MEK inhibition leads to lysosome-mediated Na^+/I^- symporter protein degradation in human breast cancer cells

Zhaoxia Zhang^{1,2,*}, Sasha Beyer^{1,3,*} and Sissy M Jhiang^{1,2,3}

¹Department of Physiology and Cell Biology

²The Ohio State Biochemistry Graduate Program

³The Integrated Biomedical Science Graduate Program, The Ohio State University, 304 Hamilton Hall, 1645 Neil Avenue, Columbus, Ohio 43210, USA

[†]Z Zhang is now at Chemical Abstracts Service, A division of the American Chemical Society, 2540 Olentangy River Road, Columbus, Ohio 43202, USA

[‡]S Beyer is now at University of Toledo College of Medicine, Toledo, Ohio 43614, USA

Correspondence
should be addressed
to S M Jhiang

Email
jhiang.1@osu.edu

Abstract

The Na^+/I^- symporter (NIS (SLC5A5)) is a transmembrane glycoprotein that mediates active iodide uptake into thyroid follicular cells. NIS-mediated iodide uptake in thyroid cells is the basis for targeted radionuclide imaging and treatment of differentiated thyroid carcinomas and their metastases. Furthermore, NIS is expressed in many human breast tumors but not in normal non-lactating breast tissue, suggesting that NIS-mediated radionuclide uptake may also allow the imaging and targeted therapy of breast cancer. However, functional cell surface NIS expression is often low in breast cancer, making it important to uncover signaling pathways that modulate NIS expression at multiple levels, from gene transcription to posttranslational processing and cell surface trafficking. In this study, we investigated NIS regulation in breast cancer by MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) signaling, an important cell signaling pathway involved in oncogenic transformation. We found that MEK inhibition decreased NIS protein levels in all-trans retinoic acid/hydrocortisone-treated MCF-7 cells as well as human breast cancer cells expressing exogenous NIS. The decrease in NIS protein levels by MEK inhibition was not accompanied by a decrease in *NIS* mRNA or a decrease in *NIS* mRNA export from the nucleus to the cytoplasm. NIS protein degradation upon MEK inhibition was prevented by lysosome inhibitors but not by proteasome inhibitors. Interestingly, NIS protein level was correlated with MEK/ERK activation in human breast tumors from a tissue microarray. Taken together, MEK activation appears to play an important role in maintaining NIS protein stability in human breast cancers.

Key Words

- NIS
- MEK
- lysosome
- breast
- cancer

Endocrine-Related Cancer
(2013) 20, 241–250

Introduction

Breast cancer is the most common type of cancer in women living in the United States and is the second most common cause of cancer death in women (American

Cancer Society 2012). Because of routine clinical examination and increased use of mammographic screening, diagnosis is currently made at an earlier stage

of the disease. Conventional treatments for breast cancer include surgery, radiation therapy, chemotherapy, hormone therapy, and/or MAB therapy. However, despite improvements in breast cancer treatments, the 5-year survival rate for breast cancer patients with distant metastases remains low at 23% (American Cancer Society 2012). Accordingly, the development of non-invasive imaging modalities that allow early detection and monitoring of recurrence and metastases as well as alternative targeted therapy with minimal side effects for breast cancer patients remains warranted.

The Na⁺/I[−] symporter (NIS (SLC5A5)) is a transmembrane glycoprotein that mediates active iodide uptake in the thyroid gland, as well as in extra-thyroidal tissues such as salivary gland, gastric mucosa, and lactating mammary gland. NIS-mediated iodide uptake in thyroid cells is the basis for targeted radionuclide imaging and treatment of differentiated thyroid carcinomas and their metastases. The finding that NIS is also expressed in breast cancer inspires the possibility that patients with breast cancer may also benefit from NIS-mediated radionuclide imaging and therapy (Tazebay *et al.* 2000, Wapnir *et al.* 2003). However, the level of cell surface NIS is too low for effective NIS-mediated radionuclide therapy of breast cancer (Wapnir *et al.* 2004, Beyer *et al.* 2009). A better understanding of NIS regulation in breast cancer is necessary to realize NIS-mediated radionuclide imaging and ablation of breast cancer, as NIS expression/activity needs to be selectively upregulated in breast cancer to achieve clinical benefits.

MAPK/ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling has been recognized to play a role in oncogenic transformation. This signaling pathway is activated by cell surface growth factor receptors and induces cellular proliferation, differentiation, and survival. MEK1/2-ERK1/2 signaling pathway has been proposed to be a potential molecular target for cancer therapies. PD0325901 and AZD6244 are small molecule inhibitors of MEK that have shown antitumor activity in tumor xenograft models and have now entered into phase I/II human clinical trials for breast cancer, melanoma, and non-small-cell lung carcinoma, among other cancer types (reviewed in Roberts & Der (2007)). MEK inhibitor has been recently shown to restore radioiodine accumulation in mouse thyroid cancers with conditional BRAF activation (Chakravarty *et al.* 2011) and in non-avid thyroid cancer lesions in some patients (Ho *et al.* 2012).

