Biochemical and Biophysical Research Communications xxx (2017) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel transmembrane protein defines the endoplasmic reticulum stress-induced cell death pathway

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ARTICLE INFO

Article history:
Received 22 February 2017
Accepted 7 March 2017
Available online xxx

Keywords:
RNAi
Mitochondrial membrane potential
Cell death
Endoplasmic reticulum stress

ABSTRACT

Mitochondrial membrane potential ($\Delta\Psi_m$) maintenance is physiologically critical in cells; its loss causes apoptotic signalling and cell death. Accumulating DNA mutations and unfolded proteins in stressed cells activate signalling pathways for cell death induction. Cancer cells often fail to die even in the presence of some death signalling proteins. Here, we report a short hairpin RNA (shRNA) with an artificial sequence, denoted Psi1 shRNA, which leads to $\Delta\Psi_m$ loss in HCT116 cells. The Psi1 shRNA target gene was shown to encode transmembrane protein 117 (TMEM117). *TMEM117* knockdown led to $\Delta\Psi_m$ loss, increased reactive oxygen species levels, up-regulation of an endoplasmic reticulum (ER) stress sensor C/EBP homologous protein and active caspase-3 expression, and cell growth impairment, altering homeostasis towards cell death. TMEM117 levels were down-regulated in response to the ER stressor thapsigargin and decreased when cells showed $\Delta\Psi_m$ loss. These results suggested that *TMEM117* RNAi allowed apoptotic cell death. Therefore, TMEM117 probably mediates the signalling of $\Delta\Psi_m$ loss in ER stress-mediated mitochondria-mediated cell death.

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1. Introduction

Gene knockdown technology increases the potential for functional annotation of genes and RNAi-based treatments of human diseases [1]. This technology has also enabled the development of a new approach that is suitable to improve research in the field of genetics. Novel gene functions have been successfully identified through large-scale genetic screening using RNAi libraries [2,3], demonstrating the potential of this strategy. New RNAi library construction methods using random oligonucleotides may facilitate RNAi screens [4].

Cells possess intrinsic mechanisms of mitochondria-mediated cell death to respond to stressful alteration of cell homeostasis [5]. When stress exceeds a critical threshold for damage, such as endoplasmic reticulum (ER) stress, nuclear DNA damage, and lysosome permeabilization, cell death signalling finally activates mitochondrial membrane permeabilization machinery followed by intrinsic proteolysis to regulate organismal homeostasis. However, moderate ER stress has a profound effect on cancer cell survival [6]. In tumour growth, exposure to hypoxic and low nutrient environments often induces impaired folding of proteins in the ER following the activation of the unfolded protein response (UPR) and the integrated stress response (ISR). UPR activates the defensive response of removing damaged organelles by increasing autophagy. ISR controls oxidative stress by increasing antioxidant synthesis. Thus, tumours acquire profound effects in cancer cell proliferation. Cancer cell viability is supported by the stabilisation of mitochondria, where anti-apoptotic BCL-2 proteins and glycolysis are upregulated [7]. It is postulated that tumour cells show phenotypes of chronic ER stress while normal cells do not harbour a chronically active ER stress defence system.

Destabilisation of mitochondria is effective for cancer cell treatment. Approaches include both the disruption of mechanisms protecting mitochondria and the enhancement of pro-apoptotic pathways [8]. Some compounds directly affect mitochondrial proteins for anti-cancer cell proliferation because each component of the mitochondrial permeability transition pore complex is dispensable to protect from the dissipation of mitochondrial membrane potential ($\Delta\Psi_m$) [9]. Gene knockdown technology has been effective for destabilising mitochondria. For example, *Bcl-2*

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http://dx.doi.org/10.1016/j.bbrc.2017.03.017
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Please cite this article in press as: T. Tamaki, et al., A novel transmembrane protein defines the endoplasmic reticulum stress-induced cell death pathway, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.03.017

knockdown led to the apoptotic cell death of pancreatic cancer cells [10]. Enhancement of pro-apoptotic pathways includes indirect induction of $\Delta\Psi_m$ loss. The ER chaperon system GPR78/BiP has been shown to inhibit the activation of pro-apoptotic ER proteins [11]. Conversely, destabilisation of GPR78/BiP led to apoptosis. Thus, cancer cell death could be initiated by a molecule that is responsible for cells surviving from intrinsic mechanisms of mitochondria-mediated cell death.

RNAi has potential for RNAi-based pharmaceuticals, in which cancer cell viability is affected by loss of target molecule function [12]. We have previously developed a web application to support the sequence analysis required for RNAi screening [13]. Compared to the use of conventional RNAi libraries, this system facilitates the use of an RNAi library that includes many non-natural sequences, which can be prepared inexpensively using random oligonucleotides. The diversity of siRNA sequences in the library would maximize the possibility of RNAi screening. Here, we demonstrate a reverse genetic approach for molecules that affect $\Delta\Psi_m$ using a cell-based assay. This report presents evidence to suggest that the shRNA target molecule is a novel transmembrane protein involved in the ER stress-mediated cell death pathway.

2. Materials and methods

2.1. Cell culture and transfection

HCT116 cells (Public Health England Culture Collection, UK) were grown in McCoy's 5A medium (Life Technologies, CA, USA) with 10% foetal bovine serum at 37 °C. For HeLa cells (a kind gift from Dr. A. Sato), RPMI-1640 medium (Sigma-Aldrich, MO, USA) was used. Electroporation was performed using 5×10^5 cells with 2 μg of plasmid by the Gene Pulser Xcell system (Bio-Rad, CA, USA). Lipofection was performed using 1×10^5 cells with siRNA by Screen*Fect* A (Wako, Japan).

