Iodide Transporter NIS Regulates Cancer Cell Motility and Invasiveness by Interacting with the Rho Guanine Nucleotide Exchange Factor LARG

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Abstract
A number of solute carrier (SLC) proteins are subject to changes in expression and activity during carcinogenesis. Whether these changes play a role in carcinogenesis is unclear, except for some nutrients and ion carriers whose deregulation ensures the necessary reprogramming of energy metabolism in cancer cells. In this study, we investigated the functional role in tumor progression of the sodium/iodide symporter (NIS; aka SLC5A5), which is upregulated and mislocalized in many human carcinomas. Notably, we found that NIS enhanced cell migration and invasion without ion transport being involved. These functions were mediated by NIS binding to leukemia-associated RhoA guanine exchange factor, a Rho guanine exchange factor that activates the small GTPase RhoA. Sequestering NIS in intracellular organelles or impairing its targeting to the cell surface (as observed in many cancers) led to a further increase in cell motility and invasiveness. In sum, our results established NIS as a carrier protein that interacts with a major cell signaling hub to facilitate tumor cell locomotion and invasion. Cancer Res; 72(21): 5505–15. ©2012 AACR.

Introduction
The members of solute-linked carrier family 5 (SLC5) mediate the secondary active transport of nutrients and ions. Modifications to their transport activities have been associated with carcinogenesis (1). The greater nutrient needs of cancer cells are met by an upregulation of some SLC5 carriers. Glucose uptake into cancer cells is facilitated by a physical interaction between the sodium/glucose transporter SLC5A1 (SGLT1) and the EGF receptor (EGFR; ref. 2). Downregulation of the sodium/monocarboxylate transporter SLC5A8 (SMCT) in colorectal cancer entails the reduced delivery of one of its substrates (butyrate) and would account for the proposed tumor suppressor activity of this carrier (3, 4). The sodium/iodide symporter SLC5A5 (NIS) is mostly known for taking up iodide into the thyroid and lactating breast (5–7). The regulation of NIS expression and function in these organs is conducted via hormone-dependent signaling pathways (8–10). NIS thereby plays a key role in vital hormone-dependent physiologic processes, such as the biosynthesis of thyroid hormones, and the feeding of fetuses and newborns with iodide. NIS-mediated iodide uptake also forms the basis for the diagnostic nuclear imaging and radioiodine therapy for thyroid diseases. Radioiodine therapy following NIS gene transfer was shown to be efficient in nonthyroid, especially, liver cancer models. NIS is expressed in many cancers, among them cancer of the thyroid, breast, and liver (7, 11–14). The role of iodide in thyroid carcinogenesis has been investigated but remains unclear (15). In breast cancers, NIS expression is induced by retinoic acid (16) and is regulated by the phosphoinositide-3 kinase and p38 mitogen-activated protein kinase (MAPK) signaling pathways (17). A transcription factor (npx 2.5) activates NIS specifically in lactating and cancer breast cells (18). In fact, in many cancers, NIS is localized intracellularly and, as a result, mediates no sodium-dependent transport. Here, we investigate mechanisms other than solute transport through which the deregulation of NIS may influence the behavior of cancer cells. We show that NIS interacts with the leukemia-associated RhoA guanine exchange factor (LARG) to activate RhoA, thereby enhancing cell migration and invasion. This new function is unaffected by solute transport and is most effectively conducted by the intracellular fraction of NIS.
Materials and Methods

Yeast two-hybrid analysis

Human NIS (amino acids 547–643, GenBank accession number GI: 164663746) was PCR-amplified and cloned into pB27 as a C-terminal fusion to LexA (N-LexA-SLC5A5-C). The yeast 2-hybrid screening was conducted by HybriGenics. The insert was sequenced and used as a bait to screen a random-placed placenta cDNA library constructed into pP6. A total of 76.9 million clones (more than 7-fold the complexity of the library) were screened using a mating approach with Y187 (Mat-alpha) and L40DGal4 (Mat-a) yeast strains. Hundred His-positive colonies were selected on a medium lacking tryptophan, leucine, and histidine, and supplemented with 50 mmol/L 3-aminotriazole to handle bait autoactivation. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5′ and 3′ junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (National Center for Biotechnology Information) using a fully automated procedure. A confidence score (PBS, for Predicted Biological Score) was attributed to each interaction (19).

