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Unliganded estrogen receptor-α activates transcription of the mammary gland Na^+/I^- symporter gene

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Abstract

The function of sodium iodide symporter (Na^+/I^- symporter, or NIS) in mammary epithelial cells is essential for the accumulation of I^- in milk; the newborn's first source of I^- for thyroid hormone synthesis. Furthermore, increased mammary gland NIS expression has previously been shown in human breast cancer. Several hormones and factors including all-*trans*-retinoic acid (tRA) regulate the expression of NIS. In this study, using breast cancer cell lines, we established that tRA-responsive NIS expression is confined to estrogen receptor-α (ERα) positive cells and we investigated the role of ERα in the regulation of NIS expression. We showed that the suppression of endogenous ERα by RNA interference downregulates NIS expression in ERα positive mammary cells. Besides, in an ERα negative cell line, reintroduction of ERα resulted in the expression of NIS in a ligand-independent manner. We also identified a novel estrogen-responsive element in the promoter region of NIS that specifically binds ERα and mediates ERα-dependent activation of transcription. Our results indicate that unliganded ERα (apo-ERα) contributes to the regulation of NIS gene expression.

Keywords: Iodide transport; NIS; Estrogen receptor; Mammary gland; Breast cancer

In mammary gland lactocytes, sodium/iodide (Na^+/I^-) symport via NIS is required to secrete I^- in mother's milk [1]. I^- in milk is used by the newborn in thyroid hormone biosynthesis, and thus it plays an essential role in post-natal development of skeletal muscles, nervous system, and lungs [2]. In vivo experiments in mice have previously demonstrated that in normal physiology, NIS expression is strictly linked to mammary development in gestation, and to lactation [1]. Non-lactating mammary gland tissue in female mice does not express NIS unless animals receive subcutaneous oxytocin treatments for three consecutive days. On the other hand, a similar treatment in ovariectomized mice is not sufficient for NIS upregulation. In these surgically treated animals, administration of 17-β-estradiol (E2) together with oxytocin is essential for functional expression of NIS. The fact that E2 treatment was only essential in ovariectomized animals, whereas lactogenic hormones were sufficient for functional NIS expression in surgically untreated mice, suggested that ovary functions and endogenous estrogens are essential in upregulating NIS expression [1]. Unlike in non-lactating mammary gland tissue, in transgenic mice bearing experimental breast cancers triggered by *Erb-B2/neu* and *ras* oncogenes, functional expression of NIS significantly increases [1]. In the same study, human breast cancer specimens were also analyzed, and an increased NIS expression was detected in human invasive breast cancer and ductal carcinoma in situ, as compared to no expression of NIS in healthy breast samples obtained from reductive mammoplasty operations [1].

Recent studies with an ERα+ mammary cell line model, MCF-7, have led to the identification of additional hormones or ligands that control transcriptional regulation of NIS. In this cell line, the symporter gene was shown to be inducible in response to 9-*cis*-retinoic acid (9cRA) and all-*trans*-retinoic acid (tRA), ligands that were previously

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known to induce I^- transport activity in dedifferentiated thyroid tumor metastatic tissues in humans [3–5]. Kogai et al. [3] have shown that tRA have upregulated both NIS expression and iodide transport in MCF-7 cells in a dose-dependent manner. The absence of a similar increase in NIS mRNA levels in the ERα− MDA-MB-231 cell line after tRA treatment has led the authors to consider that ERα positivity of MCF-7 may lead to increased levels of retinoic acid receptor (RAR) in the presence of E2, which may provide cellular conditions favorable for NIS expression [3]. A correlation between the ERα status of mammary cell lines and 9cRA (a ligand for both RAR and retinoid x receptor (RXR) heterodimers and RXR/RXR homodimers) inducibility of NIS gene was also previously indicated [5]. In a separate study, Nkx2.5, a homeobox transcription factor, was indicated as the mediator of tRA-responsive NIS expression in MCF-7 cells [6].

