Rapid Communication

J. Biochem. **136**, 421–425 (2004)
DOI: 10.1093/jb/mvh139

Establishment of Stable hFis1 Knockdown Cells with an siRNA Expression Vector

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Received June 2, 2004; accepted July 9, 2004

Yeast Fis1p participates in mitochondrial fission, together with Dnm1p and Mdv1p. Recently, human Fis1 (hFis1) was reported to be involved in mitochondrial fission, together with Drp1. We established stable transformants with an hFis1 siRNA expression vector. In the stable hFis1 knockdown cells, hFis1 expression was suppressed to approximately 10%, and mitochondrial fission, induced by cisplatin treatment, was delayed. In addition, mouse Fis1 (mFis1) expression promoted mitochondrial fission and cell death in the hFis1 knockdown cells, suggesting that mFis1 complements the function of hFis1. These hFis1 siRNA expression vectors may be useful for studying the molecular function of mammalian Fis1.

Key words: Fis1, knockdown, mitochondrial fission, RNAi, siRNA.

Abbreviations: BGH, bovine growth hormone; CFP, cyan fluorescent protein; CMV, cytomegalovirus; EGFP, enhanced green fluorescent protein; FANTOM, Functional Annotation of Mouse; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hFis1, human Fis1; mFis1, mouse Fis1; polyA, polyadenylation; RNAi, RNA interference; RT-PCR, reverse transcription–PCR; siRNA, small inhibitory RNA.

Mitochondrial morphology is regulated through fusion and fission mechanisms (*1*). In the yeast *Saccharomyces cerevisiae*, the fission of the outer mitochondrial membrane is regulated by three proteins: Dnm1p, Fis1p and Mdv1p (*2*). The deletion of any of these genes blocks the fission mechanism and leads to a fused mitochondrial network (*3*, *4*). The mammalian homologue of Dnm1p, Drp1 (also called DLP1), is a mediator of mitochondrial fission, similar to yeast Dnm1p (*5–7*). Recently, studies on the function of human Fis1 (hFis1) were published (*8*, *9*). Yoon *et al.* reported that hFis1 is a mitochondrial membrane protein that regulates mitochondrial fission in mammalian cells through interaction with Drp1. James *et al.* also reported that hFis1 is part of the mitochondrial fission machinery, and suggested that it is involved in the regulation of apoptosis. These studies mainly utilized hFis1 overexpression experiments. In addition, Yoon *et al.* used an antisense suppression technique, and a 50% reduction of hFis1 expression was observed.

Vector-based small inhibitory RNA (siRNA) expression systems that can induce RNA interference (RNAi) in mammalian cells have been reported (*10–17*). These RNAi systems have made it possible to suppress gene expression over a long period. In the present study, we employed a vector-based siRNA expression system to analyze mammalian Fis1.

A cDNA clone of the hFis1 gene (GenBank accession No. BC003540) was obtained from a human liver Marathon Ready cDNA library (Clontech, BD Biosciences, Palo Alto, CA, USA) by PCR. The hFis1 DNA fragment was inserted into the modified pENTR vector, based on pENTR1A (Invitrogen, Carlsbad, CA, USA), between the *Bam*HI and *Eco*RI sites. Expression vector pEXP26/FLAG-hFis1/IRES-EGFP, bearing the FLAG-tagged hFis1 and EGFP as an expression marker, was constructed using Gateway technology with pENTR/hFis1 and pDEST26-FLAG/IRES-EGFP based on pDEST26 (Invitrogen). A mouse Fis1 (mFis1) clone was obtained from the FANTOM RIKEN full-length cDNA clones (*18*, *19*) (FANTOM clone ID: 2010003O14). The expression vectors, pEXP26/FLAG-mFis1/IRES-EGFP, were constructed in the same way as the hFis1 expression vectors. The DNA fragments of CFP, containing the CMV enhancer-promoter and the BGH polyA signal, were inserted into siRNA expression vector pcPURU6i (*20*), between the *Eco*RI and *Bgl*II sites, to yield plasmid pcPURU6iCFP. CFP fluorescence is a useful marker for distinguishing cells transfected with the siRNA expression vector and for evaluating the transfection efficiency. We selected two hFis1 target sequences for siRNA in the hFis1 coding sequence (Seq1, GTACAATGATGACATCCGTAA; Seq2, GTACGTCCGCGGGTTGCTGCA). The 21 nucleotide target sequences should start from G after A for transcription with the U6 promoter. The sense sequence and