The role of MEK signaling on NIS modulation in breast cancer is unknown. In this study, we investigated the effects of MEK inhibitor on NIS expression in human

breast cancer cells. In contrast to thyroid cells, MEK inhibition decreased NIS protein levels by promoting lysosome-mediated NIS protein degradation in human breast cancer cell lines. Interestingly, NIS protein levels were correlated with pERK levels, an indicator of MEK/ERK activation, in human breast tumors. Taken together, MEK activation appears to play an important role in maintaining NIS protein stability in human breast cancers.

Materials and methods

Cell culture

MCF-7 breast cancer cells were maintained in 44.5% DMEM (Invitrogen) and 44.5% F-12 (Invitrogen) supplemented with 10% FCS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). T47D breast cancer cells were maintained in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin. SK-BR-3 breast cancer cells were maintained in RPMI-1640 (Invitrogen) with 10% fetal bovine serum and 1% penicillin/streptomycin.

Reagents

U0126 (Promega) and U0124 (Promega) were dissolved in DMSO at 10 mM before adding them to the media. Leupeptin and chloroquine (Sigma) were dissolved in distilled water at 10 mM. All-trans retinoic acid (tRA) (Sigma) was dissolved in DMSO at 10 mM. Hydrocortisone (H) (Sigma) was dissolved in DMEM at 1 mM. MG132 and β -lactone (Sigma) were dissolved in DMSO at 5 mM.

Western blot analysis

Proteins extracted from whole cells were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane. After the membrane was blocked in 5% nonfat dry milk overnight at 4 °C, human NIS, ERK1/2, phosphorylated ERK1/2 (pERK1/2), p53, and β -actin were detected using custom-made polyclonal rabbit anti-human NIS antibody (1:1000 dilution), sc-94 polyclonal rabbit anti-ERK1/2 antibody (Santa Cruz Biotechnology, 1:250 dilution), sc-7976 polyclonal rabbit anti-pERK1/2 antibody (Santa Cruz Biotechnology, 1:250 dilution), polyclonal rabbit anti-p53 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:300 dilution), and monoclonal mouse anti- β -actin antibody (Abcam, Cambridge, MA, USA; 1:2000) respectively. Primary antibodies were detected using appropriate HRP-conjugated secondary antibodies. The signal was then detected by enhanced chemiluminescence detection reagents. NIS protein levels were quantitated by densitometry analysis using ImageL software and normalized with β -actin.

Radioactive iodide uptake assay

MCF-7 cells were seeded in 12-well plates for 24 h. Cells were then treated as indicated before radioactive iodide uptake (RAIU) assay. RAIU was performed as described previously (Zhang *et al.* 2005). Briefly, cells were incubated with 2.0 μCi NaI^{125} in 5 μM nonradioactive NaI for 30 min at 37 °C with 5% CO_2 . Cells were then washed twice with cold Hanks' balanced salt solution and lysed with 95% ethanol for 20 min. The cell lysate was collected and radioactivity was counted by a γ -counter (Packard Instruments). RAIU values were normalized with corresponding DNA values acquired by diphenylamine assay. Experiments were performed in triplicate. RAIU values in the presence of NIS inhibitor, perchlorate, was determined simultaneously as controls for cellular radioactivity not mediated via NIS.

RNA analysis

MCF-7 cells were seeded in 60 mm dishes for 24 h. Cells were then treated with or without tRA/H and pharmacological inhibitors, as indicated. The total RNA was isolated using TRIzol reagent (GibcoBRL) according to the manufacturer's protocol. Cellular fractionation before RNA extraction was performed using hypotonic lysis as described previously with some modification (Phelps *et al.* 2005). Cells were washed with PBS and detached with 0.25% trypsin treatment. Cells were pelleted by centrifugation at 300 *g* for 5 min at 4 °C. Cell pellets were resuspended in 200 μl cold hypotonic lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM CH_3COOK , 1.5 mM CH_3COOMg , and 2 mM dithiothreitol) containing 1 μl RNase OUT and put on ice for 5 min. Cell lysates were centrifuged at 300 *g* for 2 min at 4 °C to separate the cytoplasmic extract from the nuclear pellet. Then cytosolic RNA and nuclear RNA were isolated using the same method as the total RNA isolation.