In the present study, we investigated roles of E2 and ERα in transcriptional regulation of NIS in mammary cell lines. We first established a correlation between ERα status of mammary cell lines and tRA-responsive NIS expression. Then, we studied the roles of E2 and ERα in NIS regulation using two RA-responsive mammary cancer cell models such as the MCF-7 and MDA-MB-231 cell lines. We established that in a previously ERα− mammary cell line, MDA-MB-231, both transient and stable expression of ERα activates basal expression of NIS in an estrogen-independent manner (in the apo-ERα state; [7]). Furthermore, suppression of the endogenous ERα gene in MCF-7 cells by RNA interference method downregulated tRA induced NIS expression, indicating the role of ERα in regulation of the symporter gene. Subsequently, we have identified a novel ERE sequence located in close proximity (9 base pairs upstream) of the TATA element in NIS gene promoter. By chromatin immunoprecipitation (ChIP) experiments, we obtained strong evidences in support of a physical interaction between this novel *cis*-acting element and ERα in MCF-7 cells. Our results indicated a functional interaction between the unliganded ERα and RA-responsive pathways in NIS regulation in the mammary gland.

Materials and methods

Plasmids. The expression vector for ERα (pCMV-ERα) was prepared by inserting the *Eco*RI fragment (containing ERα coding sequence) form the plasmid pSG5ERpuro (kindly provided by Patrick Balaguer, Montpellier) into pcDNA3.1C (Invitrogen). The luciferase reporter vectors, pGL3E1bLuc and phRL-TK, were kindly provided by Roberto Di Lauro, Naples. The reporter pPS2XERE was prepared by ligating a synthetic double strand (ds) oligonucleotide containing two tandem copies of the pS2 ERE into *Mlu*I/*Xho*I sites of pGL3E1bLuc. The DNA sequence of this oligonucleotide was 5′-CGC GTA AGG TCA CGG TGG CCA CAC GCG TAA GGT CAC GGT GGC CAC CCC GTC-3′. Likewise, pNIS2XERE was created by inserting a synthetic ds oligonucleotide containing two tandem copies of the putative NIS ERE (5′-CGC GTA GGC GGA GTC GCG GTG ACC CGG CGG AGT CGC GGT GAC CCG GGA GC-3′) into the *Mlu*I/*Xho*I sites of pGL3E1bLuc. Oligonucleotides for the knockdown of ERα were designed and supplied by Oligoengine, WA (N-19 targets 458 and 499 on NM_000125 were sh-ER458: 5′-TTC AGA TAA TCG ACG CCA G-3′, and for sh-ER499: 5′-GTA CCA ATG ACA AGG GAA G-3′). These shRNA oligos were then cloned in the *Bgl*II/*Xho*I sites of pSuper-GFP/Neo (pSR, Oligoengine, WA) to generate the two knockdown constructs, pSR-ER-458 and pSR-ER-499.

Cell culture. Human mammary gland cell lines BT-474, T-47D, BT-20, MDA-MB-453, MDA-MB-468, *hTERT*-HME1, MCF-7, MDA-MB-231, and MDA-66 were used in this study. All-*trans*-retinoic acid (tRA) and 17-β-estradiol (E2) were purchased from Sigma. tRA was dissolved in DMSO, E2 was dissolved in ethanol as 10 mM stock solutions, and stored protected from light at −20 °C. All cell lines were maintained in high glucose Dulbecco's modified Eagle's medium [Gibco, supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S) and 1% L-glutamine (Biochrom)], abbreviated in the text as reg-DMEM, at 37 °C in a 5% CO_2 incubator. MDA-66 was maintained in the above medium with the addition of 0.4 mg/ml Hygromycin (Roche). In general, hormone induction experiments were performed in sf-DMEM (a phenol red-free DMEM (Sigma) supplemented with 10% dextran-coated-charcoal stripped FBS, 1% P/S and 1% L-glutamine) unless otherwise mentioned. Two days before the addition of hormones, cells were fed with sf-DMEM in order to deplete the culture media from endogenous steroids and retinoids. tRA was applied to a final concentration of 1 μM for 12 h, while E2 was applied to a final concentration of 10 nM for 3 h. Cells were harvested by trypsinization and cell pellets were divided into two tubes to be used for both RNA and protein extract preparations.