Cell culture

Human hepatocarcinoma HuH2.2 (from P. Pinaud, Pasteur Institute, Paris, France; ref. 20) and HuH7 (21), cholangiocarcinoma CCLP1 (22; from P. Pinaud, Pasteur Institute, Paris, France), mammary carcinoma T47D [American Type Culture Collection (ATCC) HTB-133], and embryonic kidney HEK293 (ATCC CRL-1573) cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) GlutaMax. Human thyroid carcinoma TCPC-1 line (from C. Dupuy, Gustave Roussy Institute, Villejuif, France) was maintained in RPMI-1640. The culture media were supplemented with 10% fetal calf serum (FCS, PAA Laboratories, Les Mureaux, France), penicillin (200,000 U/mL), and streptomycin (100 mg/mL; P/S: Invitrogen). Breast cancer cells from ascites due to peritoneal carcinoma (kindly provided by Dr. R. Conforti, Department of Medical Oncology, Institut Gustave Roussy, Villejuif, France) were maintained in a mixture of F-12 and DMEM supplemented with 10% FCS, antibiotics, 1 mmol/L Na pyruvate, 1% insulin–transferrin–selenium supplement (Invitrogen), 1% nonessential amino acids (Invitrogen). Collection of ascites fluid was done with informed consent, and the protocol was approved by the Institutional Ethics Review Committee of the Institut Gustave Roussy. All the culture media except F-12 were approved by the Institutional Ethics Review Committee of the Medical Oncology, Institut Gustave Roussy, Villejuif, France). Breast cancer cells from ascites due to peritoneal carcinoma (kindly provided by Dr. R. Conforti, Department of Medical Oncology, Institut Gustave Roussy, Villejuif, France) were maintained in a mixture of F-12 and DMEM supplemented with 10% FCS, antibiotics, 1 mmol/L Na pyruvate, 1% insulin–transferrin–selenium supplement (Invitrogen), 1% nonessential amino acids (Invitrogen). Collection of ascites fluid was done with informed consent, and the protocol was approved by the Institutional Ethics Review Committee of the Institut Gustave Roussy. All the culture media except F-12 were approved by the Institutional Ethics Review Committee of the Medical Oncology, Institut Gustave Roussy, Villejuif, France).

Antibodies

The rabbit polyclonal anti-NIS antibody called serum R14 (13) has been purified by affinity chromatography after immobilizing a human NIS peptide (624–643) to the column. Mouse monoclonal antibody against the 625–643 C-terminal epitope of NIS (clone FP5A) was purchased from Lab Vision. Goat polyclonal antibody against an N-terminal extracellular domain of NIS (clone N15), rabbit polyclonal antibody against LARG (clone H70), and rabbit polyclonal antibody against actin were purchased from Santa Cruz Biotechnology, Vimentin, N-cadherin, and E-cadherin were purchased from Dako, Abcam, and BD Biosciences, respectively.

DNA constructs and siRNAs

PCR products of NIS Δter using pcDNA5-NIS (kindly provided by Dr. T. Pourcher, Nice Sophia Antipolis University, Nice, France) as a template were subcloned into pEGM-T (Promega) or pcDNA3 plasmids (Invitrogen). The following sequences were used: a forward primer carrying a HindIII restriction site 5′-AAGCTTCACTAGGGCCGGAGACCGGG-GA-3′ and a reverse primer carrying a BglII restriction site 5′-GGACTCCAGGAGTCTTTAGA-3′; a forward primer carrying a BglII restriction site 5′-TCGAAGATCTGCTGGAGTTCGAGCTCCG-TNLI (forward primer carrying a BamHI restriction site 5′-CTTAGCTTCGGGATCC-3′) and a reverse primer carrying a BamHI restriction site 5′-CTAGCTACTCCGGGCGTCTGTCGACC-3′) were used as the cDNA as a template was digested with BamHI and Xhol, purified on agarose gel and ligated into the BamHI–Xhol pcDNA3, yielding the pcDNA3/NISATL plasmid. The PCR products of NIS-Cter (forward 5′-CAGGATCCAGCTGCCTGACAGGCCCCACC-3′, reverse 5′-CGCCCTCGCTGCTGGTGACACC-3′) and NIS-CterΔTNL (forward 5′-CAGGATCCAGCTGCCTGACAGGCCCCACC-3′, reverse 5′-CGCCCTCGCTGCTGGTGACACC-3′) were cloned into pGEX-4T-1 vector (GE Healthcare) by BamHI and Xhol sites to produce GST-NISΔter and GST-NISAtTNL fusion proteins. All plasmid inserts were verified by DNA sequencing. The pCEFL-LARG and pGFP-LARG plasmids were kindly provided by Dr. J.S. Gutkind (NIH, Bethesda, MD; refs. 23, 24). For siRNA experiments, the following sequences were designed as NIS target: GCAAAUGAGUCAUGACUU (3′-untranslated region) and GGUCAAGGGUGAAGAU (open reading frame region). Pools of siRNAs directed against human NIS and nontargeting siRNAs (ON-TARGETplus SMART pools) were purchased from Dharmacon. The following sequences were previously reported as LARG target (25, 26): GAAACUGUCCGACAUCC and AAACCAAUAUAAGAGCU. Pools of siRNAs directed against human LARG and nontargeting siRNAs were purchased from Thermo Scientific.