For the Taqman quantitative PCR (qPCR) analysis of NIS transcripts, 1 μg RNA was used for the reverse transcription reaction (Applied Biosystems). Quantitative PCR was performed in 20 μl reaction volumes containing 1 $\times$ Power SYBR Green PCR Master Mix (Applied Biosystems), cDNA (from 100 ng of RNA), and primers. The paired primers used for amplifying hNIS were NIS-F 5'-CCGGATCAACCTCATG-GACT-3' and NIS-R 5'-CTGAGGGTGCCACTGTAAG-3'. The paired primers for amplifying GAPDH were GAPDH-F 5'-CATCATCTCTGCCCCCTCTGCTG-3' and GAPDH-R 5'-GCAATGCCAGCCCCAGCGTCAAAGG-3'. Reaction mixtures were incubated at 50 °C for 2 min followed by an initial activation at 95 °C for 15 min and then subjected to

40 PCR cycles of denaturation (95 °C for 15 s), annealing (56 °C for 30 s), and extension (60 °C for 1 min) using ABI 7900HT instrument (Applied Biosystems). The relative amount of hNIS cDNA or GAPDH cDNA of various samples was measured against the standard curves created by serial dilutions of plasmids containing hNIS or GAPDH respectively. GAPDH was used to normalize the quantification of hNIS. The data were presented as hNIS copies per 10^6 molecules of GAPDH. Each experiment was performed in triplicate.

Adenovirus-mediated transduction of dominant negative MEK1 or FLhNIS

Cells were seeded for 24 h, washed with PBS twice, and followed by incubation with recombinant adenovirus (MOI=5) in cultured medium with 2% FBS for 3 h. Transduced cells were then washed with PBS twice and cultured in regular media for additional 24 h before conducting RAIU assay. Recombinant adenovirus carrying LacZ or dominant negative MEK1 (A217/A221) was acquired from Cell Biolabs (San Diego, CA, USA). Recombinant adenovirus carrying FLhNIS was engineered in our laboratory (Lin *et al.* 2004). For experiments treated with U0126, U0126 was added 24 h post-transduction.

Immunohistochemistry

The paraffin-embedded formalin-fixed human breast cancer tissue microarray slides were requested through The Ohio State University (OSU) Wexner Medical Center's (WMC) Tissue Archives Service. Access to and use of these samples were covered under an exempt Institutional Review Board (IRB)-approved protocol allowing for use of anonymous archival tissues in research. This array consisted of randomly selected breast tissues from patients diagnosed with breast carcinoma at The OSU Medical Center from 1992 to 1994. All tumor specimens were primary, although 29% of the patients also had local lymph node metastases. None had known distant metastases. The average age of the patients is 42.26 years. Eighty-nine percent of them are Caucasian and 11% are African-American.

Immunohistochemical staining for NIS or pERK was performed as described previously (Knostman *et al.* 2007). Slides were incubated with rabbit polyclonal antihuman NIS primary antibody (1:500) or anti-pERK (1:100) antibody (Cell Signaling Technology, Danvers, MA, USA) for 1 h. The slides were then incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody

Table 1 Correlation of ERK activation and NIS expression in human breast cancers.

Tumor classification	NIS positive		NIS negative	
	n	%	n	%
pERK positive	9/12*	75	3/12	25
pERK negative	5/19	26	14/19*	74

* $P=0.01$.

(Bio-Rad; 1:250) for 30 min and detected by chromogen DAB (Dako, Carpinteria, CA, USA).

Thirty-one of the breast tumors on the array had suitable integrity for interpretation of both NIS and pERK expressions. The microarray consisted of two cores from each tumor, each core 2 mm in diameter and sectioned to 4 μ m thickness, that corresponded to different regions of

the tumor. The intensity of cell surface/cytoplasmic NIS immunostaining in both cores was evaluated by three investigators independently, including an experienced pathologist. Absent staining was scored as 0; weak staining was scored as 1+; weak to moderate staining was scored as 2+; and strong NIS staining was scored as 3+. Cases with scores of 2+ and 3+ were considered positive for NIS immunostaining.

Statistical analysis

Statistical comparison of RAIU was performed using paired Student's *t*-test. Differences were considered significant at $P<0.05$. Fisher's exact test was conducted to show the correlation between ERK activation and NIS expression by immunohistochemical staining in human breast cancers (Table 1).

Figure 1

MEK inhibition decreases NIS protein levels and NIS-mediated radioactive iodide uptake (RAIU) in tRA/H-treated MCF-7 human breast cancer cells. (A) Western blot analysis showed that MEK inhibitor U0126 (left panel) or recombinant adenovirus carrying dominant negative MEK1 A217/A221 (right panel) decreased NIS protein levels in MCF-7-tRA/H cells. MCF-7 cells treated with tRA/H were cultured in the presence of DMSO, U0126, or inactive analog U0124 for 24 h or were infected with recombinant adenovirus carrying LacZ (rAdLacZ) or dominant negative MEK1 (rAddNMEK1) at a multiplicity of infection (MOI) of 5 for 24 h before western blot analysis. β -Actin served as a loading control. The results are

representative of at least two independent experiments. (B) Bar graph indicates the densitometry values of NIS protein level normalized with β -actin of cells under various treatments (represented as percentage relative to MCF-7/tRA/H or MCF-7/tRA/H/Lac-Z control). (C) U0126 decreased RAIU in a dose-dependent manner in tRA/H-induced MCF-7 cells. The results are representative of three independent experiments performed in triplicate and the mean $\pm$ s.d. are shown. Asterisk indicates statistically significant difference ($P<0.05$). Parallel iodide uptake experiments were conducted in the presence of perchlorate NIS inhibitor to examine RAIU activity not contributed by NIS-mediated iodide uptake.