Luciferase reporter assay. Cell lines were transfected with plasmid DNAs using FuGENE-6 reagent (Roche). FuGENE:DNA ratios were determined experimentally to be 3:1 for MCF-7 and 6:1 for both MDA-MB-231 and MDA-66. Cells were seeded in 24-well plates in reg-DMEM; so that they reach confluence at the time of the assay. Two days later, and 1 h prior to transfection, cells were washed twice with PBS, and the medium was replaced with sf-DMEM lacking antibiotics. Transfection was carried out with 200 ng of reporter vector plus 3 ng phRL-TK to normalize for transfection efficiency. Two days post transfection, medium was changed with fresh sf-DMEM containing 10 nM E2 (or EtOH as vehicle control) and continued incubation for 6 h. Then the cells were harvested and luciferase assay was performed using the Dual-Glo Luciferase Assay system (Promega). Luciferase values for all samples were normalized by first subtracting the background of no-transfection control and then dividing firefly luciferase values over those of *Renilla* luciferase. Fold induction is relative to the value of the empty vector pGL3E1bLuc.

Transient transfection with ERα. MDA-MB-231 cells were transfected with pCMV-ERα in 100 mm dishes, using FuGENE-6 (as described above) and 5 μg of the expression vector. Two days after transfection, media were replaced with fresh sf-DMEM containing 10 nM E2 or 1 μM tRA, or a combination of both hormones. After hormone induction, cells were rinsed with cold PBS, and harvested by trypsinization, cell pellets were divided into two tubes; RNA and protein extracts were prepared from the same sample.

RNA, cDNA, and semi-quantitative RT-PCR. The expression level of NIS, pS2, and GAPDH was monitored by semi-quantitative RT-PCR. RNA were prepared using the Nucleospin RNA II kit (Macherey–Nagel) as recommended by the manufacturer. In general 2 μg of total RNA were used for cDNA synthesis using the Revert-Aid First Strand cDNA Synthesis Kit (Fermentas). Primers for semi-quantitative RT-PCR amplified corresponding transcripts from positions spanning two or more exonic sequences. PCR primers were RT-NIS-F: 5′-CTC ATC CTG AAC CAA GTG AC-3′, RT-NIS-R2: 5′-TAC ATG GAG AGC CAC ACC A-3′, RT-pS2-F: 5′-CCA TGG AGA ACA AGG TGA TCT GC-3′, RT-pS2-R2: 5′-GTC AAT CTG TGT TGT GAG CCG AG-3′, GAPDH-F: 5′-GGC TGA GAA CGG GAA GCT TGT CAT-3′, GAPDH-R: 5′-CAG CCT TCT CCA TGG TGG TGA AGA-3′. PCR amplification was performed in 25 μl reaction volumes containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 10 pmol of each primer, and 1 U *Taq* DNA polymerase (Fermentas). Thermal cycler conditions were an initial denaturation step at 95 °C for 3 min; a loop cycle of 95 °C, 30 s/61 °C, 30 s/72 °C, 30 s; and a final extension at 72 °C for 10 min. The cycle number varied for each transcript amplified, for NIS it was 40 cycles, pS2 in MCF-7 was 15 cycles,

and in MDA-MB-231/MDA-66 was 40 cycles. Cycle number for GAPDH was 19. PCR products were resolved on 2% agarose gels stained with ethidium bromide and visualized using the Gel Doc-2000 supported with the Multi-Analyst Ver.1.1 image analysis software (Bio-Rad).

Western blot analysis. The expression of ERα, RARα, and calnexin was examined by Western blot analysis. Cell pellets were incubated in lysis buffer for 30 min (50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 0.1% Nonidet P-40, and 1× protease inhibitor cocktail (Roche)), cell extracts were cleared by centrifugation, and protein content was quantified using Bradford assay. 20 μg of whole cell extracts was denatured in gel loading buffer (50 mM Tris–HCl, pH 6.8, 1% SDS, 0.02% bromophenol blue, 5% 2-mercaptoethanol, and 10% glycerol) at 95 °C for 5 min, resolved by SDS–PAGE using a 10% gel, and electro-transferred onto PVDF membranes (Millipore). The membranes were blocked in Blotto (Tris-buffered saline containing 0.5% Tween 20 and 5% nonfat milk powder) for 1 h at room temperature. The membranes were incubated with mouse monoclonal anti-hERα F-10 (1:500, Santa Cruz) for 16 h at 4 °C, washed three times with Blotto, and incubated with peroxidase-conjugated goat anti-mouse (1:2000, Sigma) for 1 h, immunocomplexes were then detected using ECL-plus (Amersham), and exposed to X-ray films (AGFA) for 1 min. The films were then developed using a hyper-processor developer (Amersham). Membranes were then washed three times with Blotto, re-incubated with rabbit monoclonal anti-hRARα C-20 (1:1000, Santa Cruz) for 16 h, and then stained with goat anti-rabbit (1:2000, Sigma). The same protocol was repeated for the internal control calnexin using a rabbit anti-calnexin (1:5000, Sigma).

