative controls. After washing, the coverslips were mounted using the Prolong Antifade kit with DAPI. TUNEL-positive cells were identified by fluorescence microscopy analysis using an excitation wavelength of 488 nm.

Cytochrome c Release from Mitochondria

Preparation of Cytosolic and Mitochondrial Fractions—ECs were gently scraped from the culture dish and collected by centrifugation at 500 g for 10 min. The cell pellets were washed twice in ice-cold PBS, pH 7.4, and then resuspended in buffer containing 250 mM sucrose, 40 mM Tris/HCl, pH 7.5, 10 mM EDTA, 1 mM glycerol, 1 mM dithiothreitol, and mixture of protease inhibitors and the resulting supernatant (cytosolic fraction) were used to analyze by immunoblotting the levels of cytochrome c.

Immunoblotting—Protein concentration of both cytosol fraction and mitochondrial extract were determined by BCA. Equivalent protein amounts (25 μg/lane) were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-cytochrome c, anti-BAX, or anti-BAK mAbs. The bands of immunoreactivity were visualized using appropriate reporter Abs horseradish peroxidase-conjugated and ECL. Densitometric analysis was performed using ImageJ1.37v software.

Cross-linking of Mitochondrial Fractions—In parallel experiments, mitochondria-enriched fraction from controls and ITSN-1 RNAi-treated ECs were treated with dimethyl sulfoxide-containing control buffer or with 10 mM BMH cross-linker. After 30 min, the cross-linking reaction was stopped with 1 M Tris/HCl, pH 7.5, to a final concentration of 20 mM, and the mitochondrial membranes were solubilized in electrophoresis sample buffer and further analyzed by SDS-PAGE and immunoblotting with anti-BAX mAbs.

Permeability Transition Pore Assay

The mitochondrial permeability transition pore (MTP) opening was measured in intact ECs by monitoring the fluorescence of mitochondrial confined calcein-AM, including the appropriate use of CoCl₂ to quench cytosolic fluorescence. Briefly, 1 × 10⁶ control and ITSN-1 RNAi-treated ECs (48 h post-transfection) were prepared as single cell suspension in Hanks’ balanced salt solution (HBSS) containing Ca²⁺ and loaded with calcein-AM for 15 min at 37 °C according to the manufacturer’s instructions. In parallel experiments, similar cell samples were loaded with calcein-AM in the presence of CoCl₂. After removal of excess stain and quenching reagent, the cell pellets were resuspended in ~400 μl of HBSS containing Ca²⁺ and analyzed for calcein-AM fluorescence by flow cytometry using a BD Biosciences FACscan flow cytometer in conjunction with winMDI 2.8 software (excitation/emission 494/517 nm).

Assessment of the Mitochondrial Membrane Potential

Changes in the mitochondrial membrane potential (ΔΨ) were determined by flow cytometry using TMRE staining as in Ref. 16. Briefly, aliquots of 10⁶ control and ITSN-1s-depleted ECs (48 h post-transfection) were prepared as a single cell suspension in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum and stained for 20 min at 37 °C with 25 nM TMRE. Cells stained with TMRE were visualized by flow cytometry using the BD Biosciences FACscan flow cytometer (excitation/emission 549/574 nm).

Mitochondrial Superoxide Generation

The levels of mitochondrial reactive oxygen species (ROS) were analyzed by fluorescent microscopy and flow cytometry. For fluorescence microscopy, control and ITSN-1 RNAi-treated ECs (48 h post-transfection) grown on coverslips were briefly washed with HBSS containing Ca²⁺ and Mg²⁺ and then incubated with MitoSOX for 10 min at 37 °C, protected from light. The cells were washed, counterstained with DAPI, and used for imaging.

For flow cytometry, controls and ITSN-1 RNAi-transfected ECs were trypsinized and prepared as single cell suspension in HBSS with Ca²⁺ and Mg²⁺ (HBSS/Ca/Mg). As controls, non-transfected ECs or ECs transfected with the nontargeting siRNA sequence were used.
Cells were loaded with the MitoSOX Red reagent as above, washed gently, and then resuspended in ~400 µl of HBSS/Ca/Mg. The fluorescence was analyzed by flow cytometry as above.

**Kinase Assay**

The cells were washed briefly in PBS, centrifuged, and lysed in kinase buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40, 1% glycerol, 0.2 mM sodium vanadate, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml aprotinin, and 0.5 µg/ml leupeptin). Equal amounts of protein (typically, 30 µg of total protein) were subjected to 5–20% SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblotting for phosphorylated forms of MAPKs with Abs specific for phospho-Erk1/2, -MEK1/2, -p38, and -JNK, followed by the appropriate horseradish peroxidase-conjugated reporters.