Figure 2

MEK inhibition does not decrease steady NIS mRNA levels or decrease export of NIS mRNA to cytoplasm in MCF-7-tRA/H cells. (A) Real-time RT-PCR showed that U0126 did not decrease *NIS* mRNA levels in MCF-7-tRA/H cells. MCF-7 cells treated with tRA/H were cultured in the presence of DMSO or U0126 for 24 h. *NIS* mRNA level was normalized with GAPDH and the values of *NIS*/GAPDH mRNA ratio of MCF-7 cells without treatment were arbitrarily assigned. The results are representative of three independent experiments performed in triplicate and the mean $\pm$ s.d. are shown. (B) Real-time RT-PCR showed that U0126 did not significantly decrease *NIS* mRNA levels in cytosolic fraction in MCF-7-tRA/H cells. Cytosolic *NIS* mRNA level was normalized with GAPDH and the values of cytosolic *NIS*/GAPDH mRNA ratio of MCF-7-tRA/H cells were arbitrarily assigned. The results are representative of two independent experiments performed in triplicate and the mean $\pm$ s.d. are shown.

Results

MEK inhibition decreases NIS protein levels and iodide uptake in tRA/H-treated MCF-7 human breast cancer cells

It has previously been shown that a combination treatment of tRA and hydrocortisone induces NIS protein levels and RAIU in MCF-7 human breast cancer cells (Dohan *et al.* 2006). To investigate the roles of MEK signaling on tRA/hydrocortisone (tRA/H)-induced NIS expression, MCF-7 cells were treated with MEK inhibitor, U0126, in the presence of tRA/H treatment. As shown in the left panel of Fig. 1A and B, U0126 decreased NIS protein levels in a dose-dependent manner in

MCF-7-tRA/H cells, yet U0124, an inactive U0126 analog, did not have any effects on NIS protein levels. Consistent with decreased NIS protein levels, U0126 dose dependently decreased NIS-mediated iodide uptake in MCF-7-tRA/H cells (Fig. 1C). PD98059 (50 μM), another MEK inhibitor with a distinct structure from U0126, also decreased NIS protein levels in MCF-7-tRA/H cells (data not shown). Moreover, NIS protein levels were decreased in MCF-7-tRA/H cells infected with recombinant adenovirus carrying dominant negative MEK1 (A227/A221) compared with cells infected with recombinant adenovirus carrying LacZ as a control (right panel in Fig. 1A). Taken together, MEK inhibition decreases NIS protein levels in MCF-7-tRA/H cells. Note that neither β-actin nor ERK1/2 protein levels were decreased by MEK inhibition.

MEK inhibition does not decrease NIS mRNA levels or decrease export of NIS mRNA to cytoplasm in MCF-7-tRA/H cells

To examine whether the decrease in NIS protein levels by MEK inhibition is contributed by decreased *NIS* mRNA levels, we investigated *NIS* mRNA levels in the presence or absence of U0126. As expected, tRA/H increased *NIS* mRNA levels; however, U0126 did not decrease *NIS* mRNA levels in MCF-7-tRA/H cells (Fig. 2A). We then further examined whether U0126 decreases *NIS* mRNA export from nucleus to cytoplasm. As shown in Fig. 2B, U0126 had no significant effect on cytosolic *NIS* mRNA levels. To ensure that the cytosolic RNA fraction is not contaminated with nuclear RNA, we showed that U6 nuclear RNA was predominantly detected in nuclear RNA fraction via RT-qPCR analysis (data not shown). These findings indicate that the decrease in NIS protein levels by U0126 was not due to a decreased transcription rate, mRNA stability, or mRNA export from nucleus.