Suppression of ERα by shRNA. MCF-7 cells were transfected as described above, using pSR-ER458, pSR-ER499, and the empty vector control pSR. After transfection, cells were washed, diluted, and transferred to 24-well plates for selection with DMEM containing 0.5 mg/ml Geneticin (Sigma). Three weeks later, stably transfected colonies (expressing the EGFP marker) were transferred to new culture dishes and were allowed to grow for further analysis. The presence of the knockdown construct was confirmed by PCR using genomic DNA isolated from each clone as a template, and the pSR insert screening primers from Oligoengine (F: 5′-GGA AGC CTT GGC TTT TG-3′ and R: 5′-CGA ACG TGA CGT CAT C-3′). The level of ERα suppression was analyzed by Western blot using ERα antibodies as described above. Clones with lowest ERα expression as compared to the empty vector transfected clones were selected for tRA induction experiments.

Chromatin immunoprecipitation analysis. Chromatin immunoprecipitation (ChIP) was performed essentially as described by the supplier of the reagents (Santa Cruz Biotechnology; protocol No. 12 on http://www.scbt.com) with the following modifications. MCF-7 cells (150 mm dish) cultured in sf-DMEM were treated with 10 nM E2. Formaldehyde cross-linking (1% (v/v)) was done for 10 min at room temperature. Cross-linking was terminated by the addition of glycine to a final concentration of 125 mM. Cells were scraped and the pellets were resuspended in 6 ml lysis buffer (5 mM Pipes, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, and 1× protease inhibitor cocktail) for 10 min on ice. The cell lysate was washed once with ice-cold PBS, resuspended in 1.9 ml high salt lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1× protease inhibitor cocktail), and sonicated in the ultrasonic processor UP50H (Hielscher Ultra-sonics, Germany) three times, 7 s each at 60% amplitude and a continuous cycle. At this point 100 μl of chromatin solution was removed and labeled "Input." Chromatin solution was precleared by adding 100 μl of protein A–Sepharose 6 MB (Sigma) as 50% slurry containing 0.5 mg/ml BSA, 200 μg/ml sonicated salmon sperm DNA in TE, pH 8.0, for 30 min at 4 °C. Immunoprecipitation was performed at 4 °C for 16 h using anti ERα antibodies and anti FGFR-1 C-15 (Santa Cruz) antibodies (as negative control) as recommended by the supplier, then immunocomplexes were incubated with 100 μl protein A–Sepharose (50% slurry) for 2 h at 4 °C. Afterwards, beads were collected, washed and eluted as recommended in the Santa Cruz protocol. All samples, including the input, were reverse cross-linked by incubating at 65 °C with proteinase K for 16 h, DNA was isolated by phenol/chloroform extraction and ethanol precipitation. Isolated DNA was used for PCR amplification of ERα-precipitated fragments for NIS-ERE (NIS-ChIP-F: 5′-TGG CCT GTC TGT CCC AGT CCA GGG CTG A-3′ and NIS-ChIP-R: 5′-GGG TTG CAG ATT TAT TGG GC-3′). NF1-F: 5′-TGC TAC TCT TTA GCT TCC TAC-3′ and NF1-R: 5′-CCT TAA AAG AAG ACA ATC AGC C-3′ were used as ERE-unrelated control.

Results and discussion

tRA-responsive NIS expression is correlated with the presence of a functional ERα

