Homo sapiens Systemic RNA Interference-defective-1 Transmembrane Family Member 1 (SIDT1) Protein Mediates Contact-dependent Small RNA Transfer and MicroRNA-21-driven Chemoresistance

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Molecular Bases of Disease: *Homo sapiens* Systemic RNA Interference-defective-1 Transmembrane Family Member 1 (SIDT1) Protein Mediates Contact-dependent Small RNA Transfer and MicroRNA-21-driven Chemoresistance

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**Homo sapiens** Systemic RNA Interference-defective-1 Transmembrane Family Member 1 (SIDT1) Protein Mediates Contact-dependent Small RNA Transfer and MicroRNA-21-driven Chemoresistance

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| Background: | The SID family is a highly conserved group of transmembrane channel-like proteins. |
| Results: | SIDT1 facilitates rapid contact-dependent intercellular small RNA transfer and mediates chemoresistance driven by microRNA-21 in human adenocarcinoma cells. |
| Conclusion: | By mediating small RNA transfer, SIDT1 contributes to cancer chemoresistance mechanisms. |
| Significance: | A better understanding of non-cell-autonomous RNA-based intercellular communication may yield novel anti-cancer therapeutics. |

Locally initiated RNA interference (RNAi) has the potential for spatial propagation, inducing posttranscriptional gene silencing in distant cells. In *Caenorhabditis elegans*, systemic RNAi requires a phylogenetically conserved transmembrane channel, SID-1. Here, we show that a human SID-1 orthologue, SIDT1, facilitates rapid, contact-dependent, bidirectional small RNA transfer between human cells, resulting in target-specific non-cell-autonomous RNAi. Intercellular small RNA transfer can be both homotypic and heterotypic. We show SIDT1-mediated intercellular transfer of microRNA-21 to be a driver of resistance to the nucleoside analog gemcitabine in human adenocarcinoma cells. Documentation of a SIDT1-dependent small RNA transfer mechanism and the associated phenotypic effects on chemoresistance in human cancer cells raises the possibility that conserved systemic RNAi pathways contribute to the acquisition of drug resistance. Mediators of non-cell-autonomous RNAi may be tractable targets for novel therapies aimed at improving the efficacy of current cytotoxic agents.

RNA interference (RNAi) is initiated locally by double-stranded RNA (dsRNA) but has the capacity to propagate systemically (sysRNAi),² inducing non-cell-autonomous posttranscriptional gene silencing in distant cells. Although best described as an antiviral mechanism in plants (1–3), sysRNAi also occurs in animals (4). In *Caenorhabditis elegans*, sysRNAi is dependent on a member of the systemic RNA interference-defective (SID) family of channels, SID-1 (5). SID-1 was initially identified following a screen of *C. elegans* mutants lacking the wild-type sysRNAi phenotype (5, 6). However, a range of organisms, including mice and humans, exhibit striking SID gene conservation (7–9).

SID channels have relative specificity for small RNA molecules (10, 11). Although organism-wide sysRNAi phenomena are not apparent in mammals, both SID-1 and its human orthologue SIDT1 (SID-1 transmembrane family member 1) have been shown to facilitate small interfering RNA (siRNA) uptake in human systems (12–14). The increased uptake of extracellular siRNA into human cells that SIDT1 mediates can result in highly specific posttranscriptional gene silencing (12, 13). We previously demonstrated that SIDT1 functions as a transmembrane channel for siRNA and localizes to the plasma membrane in human cells (12). This observation led us to hypothesize that SIDT1 might also play a role in the complex contact-dependent intercellular communication that is not only essential for normal histogenesis but, when dysregulated, also drives malignant progression and therapeutic resistance.