Quantitative real-time RT-PCR

Total RNAs were isolated using TRIzol reagent (Invitrogen) and then reverse transcribed into cDNA using a RevertAid First Strand cDNA Synthesis Kit (Fermentas), amplified and quantified by detection of SYBR Green (Roche Diagnostics). They were quantified by spectrometry, and their quality was assessed by electrophoresis. Real-time PCR and melting curve analysis were done in a LightCycler 3.0 (Roche). We used pcDNA5-NIS and pCEFL-LARG vectors as calibration standards for quantification, and β-glucuronidase (GUS) as a housekeeping gene for normalization. The following primers

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were purchased from Invitrogen: NIS, forward; (5'-CTTCTGT-ACTCGGTCCTCAC-3') and reverse; (5'-TCCAGAATGTATA-GCGGGTCT-3'). LARG, forward (5'-GAGACCTCGTTATCGAT-CAGATT-3') and reverse (5'-ACGAGACACAGTGGATTAC-TTTCAG-3'); GUS, forward (5'-CTCATTTGGAATTTGCGCAGTT-3') and reverse (5'-CCAGGTAAGATCCCTCTTTA-3').

**Protein purification and pull-down assays**

The glutathione S-transferase (GST)–fusion proteins were expressed into *Escherichia coli* BL21 cells (Invitrogen) and purified using standard techniques. Cells were lysed with a buffer containing 20 mmol/L Tris, 150 mmol/L NaCl, 0.5 mmol/L EDTA, 0.2% NP40, and protease inhibitors, centrifuged at 13,000 × g for 15 minutes at 4°C, and the precleared lysates were mixed with GST-NIS Ac, GST-NISATNL, GST-PDZLARG, or GST-bound glutathione sepharose for 2 hours at 4°C. Bead-bound complexes were washed 5 times with a buffer containing 20 mmol/L HEPES, 150 mmol/L NaCl, 10% glycerol, 0.1% Triton X100, and protease inhibitors, eluted in Laemmli buffer, boiled for 5 minutes, and subjected to 8% SDS-PAGE.

**Pull-down assays for activated RhoA GTPase**

Rho activity was measured using a RhoA pull-down kit purchased from Cytoskeleton. Cells were homogenized in the cell lysis buffer containing a protease inhibitor cocktail. Lysates were cleared by centrifugation and the soluble fraction was incubated with 50 μg of GST-Rhotekin–Rho-binding domain, a fusion protein that specifically pulls down activated Rho, for 1 hour at 4°C. Beads were collected by centrifugation (for 1 minute at 3,000 × g and 4°C), washed, resuspended in Laemmli buffer, resolved by SDS-PAGE, and then analyzed by Western blot analysis using a monoclonal anti-RhoA antibody. Relative variations in normalized RhoA-GTP levels is defined as the ratio between the level of active GTP-bound forms of RhoA and the level of total RhoA in NIS-transfected cells, divided by the same ratio in control cells.

**DNA plasmid and siRNA transduction, immunoblot, immunoprecipitation**

Cells were transfected with 10 μg of DNA plasmid using the calcium phosphate precipitation method. At 48 hours post-transfection, total protein extracts were prepared with a buffer containing 30 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L CaCl2, 150 mmol/L MgCl2, 0.5% NP40, and protease inhibitors. For immunoprecipitation, cleared lysates were incubated with the antibodies indicated in the figures. Equal amounts of extracts were separated on 8% SDS-PAGE, transferred onto nitrocellulose (GE Healthcare) and processed for immunoblotting. Incubations were conducted with a buffer containing 20 mmol/L Tris–HCl pH 7.5, 137 mmol/L NaCl, 0.05% Tween 20, and 5% nonfat dry milk. The horseradish peroxidase–conjugated secondary antibodies were used at a dilution of 1:2,000 (GE Healthcare). The chemiluminescence ECL Plus reagent was purchased from GE Healthcare. For siRNA knockdown, cells were transfected with 50 nmol/L NIS siRNA using electroporation (Electroporator Micropulser, Bio-Rad Laboratories) or 50 nmol/L LARG siRNA using HiPerFect Reagent (Qiagen).