**RESULTS**

**Knockdown of ITSN-1s Protein Expression**—siRNA targeting the *ITSN-1* gene was employed to assess the function of...
ITSN-1s in ECs. Down-regulation of ITSN-1s protein expression was first achieved using SMARTpool ITSN-1 RNAi reagents that comprise four selected siRNA duplexes targeting the *ITSN-1* gene. Since ITSN-1L is neuron specific and thus not expressed in lung endothelium, isoform specificity for the siRNA sequence is not required. Each siRNA duplex in the SMARTpool was tested for the depletion efficiency of ITSN-1s. The siRNA duplex GGACAUAGUUGUACUGAAAUU (sense sequence) and 5’-UUUCAGUAACUAUGUUCCUU (antisense sequence) was found to be the most efficient in suppressing ITSN-1s expression; thus, this sequence was further used for the silencing experiments. The depletion efficiency of ITSN-1s was first evaluated by Western blotting using the anti-ITSN pAb raised against the GST-human ITSN-(1–440) as described under “Experimental Procedures.” A significant decrease in ITSN-1s/2s immunoreactivity 48 h post-transfection was detected in ITSN-1 RNAi-transfected ECs (Fig. 1A, lane c, top), by reference to control non-transfected cells (Fig. 1A, lane a) or ECs transfected with the control siRNA sequence (Fig. 1A, lane b), due to efficient silencing of ITSN-1s protein expression. A weak ITSN immunoreactivity was still detected in transfected cells due to ITSN-2s and remnants of ITSN-1s. No detectable changes in ITSN-2L or caveolin-1 protein expression were observed (Fig. 1A), indicating the specific nature of ITSN-1 knock down. Actin immunoblotting (Fig. 1A) was used to confirm the equal protein loading.

To confirm that indeed the RNAi sequence silenced specifically *ITSN-1s*, the Western blot analysis was complemented by RT-PCR to evaluate the mRNA levels of ITSN isoforms in controls and RNAi-transfected ECs. As shown in Fig. 1B, ITSN-1 RNAi transfection did not affect the mRNA levels of ITSN-2 isoforms (Fig. 1B, lane c) by reference to controls (Fig. 1B, lanes a and b), whereas in ECs transfected with ITSN-1 RNAi, the ITSN-1s mRNA levels were significantly decreased (Fig. 1B, lane c). The control siRNA sequence did not affect the level of ITSN-1s mRNA (Fig. 1B, lane b). RT-PCR analysis expectedly showed that ITSN-1L was not present in the lung ECs (not shown).

Since the levels of ITSN-2 protein expression are not affected by ITSN-1 RNAi, one can assume that the decreased immunoreactivity of short ITSNs detected in the lysates prepared from ITSN-1 RNAi-transfected ECs is caused exclusively by ITSN-1s down-regulation. Quantitative analysis applied on similar ECL-developed immunoblots obtained from four different experiments indicated that greater than 85% of the total amount of ITSN-1s protein was knocked down in ECs transfected with RNAi targeting the *ITSN-1* gene (Fig. 1C).

Control experiments to achieve efficient transfection and cell culture conditions were carried out and monitored using siRNA GloCyto-}

**Figure 2.** ECs depleted of ITSN-1s show genomic DNA fragmentation. A, ECs transfected with ITSN-1 RNAi show TUNEL-positive nuclei at 48 h post-transfection. B, DAPI staining revealed nuclear fragmentation and extensive blebbing of the nuclear membrane (b1 and b2). C, TUNEL staining of MEFs transfected with ITSN-1s RNAi at 48 h post-transfection. The majority of nuclei of MEFs depleted of ITSN-1s showed TUNEL-positive staining at 48 h post-transfection. D, DAPI staining of the nuclei of MEFs. E, ECs transfected with the control siRNA show no TUNEL-positive staining at 48 h post-transfection. F, DAPI staining of the nuclei of ECs transfected with the control siRNA. Bars, 20 μm (A–F); 5 μm (b1 and b2).
changes caused by suppression of ITSN-1s expression are not secondary effects caused by siRNA transfection.

Next, we examined the morphology of ITSN-1s RNAi-transfected ECs at the EM level. One of the most obvious morphological changes caused by transfection of ECs with ITSN-1 RNAi was the presence of numerous small round mitochondria often associated with an extensive endoplasmic reticulum network (Fig. 3B). In ECs transfected with the control siRNA sequence (Fig. 3A, a1) or nontransfected ECs (not shown), mitochondria were elongated, oval structures with narrow cristae. A detailed analysis showed mitochondria with pleiomorphic cristae, blebbing, and swelling after transfection of ECs with ITSN-1 RNAi (Fig. 3B, b1 and b2). Morphometric analysis indicated a 2.4-fold increase in the number of mitochondria in ITSN-1 RNAi-transfected cells compared with nontransfected ECs or cells transfected with the control siRNA sequence (Fig. 3F).