By immunoblot experiments, we have screened eight different human mammary gland cell lines that we have in our collection for the presence of ERα (Fig. 1A). Cells were cultured in regular DMEM containing 10% FBS (abbreviated as reg-DMEM in this text). In parallel experiments, we either treated them with 1 μM tRA or with vehicle (DMSO) for 12 h before analyzing NIS expression by RT-PCR (Fig. 1C). We have also monitored the expression of pS2, an estrogen-responsive gene widely used as a marker to monitor the functionality of ERα and/or E2 treatments in ERα+ cell lines [8,9]. Expression of pS2 in cells not treated with E2 but cultured in reg-DMEM (Fig. 1B) was probably due to the well-known estrogenic activity of phenol red, a pH indicator dye [10,11]. Close correlation between ERα and pS2 expressions suggested that, as expected, pS2 gene expression was a reliable indicator of ERα activity (Fig. 1A and B). As a result of these analyses, we confirmed that BT-474, T-47D, and MCF-7 were both physically and functionally ERα+ (Fig. 1A). Remaining cell lines such as BT-20, MDA-MB-453, MDA-MB-468, *hTERT*-HME1, and MDA-MB-231 were classified as ERα−. Basal expression (uninduced by tRA) of NIS was detected in all three cell lines with strong ERα positivity (BT-474, T-47D, and MCF-7) and in one ERα− cell line (BT-20). On the other hand, tRA-induced NIS expression was strictly specific to cell lines that were expressing both ERα and RARα, and not to cell lines that were only RARα+ (Fig. 1C). A similar result suggesting a correlation between ERα status of mammary cell lines and 9cRA (a RAR/RXR-specific ligand) induction of NIS gene has also previously been shown [5]. However, in our studies, we have also observed a basal expression of NIS in ERα+ mammary cell lines, as opposed to no detectable expression in ERα− cells (Fig. 1). This suggested that besides playing its indirect role in modulating RAR-dependent regulation [12], ERα also plays a role in regulation or initiation of basal NIS expression (see below). The BT-20 cell line could be considered as an exception, as ERα negativity and lack of basal NIS expression did not correlate (Fig. 1). In previous studies, where human breast tumor samples were analyzed, NIS expression was also seen in ERα− tumors [1,13]. Thus, BT-20 cells might provide a model for ERα− independent NIS gene activation in human breast cancer.