**Cell migration assays**

Migration of transfected cells was assayed using a 8-μm-pore-size Transwell Boyden chamber (Millipore). The cells were seeded in the upper insert of a chamber at 4 × 10⁶ cells per well. In the bottom well, 0.3 mL of conditioned medium was diluted in 0.7 mL of fresh cell medium. Conditioned medium was collected from Panc-1 pancreatic cancer cells (27) incubated in DMEM for 5 days and filtered through a 0.22-μm filter. Nonmigrated cells were removed from the top compartment with a cotton swab 7 (for pcDNA-NIS transfection) or 18 hours (for siRNA NIS transfection) after seeding. Cells attached to the bottom side of the filters were stained using the RAL-555 Kit (Reactifs RAL), photographed with a ×10 objective, and then counted at the rate of 10 fields per filter. Each condition was run in triplicate. Data are mean ± SEM from 3 independent experiments. For wound-healing assays, confluent-transfected cell monolayers were scraped with a fine sterile pipette tip, placed in a heated chamber (5% CO₂, 37°C) and monitored by time-lapse videomicroscopy (Axiolab 200M; Carl Zeiss). Migration inhibition tests were conducted after adding the Rho-associated coiled-coil kinase (ROCK) inhibitor Y-27632 at 30 μmol/L, as from 1 hour before scrapping. Images were acquired with 30-minute time intervals for 20 hours. The wound margin area was determined by image processing (binarization and thresholding) using the ImageJ software at each time point. The average wound-healing speeds were calculated as the slopes of the best-fit linear regression line through the data using the KaleidaGraph 4.0 software. Each condition was run in duplicate. Data are mean ± SEM from 3 independent experiments.

**Invasion assays**

Modified Boyden chambers with filter inserts (pore size of 8 μm) coated with Matrigel (Becton Dickinson) in 24-well dishes were used for invasion assays. Each clone was plated in duplicate for each experiment. Cells were deposited on filters 15 hours after transfection by pcDNA-NIS vectors, or 120 hours after siRNA electroporation. The migrated cells were stained using the RAL-555 Kit 15 (for pcDNA-NIS transfection) or 36 hours (for siRNA NIS transfection) after deposition, photographed with a ×20 objective, and then counted at the rate of 10 fields per filter. Each condition was run in triplicate. Data are mean ± SEM from 3 independent experiments.

**Immunofluorescence**

Cancer cells were fixed in cold methanol for 5 minutes at −20°C and then cold acetone for 1 minute at room temperature. Coverslips were incubated with a primary antibody diluted in PBS for 45 minutes at 37°C, followed by alexa–conjugated donkey antirabbit or anti-mouse secondary antibodies. Samples were analyzed using an inverted microscope (Axio Imager.M1 Zeiss). Sequential images were taken at 0.4 μm intervals using a 510 LSM confocal microscope (Zeiss).
Immunohistochemistry

Formalin-fixed paraffin-embedded 5-µm-thick sections from 15 liver metastasis from human breast, pancreas, colorectal, and biliary cancers were incubated with FP5A monoclonal NIS antibody (Labvision). Incubation with primary antibody was followed by incubation with peroxidase-conjugated mouse antibody.

In vitro pertechnetate uptake

Technetium (99m-Tc) pertechnetate is a short half-life radioactive substrate of NIS commonly used in nuclear imaging. Pertechnetate uptake assays were conducted 36 hours after transfection as previously described (28, 29). The radioactive B-Hanks balanced salt solution (Life Technologies) contained 1 µCi (37 kBq) of (99m-Tc) pertechnetate and 10 µmol/L of NaI without or with 30 µmol/L NaClO₄ per well.

Results

NIS is expressed in liver metastases and invading single cells

A first clue to the role of NIS in tumor cell locomotion was provided by the observation of pronounced NIS expression in secondary tumors as well as individual metastatic cancer cells. NIS expression was studied in liver metastases from 15 different human carcinomas by immunohistochemistry. The laboratory made R14 anti-NIS antibody revealed NIS at the basolateral plasma membrane of normal human thyroid follicles (Fig. 1A), as reported (13). NIS was strongly expressed in 12 of 15 liver metastases, displaying an intracellular localization in 5, and a mixed intracellular/plasma membrane localization in 7 tumors, without this localization being correlated with the site of the primary cancer (Fig. 1B).

