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His-87 ligand in mitoNEET is crucial for the transfer of iron sulfur clusters from mitochondria to cytosolic aconitase

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ABSTRACT

MitoNEET is the first identified iron sulfur protein that located in the mitochondrial outer membrane. We showed that knockdown of mitoNEET did not affect the iron sulfur protein expression in mitochondria and cytoplasm, but significantly reduced the cytosolic aconitase activity. The reduction of aconitase activity was rescued by transfection of wild type mitoNEET, but not by mitoNEET mutants H87C and H87S. Our results confirm the observation that mitoNEET is important in transferring the iron sulfur clusters to the cytosolic aconitase in living cells and the His-87 ligand in mitoNEET plays important role in this process.

1. Introduction

Iron-sulfur (Fe-S) cluster proteins play important roles in many essential processes, such as enzymatic catalysis, respiration, nitrogen fixation, DNA repair, ribosome biogenesis and iron homeostasis [1–3]. Fe-S cluster biogenesis disorder has been attributed to many human diseases, such as Friedreich's ataxia [4]. In mammalian cells, iron-sulfur clusters are assembled in mitochondria by the iron sulfur cluster (ISC) assembly machinery. ISC system assembles nascent iron sulfur clusters using iron and L-cysteine, and transfers them to the target proteins [5]. The ISC system is highly conservative from prokaryotes to eukaryotic organisms. Although it has been known that Fe-S clusters are mainly produced in the mitochondria, how these assembled iron-sulfur clusters are transported to target proteins in cytoplasm is not clear. So far, the only known components of the mitochondrial ISC export machinery are ATP-binding cassette (ABC) transporter system including ABC7 and MTABC3 [6–8].

Human mitoNEET (mNT) is nearly discovered as a target of type II diabetes drugs pioglitazone [9,10]. The protein is the first drug binding iron sulfur proteins which contains a unique CDGSH-type zinc finger domain [11]. MitoNEET is located to the outer mitochondrial membrane (OMM) via an amino-terminal anchor sequence tethered to the mitochondrial outer membrane. The CDGSH domain of mitoNEET is oriented toward cytoplasm [11]. Biochemical studies have shown that mNT contains a [2Fe-2S] cluster [12]. Protein crystallographic studies revealed that mNT is a homodimer with each monomer containing a [2Fe-2S] center. Distinct from the classic 4-Cys ferredoxin and 2-Cys, 2-His Rieske [2Fe-2S] clusters, mNT represents a novel hybrid [2Fe-2S] cluster [13]. The [2Fe-2S] cluster center in mNT is hosted by three cysteines (Cys-72, Cys-74 and Cys-83) and one histidine (His-87) in the unique CDGSH domain [14–17]. His-87 has been shown to mostly affect Fe-S cluster redox and stability properties [9,13,18,19], and low pH can increase its instability [20]. It shares the same unique [2Fe-2S] ligation and protein fold with the proteins of Miner1 and Miner2 [11].

Increasing evidence indicated that mNT may be involved in diverse biological processes, including autophagy, apoptosis, aging, diabetes, and reactive oxygen homeostasis [21–24]. It has been shown that mNT may transfer its [2Fe-2S] clusters to an apo-acceptor protein *in vitro* [25–27], and that NADPH inhibits the cluster transportation [28]. In addition, mNT can also transfers [2Fe-2S] clusters to Anamorsin, a protein required for cytosolic Fe-S cluster biogenesis [29]. Furthermore, a recent study [27] revealed

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that mNT may transfer the [2Fe-2S] clusters only to the iron regulatory protein 1 (IRP1)/cytosolic aconitase in living cells. However, in their studies, transient transfection was used to interfere mNT expression in cells, and only cytosolic iron-sulfur protein GPAT and aconitase were measured.

In this study, we have constructed stable MCF-7 and HepG2 cell lines in which mNT is depleted using shRNA. We confirm that mNT may mediate the transfer of iron sulfur clusters to the cytosolic aconitase. Furthermore, we find that mutations of the His-87 ligand in mNT block the transfer of iron-sulfur clusters to cytosolic aconitase in the cells, suggesting that the His-87 ligand in mNT is crucial for the iron sulfur cluster transfer to the cytosolic aconitase.

2. Materials and methods

2.1. Cell cultures

Human Epithelial Breast Michigan Cancer Foundation-7 (MCF-7) cells and liver hepatocellular carcinoma (HepG2) cells were cultured in DMEM (Sigma, USA) containing 4.5 g/liter glucose and 1 mM stable L-glutamine and 10% fetal bovine serum (FBS) (HyClone, USA) and supplemented with 1% penicillin-streptomycin. Cells were maintained in 100-mm Petri dishes (Corning Incorporated, USA) in an incubator (HERAcell 150i; Thermo Scientific) controlled at 37 °C and 5% CO_2.

2.2. Vector construction

PGPU6/GFP/Neo-CISD1-homo plasmid vector (shRNA) to knockdown mNT and the same plasmid with a scrambled sequence cassette (control-shRNA) in this study were synthesized by Shanghai GenePharma Co. Ltd.