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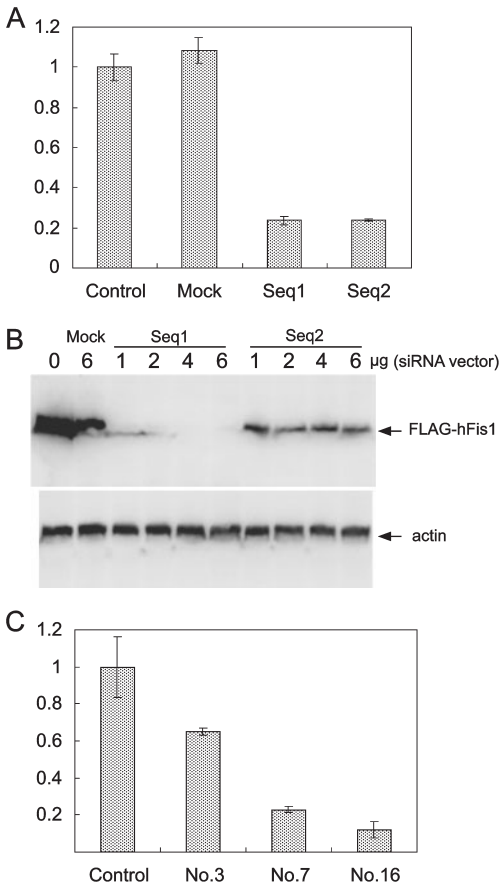

Fig. 1. **RNAi experiments on hFis1, using siRNA expression vectors in HeLa cells.** (A) Transient suppression of hFis1 mRNA expression in HeLa cells transfected with siRNA expression vectors. The medium was changed to that containing 2.5 μg/ml puromycin at 31 h after transfection. After 17 h, the cells were harvested and RNA was prepared. Samples were analyzed by real-time RT-PCR (TaqMan) for the relative quantitation of hFis1 mRNA, using the comparative C_T method with endogenous GAPDH mRNA. "Control" in the graph shows the level of hFis1 mRNA in non-transfected HeLa cells. "Mock" shows the level of hFis1 mRNA in cells transfected with pcPURU6iCFP, a control vector without the siRNA sequence. Seq1 and Seq2 show the hFis1 mRNA levels in cells transfected with pcPURU6iCFP/Seq1 and pcPURU6iCFP/Seq2, respectively. These values were normalized by the control value. (B) Transient suppression of FLAG-hFis1 protein expression in HeLa cells transfected with siRNA expression vectors. Samples were analyzed by Western blotting with an anti-FLAG M2 antibody at 41 h after co-transfection with the FLAG-hFis1 expression vector (pEXP26/FLAG-hFis1/IRES-EGFP) and the siRNA expression vector (pcPURU6i/Seq1 or pcPURU6i/Seq2). "Mock" shows the results for cells transfected with the control vector (pcPURU6i) instead of the siRNA expression vector. Various amounts of the siRNA expression vector DNA were used, as shown. The lower panel shows the actin expression level in each sample, as a standard. (C) Stable RNAi experiments on hFis1 with the siRNA expression vector (pcPURU6iCFP/seq1). The expression levels of hFis1 mRNA in clone Nos. 3, 7 and 16 of stable transformants with the siRNA expression vector and in control HeLa cells are shown. Samples were analyzed by real-time RT-PCR (TaqMan) for the relative quantitation of hFis1 mRNA, using the comparative C_T method with endogenous GAPDH mRNA.

antisense sequence were connected by a stem loop sequence (TAGAATTACATCAAGGGAGAT), and T_4 was added to the 3′-terminus for transcription termination (Seq1, GTATAATGATGATATTTGTAA-TAGAATTACA-TCAAGGGAGAT-TTACGGATGTCATCATTGTAC-TTTT; Seq2, GTATGTCTGTGGGTTGTTGCA-TAGAATTACAT-CAAGGGAGAT-TGCAGCAACCCGCGGACGTAC-TTTT). To prevent unexpected recombination and mutation, four T's were substituted for four C's in the sense sequences of the target (underlined letters). For construction of siRNA expression vectors, the sense and antisense oligonucleotides were annealed and inserted into the *Bsp*MI site of pcPURU6iCFP and pcPURU6i under U6 promoter control. These vectors have a puromycin resistance gene for selection in mammalian cells.