MEK inhibition leads to lysosomal-mediated NIS protein degradation in MCF-7-tRA/H cells

To examine whether MEK inhibition leads to proteasomal- or lysosomal-mediated NIS degradation, NIS protein levels were investigated in U0126-treated MCF-7-tRA/H cells in the presence of proteasome or lysosome inhibitors. As shown in Fig. 3A and B, MG132 and β-lactone proteasome inhibitors had no effect on NIS protein levels. The efficacy of proteasome inhibition was demonstrated by increased p53 protein levels, a protein with well-characterized mechanism of proteasomal degradation. While lysosome inhibitor leupeptin or chloroquine alone had little effect

Figure 3

The decrease in NIS protein levels by MEK inhibition is mediated by lysosomal but not proteasomal pathway in MCF-7-tRA/H cells. (A) Western blot analysis showed that proteasome inhibitors did not prevent the decrease in NIS protein levels in U0126-treated MCF-7-tRA/H cells. MCF-7 cells treated with tRA/H were cultured in the presence of DMSO or U0126 for 24 hours, along with or without proteasome inhibitors MG132 or β-lactone for 8 h. β-Actin served as a loading control and p53 was included to ensure the efficacy of proteasome inhibition. The results are representative of two independent experiments. (B) Bar graph indicates the densitometry values of NIS protein level normalized with β-actin of cells

under various treatments (represented as percentage relative to MCF-7/tRA/H). (C) Western blot analysis showed that lysosome inhibitors prevented the decrease in NIS protein levels in U0126-treated MCF-7-tRA/H cells. MCF-7 cells treated with tRA/H were cultured in the presence of DMSO or U0126 for 24 h, along with or without lysosome inhibitors leupeptin or chloroquine for 8 hours. β-Actin served as a loading control. The results are representative of three independent experiments. (D) Bar graph indicates the densitometry values of NIS protein level normalized with β-actin of cells under various treatments (represented as percentage relative to MCF-7/tRA/H).

on NIS protein levels in MCF-7-tRA/H cells, NIS reduction by U0126 was prevented by co-treatment with lysosome inhibitor (Fig. 3C and D). These findings indicate that the decrease in NIS protein levels by U0126 was largely contributed by lysosomal-mediated NIS protein degradation in MCF-7-tRA/H cells. Note that lysosome inhibitor did not alter pERK nor total ERK1/2 level.

MEK inhibition leads to lysosomal-mediated NIS protein degradation in MCF-7, SK-Br-3, and T47D breast cancer cells expressing exogenous NIS

To investigate whether the finding that MEK inhibition leads to lysosomal-mediated NIS protein degradation is

restricted to MCF-7-tRA/H cells, we examined the effect of U0126 in the presence and absence of lysosome inhibitor on NIS protein levels in three human breast cancer cell lines infected with recombinant adenovirus carrying CMV promoter-driven NIS cDNA. In addition to estrogen receptor-positive MCF-7 cells, we included other estrogen receptor-positive T47D cells and the estrogen receptor-negative SK-Br-3 cells. As shown in Fig. 4, U0126 leads to lysosomal-mediated NIS protein degradation, which is prevented at least partially by the presence of leupeptin in all three human breast cancer cell lines. These results suggest that MEK signaling plays an important role in modulating NIS protein stability in various human breast cancer cells.

Figure 4

MEK inhibition leads to lysosomal-mediated NIS protein degradation in MCF-7, SK-Br-3, and T47D breast cancer cells expressing exogenous NIS. Western blot analysis showed that leupeptin prevented the decrease in NIS protein levels in U0126-treated MCF-7 cells (A), SK-Br-3 (B), and T47D (C) human breast cancer cells infected with recombinant adenovirus carrying hNIS (rAdFLhNIS). Cells infected with rAdFLhNIS were treated with U0126

for 24 h, along with or without leupeptin for 8 h. β -Actin served as a loading control. The results are representative of two independent experiments. (D) Bar graph indicates the densitometry values of NIS protein level normalized with β -actin of cells under various treatments (represented as percentage relative to the corresponding untreated rAdFLhNIS-transduced cells).

NIS expression is associated with MEK/ERK activation in human breast tumors

To examine whether MEK activation is associated with the presence of NIS protein in human breast tumors, we performed immunohistochemical staining against pERK as an indicator of MEK activation, as well as NIS, in a tissue microarray consisting of 31 human breast tumors. Among the 31 tumors examined, 14 are NIS positive and 19 are NIS negative; 12 are pERK positive and 19 are pERK negative. As shown in Fig. 5, MEK activation status

appears to correlate with NIS protein levels, i.e. pERK-negative tumors are generally NIS negative, and pERK-positive tumors are generally NIS positive. Note that NIS-positive tumors were three times more likely than NIS-negative tumors to have ERK phosphorylation, and Fisher's exact test confirmed a significant association between NIS and pERK expression among breast tumors ($P=0.01$) (see Table 1). By contrast, NIS expression was not associated with tumor estrogen or progesterone receptor status as reported in our previous study

Figure 5

NIS expression is correlated with MEK/ERK activation in human breast tumors. Immunohistochemical analysis showed that the majority of human breast tumors that were positive for pERK were also positive for NIS, whereas the majority of those breast tumors that were negative for pERK

were also negative for NIS. Immunohistochemical staining for pERK (a, b, c and d) and corresponding NIS proteins (e, f, g and h) in four representative breast cancer specimens are shown. Magnification = 400 $\times$.