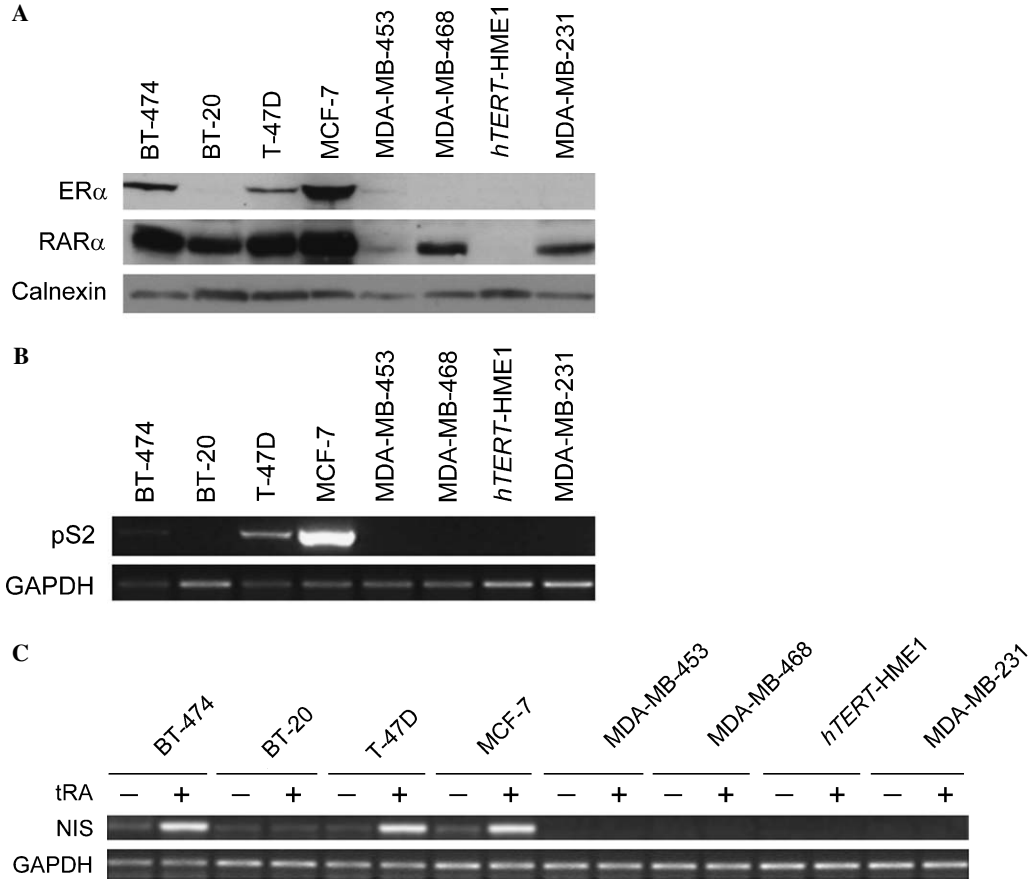

Fig. 1. ERα positivity in mammary gland cell lines is correlated with tRA-responsive NIS expression. (A) Immunoblot analysis of ERα and RARα expression in a variety of mammary gland cell lines, as indicated on top of each lane. Cells were grown in reg-DMEM, total proteins were extracted, and electrophoresed samples were blotted using anti-human-ERα antibody and anti-human-RARα antibody, respectively. Calnexin expression was also monitored with a similar method, and it was used as a gel loading control. (B) RT-PCR analysis of pS2 expression in cell lines grown in reg-DMEM in the absence of E2 or tRA. cDNA was prepared using total RNA isolated from cell lines. Then pS2 and GAPDH specific primers are used in PCR experiments, and accumulation of corresponding gene products was visualized. pS2 is a gene under control of ERα, and its expression was considered as an indicator of ERα activity. (C) Cell lines grown either in the presence (+) or absence (−) of 1μM tRA were collected, and tRA-responsive NIS gene expression was monitored by RT-PCR as described in (B). Amplification of GAPDH gene cDNA was used as an internal control both in (B) and in (C).

Suppression of ERα by shRNA downregulates NIS expression

To determine the functional relevance of ERα in NIS gene regulation, we suppressed endogenous ERα in MCF-7 by RNA interference (RNAi) method. For this, we used two alternative small hairpin RNA (shRNA) probes targeting different regions of ERα mRNA (sh-ER499 and sh-ER458; see Materials and methods). Cells were stably transfected either with empty vectors (pSuper-*GFP*/*Neo*, OligoEngine, WA) carrying the GFP and the neomycin resistance (Neo^R) marker genes, or with similar vectors carrying shRNA N-19 target sequences in addition to these two markers. Then, colonies originated from transfected cells that were both resistant to neomycin and that were green fluorescing were isolated and cultured separately. By Western blots, we monitored the level of ERα suppression in a number of different cell colonies expressing sh-ER458 as compared to colonies transfected with empty pSR vector (Fig. 2A). We then selected colonies with significant suppression for further studies (such as colonies 458-12 and 458-13, Fig. 2A). We also noticed that one of the two shRNAs was more potent (sh-ER458) in suppressing the endogenous ERα gene as compared to the other one (sh-ER499, results not shown). Subsequently, we treated these ERα suppressed MCF-7 colonies either with tRA or with vehicle (DMSO), and analyzed both tRA induced and basal NIS expression (Fig. 2B). In these studies, for both 458-12 and 458-13, we observed about 60% decrease in basal and about 45% decrease in tRA induced expression of NIS as compared to empty vector transfected controls (Fig. 2B); results indicating that ERα plays a role in both basal and tRA induced NIS gene expression. The partial decrease at NIS mRNA levels in response to severe downregulation of ERα may indicate redundant functions between ERα and other factors such as RARs and Nkx2.5 regulating this gene [3,6]. Interestingly, previous studies have shown that in a rat thyroid cell line model, FRTL-5, activation of ER pathway by E2 downregulates thyroid NIS gene expression [3,14]. This suggests that com-