Small RNAs have a capacity to convey highly specific sequence-encoded signaling information (15). The microRNA (miRNA) system plays critical roles in the genesis, progression, and cytotoxic drug resistance of a range of human malignancies (16). Both the functional complexity of the “miRNome” and the diversity of miRNA targets suggest that regulation of gene function by miRNAs can be extremely subtle and adaptable (17). Within the tumor microenvironment, contact-dependent intercellular communication that is critical to the development of chemoresistance (18–20) is directly influenced by perturbation of the miRNome (21). This form of intercellular commu-
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| TABLE 1: Oligonucleotide sequences |
|-----------------------------------|
| **Oligonucleotide**               | **Sequence**                          |
| miR-21                            | 5'-UAGCUUAUAGACUGAUGGUGA-3'           |
| miR-21 reporter oligonucleotides   | 5'-GGAGTAAAGCCTGTAGCTAACAtAGTGCTAGTGTAGATAGGAACTGTTT-3' |
| miR-21-resistant single-base mismatch control oligonucleotides | 5'-AACTATGTTTACGACCTGTTGATAGCTGACTTACGCGAATG-3' |
| SIDT1-specific siRNA 1 target sequence | 5'-CUUCAGAUGCAUGAUUAU-3' |
| SIDT1-specific siRNA 2 target sequence | 5'-CUACUGGGAUAGAAGUUU-3' |
| SIDT1-specific siRNA 3 target sequence | 5'-GAGGAAUUGGGGACAAUUU-3' |
| Cy3/control siRNA                  | 5'-UAGGCACUAACACAUCAAUU-3' |

shRNA, Oligonucleotides, Plasmids, and Transfection and Electroporation—pCMV-AC, pCMV-AC-tGFP, pCMV6-AC-tGFP-SIDT1 (NM_017699), and pCMV6-Connexin-43/GJA1 (NM_000165) plasmids originated from Origene. Turbo green fluorescent protein (tGFP) was excised by NotI/Pmel digestion, fill-in, and ligation to derive pCMV-AC-SIDT1. Virus-incompetent pTRIPZ-based shRNA vectors (Open Biosystems) were used for microRNA expression. A miR-21 dual luciferase reporter construct was engineered using oligonucleotides designed to include Sgfl and Pmel sites (see Table 1 for oligonucleotide sequences). A miR-21-resistant single base mismatch insert served as a control. Oligonucleotides were directionally cloned into the corresponding sites of the ppsiCHECK2 vector (Promega), in accordance with the manufacturer’s protocol. Renilla luciferase substrate luminescence was normalized to that of firefly luciferase substrate to allow quantification of Renilla luciferase-miR-21 target sequence mRNA degradation by miR-21. The Dual-Glo luciferase assay system (Promega) was read using a VICTOR3-1420 multilabel reader (PerkinElmer Life Sciences). Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Stable cell lines were derived using G418 (0.3 mg/ml) or puromycin (5 μg/ml; both from Sigma) selection, as appropriate. All constructs were verified by sequencing. SIDT1-specific siRNA and mismatch control and Cy3-siRNA (supplemental material) were obtained from Dharmaco, Sigma, and Eurogentec. Lucifer yellow introduction was performed by electroporation in accordance with the manufacturer’s cell type-specific protocols using the Nucleofector™ system (Lonza). Gap junction intercellular communication was quantified by flow cytometric quantification of Lucifer yellow transfer as described previously (30). Recipient cells were labeled with far red membrane linker as described above.

Direct Coculture and Flow Cytometric Analysis of Intercellular siRNA Transfer—Cocultured labeled cell subpopulations were encouraged to conjugate by centrifugation at 500 rpm for 1 min and cocultured at 37 °C or 4 °C. Following coculture, cells were washed and resuspended in 5 ml EDTA/PBS and kept on ice. Multiparametric flow cytometry data were obtained from 10,000 single cell events with stringent doublet exclusion gating FACSARia™ II using FACSCdiva software (BD Biosciences). Flow cytometry data were analyzed using FlowJo V8 (TreeStar). Viable single cells were identified based on forward scatter and side scatter characteristics (width, height, and area).
### RESULTS

**Analysis of Stable HEK293-derived Transfectant Cell Lines**—HEK293 cells were transfected with pCMV-based plasmids encoding SIDT1 alone or in combination with tGFP and the aminoglycoside 3'-phosphotransferase neomycin resistance selection marker. Levels of SIDT1 expression were quantified by Western blotting in the following stable transfectants, which were derived using G418 selection: HEKSIDT1, which overexpresses SIDT1; HEKSIDT1/tGFP, which overexpresses SIDT1 and tGFP; and HEKSIDT1-tGFP, which overexpresses a fusion protein comprising SIDT1 and C terminus tGFP. Stable tGFP (HEKtGFP) and empty vector transfectants (HEKVector) served as controls. The electrophoretic migration of SIDT1 and SIDT1-tGFP fusion protein was consistent with predicted respective molecular masses of 94 and 120 kDa (Fig. 1a). The transfectant cell lines demonstrated no significant differences in respective rates of cellular proliferation or fraction of apoptotic cells (TUNEL) under standard culture conditions (supplemental Fig. S1).