(Knostman *et al.* 2007). Taken together, our data suggest that MEK activation plays an important role in maintaining NIS protein stability in most breast tumors.

Discussion

In this study, we found that MEK inhibition decreased NIS protein levels as well as NIS-mediated iodide uptake in tRA/H-treated MCF-7 breast cancer cells. The decrease in NIS protein levels by MEK inhibition was not due to decreased *NIS* mRNA levels or decreased *NIS* mRNA export from nucleus to cytoplasm but due to lysosome-mediated NIS degradation. This is the first report to describe NIS modulation by lysosomal-mediated degradation. The fact that MEK inhibition decreased both tRA/H-induced endogenous NIS in MCF-7 cells and exogenous NIS expressed in all three examined breast cancer cell lines indicates that MEK activation plays an important role in maintaining NIS protein stability in breast cancers. This novel finding is further supported by the correlation of NIS expression with MEK/ERK activation in a tissue microarray composed of 31 human breast tumors.

Kogai *et al.* (2000) first reported that tRA induces *NIS* mRNA, NIS protein, and NIS-mediated RAIU in MCF-7 human breast cancer cells, which was later confirmed by several other groups (Dohan *et al.* 2006, Unterholzner *et al.* 2006). Corticosteroids (dexamethasone or hydrocortisone) alone had minimal effect on NIS-mediated RAIU activity in MCF-7 cells; however, they greatly enhance NIS

expression/activity in tRA-treated MCF-7 cells (Kogai *et al.* 2005, Unterholzner *et al.* 2006). NIS induction by tRA is primarily contributed by increased transcription of *NIS* (Kogai *et al.* 2000), and corticosteroid appears to stabilize tRA-induced *NIS* mRNA (Kogai *et al.* 2005).

A recent publication by Kogai *et al.* (2012) showed that p38 kinase inhibitor, but not MEK inhibitor, reduces NIS-mediated RAIU activity in tRA-treated MCF-7 cells. In contrast to reduced NIS protein stability in the presence of MEK inhibitor, p38 kinase inhibitor reduces *NIS* mRNA levels, most likely by reducing *NIS* transcription. In agreement with their finding, we found that RAIU was not significantly decreased by U0126 at 10 μ M but only significantly decreased by U0126 at a concentration > 20 μ M in MCF-7-tRA/H cells (Fig. 1C). Accordingly, we chose 20 μ M U0126 to examine the mechanisms underlying NIS reduction by U0126, as NIS protein degradation was consistently found in cells treated with 20 μ M U0126. Similarly, iodide uptake was not reduced in MCF-7/tRA/H cells treated with 10 μ M PD98059 (Kogai *et al.* 2012), yet we found a significant reduction in iodide uptake when cells were treated with a higher dose of 50 μ M PD98059. Taken together, MEK signaling needs to be inhibited to a certain extent before eliciting lysosomal-mediated NIS protein degradation in human breast cancer cells. Note that pERK level was slightly decreased by tRA/H treatment in MCF-7 cells (Figs 1A and 3A).

In thyroid cells, it has been shown that MEK activation decreases NIS expression/activity and that MEK inhibition

increases NIS protein levels (Riesco-Eizaguirre *et al.* 2006, Vadysirisack *et al.* 2007). In human breast cancer cells, MEK inhibition targeted NIS proteins to lysosomal degradation. Not only did this study uncover a novel mechanism for NIS modulation but we also showed that MEK signaling plays a very different role in NIS modulation between breast cancer cells and thyroid cells. This finding may help to explain why NIS is often reduced in thyroid carcinomas but increased in breast carcinomas compared with their respective normal tissues, despite that MEK is activated in both thyroid and breast cancers. It would be most interesting to investigate whether differential NIS modulation by MEK signaling between thyroid and breast cancer is due to differences in modifications of NIS itself or differences in modifications of lysosomal machinery.

Interestingly, Imai *et al.* (2009) recently showed that MEK inhibition resulted in lysosomal-mediated protein degradation of another plasma membrane protein in breast cancer cells, known as the breast cancer resistance protein. MEK inhibition has also been reported to result in degradation of another cell surface protein in MCF-7 cells, known as the multi-drug resistance protein 1 P-glycoprotein, although this mechanism of protein degradation is yet to be elucidated (Katayama *et al.* 2007).