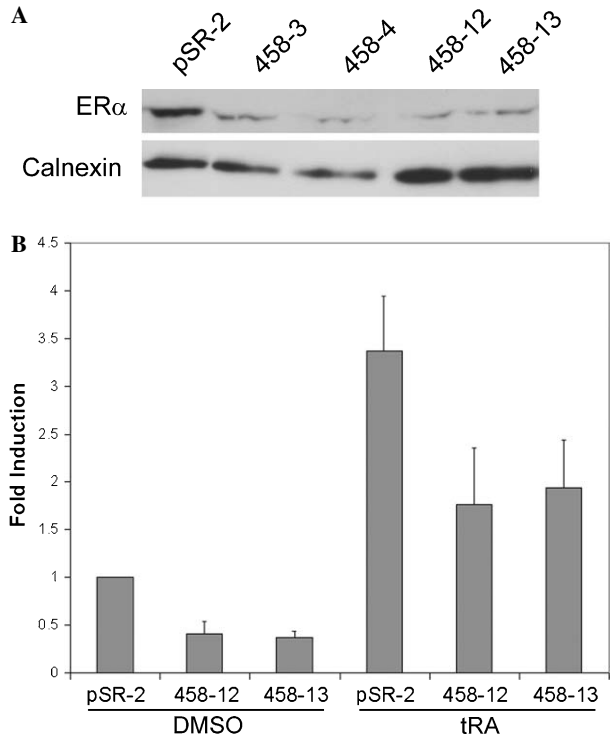

Fig. 2. Suppression of ERα by shRNA downregulates NIS expression in MCF-7 cells. MCF-7 cells were transfected either with empty vector pSR, with pSR-ER-458, or with pSR-ER499. Following a double selection procedure based on Geneticin (0.5 mg/ml) resistance and EGFP expression, clones were isolated and further analyzed. (A) A representative Western blot result showing the effect of sh-ER458 on the levels of ERα in four individual clones compared to an empty vector clone pSR-2. Clones 458-12 and 458-13 showed significant ERα suppression and were selected for tRA induction. (B) Clones 458-12 and 458-13 were grown in reg-DMEM and treated with 1μM tRA or with DMSO (5 μl in 10 ml culture medium) for 12 h. After RNA isolation, cDNA was prepared using 2 μg total RNA and subsequently used as a template for semi-quantitative RT-PCR analysis using NIS specific primers. Data represent the fold induction (average of four independent experiments) of NIS in 458-12 and 458-13 clones normalized to GAPDH control, and relative to the empty vector pSR.

mon molecular elements may exert opposite regulatory effects on NIS gene expression in thyroid and in the mammary gland. As pregnancy is a physiological state which is associated with increased needs for thyroid hormone (TH) synthesis [15], a potential reduction of the iodide available for TH synthesis of the mother could explain increased hypothyroidism cases in pregnancy and during lactation [16]. On the other hand, above-described regulatory actions of ERα on NIS gene expression might also provide an additional possible explanation to increased hypothyroidism cases in pregnancy and lactation when I^- uptake substantially increases in mammary glands [1].

Ectopic ERα expression in MDA-MB-231 upregulates NIS expression

MDA-MB-231 cell line expresses RARα, a major component of tRA signaling mechanism as detected by immunoblots using anti-human RARα antibodies (Fig. 1A). Furthermore, these cells respond to tRA, as assessed by the RAR controlled RIP140 gene expression ([17]; and results not shown). However, although RA signaling pathway is intact, MDA-MB-231 cells do not express NIS in response to either tRA or any other ligand known to induce NIS in other cell systems ([3]; and data not shown). Because tRA-responsive NIS expression was only detected in both ERα+ and RARα+ cell lines (Fig. 1C), we investigated whether introduction of human ERα gene could restore tRA-responsiveness of NIS expression in MDA-MB-231. For this purpose, we first transiently introduced an ERα expression vector (pCMV-ERα) to this ERα– mammary cell line and studied ligand-responsive NIS expression. Note that, in order to precisely control the concentration of supplemented steroids and other ligands, cells were cultured in phenol-red free DMEM supplemented with dextran-coated-charcoal treated steroid-free FBS (abbreviated as sf-DMEM). Transfected cells expressed ERα at levels comparable to those in MCF-7 cells, and the receptor was functional as assessed by the increase in pS2 expression levels in response to E2 (Fig. 3A). Interestingly, introduction of ERα significantly increased NIS gene expression, although it did not restore tRA-responsiveness of this gene. We noticed that the ERα-activated NIS expression in MDA-MB-231 was even higher than the basal levels in the ERα+ cell model, MCF-7. On the other hand, when compared to tRA induced levels of NIS expression in MCF-7 cells, this ERα-activated expression of NIS was about three times lower (Fig. 3A). A dose–response curve established using MCF-7 (and MDA-66, see below) cells has indicated that, as also reported by others, a concentration of 10 nM E2 was the optimal ligand concentration to be used for the highest level of pS2 gene induction in mammary cells cultured in vitro (results not shown, see [18,19]). Therefore, whenever we studied the effects of E2 we have added this ligand to cell culture medium at a 10 nM concentration. Treatment of ERα transfected cells with E2 (10 nM), tRA (1 μM), or E2 together with tRA did not lead to a further increase in this ERα activated NIS expression (Fig. 3A). In order to further confirm these results, we also used a genetically modified MDA-MB-231 cell line that was stably transfected with a vector expressing human ERα gene [19]. First, we confirmed ERα and RARα status of this cell line named as MDA-66 (Fig. 3B). Then, we analyzed functionality of these receptors by assessing modulations in expressions of tRA- and E2-responsive genes such as RARα, pS2, and RIP140 (Fig. 3B and C, and results not shown, respectively). As expected, an E2-responsive upregulation of RARα [12] was clearly observed in MDA-66. This result was an additional evidence indicating that in these cells E2 signaling mechanism was intact (Fig. 3B). Subsequently, we studied both the basal expression and E2/tRA responses of NIS gene in these cells (Fig. 3C). In accordance with the results obtained in MDA-MB-231 cells that were transiently transfected with ERα, in

