Research Article

Perturbing the Normal Level of SIDT1 Suppresses the Naked ASO Effect

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1. Introduction

Antisense oligonucleotide (ASO) therapeutics can be taken up by living cells without carrier molecules, a large part of incorporated ASOs are trapped in the endosomes and do not exert therapeutic effects. To improve their therapeutic effects, it would be important to elucidate the mechanism of cellular uptake and intracellular trafficking of ASOs. In this study, we investigated how SIDT1 affects cellular uptake and intracellular trafficking of ASOs. Fluorescence microscopic analysis suggested that most of naked ASOs are trafficked to the lysosomes via the endosomes. The data obtained from flow cytometry and fluorescence microscopy together showed that although the SIDT1 level barely affects the total cellular uptake of ASOs, it appears to affect the intracellular trafficking of ASOs. We also showed that SIDT1 exists mainly in the endoplasmic reticulum and that perturbing the normal level of SIDT1 suppresses the antisense effect of the naked ASO targeting miR-16.

2. Materials and Methods

2.1. Cell Culture. Mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco’s modified Eagle’s medium (Thermo...
2. Preparation of Oligonucleotides and Plasmids. The fully 2'-O-methylated, 5'-Alexa568-labeled, 3'-phosphorylated ASO A-15 (5'-AAAAAAGAAAAAAA-3') and the fully 2'-O-methylated, 5' and 3'-phosphorylated miR-16-targeting ASO antimir-16 (5'-CGCCAAUAUUACGUG CUGCUA-3') were chemically synthesized and subsequently purified by high-performance liquid chromatography as a custom service by Nippon Bioservice (Saitama, Japan).

The following primers used for quantitative RT-PCR (qPCR) were obtained from Fasmac or Hokkaido System Science (Hokkaido, Japan): β-actin forward primer, 5'-CTGTCGTACATCAAAGAGAA-3'; β-actin reverse primer, 5'-CAATAGTGTACCTTGCCGT-3'; miR-16 reverse transcription primer, 5'-GTCGTATCCAGCAGGGTGCGTACATCAAAGAGAA-3'; β-actin reverse primer, 5'-GGTCCGAGGTATTCGCACTGGATACGACCGCCAA-3'; miR-16 forward primer, 5'-CGGCCTAGCAGACGTCGTATCCAGCAGGGTGCGTGACATCAAAGAGAA-3'; miR-16 reverse primer, 5'-GGTCCGAGGTATTCGCACTGGATACGACCGCCAA-3'.

The siRNAs ssSIDT1#1 (the sense sequence: 5'-GCCUCUGUUCAGUU-3'), ssSIDT1#2 (the sense sequence: 5'-CUUAGGGGACCGAACUCCU-3'), and ssControl (the sense sequence: 5'-GCCACAGUGCUAUCAAUAAT-3'; miR-16 forward primer, 5'-GGTCCGAGGTATTCGCACTGGATACGACCGCCAA-3'; miR-16 reverse primer, 5'-GGTCCGAGGTATTCGCACTGGATACGACCGCCAA-3') were chemically synthesized by Nippon Bioservice (Saitama, Japan).

The following primers used for quantitative RT-PCR (qPCR) were obtained from Fasmac or Hokkaido System Science (Hokkaido, Japan): β-actin forward primer, 5'-CTGTCGTACATCAAAGAGAA-3'; β-actin reverse primer, 5'-CAATAGTGTACCTTGCCGT-3'; miR-16 reverse transcription primer, 5'-GTCGTATCCAGCAGGGTGCGTACATCAAAGAGAA-3'; β-actin reverse primer, 5'-GGTCCGAGGTATTCGCACTGGATACGACCGCCAA-3'; miR-16 forward primer, 5'-GGTCCGAGGTATTCGCACTGGATACGACCGCCAA-3'; miR-16 reverse primer, 5'-GGTCCGAGGTATTCGCACTGGATACGACCGCCAA-3'.