The sorting of transmembrane protein to endosome-lysosome is mediated by signals present in the cytosolic fragments of protein. Two major classes of these signals are 'tyrosine-based' and 'dileucine-based' sorting signals (Bonifacino & Traub 2003). Dileucine-based sorting signals are represented by the motifs, [DE]XXXL[LI] and DXXLL. Human NIS contains 12 dileucines (LL) and six leucine-isoleucine (LI), in which two (L282L283 and L562L563) of these 12 LL and three (L283I284, L370I371, and L546I547) of these six LI are predicted to be located intracellularly. However, these intracellular LL and LI do not match with the [DE]XXXL[LI] or DXXLL motif. Tyrosine-based sorting signal is represented by the NPXY and YXXØ consensus motifs (X stands for any amino acid and Ø stands for an amino acid residue with a bulky hydrophobic side chain). YXXØ signals are much more widely involved in the targeting of transmembrane proteins to lysosomes and lysosome-related organelles (Bonifacino & Traub 2003). YXXØ sorting signal is recognized by specific adaptor protein complexes and the recognition is believed to be regulated by phosphorylation/dephosphorylation of the tyrosine amino acid residue in the YXXØ motif or its adjacent amino acid residues. In addition, ubiquitin and acidic clusters or specific folded structures can also serve as endosome-lysosome sorting signals (Bonifacino & Traub 2003).

In hNIS, five tyrosines (Y110, Y118, Y120, Y137, and Y178), which are located in intracellular domain, may serve as 'tyrosine-based YXXØ' lysosome sorting signal. Using PPSP, GPS and Motif Scan to analyze the predicated intracellular domains of hNIS, T442, T596, and T627 are identified as possible ERK phosphorylation sites. However, these three amino acid residues are not adjacent to the five possible YXXØ motifs. Our initial study found that both phosphor-mimic and phosphor-defective T442 mutants resulted in decreased iodide uptake (data not shown). This result suggests that structural geometry, not phosphorylation status of T442, modulates NIS activity. Thus, further studies are required to determine whether lysosome-mediated NIS degradation is modulated by NIS phosphorylation.

In conclusion, this study uncovers a novel mechanism of NIS modulation by lysosomal degradation, and MEK activation appears to contribute to NIS protein stability in human breast cancer. Further investigation on the underlying mechanisms in which MEK signaling leads to increased NIS stability may identify useful targets to selectively increase radionuclide uptake in breast cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported in part by National Institutes of Health P01 CA124570 (PI: M Ringel; Project 3 leader: S M Jhiang), R01 CA125481 (PI: S M Jhiang), R01 EB001876 (PI: S M Jhiang), and Nucleic Acids Shared Resource for The Ohio State University Comprehensive Cancer Center P30 CA16058 (PI: M Caligiuri).

References

- American Cancer Society 2012 Breast Cancer facts and figures. American Cancer Society: Atlanta. Available from <http://www.cancer.org>.
- Beyer SJ, Jimenez RE, Shapiro CL, Cho JY & Jhiang SM 2009 Do cell surface trafficking impairments account for variable cell surface sodium iodide symporter levels in breast cancer? *Breast Cancer Research and Treatment* **115** 205–212. (doi:10.1007/s10549-008-0059-5)
- Bonifacino JS & Traub LM 2003 Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annual Review of Biochemistry* **72** 395–447. (doi:10.1146/annurev.biochem.72.121801.161800)
- Chakravarty D, Santos E, Ryder M, Knauf JA, Liao XH, West BL, Bollag G, Kolesnick R, Thin TH, Rosen N *et al.* 2011 Small-molecule MAPK inhibitors restore radioiodine incorporation in mouse thyroid cancers with conditional BRAF activation. *Journal of Clinical Investigation* **121** 4700–4711. (doi:10.1172/JCI46382)
- Dohan O, De la Vieja A & Carrasco N 2006 Hydrocortisone and purinergic signaling stimulate sodium/iodide symporter (NIS)-mediated iodide transport in breast cancer cells. *Molecular Endocrinology* **20** 1121–1137. (doi:10.1210/me.2005-0376)