PSinEGFPiresPURO-mitoNEET (pSin-mNT) plasmid vector construction was used to express mNT. Homo-mNT sequence was amplified with the forward primer (5′-AGCGAATTCGCCACCATGAGTCTGACTTCCAGT), and the reverse primer (5′-AGCGGATCCCAGGTAACTTCACGACAAGCTG). Through standard molecular cloning procedure, we made homo-mNT DNA fragment and linear pSinEGFPiresPURO vector connected together to complete construction of plasmid vector.

Several nucleotide mutations were introduced into the homo-mNT sequence to produce mNT mutants. PSin-mNT plasmid was mutated following the protocol offered in the Mut Express II Fast Mutagenesis Kit (Vazyme Biotech Co., Ltd, China). Mutations of mNT His-87 to Ser (H87S) plasmid was achieved through using the forward primer as 5′-TCCCATTCTGTGATGGGGCTTCCACAAAACATAACGA, while mutations of mNT His-87 to Cys (H87C) was gained through the forward primer as 5′-TCCCATTCTGTGATGGGGCTTGCACAAAACATAACGA.

2.3. Transfection

2×10^5 MCF-7 or HepG2 cells were plated in 6-well plates for 24 h before transfection. ShRNA or control-shRNA transfection was performed with 2 μg DNA though jetPRIME-DNA Transfection reagent (Polyplus, the French Republic) according to the manufacturer's instructions. Cells were then treated with 1000 μg/ml G418 solution after 2 days of the infection for 14 days. The single clone with G418 resistance was selected and expanded. The knockdown efficacy was evaluated with qRT-PCR and western blotting.

To overexpress mNT, cells were transfected with pSin-mNT plasmids or related mutation constructs [30]. Briefly, 2 μg DNA was added into 200 μl DMEM without FBS and sit for 5 min, PEI was added to the diluted DNA. After 20 min of incubation at room temperature, the mixture was added to cells. Expression of genes was detectable after 24–48 h.

2.4. Cell proliferation assay

Cell proliferation viability was assessed by a Cell Counting Kit-8 (CCK8) assay (Beyotime, China). MCF-7 or HepG2 cells (3×10^3 cells/well) were plated into 96-well plates. After cells growth for 6, 24, 48, 72 and 96 h, the CCK8 reagent was added to each well and cells were incubated for 2 h at 37 °C. The absorbance at 450 nm was measured.

2.5. Western blotting

Cells were lysed in RIPA buffer which contains 1% phenylmethylsulfonyl fluoride (PMSF). BCA protein assay kit (Beyotime, China) was used to determine protein concentrations. Equal amount of proteins (50 μg) were separated on SDS-PAGE and transferred to PVDF membranes. The primary antibodies were anti-CISD1 (Abcam, ab118027), anti-aconitase 1 (Abcam, ab126595), anti-aconitase 2 (Abcam, ab129069), anti-lipoic acid (Abcam, ab58724), anti-DPD (Abcam, ab134922), anti-VDAC (Abcam, ab15895) and anti-β-actin (Beyotime, China).

2.6. Real-time qRT-PCR

Total RNA was isolated from cells using TRIzol reagent (Beyotime, China) according to the manufacturer's instructions. Synthesis of cDNA was performed by using a PrimeScript™ RT reagent kit (TaKaRa Bio, Japan). To determine mNT RNA expression levels, quantitative real-time PCR was performed using the SYBR Premix Ex Taq II in a biorad CFX96 touch. The primer sequence for mNT was 5′-GATCGCAGCAGTTACCATTGC (forward) and 5′-GCATGTACTATCTTGGGGTTGTC (reverse).

2.7. Enzymatic activity

The aconitase activity was measured by using the Aconitase Activity Colorimetric Assay Kit (BioVision, USA) and following the instructions from the manufacturer. In eukaryotes, there are two forms of the enzyme, a mitochondrial aconitase (aconitase 2) and a cytosolic aconitase (aconitase 1). Briefly, cells homogenized in 0.1 ml cold assay buffer, centrifuged at 800 × g for 10 min at 4 °C, then centrifuged the supernatant at 20,000 × g for 15 min and collected the supernatant for aconitase 1 assay, dissolved the pellet into 0.1 ml cold assay buffer for aconitase 2 assay. In the assay, citrate was converted by aconitase into isocitrate, which was further processed resulting in a product that converted a nearly colorless probe into an intensely colored form ($\lambda = 450$ nm). Complex I activity was measured as previously described [31]. The specific activity of complex I was calculated by subtracting total complex I activity and rotenone-resistant activity. Mitochondria were prepared and purified from MCF-7 cells using Cell Mitochondria Isolation Kit (Beyotime, China) as the manufacturer's instructions. The specific activity of lipic acid synthetase [32] and DPD [33] were reflected by the protein expression of the function products. Densitometry analysis of protein levels were used as the activity.

2.8. Statistical analysis

All the experiments were performed at least three times. Data were analyzed using one-way analysis of variance. Differences between groups were considered significant if the p value was less than 0.05.

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