2.2. ShRNA library and siRNA preparation

The shRNA library was prepared according to a previous report [4]. Plasmid pGE-1negative (Agilent Technologies, CA, USA) was used for a control shRNA. Psi1 siRNA (GGUGCGUCUCAUGGCUACUdTdT and AGUAGCCAUGAGACGCACCdTdT) were chemically synthesized (Hokkaido System Science, Japan). Control siRNA (CAGUCGCGUUUGCGACUGGdTdT and CCAGUCGCAAACGCGACUGdTdT) were used [14]. The siRNA sequence for *TMEM117* (GenBank RefSeq no. NM_032256) was designed using siDirect [15]. Synthetic siRNA was prepared for Tmem117psi1 (GGUGCUUCUAUGGCUACUUdTdT and AAGUAGCCAUAGAAGCACCdTdT), Tme m117_1 (GGUUGUACUUGUGAUUACAdTdT and UGUAAUCACAAGUACAACCdTdT) and Tmem117_2 (GAAUAUCGUAUUCACAUAAd TdT and UUAUGUGAAUACGAUAUUCdTdT).

2.3. Flow cytometry

For the $\Delta\Psi_m$ assay, cells were incubated with 200 nM JC-1 (Life Technologies) in PBS at 37 °C for 30 min. JC-1 fluorescence (530 and 610 nm) was analysed using FACSAria (Becton Dickinson, NJ, USA) at a 488-nm excitation. For the ROS assay, cells were incubated with 20 nM carboxy-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (carboxy-H_2DCFDA) (Life Technologies) in PBS for 60 min at 37 °C. DCF fluorescence (530 nm) was examined at a 488-nm excitation. Cells were incubated with 100 nM lipid peroxidation sensor BODIPY 581/591 C11 (Life Technologies) in culture medium for one day. BODIPY 581/591 C11 fluorescence (510 and 590 nm) was examined at a 488-nm excitation. For the caspase-3 assay, the NucView 488™ Caspase-3 assay kit for live cells (Biotium, CA, USA) was used. The reagents *m*-chlorophenylhydrazone (CCCP), gamma-glutamylcysteine synthetase inhibitor buthionine sulfoximine (BSO), thapsigargin, and α-tocopherol were purchased from Wako.

2.4. Sequence analysis

The pGE-1 vector sequencing primers CGTCGATTTTTGTGATGCTCGTCAG and GAAGCATTTATCAGGGTTATTGTCTCATG were used. Sequence analysis was performed using the SPICE web application [13].

2.5. Western blotting

Cells were dissolved with lysis buffer (62.5 mM Tris-HCl; pH 6.8, 2% SDS). Rabbit antibodies to TMEM117 (Abgent, CA, USA), caspase-3 (5A1E; CST, MA, USA), caspase-8 (18C8; CST), and phospho PERK (Poly6494; BioLegend, CA, USA) were used. Mouse antibodies to β-actin (2F3; Wako), CHOP (L63F7; CST) and XBP-1s (BioLegend) were used. Secondary antibodies to rabbit and mouse were detected using colorimetric or chemiluminescent reagents.

2.6. Cell viability assay

Cells grown in 96-well plates were incubated with WST-8 (Dojindo, Japan). WST-8 formazan absorbance was measured using a microplate reader (Bio-Rad, CA, USA) with a 450-nm filter.

2.7. Statistical analysis

An unpaired Welch's *t*-test was performed to calculate *P*-values in comparison with the corresponding control.

3. Results

3.1. High-throughput shRNA library screening

To perform a screening, 1.5×10^5 types of shRNA were tested as a pooled RNAi library. The sequence diversity was enormous compared to conventional RNAi libraries, which included 3×10^2–1×10^4 kinds of siRNA sequences. However, most of the shRNA sequences tested were non-natural to the human genome [13]. Therefore, the expectancy of knockdown by a shRNA in the RNAi library was presumed to be very low. Transfected HCT116 cells were cultured for two days and subsequently labelled with the fluorescent probe JC-1 for $\Delta\Psi_m$ measurement. Proper monitoring of $\Delta\Psi_m$ was demonstrated by a decrease in the JC-1 red/green fluorescence ratio in cells incubated with CCCP, a mitochondrial-uncoupling reagent (Fig. 1a). The $\Delta\Psi_m$-sensitive colour shift was CCCP dose-dependent (Fig. 1b). Thus, cells showing loss of $\Delta\Psi_m$ were sorted using fluorescence activated cell sorter (FACS) to recover the shRNA-encoding DNA for the second screen. Under our experimental conditions, the transfection efficiency calculated using a green fluorescent protein expression plasmid was 40–60%, indicating that half of the cells were effective for testing shRNA on $\Delta\Psi_m$.

Interestingly, cells transfected with the shRNA library showed a decrease in the JC-1 red/green fluorescence ratio (Fig. 1c). For example, 15.7% of cells exhibited $\Delta\Psi_m$ loss. In contrast, $\Delta\Psi_m$ loss was observed in only 9.1% of control cells. Untransfected cells showed $\Delta\Psi_m$ loss in 8.3% of cells. To recover the shRNA-encoding DNA, 68,000 cells were sorted from the 450,000 cells. Next, the pooled DNA was subjected to a second round of screening. Unexpectedly, the cell population showing loss of $\Delta\Psi_m$ did not increase robustly. For example, 17.1% of transfected cells showed decreased $\Delta\Psi_m$, while 15.4% and 6.9% of control and untransfected cells,

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