主論文の要約

Association of poly(*rC*)-binding protein-2 with sideroflexin-3 through TOM20 as an iron entry pathway to mitochondria

（TOM20 を介した poly(*rC*)-binding protein-2 と sideroflexin-3 の結合がミトコンドリアへの新たな鉄輸送経路である）

名古屋大学大学院医学系研究科　総合医学専攻
病理病態学講座　生体反応病理学分野
（指導：豊國 伸哉　教授）
米 丹阳

【Introduction】

Iron is an indispensable element for all life forms. Mitochondria, as the largest iron-utilizing organelles within cells, integrate iron into heme and Fe-S clusters, serving as crucial cofactors involved in various biological metabolisms. PCBP2, the primary intracellular iron chaperone, has been previously demonstrated in studies to receive iron from DMT1 and assist DMT1 in transporting divalent iron out of lysosomes. However, the subsequent processes through which iron is delivered to and utilized in mitochondria remain unclear. In this study, we primarily employed two leukemia cell lines, KU812 and K562, to screen for mitochondrial proteins that can specifically bind with PCBP2, further exploring the transport process of iron from cytosol to mitochondria.

【Materials and Methods】

Human chronic myelogenous leukemia cell lines, KU812 and K562 cells were cultured in RPMI1640 supplemented with 10% FBS. Human cervical adenocarcinoma cells, human embryonic kidney cells and MEFs were cultured in DMEM supplemented with 10% FBS.

1） Glutathione *S*-transferase pulldown assay

The E.coli BL21 transformed with pGEX-2T-PCBP2 plasmids were grown, pelleted, and resuspended as cell lysates. The PCBP2 proteins isolated by pGEX-2T-PCBP2 beads from the cell lysates were cultured with mitochondrial lysates of KU812 cells. After the beads were analyzed by silver staining, the PCBP2 protein complexes eluted from the beads then were analyzed by LC-MS.

2） Proteinase K digestion and Immunofluorescence assays

The isolated mitochondria from Hela cells were incubated in a 1, 2, and 5 μg/ml proteinase K solution on ice for 15 min and the digestion was stopped by the addition of 2 mM phenylmethylsulfonylfluoride (PMSF).

HeLa cells transfected with pMXs_FLAG-SFXN3 plasmids were seeded on the coverslip. The cells were co-stained with anti-flag antibody and anti-TOM20/COX4 antibody. For super-resolution microscopy, the secondary antibody STAR ORANGE/RED -conjugated goat anti-Mouse/rabbit IgG IgG were used.

3） Binding assay

・Proximity ligation assay (PLA)

HeLa cells cultured on coverslips were co-incubated with mixed antibodies and with PLA probes by using Duolink In *Situ*-Fluorescence according to the manufacture’s protocol.

・Co-immunoprecipitation assay

Mitochondria fractions from HeLa cells expressing FLAG or FLAG-SFXN3 lysed in co-IP buffer. Lysate was co-immunoprecipitated with anti-FLAG mAb-conjugated protein G beads and then analyzed by immunoblot.

4） Intracellular catalytic Fe(II) and Its Associated Proteins assay

・Catalytic Fe(II) and heme assay

K562 cells were transfected with human SFXN3/PCBP2 siRNAs and then incubated with FerroOrange, Mito-FerroGreen and H-FluNox-1 probes. The fluorescence intensity was measured by Cytometer. MEF and HeLa cells were treated with FAC and stained with Mito-FerroGreen. Images were randomly obtained on FV1000.

・Hemoglobin Assay

K562 cells were treated with a variety of siRNAs as described below. On the day 4, the cells were harvested and lysed. The supernatant was used to measure hemoglobin by using the Sigma hemoglobin assay kit.

・Transferrin (Tf)-uptake assay

MEF cells plated on glass coverslips were incubated with 20 μg/mL of Alexa568-conjugated-Transferrin. Following incubation, cells were fixed and mounted. Nine images were obtained by FV1000 microscope.

・Immunoblot analysis

The cells were lysed with RIPA buffer. The proteins were separated and analyzed by SDS-PAGE with the specific antibodies

・Mitochondrial functional assay

K562 cells transfected with siRNAs were performed by Seahorse XFe24 Analyzer following the manufacturer's protocol.

5） Ferroptosis assay

HeLa cells treated with RSL3 were analyzed by the trypan blue exclusion assay.

【Results】

1） Glutathione *S*-transferase pulldown assay

Silver staining results showed several bands indicating the mitochondrial proteins can bind PCBP2 proteins (**Figure 1A).** Mitochondrial proteins of 234 distinct identifies to interact with PCBP2 were identified with LC-MS.