In addition, we observed in transfected cells swelling and unstacking of the Golgi apparatus (Fig. 3D). In healthy ECs, the Golgi apparatus consisted of a system of multiple flattened membranous cisternae, situated in close apposition to each other as stacks (Fig. 3C, arrows). In contrast, in ECs depleted of ITSN-1s, individual cisternae were swollen and disassembled into clusters of vesicles and tubules dispersed throughout the cell (Fig. 3D).

We also observed in ITSN-1s RNAi-transfected ECs widespread network of the endoplasmic reticulum. As shown in Fig. 3B, the endoplasmic reticulum tubules extend throughout the cytosol from one front to the other of the cell, in association with small and round mitochondria. The morphological analysis of ITSN-1s RNAi-transfected ECs also revealed a dramatic reduction in the number of caveolae (Fig. 3E) by comparison with control cells (Fig. 3A, a2). Occasionally, ITSN-1 RNAi-transfected ECs displayed caveolae open to the cell surface or caveolae free in the cytosol (not shown).

**ITSN-1s Knockdown Induces Release of Cytochrome c Mitochondria—**Studies have shown that the involvement of mitochondria in apoptosis includes a critical step involving the release of cytochrome c from the mitochondrial IMS into the cytosol (17, 18). To test if down-regulation of ITSN-1s protein levels by siRNA induced cytochrome c efflux, we isolated mitochondria and cytosolic fractions (postmitochondrial supernatants) from ITSN-1 RNAi-transfected ECs and control cells as in (15) and analyzed the levels of cytochrome c by Western blotting. A strong cytochrome c immunoreactivity was detected in the cytosolic fraction prepared from ITSN-1 RNAi-transfected ECs by comparison with control cells (Fig. 4A, lane c versus lanes a and b), indicative of its release from the mitochondrial IMS to the cytosol. In contrast, cytochrome c was confined to mitochondrial IMS.
Intersectin-1s Promotes Cell Survival

**A**

![Diagram](image)

**B**

![Graph](image)

**C**

![Image](image)

**D**

![Image](image)

**FIGURE 4.** siRNA-mediated down-regulation of ITSN-1s induces release of cytochrome c from mitochondria. **A**, total protein (25 μg) of cytosol and mitochondrial pellet prepared from nontransfected ECs (lanes a and d'), ECs transfected with the control siRNA (lanes b and b'), and ECs transfected with ITSN-1s RNAi (lanes c and c') were subjected to 5–20% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with anti-cytochrome c mAb. **B**, densitometric analysis of cytochrome c release from the mitochondrial IMS to the cytosol in controls and ECs transfected with ITSN-1 RNAi. Values shown are mean ± S.D. from three independent experiments. **C** and **D**, double fluorescence microscopy of control ECs and ITSN-1s RNAi-transfected cells at 48 h post-transfection indicates that under control conditions (C) immunofluorescent staining using anti-cytochrome c mAb/Alexa 488 anti-mouse IgG (c1) and MitoTracker Red CMXRos (c2) significantly overlapped (c3). Cytochrome c fluorescent staining is diffuse in ITSN-1 RNAi-transfected cells (D, d1). Note also the mitochondrial fragmentation in transfected cells (d2, d2') by comparison with controls (c2, c2'). cyt c, cytochrome c. Bars, 5 μm.

The little cytochrome c found in the cytosolic fraction of nontransfected cells (Fig. 4A, lane a') or cells transfected with the control siRNA sequence (Fig. 4A, lane b') is expected (15) and is most likely explained by the presence of a small population of unhealthy cells and possible contamination with mitochondria during the preparation procedure. Quantification of cytochrome c amounts present in mitochondrial pellets and cytosolic fractions of controls and ITSN-1s RNAi-transfected ECs by densitometry analysis (Fig. 4B), applied on similar ECL-developed immunoblots obtained from three different experiments, indicated that greater than 70% of the total amount of cytochrome c was detected in the cytosolic fraction of ECs depleted of ITSN-1s. Consistent with these data, morphological analysis by fluorescence microscopy indicated in control cells cytochrome c staining (Fig. 4C, c1) characteristic of the subcellular distribution of mitochondria (Fig. 4C, c2) and significant co-localization with the mitochondrial marker MitoTracker Red CMXRos (Fig. 4C, c3). In ITSN-1s RNAi-transfected cells, the cytochrome c immunostaining was diffuse, indicative of its release in the cytosol (Fig. 4D, d1). We also observed altered mitochondrial staining in ITSN-1s RNAi-transfected ECs (Fig. 4D, d2, inset d2') by comparison with control ECs (Fig. 4C, c2 and inset c2'), indicating mitochondrial fragmentation. These results demonstrate that down-regulation of ITSN-1s protein expression induced the release of cytochrome c from the mitochondrial IMS into the cytosol.