The mouse SIDT1 (NP_932151.2) expression plasmids pCI-neo-SIDT1 and pEGFP-N1-SIDT1 were prepared as described previously [10].

2.3. Transfection. Transfection experiments were conducted using Lipofectamine 3000 reagent (Thermo Fisher Scientific, L3000015) in accordance with manufacturer's protocol (forward transfection). Cells were cultured for 72 and 48 hours after transfection with siRNA and plasmid, respectively, and then used for various assays.

2.4. Quantitative RT-PCR. Total RNA was extracted from MEFs using TRIzol (Thermo Fisher Scientific, 15596018) or RNAiso Plus (Takara, 9108), and cDNA was synthesized using PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, RR047A), in accordance with manufacturer's protocols. β-Actin mRNA and miR-16 levels were quantified using a CFX96TM Real-Time System (Bio-Rad) or Thermal Cycler Dice Real Time System (Takara) with SYBR Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, RR0820A) [11].

2.5. Confocal Fluorescence Microscopy. MEFs prepared in 35 mm glass-bottomed dishes (IWAKI, 3910035 or ibidi, 81158) were cultured as above. After treating with various reagents for the indicated cells, the cells were washed with PBS and analyzed using a FLUOVIEW FV10i or FV1000 confocal microscope (Olympus). The fluorescence intensity was quantified with ImageJ (NIH). Each field of view was comprised of ~100–150 cells, and at least 3 fields of view per condition were quantified. ER-Tracker™ Red (Thermo Fisher Scientific, E34250) was used to stain the ER, and LysoTracker™ Green DND-26 (Thermo Fisher Scientific, L7526) and LysoTracker™ Red DND-99 (Thermo Fisher Scientific, L7528) were to stain the lysosomes.

2.6. Flow Cytometry. MEFs were cultured for 6 or 24 hours after adding 500 nM of naked A-15 to the medium. Then, the cells were washed with PBS and analyzed with a flow cytometer, SH800Z (SONY).

2.7. Western Blotting. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting were performed according to standard procedures [11]. Antibodies used were the polyclonal antibody against SIDT1 (Sigma Aldrich, SAB2501860) and the monoclonal antibody against β-actin (Sigma Aldrich, A4700).

2.8. Statistical Analysis. For comparison of two groups, the statistical significance of difference was evaluated by Student’s t-test.

3. Results

3.1. Naked ASOs Are Mainly Localized to the Lysosomes. First of all, we examined subcellular localization of naked ASOs incorporated into MEFs by confocal fluorescence microscopy. MEFs were cultured in the presence of a naked
ASO, A-15. We observed that A-15 was taken up by MEFs, and on average, ~95% of A-15 molecules incorporated appeared to be localized to the lysosomes (Figure 1). This result suggests that most of the naked ASOs are trafficked to the lysosomes via the endosomes.

### 3.2. The Effect of SIDT1 on Total Cellular Uptake of the Naked ASO

We examined whether overexpression of SIDT1 enhances total uptake of the naked A-15 into cells. MEFs, which were transfected with the SIDT1-expressing plasmid, were incubated with or without A-15 and analyzed by flow cytometry (Figures 2(a) and 2(b)). Although Alexa568 signals were not completely separated from autofluorescence, the distribution in the SIDT1-overexpressing cells was slightly shifted to the right compared with that in the control cells (Figure 2(b)).