HeLa cells were grown in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (ICN Biomedicals, Aurora, OH, USA) under a 5% CO_2 atmosphere at 37°C. Cells were transfected with the siRNA expression vectors using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. Total cellular RNA was isolated and purified using Isogen (Nippon Gene, Tokyo, Japan), and reverse transcription was performed using the MuLV reverse transcriptase in the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA). The hFis1 mRNA expression level was determinded by TaqMan real-time PCR analysis, using Assays-on-Demand Gene Expression Product for hFis1 (Assay ID: Hs00211420_m1) (Applied Biosystems) containing hFis1 specific primers and a FAM-MGB labeled TaqMan probe. A human GAPDH Endogenous Control (VIC-MGB Probe) (Applied Biosystems) was used as an endogenous control for mRNA quantitation. The reactions were carried out using the TaqMan Universal PCR Master Mix (Applied Biosystems) and were assayed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. The relative quantitation of mRNA was performed by the comparative C_T method ($2^{-\Delta\Delta C_T}$ method) (*21*).

First, HeLa cells were transiently transfected with the siRNA expression vectors. To evaluate the efficacy of RNAi, the hFis1 mRNA was quantitated by the TaqMan real-time PCR method. The endogenous hFis1 mRNA was clearly suppressed to about 20%, as compared to in mock-infected cells, with both the Seq1 and Seq2 siRNAs (Fig. 1A). The siRNA expression vectors also efficiently suppressed the expression of the FLAG-hFis1 protein (Fig. 1B). The Seq1 siRNA suppressed the expression of FLAG-hFis1 more than the Seq2 siRNA. With 4 μg of the Seq1 siRNA expression vector DNA, the expression of FLAG-hFis1 was not detected. These results indicated that the siRNAs derived from the siRNA expression vectors efficiently suppressed the expression of hFis1. However, no obvious changes in the mitochondrial morphology of the CFP-positive cells transfected transiently with the siRNA expression vectors were observed in one week after transfection (not shown). One reason might be the lag time from RNAi suppression of the target mRNA to degradation of the endogenous target protein. If the target protein is stable, then it may take a longer time to degrade the target endogenous protein completely, and we may not be able to see a change in the phenotype during the time frame of the transient transfection.

The mitochondria in the yeast Fis1p deletion mutant reportedly adopted a fused morphology because of the