- Ho AL, Leboeuf R, Grewal RK, Sherman EJ, Deandreis D, Pentlow KS, Tuttle RM, Fury MG, Ricarte-Filho JCM, Pfister DG *et al.* 2012 Reacquisition of RAI uptake in RAI-refractory metastatic thyroid cancers by pretreatment with the MEK inhibitor selumetinib. *Journal of Clinical Oncology* **30** (suppl) abstr 5509.
- Imai Y, Ohmori K, Yasuda S, Wada M, Suzuki T, Fukuda K & Ueda Y 2009 Breast cancer resistance protein/ABCG2 is differentially regulated downstream of extracellular signal-regulated kinase. *Cancer Science* **100** 1118–1127. (doi:10.1111/j.1349-7006.2009.01154.x)
- Katayama K, Yoshioka S, Tsukahara S, Mitsuhashi J & Sugimoto Y 2007 Inhibition of the mitogen-activated protein kinase pathway results in the down-regulation of P-glycoprotein. *Molecular Cancer Therapeutics* **6** 2092–2102. (doi:10.1158/1535-7163.MCT-07-0148) (doi:10.1158/1535-7163.MCT-07-0148)
- Knoston KA, McCubrey JA, Morrison CD, Zhang Z, Capen CC & Jhiang SM 2007 PI3K activation is associated with intracellular sodium/iodide symporter protein expression in breast cancer. *BMC Cancer* **7** 137–147. (doi:10.1186/1471-2407-7-137)
- Kogai T, Schultz JJ, Johnson LS, Huang M & Brent GA 2000 Retinoic acid induces sodium/iodide symporter gene expression and radioiodide uptake in the MCF-7 breast cancer cell line. *PNAS* **97** 8519–8524. (doi:10.1073/pnas.140217197)
- Kogai T, Kanamoto Y, Li AI, Che LH, Ohashi E, Taki K, Chandraratna RA, Saito T & Brent GA 2005 Differential regulation of sodium/iodide symporter gene expression by nuclear receptor ligands in MCF-7 breast cancer cells. *Endocrinology* **146** 3059–3069. (doi:10.1210/en.2004-1334)
- Kogai T, Liu YY, Mody K, Shamsian DV & Brent GA 2012 Regulation of sodium iodide symporter gene expression by Rac1/p38 β mitogen-activated protein kinase signaling pathway in MCF-7 breast cancer cells. *Journal of Biological Chemistry* **287** 3292–3300. (doi:10.1074/jbc.M111.315523)
- Lin X, Fischer AH, Ryu KY, Cho JY, Sferra TJ, Kloos RT, Mazzaferri EL & Jhiang SM 2004 Application of the Cre/loxP system to enhance thyroid-targeted expression of sodium/iodide symporter. *Journal of Clinical Endocrinology and Metabolism* **89** 2344–2350. (doi:10.1210/jc.2003-031963)
- Phelps M, Phillips A, Darley M & Blaydes JP 2005 MEK-ERK signaling controls Hdm2 oncoprotein expression by regulating hdm2 mRNA export to the cytoplasm. *Journal of Biological Chemistry* **280** 16651–16658. (doi:10.1074/jbc.M412334200)
- Riesco-Eizaguirre G, Gutierrez-Martinez P, Garcia-Cabezas MA, Nistal M & Santisteban P 2006 The oncogene BRAFV600E is associated with a high risk of recurrence and less differentiated papillary thyroid carcinoma due to the impairment of Na⁺/I[−] targeting to the membrane. *Endocrine-Related Cancer* **13** 257–269. (doi:10.1677/erc.1.01119)
- Roberts PJ & Der CJ 2007 Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* **26** 3291–3310. (doi:10.1038/sj.onc.1210422)
- Tazebay UH, Wapnir IL, Levy O, Dohan O, Zuckier LS, Zhao QH, Deng HF, Amenta PS, Fineberg S, Pestell RG *et al.* 2000 The mammary gland iodide transporter is expressed during lactation and in breast cancer. *Nature Medicine* **6** 871–878. (doi:10.1038/78630)
- Unterholzner S, Willhauck MJ, Cengic N, Schutz M, Goke B, Morris JC & Spitzweg C 2006 Dexamethasone stimulation of retinoic acid-induced sodium iodide symporter expression and cytotoxicity of 131-I in breast cancer cells. *Journal of Clinical Endocrinology and Metabolism* **91** 69–78. (doi:10.1210/jc.2005-0779)
- Vadysirisack DD, Venkateswaran A, Zhang Z & Jhiang SM 2007 MEK signaling modulates sodium iodide symporter at multiple levels and in a paradoxical manner. *Endocrine-Related Cancer* **14** 421–432. (doi:10.1677/erc.1.01263)
- Wapnir IL, van de Rijn M, Nowels K, Amenta PS, Walton K, Montgomery K, Greco RS, Dohan O & Carrasco N 2003 Immunohistochemical profile of the sodium/iodide symporter in thyroid, breast, and other carcinomas using high density tissue microarrays and conventional sections. *Journal of Clinical Endocrinology and Metabolism* **88** 1880–1888. (doi:10.1210/jc.2002-021544)
- Wapnir IL, Goris M, Yudd A, Dohan O, Adelman D, Nowels K & Carrasco N 2004 The Na⁺/I[−] symporter mediates iodide uptake in breast cancer metastases and can be selectively downregulated in the thyroid. *Clinical Cancer Research* **10** 4294–4302. (doi:10.1158/1078-0432.CCR-04-0074)
- Zhang Z, Liu YY & Jhiang SM 2005 Cell surface targeting accounts for the difference in iodide uptake activity between human Na⁺/I[−] symporter and rat Na⁺/I[−] symporter. *Journal of Clinical Endocrinology and Metabolism* **90** 6131–6140. (doi:10.1210/jc.2005-0895)

Received in final form 8 January 2013

Accepted 28 January 2013

Made available online as an Accepted Preprint

12 February 2013