**SIDT1 Mediates Contact-dependent Small RNA Transfer**—A direct coculture assay was used to investigate the role of SIDT1 in contact-dependent siRNA transfer. "Donor" (HEKSIDT1 or HEKtGFP) and "acceptor" (HEKSIDT1/tGFP or HEKtGFP) cell subpopulations were subjected to direct coculture, allowing cell-cell contact as schematized (Fig. 1b). Cy3-labeled 21-mer siRNA was introduced into donor cells by electroporation alone to eliminate potentially confounding effects of persisting transfection reagent. To mitigate against donor epifluorescence signal decay, as might occur due to Cy3-siRNA degradation, and to control for trogocytosis or cell fusion events, donor cells were co-labeled with a far red fluorescent plasma membrane linker. This linker is highly persistent (t1/2 = 12 days), is biochemically inert, does not affect cell viability or membrane function, and has an emission spectrum that is readily distinguishable from that of Cy3 (35–37). Far red label transfer to unlabeled cells was not observed during any direct or indirect coculture experiments. Potential artifact arising from contact-independent medium-borne siRNA transfer was minimized by postelectroporation washing and RNase treatment, which degrades extracellular RNA to nucleoside monophosphates (supplemental Fig. S2a) (38, 39). SIDT1-overexpressing (HEKSIDT1 and HEKSIDT1/tGFP) and control (HEKtGFP and HEKtGFP) donor and acceptor cells were cocultured at 1:1 ratios. Following coculture for 90 min, cell conjugates were disrupted to form single cell suspensions by EDTA treatment and agitation. Additional RNase treatment ensured removal of cell surface-associated Cy3-siRNA and free RNA that may have been released from lysed cells. Intercellular Cy3-siRNA transfer to acceptor cells was quantified by flow cytometry using stringent doublet exclusion. We quantified Cy3-siRNA-positive, tGFP-positive, far red-negative acceptor cells, defining a new subset of acceptor cells that had acquired Cy3-siRNA from donor cells (Fig. 1c, I–VI). Transfer of Cy3-siRNA between HEKSIDT1 donor and HEKSIDT1/tGFP acceptor cells was insensitive to RNase treatment and occurred rapidly (Fig. 1c, VII). In contrast, transfer of Cy3-siRNA between HEKtGFP donor and HEKtGFP acceptor cells was negligible (Fig. 1c, l). Direct coculture using HEKSIDT1 donor and HEKtGFP acceptor cells (Fig. 1c, II), as well as HEKtGFP donor and HEKSIDT1/tGFP acceptor cells (Fig. 1c, III), resulted in no significant difference in siRNA transfer (i.e. SIDT1 increased Cy3-siRNA acquisition regardless of whether it was overexpressed by donor or acceptor cells, indicating that facilitation of intercellular siRNA transfer by SIDT1 overexpression is bidirectional). Transfer of Cy3-siRNA from HEKSIDT1 to HEKSIDT1/tGFP was abolished by preincubation with polyclonal anti-SIDT1 (10 μg/ml; Fig. 1c, IV).

Although free Cy3-siRNA was eliminated from the culture medium, we were cognizant that nascent or RNase-resistant exosome-borne Cy3-siRNA arising from donor cells could also potentially contribute to the acceptor Cy3-siRNA signal, through contact-independent acquisition. To control for contact-independent Cy3-siRNA transfer, we performed indirect
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coculture of identical donor and acceptor cell groups, separated by permeable (0.4-μm diameter pore) Transwell insert membranes. In addition, acceptor cells were exposed to cell-free (0.4-μm filtered) donor conditioned medium. After either 90 min of indirect coculture or 90 min of exposure to donor conditioned medium, Cy3 epifluorescence was quantified by flow cytometry, as described above. No transfer of Cy3-siRNA to either HEKsidt1/tGFP or HEKtGFP acceptor cells was detected either following indirect coculture with donor cells or following exposure to donor conditioned medium (supplemental Fig. S2b). Contact-independent uptake of extracellular Cy3-siRNA therefore did not account for the Cy3 signal acquired by the acceptor cell subpopulation.