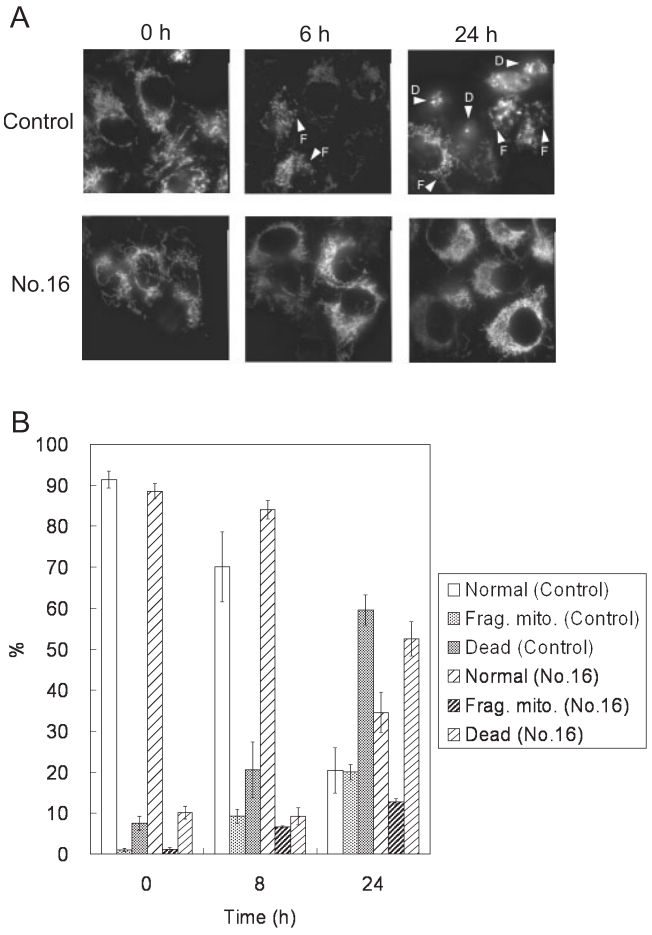

Fig. 2. **Changes in mitochondrial morphology after the addition of cisplatin.** (A) Changes in the mitochondrial morphology of the hFis1 knockdown cells (No. 16 clone), as compared to the control HeLa cells (Control), induced by treatment with cisplatin. The time after cisplatin addition is indicated. Cells were transfected with pDsRed1-mito (Clontech) to highlight the mitochondria in living cells. The cells were observed under a fluorescence microscope, IX70 (Olympus, Tokyo, Japan), with a CCD camera, Orca (Hamamatsu Photonics, Hamamatsu, Japan). The typical morphology of fragmented mitochondria is shown in the control at 6 h and 24 h (arrowhead F). Thereafter, the cells became round, detached and dead, as shown in some control cells at 24 h (arrowhead D). (B) The mitochondrial morphology of the hFis1 knockdown cells (No. 16 clone) and parental HeLa cells (Control) was assessed at the indicated time points after the addition of 100 μM cisplatin. Cells (n > 250) were counted in three wells independently at each time point. The cells were classified as having normal mitochondria (Normal), having fragmented mitochondria (Frag. mito.), or being dead (Dead), as shown in Fig. 2A. The percentages of each type of cell are shown.

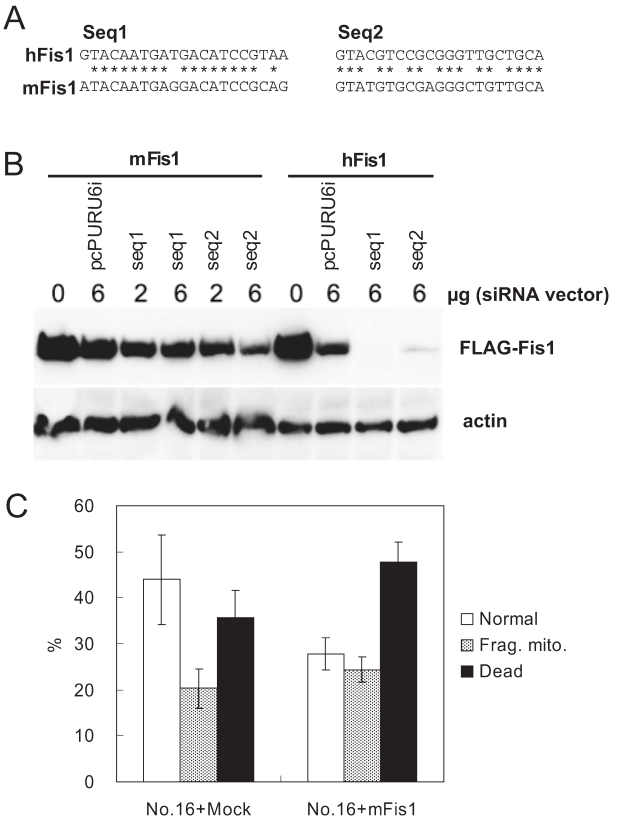

Fig. 3. **mFis1 complements the function of hFis1 in the hFis1 knockdown cells.** (A) DNA sequence comparison between hFis1 and mFis1 in the siRNA target sites. (B) The RNAi expression vectors specifically suppressed hFis1 protein expression, but did not suppress mFis1 protein expression. Samples were analyzed by Western blotting, using an anti-FLAG M2 antibody, at 24 h after cotransfection with the FLAG-hFis1 expression vector (pEXP26/FLAG-hFis1/IRES-EGFP) or the FLAG-mFis1 expression vector (pEXP26/FLAG-mFis1/IRES-EGFP) and the siRNA expression vector (pcPURU6i/Seq1 or pcPURU6i/Seq2). Various amounts of the siRNA expression vector DNA were used, as shown. (C) The mitochondrial morphology of the hFis1 knockdown cells (No. 16 clone) transfected with either the FLAG-mFis1 expression vector or mock vector was assessed. At 24 h after transfection with the mFis1 expression vector or mock vector, cisplatin (100 μM) was added, and after 17 h, the cells were observed. Cells (n > 250) were counted in three wells independently. The cells were classified as having normal mitochondria (Normal), having fragmented mitochondria (Frag. mito.), or being dead (Dead), as shown in Fig. 2. The percentages of each type of cell are shown.

inhibition of mitochondrial fission, due to the absence of Fis1p (*3*, *4*). Therefore, we attempted to establish a stable hFis1 knockdown transformant with the hFis1 siRNA expression vector (pcPURU6iCFP/Seq1) in mammalian cells. HeLa cells transfected with pcPURU6iCFP/Seq1 were selected with puromycin (1 μg/ml). After 2 weeks, we obtained puromycin-resistant cells, and selected several clones exhibiting high CFP fluorescence. The hFis1 mRNA in the selected clones was quantified using the TaqMan real-time PCR method (Fig. 1C). The mRNAs of clone Nos. 3, 7 and 16 were suppressed to approximately 60%, 20% and 10%, respectively, as compared with in the control HeLa cells, suggesting that the RNAi technique with the pcPURU6iCFP vector can establish stable gene-knockdown transformants with various suppression levels. In the hFis1 knockdown cells, however, we could hardly observe a significant change in the mitochondrial morphology (not shown).