The efficacy of the R14 anti-NIS antibody for the detection of endogenous NIS in cancer cell models was assessed by RNA interference knockdown in T47D breast cancer cells. NIS mRNA expression vanished 96 hours after siRNA NIS transfection, validating the designed siRNA target sequences. R14 yielded a plasma membrane signal due to an endogenous NIS in cells transfected with siRNA scramble, and no signal 120 hours posttransfection in most of the siRNA NIS-transfected cells (Fig. 1C). This signal was not observed with the commercial anti-NIS antibodies FP5A and N15 (data not shown). Detection of NIS by immunofluorescence was conducted in ascites-derived cancer cells from 5 patients presenting with metastatic breast cancer using R14 anti-NIS antibody. NIS was expressed in 74% of 350 counted cancer cells. The subcellular localization was exclusively intracellular (30%) or mixed intracellular/plasma membrane (44%) with a clear concentration at leading edges in a quarter of the latter (Fig. 1D).

NIS interacts with LARG

NIS, such as all the solute carriers (SLC), is a multiple-membrane-spanning protein with numerous segments exposed at the extra- and intracellular faces of the plasma membrane, endowing it with a potential for molecular interactions. The current secondary-structure model of NIS displays 13 transmembrane segments, an extracellular NH₂-terminus and a cytosolic COOH-terminus, which contains a putative density-95/discs large/zona occludens-1 (PDZ) class 1 target motif (T/S-X-V/L) capable of binding proteins through PDZ protein–protein interaction (30). We searched for NIS protein partners using the yeast two-hybrid screening of a highly complex placenta cDNA library, with the COOH-terminus of human NIS (Cter-NIS; residues 547–643) as bait. One candidate protein partner with a PBS A (very high confidence in the interaction) and 6 different picked-up fragments was found, namely, the LARG. Two other presumably true-positive (score B) partners were identified and are currently under study. The number of false-positive (score E) proteins was 5. The LARG Dbl homology domain serves as a guanine exchange factor (GEF) for RhoA but not for Rac1 or Cdc42 (31, 32). LARG also contains an N-terminal postsynaptic (PDZ) domain that has been shown to localize LARG to the plasma membrane via interactions with the histamine-H1 receptor, the insulin-like growth factor-1 (IGF-1) receptor, the semaphorin 4D/plexin-B1 receptor, the Unc5B netrin receptor, the hyaluronan receptor, or the ABCA1 transporter (33–40).

We studied LARG expression by immunohistochemistry in primary and metastatic liver cancer tissues. Like NIS (15), LARG was expressed in most of the intrahepatic cholangiocarcinomas (10/12) and liver metastases (9/11) examined (Fig. 2A). Moreover, the cellular distributions of NIS and LARG in tumors coincided, supporting the view that a NIS–LARG interaction might be effective in mammalian cells. We studied NIS–LARG binding in a set of human nonthyroid cell lines, which we showed to endogenously express both NIS and LARG. This set included cell lines from breast cancer (T47D), hepatocellular carcinoma (HuH2.2, HuH7), and cholangiocarcinoma (CCLP1; Fig. 2B). The TPC-1 thyroid papillary cancer cell line, which does not express NIS (41, 42), was used as a negative control. At the protein level, NIS was detected using the affinity-purified R14 anti-NIS polyclonal antibody (13). Immunoblots from Hek293 cells transfected with NIS cDNA displayed a pattern composed of 4 main bands centered about 80, 110, 200, and above 250 kDa, respectively, reflecting the multiple glycosylation and oligomeric forms of NIS. Some of the same bands were found in the immunoblots of nontransfected CCLP1, HuH7, HuH2.2, and T47D cell lines (Fig. 2C, input lanes). We then tested the NIS–LARG interaction by immunoprecipitation and pull-down assays. The NIS band pattern was detected in immunoprecipitates of LARG from whole-cell lysates of all 5 cell lines (Fig. 2C, + lanes). Conversely, LARG was immunodetected after NIS was immunoprecipitated using anti-Nter (N15) or anti-Cter (R14) antibodies (Fig. 2D, + lanes).

Thus, either endogenous or transiently overexpressed NIS interacts with LARG. The GST Cter-NIS fusion protein bound to endogenous LARG in Hek293 cells (+ lane), whereas Cter-NIS devoid of the PDZ-binding TNL motif (NIS ΔTNL) did not (− lane; Fig. 2E, top). Consistently, a GST-LARG PDZ fusion protein bound to full-length wild-type (WT) NIS but not to NIS ΔTNL (Fig. 2E, bottom). These observations establish that the PDZ-binding motif contained within the COOH-terminus of NIS interacts with the PDZ domain–containing LARG. We studied NIS and LARG subcellular localization by confocal microscopy in Hek293 cells stably
expressing a WT NIS and transiently transfected with a GFP-LARG vector. NIS was localized in the plasma membrane region (as illustrated by the 2 distinct peaks appearing in the intensity profiles), whereas LARG distribution covered both the cytoplasm and the plasma membrane region (Fig. 2F). This establishes that NIS and LARG lay in very close spatial positions allowing interaction between a submembrane LARG and a transmembrane NIS carrying a cytosolic C-terminus.