We tested how the total uptake of the naked A-15 is affected in SIDT1-knockdown cells. We transfected MEFs with siRNAs against the SIDT1 mRNA and confirmed that the siRNAs reduce the SIDT1 protein level (Figure 2(c)). We cultured MEFs in the presence or absence of the naked A-15 for 6 hours and analyzed them by flow cytometry. The distributions in the cytometric histograms for the SIDT1 knockdown cells were slightly shifted to the left.
Figure 3: Microscopic analysis for the cellular uptake of naked Alexa568-labeled A-15. (a) MEFs were transfected with the empty plasmid (control) or pCI-neo-SIDT1. After the 48-hour culture, the cells were rinsed with PBS and further cultured in the absence (−) or presence (+) of 500 nM of naked A-15 for 24 hours. After washing the cells with PBS, they were analyzed by confocal microscopy. The Alexa568 fluorescence intensity in the SIDT1-overexpressing cells relative to that in the control cells is shown. Error bars indicate s.d. (n = 6). (b) MEFs were transfected with siControl, siSIDT1#1, or siSIDT1#2. After the 72-hour culture, the cells were rinsed with PBS and further cultured in the absence (−) or presence (+) of 500 nM of naked A-15 for 6 hours. After washing the cells with PBS, they were analyzed by confocal microscopy. The Alexa568 fluorescence intensity in the SIDT1 knockdown cells relative to that in the control cells is shown. Error bars indicate s.d. (n = 6). (c) MEFs were transfected with the empty plasmid (control) or pCI-neo-SIDT1. After the 48-hour culture, the cells were rinsed with PBS and further cultured in the presence of 500 nM of naked Alexa568-labeled A-15 for 24 hours. After washing the cells with PBS, fluorescence images from Alexa568 and LysoTracker Green were visualized using a confocal microscope. Colocalization rates were quantified with ImageJ. Error bars indicate s.d. (n = 6).
compared with that for the control cells (Figure 2(d)). These results indicated that the SIDT1 level barely affects the total cellular uptake of the naked A-15.

3.3. Microscopic Analysis for the Cellular Uptake of the Naked ASO. We next examined microscopically how the SIDT1 level affects cellular uptake of ASO. SIDT1-overexpressing or SIDT1 knockdown MEFs were incubated with the naked A-15 and analyzed by confocal fluorescence microscopy. The level of fluorescence intensity in the SIDT1-overexpressing cells was increased on average by ~80% compared with that in the control cells (Figure 3(a)). We also observed that the level of fluorescence intensity in the SIDT1 knockdown cells was decreased on average by ~50% compared with that in the control cells (Figure 3(b)).

We confirmed that on average, ~97% of the A-15 molecules were localized to the lysosomes in the SIDT1-overexpressing MEFs like in the control cells (Figures 1 and 3(c)). As discussed below, these results together with the cytometric data suggested that although the SIDT1 level barely affects the total cellular uptake of ASO, it appears to affect the intracellular trafficking of ASO.

3.4. SIDT1 Exists Mainly in the Endoplasmic Reticulum. We performed intracellular localization analysis using the C-terminal EGFP-tagged SIDT1. We transfected MEFs with its expression plasmid, cultured them for 24 hours, and located the SIDT1 by co-staining the lysosomes or the endoplasmic reticulum (ER) with a confocal fluorescence microscope. About 65% of the EGFP-tagged SIDT1 were localized to the ER, and ~10% existed in the lysosomes (Figure 4).

3.5. Perturbing the Normal Level of SIDT1 Dampens the Naked ASO Effect. Next, we examined how the SIDT1 level affects the effect of the ASO antimir-16 targeting miR-16. The SIDT1 knockdown MEFs were incubated with or without naked antimir-16, and the level of miR-16 was analyzed by qPCR. We observed that decreasing the SIDT1 level dampens the antisense effect of antimir-16 by ~25–67% (Figures 5(a) and 5(b)). The SIDT1-overexpressing cells were also examined for the antimir-16 effect, and its antisense effect was dampened again, albeit statistically insignificantly (Figure 5(c)). These results suggest that perturbing the normal level of SIDT1 suppresses the naked ASO effect.