SIDT1 Mediates Contact-dependent Small RNA Transfer Is Gap Junction-independent—The contribution of GJIC to Cy3-siRNA acquisition was predicted to be small in HEK293 cells, given their low levels of connexin junction formation and GJIC (40–42). However, gap junction-mediated intercellular transfer of small RNAs has been reported in some cell types (43, 44). We therefore took steps to distinguish the contribution of SIDT1 to the acquisition of Cy3-siRNA transfer to be independent of GJIC. Indirect coculture and conditioned medium exposure (90 min in each case) did not result in significant acceptor Cy3-siRNA acquisition (supplemental Fig. S3b). VI, Cy3-siRNA transfer was abolished by preincubation with anti-SIDT1 antibody. VII, time course of Cy3-siRNA transfer from HEKsidt1 donor to HEKsidt1/gfp acceptor cells. Shown is a histogram representation of tGFP/Cy3-siRNA/FarRed acceptor cells at specified time points. VIII, data presented are typical of quadruplicate biological repeat experiments. In each sample, 10,000 single cell events were recorded.

FIGURE 1. SIDT1 facilitates rapid contact-dependent intercellular Cy3-siRNA transfer between donor and acceptor subpopulations. a, characterization of SIDT1 protein expression in HEK293-derived stable transfectant cell lines. Shown is representative Western blot analysis of SIDT1 expression in control HEK293 cells (HEKvector), tGFP control (HEKtGFP), SIDT1 (HEKsidt1), SIDT1 and tGFP (SIDT1/tGFP, clones 1 and 2), and SIDT1-tGFP fusion (HEKsidt1-tGFP) transfectants (clones 1–3) and β-actin loading control. Electrophoretic mobility of SIDT1 with C-terminal fusion tGFP protein (SIDT1-tGFP) corresponded to a predicted molecular mass of 120 kDa versus 94 kDa for SIDT1. b, schematic representation of siRNA transfer coculture experimental design. Donor subpopulations comprised stable HEKvector or HEKsidt1 transfectants. Acceptor subpopulations comprised HEKtGFP or HEKsidt1/tGFP transfectants. The donor subpopulation was tagged with far red membrane linker, and Cy3-siRNA was introduced by electroporation. Donor and acceptor subpopulations were directly cocultured for 90 min at 37 °C, and single cell suspensions were generated and analyzed by flow cytometry. Quantification of tGFP/Cy3-siRNA/FarRed cells allowed donor to acceptor Cy3-siRNA transfer to be measured. c, I, in the absence of SIDT1 overexpression, Cy3-siRNA transfer was negligible. II–IV, SIDT1 overexpression resulted in a marked increase in Cy3-siRNA transfer. SIDT1 overexpression in either donor or acceptor subpopulation was sufficient for this increase in Cy3-siRNA transfer to occur, indicating that SIDT1 facilitates bidirectional siRNA transfer. V, preincubation with AGA had no effect on Cy3-siRNA transfer efficiency, indicating the effects of SIDT1 overexpression on Cy3-siRNA transfer to be independent of GJIC. Indirect coculture and conditioned medium exposure (90 min in each case) did not result in significant acceptor Cy3-siRNA acquisition (supplemental Fig. S3b). VI, Cy3-siRNA transfer was abolished by preincubation with anti-SIDT1 antibody. VII, time course of Cy3-siRNA transfer from HEKsidt1 donor to HEKsidt1/tGFP acceptor cells. Shown is a histogram representation of tGFP/Cy3-siRNA/FarRed acceptor cells at specified time points. VIII, data presented are typical of quadruplicate biological repeat experiments. In each sample, 10,000 single cell events were recorded.
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FIGURE 2. A minority subpopulation increases non-autonomous miR-21 activity in adenocarcinoma cells. a, BxPC3miR21 (doxycycline-inducible miR-21) or irrelevant miRNA (BxPC3miRN/S) controls were directly cocultured with miR-21 reporter cells (BxPC3CkmiR21) at the indicated ratios (±1 μg/ml doxycycline). Renilla luciferase luminescence (levels decreased by miR-21) was normalized to firefly luciferase luminescence to allow quantitative comparison (relative luminescence units (RLU)). Doxycycline-induced BxPC3miR21 activation increased miR-21 activity in BxPC3CkmiR21 reporter cells. Coculture of BxPC3CkmiR21 with BxPC3miRN/S had no effect on normalized Renilla activity in BxPC3CkmiR21. Indirect coculture was insufficient to induce non-autonomous miR-21 activity. The first white column indicates BxPC3CkmiR21 in standard monoculture. *, p < 0.05 versus BxPC3miR21 + BxPC3CkmiR21, # no doxycycline by multifactorial analysis of variance, n = 4, b, representative Western blot analysis of SIDT1 expression in pancreatic adenocarcinoma (BxPC3, MIAPaCa2, and Capan2) and immortalized normal ductal epithelial cells (HPDE4), demonstrating differential expression of SIDT1. Densitometric quantification (means ± S.D. (error bars)) of SIDT1 signal, normalized to that of β-actin. Shown are mean values ± S.D. (n = 3); AU, arbitrary absorbance units.