Overexpression of NIS enhances cell migration and invasion through RhoA activation

NIS may stimulate RhoA activation through its direct interaction with LARG, and thereby regulate RhoA-dependent processes, such as cell growth, differentiation, polarization, and motility (43). To investigate the effect of the interaction between NIS and LARG, we conducted cell migration and invasion assays in Hek293 cells transfected with cDNAs encoding full-length NIS (NISwt), NISΔN1ΔL, NISΔCter, or W255A.
Figure 2. Physical interaction between NIS and LARG. A, anti-NIS and -LARG immunohistochemistry in representative liver samples from patients with cholangiocarcinoma (ICC) and liver metastasis from breast cancer. Like NIS, LARG is expressed in the tumor biliary ducts of ICC. Note that the diffuse intracellular distribution of LARG sometimes exhibits reinforcement at the plasma membrane. Scale bar: 500 μm. Inset, scale bar: 50 μm. Control, scale bar, 50 μm. IgG1, mouse isotype control antibody. IgG, rabbit polyclonal control serum. B, real-time PCR for LARG mRNA and NIS mRNA in breast (T47D), liver (HuH2.2, HuH7, CCLP1), and thyroid (TPC-1) cells. Data are mean values ± SEM over 3 independent experiments. C and D, coimmunoprecipitation (IP) LARG-NIS using lysates prepared from Hek293 transfected with full-length NIS (Hek293NIS) and nontransfected CCLP1, HuH2.2, HuH7, and T47D cells. Input, 20 μg of total proteins from Hek293NIS and 50 μg of total proteins from nontransfected cell lines. The exposure time was of 3 minutes for input lanes and 30 seconds for IP lanes. C, anti-NIS immunoblots from lysates immunoprecipitated with anti-LARG (+) or control IgG (-). Asterisks, main forms of immunodetected NIS. D, anti-LARG immunoblots from lysates immunoprecipitated with anti-NIS (+) or control IgG (-). The antibodies used for NIS immunoprecipitation (IP NIS) were N15 (α-Nter) or R14 (α-Cter). E, top, anti-LARG immunoblot after pull-down in nontransfected Hek293 cells using a GST-COOH NIS fusion protein (Cter-NIS) with (+) or without (−) the PDZ-binding motif (TNI). The complete Cter-NIS domain interacted with LARG, whereas no binding was detected with Cter-NIS deleted of TNI or with empty GST (Ctrl). Bottom, anti-NIS immunoblot after pull-down in Hek293 cells transfected with empty vector (Ctrl), full-length NIS (WT) or NIS devoid of TNI (ΔTNI) using empty GST (−) or GST-PDZ LARG fusion protein (+). The PDZ domain of LARG binds to full-length NIS, but not to ΔTNI. F, confocal microscopy of Hek293 cells expressing NIS and LARG. Right, a NIS-LARG cotransfected cell. Scale bar, 10 μm. Left, fluorescence intensity profiles across the cell.
Figure 3. NIS enhances cell migration and invasion by activating RhoA. Hek293 cells were transfected with empty vector (Ctrl), full-length NIS (WT), NIS deleted of the COOH-terminus (ΔCter), NIS devoid of the TNL motif (ΔTNL), a transport-defective NIS mutant (W255A), and a targeting-defective NIS mutant (G543E). Data from Boyden chamber experiments were averaged over 10 random fields. Bar charts display averages ± SEM of data from 3 or more independent experiments. Normalized data are ratios, with data from control cells serving as reference. A, anti-NIS immunofluorescence in transfected cells. Scale bar, 100 μm. Bar chart, technetium (99mTc) uptake 36 hours posttransfection. Gray bars, uptake after addition of sodium perchlorate. B, wound-healing assays. Phase contrast movie snapshots 0 and 20 hours after wound infliction. Scale bar, 1 mm. Bar chart, wound-healing speeds in the presence (+) or absence (−) of Rho kinase inhibitor Y-27632. P < 0.01 for WT versus Ctrl. C, BrdUrd incorporation monitored by optical density. Each experiment was done in quintuplicate. D and E, Boyden chamber assays. Micrographs, representative views of stained filters. Scale bar, 100 μm. Bar charts, normalized number of migrated cells. P < 0.01 for WT versus Ctrl; P < 0.001 for G543E versus WT. F, left, anti-NIS immunoblot. Right, anti-NIS immunofluorescence. Scale bar, 100 μm. G, Matrigel-coated Boyden chamber assays. Bar chart, normalized number of invading cells. Micrographs, representative fields for the counting of stained cells. P < 0.001 for WT versus Ctrl; P < 0.001 for G543E versus WT. Scale bar, 100 μm. H and I, bar charts, normalized ratio of active to total RhoA-GTP levels. H, means of 6 independent experiments. P < 0.01. I, means of 3 independent experiments. P < 0.05. Autoradiography films: representative anti-RhoaGTP immunoblot after GST–Rhotekin pull-down. J, immunoblots for EMT markers E-cadherin, N-cadherin, and vimentin. K, confocal microscopy for NIS and the EMT marker vimentin in cells transfected with WT (Hek NIS) or empty vector (Hek Ctrl). Similar upregulation and polymerization of vimentin were observed in G543E-transfected cells. Scale bar, 50 μm. *, P < 0.05; **, P < 0.01; ***, P < 0.005.
transport-defective NIS mutant (44). Detection of NIS by immunofluorescence showed that NISwt, NISΔTNL, and W255A were mostly localized at the plasma membrane, and NISΔCter in the cytoplasm, of the transfected cells (Fig. 3A). Technetium (99mTc) uptake measurements showed that the characteristic anion transport activity of NISwt was preserved in NISΔTNL, but almost absent in NISΔCter and, naturally, in W255A NIS (Fig. 3A). No NIS activity was observed in the presence of perchlorate, a competitive inhibitor of NIS. NISwt increased the wound-healing speed 2-fold, whereas NISΔTNL or NISΔCter had no significant effect, as compared with empty vector (Fig. 3B and Supplementary Videos S1–S4). Addition of the ROCK inhibitor Y-27632 suppressed the increase in wound-healing speed in NISwt-transfected cells but had no effect in control cells confirming that the RhoA-ROCK pathway is involved in the enhancement of cell migration by NIS (Fig. 3B). The acceleration of migration induced by NIS was not due to a cell proliferation peak, as shown by the lack of an increase in BrdUrd incorporation in cells overexpressing NIS (Fig. 3C). In Boyden chamber assays, 3 times as many cells expressing NISwt migrated as cells expressing no NIS, NISΔCter, or NISΔTNL. Interestingly, as many cells expressing W255A NIS migrated as cells expressing NISwt, indicating that the effect of NIS on cell migration is independent of its transport activity (Fig. 3D). Consistent with these data, the presence or absence of NIS substrates, such as iodide or perchlorate, did not affect cell migration rates (data not shown). Next, to cast light on the effect of intracellular NIS, observed in many cancers, on cell migration, we measured the migration index induced by a NIS mutant (G543E) that is retained in intracellular organelles, and thus fails to reach the plasma membrane (45). Cells expressing G543E NIS displayed a migration rate even higher than cells expressing NISwt, indicating that intracellular NIS enhances cell migration (Fig. 3E), an observation that may be relevant to cancers in which NIS is retained intracellularly. Immunoblotting showed substantial protein expression levels for both NISwt and G543E in transiently transfected cells. Immunofluorescence analysis revealed that these molecules were present all over the cell cultures, in particular, along the wound edges (Fig. 3F). Similarly, the invasion indices of NISwt- and G543E NIS-expressing cells were 2 and 6 times higher than that of control cells, respectively, as shown by Matrigel-coated Boyden chamber assays (Fig. 3G). Finally, we investigated the activation of the RhoA pathway in NIS-induced cell migration using the GST–Rhotekin pull-down assay. The amount of activated RhoA was much larger after transfection with NISwt than with NISΔCter, NISΔTNL or empty vector (Fig. 3H), and significantly larger with G543E than with NISwt (Fig. 3I). Given that the epithelial-to-mesenchymal transition (EMT) is a crucial step in the process of invasion and metastasis of epithelial tumors, we...
studied the expression of EMT markers N-cadherin and vimentin in NIS-transfected cells. Both NISwt and G543E triggered a clear cadherin shift from E-cadherin to N-cadherin expression, an increase in vimentin expression (Fig. 3f) and a marked reorganization of vimentin filaments (Fig. 3k). Overall, these results indicate that NIS, through its PDZ-binding TNL motif, activates RhoA and thereby cell migration and invasion, and that the intracellular fraction of NIS is particularly effective in this regard.