**Mitochondrial Dysfunction Induced by ITSN-1s Knockdown**—The altered mitochondrial ultrastructure and cytochrome c release, prerequisites of mitochondrial outer membrane permeabilization, directed us to analyzing the mitochondrial functional activity in ECs depleted of ITSN-1s. ITSN-1 depletion may act as an apoptotic stimulus inducing the MTP opening, leading to downstream caspase activation, ROS production, and loss in ΔΨ (19–21). The MTP may act as a good candidate pathway for the release of cytochrome c (22, 23).
cytosol of both control (a1) and cells transfected with ITSN-1 RNAi (a3), resulting in bright overall fluorescence. When calcine-AM loading is done in the presence of the quenching agent, the overall fluorescence is reduced compared with calcine-AM alone in both cell samples (a1 versus a2 and a3 versus a4), due to quenching of cytosolic calcine. However, in ITSN-1 RNAi-transfected ECs (apoptotic cells), due to Co2+ entry and intramitochondrial calcine-AM quenching, fluorescence signal was dramatically lost (a4 versus a2), indicating the activation of MTP. The key position of mitochondria in the control of programmed cell death is supported by the observation that mitochondria contribute to apoptosis signaling via the production of ROS.

The levels of mitochondrial ROS were first analyzed in control and ITSN-1 RNAi-transfected ECs by fluorescent microscopy (Fig. 5B) using the mitochondrial superoxide indicator MitoSOX Red. Superoxide anion is the major ROS species in mitochondria (27). Briefly, ECs grown on plastic coverslips were loaded with 2.5 μM MitoSOX Red for 10 min at 37 °C, protected from light. After counterstaining with DAPI, the cells were used for imaging. The results indicated increased fluorescence intensity in ECs depleted of ITSN-1s (Fig. 5B, b2), as the result of increased superoxide production and MitoSOX Red oxidation, by comparison with control ECs (Fig. 5B, b1).

Next, controls and ITSN-1 RNAi-transfected ECs prepared as single cell suspension in HBBS with Ca2+ and Mg2+ were stained with MitoSOX Red reagent as described under “Experimental Procedures.” The fluorescence analysis by flow cytometry indicated that ITSN-1s knockdown resulted in a significant increase in the cellular levels of ROS by reference to control ECs (Fig. 5B, b3). Enhanced mitochondrial ROS formation may play an important role in apoptosis induced by ITSN-1 knockdown by causing an unstable Δψ and a disturbed mitochondrial function (25, 26, 28).

Changes in Δψ were assessed in intact cells by monitoring fluorescence of the Δψ sensitive TMRE using flow cytometry. TMRE accumulates in mitochondria, and the accumulation is dependent on Δψ. Briefly, aliquots of 106 control and ITSN-1 RNAi-transfected ECs (48 h post-transfection), resuspended in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, were incubated in 25 nM TMRE for 20 min at 37 °C. Cells stained with TMRE were visualized by flow cytometry. As shown in Fig. 5C, depletion of ITSN-1 by RNA silencing of the ITSN-1 gene (c2) caused a significant fall in Δψ by reference to controls (c1).