It has been reported that treatment with apoptotic inducers, cisplatin, staurosporin and etoposide, caused mitochondrial fission, and the expression of a mutant Drp1 ($Drp1_{K38A}$) blocked the mitochondrial fission (*22*). To investigate the changes in mitochondrial morphology, cells were treated with 100 μM cisplatin (Sigma, St. Louis, MO, USA). We performed this experiment with the

transient RNAi system, however, no obvious difference in mitochondrial morphology between transient hFis1 RNAi cells and control cells was observed (not shown). Then, we focused on the stable hFis1 knockdown cells (clone No. 16), in which the expression of hFis1 was suppressed to ~10%, and observed the mitochondrial morphology (Fig. 2A). In control HeLa cells, 6 h after the addition of cisplatin, conversion from a tubular to a fragmented mitochondrial phenotype occurred in some of the cells (Fig. 2A, arrowhead F). After 24 h, the mitochondria were fragmented, giving a punctiform morphology in more cells (Fig. 2A, arrowhead F), and some dead cells were round and detached (Fig. 2A, arrowhead D). On the other hand, in the stable hFis1 knockdown cells (clone No. 16), the conversion from a tubular to a punctiform mitochondrial phenotype had not occurred in most cells by 6 h after the addition of cisplatin. After 24 h, there were more cells with a normal mitochondrial phenotype among the No. 16 cells, as compared to the control cells. Figure 2B shows the counts of cells classified as normal, with fragmented mitochondria, or dead, among the No. 16 cells and control cells. After 8 h and 24 h, there were differences between the clone No. 16 and control HeLa cells in terms of the percentages of normal and dead cells. There were more cells with a normal mitochondrial morphology among the clone No. 16 cells than among the control cells, and more cells with a fragmented mitochondrial morphology among the control cells than among the No. 16 cells. In addition, in the case of 0.5 μM staurosporin (Sigma) treatment, the morphological changes of mitochondria occurred earlier (after 2–4 h), as compared to cisplatin. A slight difference in mitochondrial morphology between control cells and the stable knockdown cells was observed (not shown). These results suggest that a deficiency in hFis1 retards mitochondrial fission, and cell death after reatment with cisplatin and staurosporin.

To confirm the results of the RNAi experiment, we attempted to rescue hFis1 by expressing its murine homologue, mFis1. The nucleotide sequences of hFis1 and mFis1 differ at several positions in the siRNA target sites (Fig. 3A). The hFis1 siRNAs acted specifically on hFis1, and only slightly suppressed the expression of mFis1 (Fig. 3B). To determine whether or not mFis1 could complement the function of hFis1 in the hFis1 knockdown cells, mFis1 was expressed in the clone No. 16 cells, and the mitochondrial morphology was observed after the addition of cisplatin. The number of mitochondria with a normal morphology decreased, and the numbers of cells with fragmented mitochondria and dead cells increased relatively (Fig. 3C), when mFis1 was expressed in the clone No. 16 cells. This suggests that mFis1 promotes mitochondrial fission and cell death in the hFis1 knockdown cells, and complements the function of hFis1. These results are consistent with the reports that hFis1 is involved in mitochondrial fission (*8*, *9*, *23*).

In the present study, we established the stable hFis1 knockdown cells with the siRNA expression vector, and showed that mFis1 complemented the hFis1 function in the hFis1 knockdown cells. Stable RNAi experiments and complementation assays with the ortholog gene are valuable techniques for the functional analysis of a gene. Mutational analyses of Fis1 can be performed by expressing mFis1 mutants in hFis1 knockdown cells. The hFis1 siRNA expression vectors described in this work may be useful for studying the molecular function of mammalian Fis1.

We thank Ms. W. Ohashi, Dr. T. Yamazaki and Dr. H. Hirota for the valuable discussions. We also thank Ms. K. Yajima and Ms. T. Nakayama for the manuscript preparation. This work was supported by the RIKEN Structural Genomics/Proteomics Initiative (RSGI), and the National Project on Protein Structural and Functional Analyses, Ministry of Education, Culture, Sports, Science and Technology of Japan.

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