BxPC3miR21 and BxPC3CkmiR21 cells were subjected to direct coculture at high total cell density (80–90% cell-cell contact) at BxPC3miR21/BxPC3CkmiR21 ratios ranging from 1:10 to 1:1000, in the presence or absence of 1 μg/ml doxycycline. Doxycycline-induced BxPC3miR21 activation (confirmed by RFP epifluorescence) led to a decrease in normalized Renilla luciferase activity of directly cocultured BxPC3CkmiR21 reporter cells, reflecting increased miR-21 activity within the BxPC3CkmiR21 reporter cell subpopulation. Direct coculture with BxPC3miRN/S had no effect on normalized Renilla activity of BxPC3CkmiR21 cells, confirming specificity for the miR-21 sequence. Significant decreases in normalized Renilla luciferase activity were observed even when BxPC3miR21 cells were present as a minority of 0.1% (Fig. 2a). This non-autonomous increase in miR-21 activity within the BxPC3CkmiR21 subpopulation was not observed when BxPC3CkmiR21 cells were subjected to indirect coculture with BxPC3miR21 cells (0.4-μm pore diameter; Transwell; Fig. 2a).

Non-autonomous miR-21 Activity Is SIDT1-dependent—Our previous observations led us to hypothesize that the SIDT1 channel could facilitate miR-21 transfer between contacting cells. SIDT1 channel protein expression varies considerably between pancreatic adenocarcinoma and immortalized normal ductal epithelial cell (HPDE4) lines. Among adenocarcinoma cells, BxPC3 expresses relatively high levels of SIDT1 (Fig. 2b). Increased miR-21 activity in BxPC3CkmiR21 induced by the doxycycline-activated minority BxPC3miR21 subpopulation was abrogated by pretreatment with siRNAs directed against different regions of the SIDT1 sequence, including the SIDT1 3'-untranslated region (3'-UTR), but not control siRNA (Fig. 3). The specificity of this siRNA-induced effect was further confirmed by a "rescue step" in which a SIDT1 expression construct lacking the SIDT1 3'-UTR target sequence or empty vector control

 tant HEK- and BxPC3-derived cell lines following pretreatment with the specific small molecule gap junction inhibitor 18-α-glycyrrhetinic acid (AGA (25 μM); supplemental Fig. S3) (46, 47). Coculture and flow cytometric analysis of Cy3-siRNA transfer was repeated following pretreatment with AGA prior to a 90-min coculture at 37 °C, as described above. Cy3-siRNA transfer was not significantly affected by AGA. Together, these observations indicate that SIDT1 overexpression does not facilitate GJIC; nor is the resulting increase in Cy3-siRNA transfer dependent on functional GJIC.

Induction of Non-autonomous miRNA Activity through Physical Contact with Subpopulation of miR-21-overexpressing BxPC3 Cells—miR-21 is a critical driver of resistance to the nucleoside analog gemcitabine in human pancreatic adenocarcinoma, an exemplar of a chemoresistant human malignancy (26, 27). The human pancreatic ductal adenocarcinoma cell line BxPC3 was selected as a model system because it exhibits relatively low levels of miR-21 activity under standard culture conditions (26).