**ITSN-1s Promotes Cell Survival**

Intersectin-1s Promotes Cell Survival

**A**

control ECs

ITSN-1 RNAi transfected ECs

**B**

MitoSOX Red staining

**C**

control ECs

ITSN-1 RNAi transfected ECs

**FIGURE 5. ITSN-1 knockdown causes significant mitochondrial dysfunction.** A, control and ITSN-1 RNAi treated ECs (48 h after siRNA transfection) were incubated with calcine-AM only (a1 and a3) and with calcine-AM in the presence of CoCl2 (a2 and a4) and analyzed by flow cytometry. The change in calcine-AM fluorescence intensity between a2 (control ECs) and a4 (apoptotic cells) indicates the activation of MTP. B, MitoSOX Red staining of control (b1) and ITSN-1 RNAi-transfected ECs (b2). Intracellular MitoSOX Red-emitted fluorescence indicates an increase in ROS levels (b3). Data are expressed as means ± S.D. of three similar experiments. MFI, mean fluorescence intensity. Bar, 14 μm (b1 and b2). C, flow cytometry analysis of Δψ in ITSN-1 depleted ECs, using the probe TMRE. Since TMRE accumulates only in mitochondria with high Δψ, the change in TMRE fluorescence intensity between c1 and c2 indicates a fall in Δψ, as a result of ITSN-1 knockdown.
integration into the mitochondrial membrane, the cytosolic fractions prepared from controls and ITSN-1s transfected ECs were examined for the presence of BAX by immunoblotting. As shown in Fig. 6A, lane c, the cytosolic fraction prepared from ITSN-1s-depleted ECs contained a lower amount of BAX by comparison with control cytosolic fraction prepared from ECs transfected with the control siRNA sequence (Fig. 6A, lane a) or nontransfected cells (Fig. 6A, lane b), consistent with significant subcellular BAX translocation following ITSN-1s RNAi transfection. Next, we utilized the membrane-permeable 16-Å chemical cross-linker BMH as in Ref. 17 to assess whether BAX activation induced by down-regulation of ITSN-1s led to the conformational change required for the formation of higher order BAX complexes within the mitochondrial membrane. Briefly, the mitochondrial fraction (isolated as described under “Experimental Procedures”) was treated with 10 mM BMH. Upon cross-linking, the mixture was analyzed by SDS-PAGE and immunoblotting with anti-BAX mAb. We detected a higher BAX immunoreactivity in the mitochondrial pellet prepared from ITSN-1s RNAi-transfected cells (Fig. 6A, lane c) by comparison with controls, either control siRNA-transfected (Fig. 6A, lane a) or nontransfected cells (lane b), consistent with BAX redistribution from cytosol to mitochondria following ITSN-1s depletion. A higher molecular mass band (42 kDa) immunoreactive to BAX Ab was detected in the mitochondrial fraction of ITSN-1 RNAi-transfected cells (Fig. 6A, lane c) after BMH cross-linking, consistent with the formation of BAX dimers. The decrease of BAX signal in the cytosolic fraction of ITSN-1s-depleted ECs, as detected by immunoblotting, corresponded to the increase in BAX in the corresponding mitochondrial fraction (Fig. 6B). In the control mitochondrial fractions, the 42 kDa band corresponding to the BAX dimer was weak by comparison with the cross-linkable BAX homodimer in the mitochondria of ITSN-1s-depleted ECs, consistent with...
the presence of a small EC unhealthy population showing BAX activation. The ~26 kDa protein band (**), immunoreactive to BAX Ab (Fig. 6A) was detected only after the cross-linking procedure. It has also been detected in other similar studies and is thought to be an associated small protein or an intrachain cross-link (29). ITSN-1 knockdown resulted in a greater than 3-fold increase in monomeric BAX associated with the mitochondrial membrane and in an approximately 3-fold increase in BMH-cross-linkable BAX homodimers (Fig. 6B).

We next analyzed BAK activation. BAK activation triggers a specific allosteric conformational change that includes exposure of its N terminus that can be recognized by specific Abs raised against this region of the protein, followed by homoligomerization in the outer mitochondrial membrane (18, 31). Detection of BAK with Abs directed against the N-terminal epitope is indicative of exposure of the N terminus region and, thus, of activation of the protein. We isolated the mitochondrial fraction from ITSN-1s RNAi-transfected cells and analyzed it by SDS-PAGE and immunoblotting for the presence of activated BAK with anti-BAK mAb (AM04). As shown in Fig. 6C, lane f, this Ab detected the presence of BAX in mitochondrial lysates prepared from ITSN-1 RNAi-transfected cells, indicating that down-regulation of ITSN-1s induced BAX activation in ECs. A weak BAX immunoreactivity was present in the mitochondrial lysates obtained from control cells (Fig. 6C, lanes d and e), again consistent with the presence of a small fraction of damaged ECs. Quantification of the amount of active BAX in the mitochondrial membranes of controls and ITSN-1s-depleted ECs (Fig. 6D) indicated a significant increase in the amount of active BAX in the mitochondrial membranes prepared from ECs depleted of ITSN-1s.

We then carried out a morphological analysis of BAX subcellular distribution by fluorescence microscopy using a BAX Ab directed against the NH2 terminus of the BAX protein, thereby specifically recognizing activated BAX. Fig. 6E shows for comparison control ECs (either transfected with the control siRNA sequence or nontransfected) and an ITSN-1 RNAi-transfected ECs immunostained with an anti-BAX Ab. In ITSN-1 RNAi-transfected ECs, BAX immunostaining showed a strong punctate distribution that partially overlapped the typical subcellular distribution of mitochondria. The merged image indicated significant overlap with the mitochondrial marker MitoTracker RedCMXROS, indicative of BAX translocation and integration into the mitochondrial membrane. However, the presence of other BAX-immunoreactive puncta that do not colocalize with the mitochondrial marker suggests the possibility of BAX localization with other subcellular membranes or partial loss of the MitoTracker staining. By contrast, control ECs show no staining or very weak nuclear membrane BAX immunoreactivity. Together, these findings are consistent with BAX association with the mitochondrial structures and, therefore, BAX involvement in the regulation of apoptosis induced by down-regulation of ITSN-1s protein expression.