2） Proteinase K digestion and Immunofluorescence assays

Partial digestion of mitochondria with proteinase K showed that PCBP2 was completely digested, TOM20 was partially digested and SFXN3 and COX4 were not digested at all (**Figure 1B**).

The localization of COX4 and SFXN3 merged efficiently, suggesting that SFXN3 is located at the inner membrane **(Figure 1C-E)**. Colocalization of TOM20 and SFXN3 confirmed that TOM20 is localized at the outer membrane and intermembrane space (**Figure 1FG**). PCBP2 and SFXN3 did not colocalize, suggesting that PCBP2 is outside of the mitochondria (**Figure 1H-J**)..

3） Binding assay

PLA analyses demonstrated that both the combinations of PCBP2/TOM20 and SFXN3/TOM20 are at least partially colocalized **(Figure 2A-D**). Co-immunoprecipitation assay showed a direct binding between FLAG-SFXN3 and TOM20 (**Figure 2E**).

4） Intracellular catalytic Fe(II) and Its Associated Proteins assay

Both siSFXN3 and siPCBP2 significantly decreased cytoplasmic and mitochondrial catalytic Fe(II) **(Figure 3A-C)**. The maximal respiration was impaired, suggesting the association of impaired mitochondrial respiration with decreased mitochondrial Fe(II) (**Figure 3D**).

Prior to iron loading, FBXL5 ([2Fe-2S] protein) expression was lower and IRP2/FTL was higher in *SFXN3* KO cells than in *MFRN1* KO cells, suggesting that mitochondrial iron entry pathways through SFXN3 and MFRN1 are distinct with more dependence on SFXN3. HO1 expression was lower prior to iron loading in *SFXN3* KO cells in comparison to *MFRN1* KO cells, suggesting the efficient blockade of mitochondrial iron entry especially for heme synthesis. HO1 expression was lower at 12 h in *MFRN1* KO cells after iron overload, suggesting a major role of SFXN3 in iron entry to mitochondria, considering that an increase in HO1 expression may result from cytosolic iron-induced stress **(Figure 4A)**. This was confirmed by the transferrin uptake study that transferrin uptake is significantly higher in *SFXN3* KO cells than in *MFRN1* KO cells under normal medium **(Figure 4BC)**. Both MFRN1 and SFXN3 contributed to the maintenance of mitochondrial Fe(II) **(Figure 4DE)**.

Prior to iron overload, *SFXN3* overexpression caused downregulation of FTL **(Figure 5AB)** in HeLa cells, suggesting the transfer of cytosolic Fe(II) to mitochondria. *SFXN3* overexpression under iron overload promoted Fe(II) accumulation in mitochondria with higher ferritin expression and less NCOA4 degradation **(Figure 5CD)**, indicating that SFXN3 is a distinct iron entry route to mitochondria.

5） Ferroptosis assay

SFXN3 overexpression sensitized Hela cells to ferroptosis induced by RSL3 **(Figure 5EF)**.

【Discussion】

We choose KU812 cells for screening and this global screening strategy sorted out TOM20, SFXN3, and TOM70 in the affinity-score sequence. Because TOM20 revealed 3-times higher score than TOM70, we decided to focus on TOM20 whereas TOM proteins often make a complex. Then we confirmed the mitochondrial localization of both the proteins with STED microscopy, which clearly showed that TOM20 is located at the outer membrane and SFXN3 at the inner membrane. Then we confirmed the mitochondrial localization of both the proteins with STED microscopy, which clearly showed that TOM20 is located at the outer membrane and SFXN3 at the inner membrane. We also observed a decrease in intracellular and mitochondrial Fe(II) by knockdown of SFXN3 and/or PCBP2 along with deterioration of mitochondrial function in K562 cells. Of note, PCBP2 did not bind to SFXN3 directly but

appears to bind indirectly with SFXN3 in the presence of TOM20. We currently understand the gap as follows (**Figure 6**). As knockout of *SFXN3* but not of *MFRN1* decreased FBXL5 and increased transferrin uptake, indicating that mitochondrial iron entry by SFXN3 is unique. Therefore, we believe iron entry pathway to mitochondria via SFXN3 is essential. What's more, *SFXN3* overexpression sensitized HeLa to ferroptosis, indicating that SFXN3 can be a novel target for cancer drug discovery to induce ferroptosis.

【Conclusions】

We for the first time discovered a novel pathway of iron entry into mitochondria from cytosol through PCBP2-TOM20- SFXN3 axis.