We stably transplanted a subpopulation of BxPC3 cells with a non-viral pTRIPZ-derived miR-21 expression construct. These cells (BxPC3miR21) generate miR-21 in a doxycycline-inducible manner. Irrelevant miRNA-generating transfectants (BxPC3miRN/S), derived from RHS4346, served as controls. A miR-21 reporter cell line (BxPC3CkmiR21) was derived from BxPC3 by stable transfection of a psiCHECK-2-based miR-21 reporter construct. This dual luciferase reporter system allows Renilla luciferase activity, which decreases in the presence of miR-21, to be normalized to firefly luciferase, controlling for variations in reporter construct abundance. A single nucleotide mismatch reporter cell line (BxPC3CkmiR21 mm) was employed to confirm the specificity of the reporter system for miR-21 (supplemental Fig. S4).
was co-transfected with SIDT1–3′-UTR siRNA 1. SIDT1 over-expression “rescued” the abrogation of miR-21 induction in the BxPc3CKmiR21 reporter subpopulation that was observed with SIDT1–3′-UTR siRNA 1 treatment (Fig. 3).

Minority Subpopulation of miR-21-overexpressing Adenocarcinoma Cells Increases Global Cellular Chemoresistance to Gemcitabine—Given the ability of a minority subpopulation of BxPc3CKmiR21 cells to increase miR-21 activity in physically contacting BxPc3CKmiR21 cells, we examined the effect of a 1% subpopulation of BxPc3CKmiR21 on the gemcitabine IC50 for the total mixed cell population. Cells were exposed to clinically relevant concentrations of gemcitabine (48–52), and the IC50 was derived for the whole cell population. The gemcitabine IC50 was increased from 6 \times 10^{-8} to 3 \times 10^{-6} M by direct coculture of doxycycline-activated BxPc3CKmiR21 with BxPc3CKmiR21 cells (ratio 1:100). Importantly, firefly luciferase activity normalized to total cell number remained constant, indicating preservation of the BxPc3CKmiR21/BxPc3CKmiR21 ratio over the duration of the experiment. The gemcitabine IC50 approximated control levels when doxycycline was withdrawn and was unaffected by direct coculture of BxPc3CKmiRNA with BxPc3CKmiR21, confirming specificity of the effect to the miR-21 sequence (Fig. 4a). Transfer of miR-21 and IC50 were unaffected by 25 \mu M AGA (Fig. 4d and supplemental Fig. S3).

SIDT1 Deficiency Attenuates Chemoresistance Induced by Minority miR-21-overexpressing Subpopulation of Adenocarcinoma Cells—The increase in global cellular chemoresistance to gemcitabine that miR-21 induced was cell contact-dependent, the IC50 being unaffected when BxPc3CKmiR21 and BxPc3CKmiR21 cells were subjected to indirect coculture (BxPc3CKmiR21/BxPc3CKmiR21 = 1:100; Fig. 4b). The miR-21-induced increase in IC50 was significantly attenuated when cells were treated with SIDT1–3′-UTR siRNA 1 but not control siRNA. A SIDT1 re-expression rescue step, as described above, confirmed that the decrease in gemcitabine IC50 induced by SIDT1–3′-UTR siRNA 1 could be abolished by restoring levels of SIDT1 (Fig. 4c). Corresponding impairment of colony forming capacity and increased caspase 3 activities were observed when BxPc3CKmiR21 and BxPc3CKmiR21 were cocultured in the presence of gemcitabine following treatment with SIDT1–3′-UTR siRNA 1. Re-introduction of SIDT1 abolished these effects on colony formation and caspase activities (Fig. 5).