**ITSN-1s RNAi-induced Apoptosis Is Dependent on the MEK/Erk Signaling Pathway**—Since accumulating evidence suggests that the function of Bcl-2 family is regulated by post-translational modifications, including phosphorylation, dimerization, and/or proteolytic cleavage, we investigated the possible involvement of MAPK in EC apoptosis induced by ITSN-1s silencing. To evaluate the contribution of MAPK in apoptosis induced by ITSN-1s RNAi knockdown, we monitored the activity of Erk1/2, p38, and JNK by Western blotting analysis using detergent extracts of control and ITSN-1s-transfected ECs and anti-phospho-Erk1/2, anti-phospho-p38, and anti-phospho-JNK Abs that specifically recognize phosphorylated and activated Erk1/2, p38, and JNK, respectively. As shown in Fig. 6F, transfection of ECs with ITSN-1s RNAi reduced Erk1/2 phosphorylation, indicative of decreased Erk activity. No detectable changes in p38 or JNK phosphorylation were detected. Since Erk activation requires direct phosphorylation by the upstream MAPK kinase MEK (32), we examined the level of activation of MEK in control versus ITSN-1s-transfected ECs. As seen in Fig. 6F, the phosphorylation of MEK, and thereby its activation, was decreased in ECs depleted of ITSN-1s, suggesting that the anti-apoptotic effect of ITSN-1s depends on the MEK/Erk1/2 signal pathway.

Bcl-XL Overexpression Prevents BAX Activation and Cell Death in Cells Transfected with ITSN-1s RNAi—To document that down-regulation of ITSN-1s protein expression promotes the mitochondria-dependent apoptosis in ECs, we used Puro-Bcl-XL retroviral vector expressing Bcl-XL. Overexpression of the antiapoptotic Bcl-XL can block the mitochondrial apoptosis by preventing the allosteric activation of BAX and BAK (33, 34). The retrovirus was produced by transient transfection into the retroviral packaging cell line PT67. Cultured ECs were infected for 4 h with the viral supernatants in the presence of 4 μg/ml Polybrene, and 48 h after infection, the cells were subjected to ITSN-1s RNAi transfection to address the protective effects of Bcl-XL on the apoptotic EC death caused by ITSN-1s depletion. As shown in Fig. 7A, ECs transfected with the retrovirus encoding Bcl-XL showed ~4-fold increase in Bcl-XL protein expression by comparison with ECs transfected with the control Puro vector. As shown above, morphological and biochemical analyses of mitochondria from ECs depleted in ITSN-1s showed BAX activation (i.e. translocation from the cytosol into the mitochondrial membrane, insertion into the mitochondrial membrane, and formation of high M, homodimers) (Fig. 6A). To examine whether Bcl-XL overexpression prevented BAX activation induced by siRNA-mediated ITSN-1s down-regulation, ECs containing either the control Puro or Bcl-XL vector were transfected as described with 100 nm ITSN-1s RNAi. At 48 h post-transfection, BAX activation was first examined by immunofluorescence microscopy using anti-BAX mAb and mitochondrial marker MitoTracker CMXROS. As shown in Fig. 7B, in ECs depleted of ITSN-1s, BAX subcellular distribution partially overlapped the typical mitochondrial staining, consistent with BAX translocation to the mitochondrial membrane. Similar immunostaining was recorded in ECs depleted of ITSN-1s, after infection with the control vector (Fig. 7B). By contrast, in ECs depleted in ITSN-1s but overexpressing high levels of Bcl-XL (Fig. 7B), BAX activation (i.e. translocation to the mitochondria) was inhibited, indicating that Bcl-XL protected ECs from mitochondria-dependent cell death caused by siRNA-mediated ITSN-1s down-regulation.

We next assessed biochemically BAX activation by cell fractionation followed by immunoblotting of the cytosolic fraction.
Intersectin-1s Promotes Cell Survival

A

B

C

FIGURE 7. Bcl-X<sub>L</sub> prevents BAX activation induced by down-regulation of ITSN-1s protein expression. A, ECs were transfected with a control Puro and the Puro-Bcl-X<sub>L</sub>-encoding retroviral vector. Bcl-X<sub>L</sub> protein levels were evaluated by Western blotting analysis of ECs lysates. B, BAX activation was evaluated in ECs transfected with ITSN-1s RNAi only and in ECs transfected with ITSN-1s RNAi after infection with the control and Bcl-X<sub>L</sub>-encoding retrovirus. BAX activation was measured with the anti-BAX Ab that detects activated BAX. C, Bcl-X<sub>L</sub> expression prevented the subcellular translocation of BAX from the cytosol to the mitochondrial membrane in ECs depleted of ITSN-1s. BAX protein levels were evaluated by immunoblot analysis of the cytosolic fraction prepared from control and Bcl-X<sub>L</sub>-overexpressing ECs at 48 h post-ITSN-1s RNAi transfection. Bars, 7 μm.

with anti-BAX pAb. As shown in Fig. 7C, the level of BAX in the cytosol from ITSN-1 RNAi-transfected ECs was lower than levels of BAX in the cytosol from Bcl-X<sub>L</sub>-overexpressing ECs. These results demonstrate that Bcl-X<sub>L</sub> protected ECs from siRNA-mediated down-regulation of ITSN-1s protein expression.