3.6. The SIDT2 Level Hardly Affects the Total Cellular Uptake of ASOs. Previously, only from the microscopic analysis, we reported that SIDT2, a homolog of SIDT1, mediated the cellular uptake of A-15 [11]. Here, we cytometrically reexamined the effect of SIDT2 on the A-15 uptake. SIDT2-overexpressing MEFs were incubated with naked A-15 and analyzed by flow cytometry. The distributions in the cytometric histograms showed almost no difference between control and SIDT2-overexpressing cells (Figure 6). This result and our previous microscopic data together suggest that the SIDT2 level hardly affects the total cellular uptake of ASOs, whereas it appears to affect the intracellular trafficking of ASOs, in a similar fashion to SIDT1.
4. Discussion

4.1. Fluorescence Microscopy and Flow Cytometry Are Complementary. From the previous reports [1, 12, 13], we have learned that fluorescence microscopic analysis for the total cellular uptake of ASOs tends to underestimate the actual amount of incorporated ASOs compared with the flow cytometric analysis. This would be because the microscope has difficulty in detecting fluorescence signals from fluorophores dispersed throughout the cytosol and the nucleosol compared with those from fluorophores concentrated in the organelles. Cytometry and microscopy would be appropriate for analyzing the total cellular uptake of ASOs and its subcellular localization/trafficking, respectively. In this study, we performed both analyses for naked ASOs and concluded that the SIDT1 level hardly affects the total cellular uptake of A-15, but affects its subcellular distribution. The higher the SIDT1 level is, the more A-15 molecules would be concentrated in the lysosomes. This observation on SIDT1 urged us to reexamine SIDT2 for its role in ASO uptake, and the current data suggests that, contrary to the previous supposition, the SIDT2 level hardly affects the total cellular uptake of A-15, but appears to affect its subcellular distribution.

4.2. Suppression of the ASO Effect by Increasing the SIDT1 Level. The antisense effect of antimir-16 was suppressed by increasing the SIDT1 level. This may be attributed to an increased amount of lysosome-trapped antimir-16 (Figures 7(a) and 7(b)). The increase in the SIDT1 level in the ER and the lysosomes may stimulate the endosome
maturation to the lysosomes and dampen the endosomal escape of ASO through the interaction of the ER with the endosomes/lysosomes somehow via SIDT1 [14, 15].

4.3. Suppression of the ASO Effect in the SIDT1 Knockdown Cells. The antimir-16 effect was dampened in the SIDT1 knockdown MEFs. This observation cannot be explained

![Graph](image1)

**Figure 6:** The SIDT2 level hardly affects the total cellular uptake of naked A-15. MEFs were transfected with the empty plasmid (control) or pCI-neo-SIDT2 for 48 hours. Then, the cells were rinsed with PBS and cultured with or without 500 nM of naked A-15. After the 24-hour culture, the cells were washed with PBS and analyzed with a flow cytometer. In the left and middle panels, yellow and red histograms are from MEFs cultured in the absence and presence, respectively, of A-15. In the right panel, the red histograms in the left and middle panels are overlaid and shown in yellow and red, respectively.

![Diagram](image2)

**Figure 7:** Aberrant levels of SIDT1 suppress the naked ASO effect. SIDT1-overexpressing (a), normal (b), and SIDT1 knockdown (c) MEFs are shown. The numbers of ASO, SIDT1, and SIDT2 molecules reflect relative amounts among in those cells, and only the ASO molecules in the cytosol and the nucleosol are supposed to exert the antisense effect.
as above by simply considering the ASO amount in the lysosomes, since the level of lysosome-trapped ASO was decreased in the SIDT1 knockdown cells. It would be likely that a part of the antimir-16 molecules were trapped in another organelle, probably the endosomes, resulting in a decreased amount of free cytosolic antimir-16 that can target miR-16 (Figures 7(b) and 7(c)), although their existence in the endosomes was not examined. One of the SIDT1 splicing variants that exists in the endosomes has been shown to be able to export dsRNA to cytosol [16]. Thus, concomitant downregulation of this variant by the siRNAs may have decreased the level of endosomal-escaped antimir-16 and dampened the antisense effect.