SIDT1 Contributes to Cell Adhesion-mediated Drug Resistance (CAM-DR)—Given the capacity for small RNA transfer observed between adenocarcinoma cells, we examined whether a similar process might contribute to the complex tumor-stromal cell interactions that can enhance chemoresistance in a range of human cancers (53). Human pancreatic stellate cells (hPSC) were isolated from surgical resection specimens, and their morphology and immunophenotype (54) were confirmed (Fig. 6a). SIDT1 was relatively highly expressed in all three hPSC lines tested (Fig. 6b). We repeated the flow cytometric Cy3-siRNA transfer assay, as before, and observed rapid Cy3-siRNA transfer between adenocarcinoma and hPSC cells that was sensitive to anti-SIDT1 antibody treatment (Fig. 6c). In order to quantify CAM-DR, we subjected BxPc3GFP cells to direct coculture with hPSC in the presence of 1 \mu M gemcitabine and measured GFP fluorescence, which correlates with adenocarcinoma cell number (27), normalizing GFP fluorescence to MTT-based total cell quantification. BxPc3GFP proliferation in the presence of clinically relevant gemcitabine levels was markedly increased by hPSC coculture. Furthermore, this effect was abrogated by treatment with 10 \mu g/ml anti-SIDT1 antibody but not control-matched immunoglobulin. Although this aspect of SIDT1 biology requires further investigation, this
result demonstrates that heterotypic intercellular small RNA transfer can occur and that SIDT1 contributes to this process.

In summary, SIDT1 facilitates rapid bidirectional, contact-dependent, RNase-insensitive transfer of Cy3-siRNA that is independent of GJIC. Contact-independent siRNA transfer was insignificant in comparison with SIDT1-mediated contact-dependent Cy3-siRNA acquisition. SIDT1-dependent Cy3-siRNA intercellular transfer is not restricted to adenocarcinoma cells and can occur between stromal cells, influencing CAM-DR.

DISCUSSION

Although organism-wide sysRNAi is not apparent in mammals, significant phylogenetic molecular conservation suggests that sysRNAi pathways may be relevant to human physiology and pathophysiology (7). The C. elegans orthologue of SIDT1, SID1, has recently been shown to be a dsRNA-gated channel capable of selective bidirectional intercellular dsRNA transfer (11). Our findings demonstrate first that SIDT1 facilitates contact-dependent small RNA transfer and non-cell-autonomous posttranscriptional regulation; second, they support the assertion that small RNA-based signaling represents a further level of adaptive capacity and complexity within the tumor microenvironment; and third, they indicate that disruption or exploitation of sysRNAi pathways may have therapeutic utility, particularly as a means of impairing the acquisition of resistance to cytotoxic agents.

Contact-dependent intercellular communication not only maintains normal tissue organization but can also drive neoplasia. However, to date, studies of SID family proteins have generally focused on these proteins as conduits for the contact-independent uptake of free small RNAs from the extracellular milieu (12, 13). In addition to characterizing the role of SIDT1 in the context of contact-dependent small RNA intercellular transfer, this study provides new evidence that contact-dependent non-cell-autonomous RNAi can shape therapeutic resistance in pancreatic cancer and that SIDT1 can act as a mediator of this form of RNA-based intercellular communication.

The miRNome is a highly complex and adaptable system, with each miRNA exerting pleiotropic effects. Recent studies illustrate miRNA biogenesis to be exquisitely sensitive to cell context, miRNA levels increasing in a contact-dependent manner (55). miR-21 was the focus of this study because it promotes chemoresistance to gemcitabine in human adenocarcinoma cells (22–25). The ability of a minority subpopulation of miR-21-overexpressing cells to influence global chemoresistance through a contact-mediated, SIDT1-dependent mechanism raises the intriguing possibility that subgroups of cells within a
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Our results demonstrate SIDT1-mediated siRNA transfer to be independent of GJIC. GJIC can increase the susceptibility of heterogeneous tumor population can influence resistance within the wider tumor microenvironment through contact-dependent non-cell-autonomous RNAi. Subpopulations of drug-tolerant tumor cells employ dynamic survival strategies that result in therapeutic resistance (34). Parallels can be drawn with microbial resistance, in which a small number of tolerant organisms can influence the “fitness” of the populations as a whole. Similarly, cells exposed to cytotoxic drug may, through small RNA-based communication, influence survival pathways within contacting cells over significant distances.

The rapid nature of SIDT1-mediated small RNA transfer is particularly striking and significantly precedes RNA transfer via contact-independent mechanisms, such as exosomal shuttling and free RNA transfer (38). Tumor cells that are capable of rapid adaptation are more likely to gain selective advantage in the presence of a toxic perturbation. Rapid small RNA transfer and resulting posttranscriptional gene regulation would allow more timely adaptive changes than those resulting from “classic” genetic mutation. This study supports the premise that small RNAs have the capacity to act as signaling intermediaries.