DISCUSSION

This study demonstrates that siRNA-mediated down-regulation of ITSN-1s protein expression in cultured lung microvascular ECs triggers the mitochondrial pathway of apoptosis through the sequential activation of proapoptotic Bcl-2 family members to the release of cytochrome <em>c</em>. The release of cytochrome <em>c</em> from the mitochondrial IMS to the cytosol is an essential event of mitochondrial cell death (35, 36). Depending on the cell type, apoptotic stimulus, and experimental conditions, several molecular mechanisms of cytochrome <em>c</em> release and mitochondrial outer membrane permeabilization involving the PTP complex activity, the BAX/BAK pore-forming properties, modulation of ionic fluxes, and hypotonicity may co-exist within one model of cell death (17, 37–39). Although activation of the PTP complex results in mitochondrial damage, the mitochondrial outer membrane permeabilization mediated by MPT-independent mechanisms occurs without apparent alterations of mitochondrial ultrastructure and functions (39). Regardless of the molecular mechanism of cytochrome <em>c</em> release, once in the cytosol, cytochrome <em>c</em> will induce caspase activation and apoptotic cell damage (36, 40). The irreversible mitochondrial outer membrane permeabilization is considered the “point of no return” during apoptosis (40). Our experimental model involving efficient and specific knockdown of ITSN-1s protein expression 48 h after siRNA transfection does not allow us to define the precise mechanism involved in the release of mitochondrial cytochrome <em>c</em>. However, the results of our study fit the basic tenets of the mitochondrial apoptotic pathway: (i) ECs depleted of ITSN-1s displayed gross changes in mitochondrial morphology, (ii) loss of ITSN-1s triggered a cascade activating the multidomain proapoptotic proteins BAX and BAK, (iii) cytochrome <em>c</em> was released from mitochondrial IMS to the cytosol, (iv) ITSN-1s depletion caused mitochondrial dysfunction, and (v) BAX activation was prevented by overexpression of the antiapoptotic protein Bcl-X<sub>L</sub>.

The proapoptotic multidomain proteins BAX and BAX are present in most cells as inactive monomers residing at the mitochondria (BAK) or cytosol (BAX) (30). Their activation by death signals involves specific allosteric conformational changes leading to BAK oligomerization and BAX translocation and insertion into the mitochondrial membrane, followed by homo-oligomerization (18). The ability of BAK and BAX to homo-oligomerize results in formation of pores in the OMM responsible for the irreversible efflux of apoptosis-inducing proteins from the mitochondrial IMS to the cytosol (35, 36). One of these proteins is cytochrome <em>c</em>, which once released in the cytosol is highly proapoptotic (41). It interacts with the apoptosis protease-activating factor-1 in an ATP-dependent manner and forms the apoptosome complex, which activates caspases and induces apoptosis (41). Emerging evidence indicates that near the time of cytochrome <em>c</em> release, mitochondria undergo rapid and excessive fragmentation (fission) into smaller and rounder units (42, 43). The allosteric activation of BAX and BAX and the subsequent mitochondrial program of apoptosis are prevented by the overexpression of the antiapoptotic Bcl-2 and Bcl-X<sub>L</sub> (44, 45). We found that ECs depleted of ITSN-1s display a 2.4-fold increase in the number of mitochondria by reference to control ECs. Gross changes in the mitochondrial morphology (i.e. blebbing of mitochondrial membrane and widening of the narrow tubular cristae) were evident in ECs as a result of ITSN-1s depletion. These morphological and biochemical studies also revealed a significant release of cytochrome <em>c</em> from mitochondrial IMS to the cytosol.
BAX and BAK are multidomain proapoptotic proteins upstream of cytochrome c release (46). In normal ECs, BAX resides in the cytosol, whereas in response to ITSN-1 RNAi transfection, BAX underwent the allosteric conformational change required for its translocation and insertion into the mitochondrial membrane. Biochemical analysis of mitochondrial pellets prepared from ITSN-1s RNAi-transfected ECs showed strong BAX immunoreactivity compared with control ECs. This observation is supported by morphological analysis of BAX subcellular distribution in ECs depleted of ITSN-1s. BAX immunostaining was co-localized with the mitochondrial marker MitoTracker CMXRos, suggesting the subcellular translocation of BAX from cytosol into the mitochondrial membrane. Moreover, BAX is inserted into membrane as a homo-oligomerized integral membrane protein cross-linkable by BMH (29). Indeed, BMH cross-linking applied on a mitochondrial pellet of ITSN-1s RNAi-transfected cells demonstrated the existence of BAX dimers, consistent with a model in which lack of ITSN-1s triggers the activation cascade, leading to proapoptotic BAX activation and oligomerization.