4.4. Locations and Functions of SIDT1. Several findings by us and other groups on the location and the function of mouse and human SIDT1 are listed in Table 1. SIDT1 is reported to exist in the plasma membrane, the ER, the lysosomes, and the Golgi apparatus [12, 13, 16, 17]. There appears to be two forms (accession numbers NP_932151.2 and EDK98036.1) in mouse cells and only one form (accession numbers NP_060169.2) that corresponds to the mouse former form in human cells. Only the mouse latter form, which has an additional 8 amino acids in the middle of the first extramembrane loop, appears to exist in the endosomes [12]. With respect to the functions involved in nucleic acids, SIDT1 in the plasma membrane, the ER, and the endosomes appears to function for oligonucleotide uptake/transport [13, 17–19], endosome maturation, and endosomal escape [12], respectively. SIDT1 has been also reported to operate to take up and traffic cholesterol [16]. Apparent discrepancies among the reports would be in part due to the differences in cell types, nucleic acid types, SIDT1 forms, and/or experimental conditions.

4.5. Perspective. Although SIDT1 appears to play various cell physiological roles including cellular uptake and intracellular trafficking of oligonucleotides, its detailed functions remain to be elucidated. To elucidate the whole picture of the productive uptake of ASOs is much more challenging. New findings on its mechanism would help develop novel ASOs that can more efficiently exert the therapeutic effect.

Table 1: Locations and functions of SIDT1.

| Species | Claimed function                  | Location                        | Splicing variant | Reference |
|---------|-----------------------------------|---------------------------------|------------------|-----------|
| Mouse   | Endosome maturation               | ER, lysosome                    | NP_932151.2      | This study|
|         | Endosomal escape of poly (I:C)    | Endosome, lysosome              | EDK98036.1       | 12        |
|         | miRNA uptake                      | Plasma membrane                 | —                | 13        |
|         | sirNA uptake                      | Plasma membrane, ER, Golgi      | NP_060169.2      | 17        |
| Human   | Inter cellular transport of miRNA | Plasma membrane                 | NP_060169.2      | 18        |
|         | Cholesterol uptake and trafficking| Plasma membrane, ER             | —                | 16        |
|         | miRNA uptake                      | Plasma membrane                 | —                | 19        |

Abbreviations

ASO: Antisense oligonucleotide
AP2M1: Adaptor-related protein complex 2 subunit mu
COP II: Coat protein complex II
ER: Endoplasmic reticulum
dsRNA: Double-stranded RNA
ssRNA: Single-stranded RNA
SID-1: Systemic RNA interference-deficient 1
SIDT2: SID-1 transmembrane family member 2
SIDT1: SID-1 transmembrane family member 1
MEF: Mouse embryonic fibroblast
qPCR: Quantitative RT-PCR
antimir-16: miR-16-targeting ASO.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare no conflict of interest associated with this manuscript.

Authors’ Contributions

Masayuki Takahashi is responsible for the conceptualization, methodology, project administration, funding acquisition, investigation, and writing—original draft; Mineaki Seki for the methodology and investigation; Masayuki Nashimoto for the conceptualization, methodology, supervision, and writing—review and editing; and Tomohiro Kabuta for the conceptualization, methodology, and supervision.

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References

[1] K. Deprey, N. Batistatou, and J. A. Kritzer, "A critical analysis of methods used to investigate the cellular uptake and subcellular localization of RNA therapeutics," *Nucleic Acids Research*, vol. 48, no. 14, pp. 7623–7639, 2020.

[2] R. S. Geary, E. Wancewicz, J. Matson et al., "Effect of dose and plasma concentration on liver uptake and pharmacologic activity of a 2′-methoxyethyl modified chimeric antisense oligonucleotide targeting PTEN," *Biochemical Pharmacology*, vol. 78, no. 3, pp. 284–291, 2009.

[3] E. Koller, T. M. Vincent, A. Chappell, S. De, M. Manoharan, and C. F. Bennett, "Mechanisms of single-stranded phosphorothioate modified antisense oligonucleotide accumulation in hepatocytes," *Nucleic Acids Research*, vol. 39, no. 11, pp. 4795–4807, 2011.