**Knockdown of NIS inhibits cell migration and invasion**

To investigate whether endogenous NIS induces a similar migration/invasive phenotype, we conducted a series of assays in T47D, CCLP1, and HuH7 cell lines subject to a knockdown of NIS by RNA interference. NIS silencing was most effective about 120 hours after siRNA transfection, yielding a decrease in NIS protein expression of 50% to 70% (Fig. 4A). Cell migration and invasion assays started at this time point. The migration index at 18 hours after cell deposition (Fig. 4B), the average wound-healing speed at 20 hours after infliction (Fig. 4C), and the invasion index at 36 hours after cell deposition (Fig. 4D), were all reduced by a factor of 2 to 4 in all the NIS-depleted cell lines as compared with siRNA control cells. In the same 3 cell lines, RhoA-GTP activity underwent a 50% diminution after NIS silencing (Fig. 4E). This establishes that both endogenous and over-expressed NIS enhance cell migration/invasion through a RhoA activation mechanism.

**A knockdown of LARG reduces the effects of NIS on RhoA activation and cell migration**

To ascertain that the Rho guanine exchange factor (Rho-GEF) LARG is actually involved in the NIS-mediated enhancement of cell migration, we studied RhoA activity and cell migration in Hek293 cells stably transfected with empty vector, NISwt or G543E and then transiently silenced for LARG by siRNA interference. LARG knockdown was almost total 72 hours after siRNA transfection in all the studied cell lines (Fig. 5A). Following transfection with siRNA scramble, cells expressing NISwt and G543E displayed a higher level of activated RhoA-GTP than those transfected with empty vector, as expected. In all the cell lines, LARG silencing led to a significant decrease in activated RhoA-GTP. This decrease was larger in NIS transfected than empty-vector control cells. The reduction of RhoA-GTP levels in LARG-depleted cells as compared with siRNA scramble cells were of about 40%, 50%, and 70% in empty-vector, NISwt, and G543E-transfected cells, respectively (Fig. 5B). The wound-healing speed normalized to siRNA scramble was reduced after LARG silencing by about 25%, 50%, and 40% in empty-vector, NISwt, and G543E-transfected cells, respectively (Fig. 5C). Similarly, the normalized number of migrated cells in Boyden chamber assays was lowered following LARG silencing by about 40%, 60%, and 50% in empty-vector, NISwt, and G543E-transfected cells, respectively (Fig. 5D). Together, these data establish that the effects of NIS on RhoA activation and cell migration are largely mediated by a NIS–LARG interaction.

**Discussion**

NIS, which is known to be functionally expressed in thyroid cells, is upregulated and defectively targeted in many primary nonthyroid cancers, in which its biologic activities, if any, are unknown. This study has shown that NIS was also upregulated and mislocalized in many secondary cancers and invading metastatic single cells. Interestingly, a clear concentration of NIS at leading edges was observed in some motile breast metastatic cells. These observations point to a role of NIS in tumor cell locomotion. We have substantiated this view by revealing that NIS interacts with LARG, which in turn activates RhoA and thereby enhances cell migration and invasion. This
effect was also displayed by a transport-defective NIS mutant, and was therefore clearly independent of NIS transport activity. The migration and invasion function of NIS relies on an interaction of the C-terminus of NIS with a specific guanine nucleotide exchange factor triggering the activation of a small RhoGTPase. In fact, the cytoplasmic COOH tail of NIS makes it likely that NIS, and, more generally, SLC5A carriers have numerous protein interaction partners capable of activating downstream signaling pathways. For instance, this might be the origin of the binding of NIS with the proto-oncogene pituitary tumor transforming gene (PTTG) binding factor that has recently been reported to influence NIS trafficking in rat thyroid FRTL-5 cells (46). Because RhoGTPases contribute to different steps of cancer progression, including invasion and metastasis (47), our finding of a NIS–LARG–RhoA pathway suggests a metastatic potential of NIS-expressing cancers through the RhoGTPase signaling. Moreover, cells expressing a NIS mutant sequestered in intracellular organelles had a more pronounced enhanced migration and invasion phenotype than cells expressing a mostly plasma-membrane NIS, which may be of importance for those cancers, in which NIS is retained intracellularly. It should however be noted that the TGF–β1/Smad signaling, which represses thyroid NIS, is responsible for the spread of BRAF-mutated thyroid cancers (48, 49). Thus, the interplay between NIS and cancer invasiveness suggested by this study certainly depends on many host and tumor factors, which could be clarified by clinicobiologic correlation studies between metastasis incidence and NIS expression and subcellular localization. Most recent research on NIS is focused on differentiation agents capable of redirecting the intracellular NIS to the plasma membrane of cancer cells to render tumors amenable to131I radiotherapy (17, 46, 50). According to this study, such a process could have the additional positive effect of slowing tumor progression if the high efficiency of intracellular NIS for facilitating tumor invasion that we have shown in this report is confirmed in clinical studies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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