Previous observations indicated that under normal conditions, BAX is a transmembrane protein of the OMM (47). It has been shown that BAX activation involves exposure of the N-terminal domain, which is masked in the inactive protein, followed by intramembrane oligomerization, resulting in permeabilization of the OMM (35, 47). Consistent with these observations, a specific BAX Ab raised against the N terminus epitope showed significant BAX immunoreactivity in the mitochondrial pellet prepared from the ECs depleted of ITSN-1s. Finally, we observed that overexpression of the antiapoptotic protein Bcl-XL prevented BAX activation and inhibited apoptotic cell death, consistent with the previous observation indicating that antiapoptotic Bcl-2 proteins prevent disruption of mitochondrial damage associated with apoptosis (48).

Moreover, ITSN-1s RNAi transfected ECs showed, 48 h post-transfection, opening of the PTP, loss in the $\Delta\Psi$, and increased ROS levels, altogether suggestive of an altered mitochondrial physiology. Previous studies examining the relationship between the PTP, $\Delta\Psi$, BAX activation, and cytochrome c release indicated a variety of mechanisms and sequence of events responsible for cytochrome c release and apoptotic cell death. Since in this study we evaluated biochemical and morphological apoptotic changes caused by ITSN-1s knock down, at 48 h after RNAi transfection (time point when ITSN-1s protein expression is efficiently down regulated), we cannot conclude on the molecular mechanism and evaluate the sequence of events leading to cytochrome c release and apoptotic cell death of ECs under these experimental conditions.

We also demonstrated that ITSN-1s down-regulation inhibits phosphorylation of the survival kinase Erk1/2 and the upstream kinase, MEK1/2. It has been shown that activation of Erk signaling promotes cell survival (49) by phosphorylation of the proapoptotic protein Bad (50). In healthy cells, phosphorylated Bad is sequestered in the cytosol in an inactive form, by interacting with the phosphoserine-binding protein 14-3-3. Once phosphorylated, Bad is translocated to mitochondria, where it dimerizes with Bcl-XL. This dimerization releases BAX from Bcl-XL, facilitating BAX homodimerization and, as a result, activation resulting in cytochrome c release from mitochondria and apoptosis (50).

Our work suggests a model in which ITSN-1s down-regulation suppresses the activation of MEK1/2 and its target Erk1/2 and that Ras-MEK1/2-Erk1/2 signaling may be critical for EC apoptosis. Our observation is supported by the finding that ITSN-1s recognizes via its SH3A the proline-rich domain of mammalian Son-of-sevenless (9), a Ras activating guanine-nucleotide exchange factors. Subsequently, Ras-GTP may interact with Raf, assumed to be the major catalyst of MEK1/2 activation (51), to activate the kinase cascade and other downstream effectors. Regardless of the molecular mechanism involved, our findings suggest that EC apoptosis caused by ITSN-1s depletion is mediated by suppression of the survival kinase Erk1/2 and its direct activator, MEK1/2.

The functional significance of ITSN-1s-dependent regulation of EC apoptosis in not clear. Proapoptotic stimuli, such as oxidants, UV light, and mediators, such TNF and LPS, may mediate endothelial apoptosis by modifying the function of ITSN-1s and thereby activate the mitochondrial pro-death pathway. We have shown in studies in mouse lung endothelia expressing the ITSN-1s siRNA apoptotic changes in pulmonary microvessel ECs similar to those observed in cultured ECs in the present study. Thus, proapoptotic stimuli may activate the apoptotic program in ECs via ITSN-1s to kill and induce detachment of the susceptible cells in order to restore vessel wall integrity by proliferation of the healthy ECs.

Recently, it has been shown that ITSN-1 is a substrate for granzyme B, a protease able to activate apoptosis by caspase-dependent and caspase-independent pathways (52). Thus, it is possible that ITSN-1 cleavage by granzyme B disrupts ITSN-1s function and promotes apoptosis. Another possible ITSN-1s depletion-dependent mechanism of apoptosis may involve the marked reduction in the caveolae vesicle trafficking and presumably nutrient delivery, observed in the siRNA-treated ECs. However, based on the finding that caveolin-1-null mice, which have no caveolae and yet show no apparent defect in cell survival (53), it is unlikely that impaired vesicle trafficking per se was a determinant of EC apoptosis in the present study. In summary, our results have shown that ITSN-1s is a crucial link between the regulatory signaling mechanisms of the mitochondrial death and upstream survival signaling pathways.

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