[4] X. H. Liang, H. Sun, J. G. Nichols et al., "COPII vesicles can affect the activity of antisense oligonucleotides by facilitating the release of oligonucleotides from endocytic pathways," *Nucleic Acids Research*, vol. 46, no. 19, pp. 10225–10245, 2018.

[5] J. D. Shih and C. P. Hunter, "SID-1 is a dsRNA-selective dsRNA-gated channel," *RNA*, vol. 17, no. 6, pp. 1057–1065, 2011.

[6] G. Jialin, G. Xuefan, and Z. Huwen, "SID1 transmembrane family, member 2 (Sidt2): a novel lysosomal membrane protein," *Biochemical and Biophysical Research Communications*, vol. 402, no. 4, pp. 588–594, 2010.

[7] C. Wu, C. Orozco, J. Boyer et al., "BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources," *Genome Biology*, vol. 10, no. 11, p. R130, 2009.

[8] A. Chapel, S. Kieffer-Jaquinod, C. Sagne et al., "An extended proteome map of the lysosomal membrane reveals novel potential transporters," *Molecular & Cellular Proteomics*, vol. 12, no. 6, pp. 1572–1588, 2013.

[9] B. Schroder, C. Wrocklage, C. Pan et al., "Integral and associated lysosomal membrane proteins," *Traffic*, vol. 8, no. 12, pp. 1676–1686, 2007.

[10] S. Aizawa, Y. Fujiwara, V. R. Contu et al., "Lysosomal putative RNA transporter SIDT2 mediates direct uptake of RNA by lysosomes," *Autophagy*, vol. 12, no. 3, pp. 565–578, 2016.

[11] M. Takahashi, V. R. Contu, C. Kabuta et al., "SIDT2 mediates gymnosis, the uptake of naked single-stranded oligonucleotides into living cells," *RNA Biology*, vol. 14, no. 11, pp. 1534–1543, 2017.

[12] T. A. Nguyen, B. R. C. Smith, K. D. Elgass et al., "SIDT1 localizes to endolysosomes and mediates double-stranded RNA transport into the cytoplasm," *Journal of Immunology*, vol. 202, no. 12, pp. 3483–3492, 2019.

[13] Q. Chen, F. Zhang, L. Dong et al., "SIDT1-dependent absorption in the stomach mediates host uptake of dietary and orally administered microRNAs," *Cell Research*, vol. 31, no. 3, pp. 247–258, 2021.

[14] P. C. Trivedi, J. J. Bartlett, and T. Pulimilkunnal, "Lysosomal biology and function: modern view of cellular debris bin," *Cell*, vol. 9, no. 5, p. 1131, 2020.

[15] Y. Meng, S. Heybrock, D. Neculai, and P. Saftig, "Cholesterol handling in lysosomes and beyond," *Trends in Cell Biology*, vol. 30, no. 6, pp. 452–466, 2020.

[16] K. M. Mendez-Acevedo, V. J. Valdes, A. Asanov, and L. Vaca, "A novel family of mammalian transmembrane proteins involved in cholesterol transport," *Scientific Reports*, vol. 7, no. 1, p. 7450, 2017.

[17] M. S. Duxbury, S. W. Ashley, and E. E. Whang, "RNA interference: a mammalian SID-1 homologue enhances siRNA uptake and gene silencing efficacy in human cells," *Biochemical and Biophysical Research Communications*, vol. 331, no. 2, pp. 459–463, 2005.

[18] M. O. Elhassan, J. Christie, and M. S. Duxbury, "*Homo sapiens* systemic RNA interference-defective-1 transmembrane family member 1 (SIDT1) protein mediates contact-dependent small RNA transfer and microRNA-21-driven chemoresistance," *The Journal of Biological Chemistry*, vol. 287, no. 8, pp. 5267–5277, 2012.

[19] Z. Zhou, Y. Zhou, X. M. Jiang et al., "Decreased HD-MIR2911 absorption in human subjects with the SIDT1 polymorphism fails to inhibit SARS-CoV-2 replication," *Cell discovery*, vol. 6, no. 1, 2020.