The absence of disseminating fluorescent protein expression to small RNAs have the capacity to act as signaling intermediaries. These observations are in keeping with the relative specificity for dsRNA exhibited by SID1 (11).

Our results demonstrate SIDT1-mediated siRNA transfer to be independent of GJIC. GJIC can increase the susceptibility of cancer cells to cytotoxic agents through connexin-mediated “bystander effects” in pancreatic cancer (56). BxPC3 cells in which GJIC is artificially increased exhibit bystander cytotoxicity when exposed to gemcitabine (56–58). Interestingly, connexin expression and GJIC are frequently decreased in cancer, re-expression of connexins commonly suppressing tumorigenicity (59). In contrast to the relatively non-selective nature of connexin-mediated communication, SIDT1 overexpression does not increase Lucifer yellow transfer. SIDT1 may therefore represent a means by which tumor cells can adapt to maintain small RNA–based intercellular communication, without experiencing greater bystander cytotoxicity that increased GJIC would incur.

Recent data from other groups indicate that non-cell-autonomous small RNA effects may be of general relevance to human cancer. Katakowski et al. (60) reported microvesicle-independent microRNA transfer between U87 human glioma cells. Although this study did not directly examine chemoresistance, the authors demonstrated non-autonomous microRNA effects in contacting cells. Zhao et al. (61) have demonstrated that SNB19 glioma cells can undergo intercellular transfer of PTEN-silencing siRNA in coculture. Interestingly, this effect was only observed in direct (contact-dependent) but not indirect (contact-independent) coculture. The PTEN tumor suppressor mRNA is a target of miR-21 and a number of other microRNAs. PTEN deficiency is clinically associated with chemoresistance in a range of human cancers. These data also suggest that non-autonomous gene silencing can result in molecular events that promote clinical chemoresistance.

We have demonstrated that SIDT1-dependent small RNA transfer can also operate between adenocarcinoma and stromal cells, in this case pancreatic stellate cells. The important chemoprotective effects of direct tumor-stromal cell contact are increasingly recognized (27). CAM-DR that develops in the tumor microenvironment may represent a future therapeutic opportunity. Follicular dendritic cells can protect B-cell lymphoma cells from drug-induced apoptosis through contact-mediated microRNA-dependent mechanisms (21, 62). miR-181a was found to be increased by direct contact between dendritic and lymphoma cells but not when cells were cultured under indirect coculture conditions, suggesting that free RNA, RNA bound to proteins (e.g. Argonaute or lipoproteins), or exosomal RNA transfer is less likely to mediate the effects on chemoresistance in this setting. This is in keeping with our observations. Although the possibility that the non-autonomous increase in miR-181a levels could result from intercellular RNA transfer was not explored, interestingly, SIDT1 is also relatively overexpressed in dendritic cells.

The clinical implications of SIDT1 expression levels are likely to be complex and will be influenced by the prevailing miRNome within the tumor. Preliminary studies of primary breast cancer mRNA and microRNA expression arrays are consistent with the hypothesis that SIDT1 may have a “permissive” role in some of the phenotypic effects of miR-21 overexpression. High levels of miR-21 expression result in poorer survival for patients with SIDT1-overexpressing tumors relative to those with low SIDT1 expression (supplemental Fig. S5). Further clinical studies are ongoing to address this question.
SIDT1 Mediates Contact-dependent Small RNA Transfer

In conclusion, SIDT1 mediates contact-dependent siRNA and miRNA transfer and non-cell-autonomous RNAi, which can enhance pancreatic adenocarcinoma chemoresistance to gemcitabine that is driven by miR-21. SIDT1-dependent small RNA transfer may also contribute to CAM-DR. Although systemic RNAi in humans appears not to be the organism-wide phenomenon observed in C. elegans, comparable processes may support adaptation to perturbations and selective pressures within the tumor microenvironment, such as those induced by cytotoxic therapy. Therapeutic exploitation of sysRNAi pathways may have utility in human cancer and warrants further experimental evaluation.

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