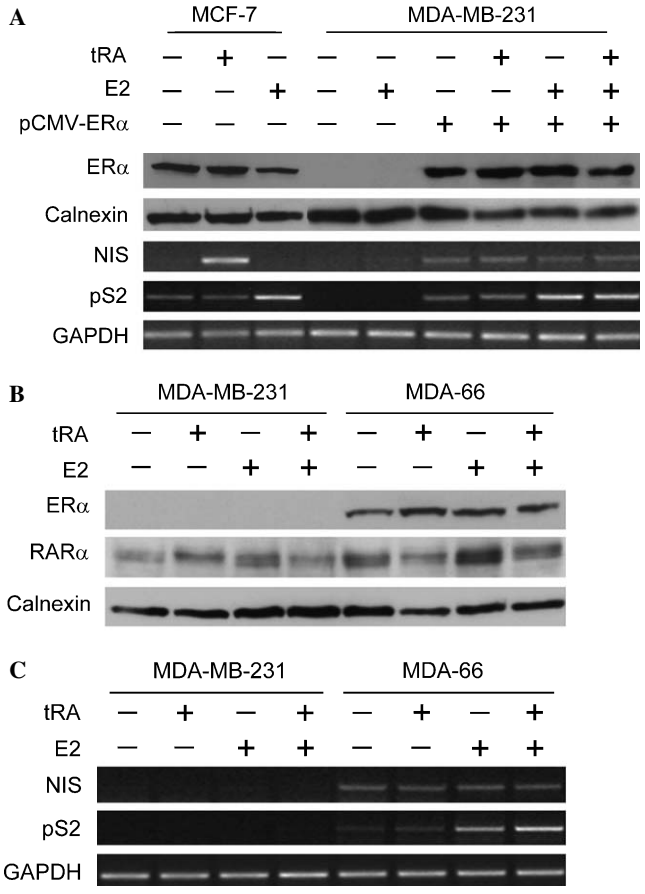

Fig. 3. Transient and stable expression of ERα in MDA-MB-231 cells leads to a higher basal expression of NIS in a ligand-independent manner. (A) MDA-MB-231 cells were transiently transfected with a plasmid vector expressing ERα under control of the CMV promoter [lanes pCMV-ERα(+)] in sf-DMEM. Forty-eight hours later cells were treated with 10 nM E2 (3 h), 1 μM tRA (12 h), or 10 nM E2 together with 1 μM tRA (12 h). Then, cells were harvested, divided to two, and one-half was used for extracting total proteins, and the other half was used for RNA extractions. ERα expression status in transfected cells was compared with those in MCF-7 cells by immunoblots using anti-human-ERα specific antibodies. Calnexin expression was used as a loading control. NIS and pS2 expressions in transfected cells and in MCF-7 cells in response to ligand treatments were assessed by RT-PCR. The ERα-responsive pS2 gene was used to monitor functionality of E2 and ERα. GAPDH expression was used to monitor the efficiency of the RT-PCR method, as an internal control. (B) MDA-MB-231 cells stably transfected with hERα expressing vector (named MDA-66), as well as untransfected cells were cultured in sf-DMEM in presence of 10 nM E2, 1 μM tRA, or both ligands. Then, they were harvested, and cell pellets were collected for immunoblot analysis and RT-PCR analysis (B). Total proteins were extracted from pellets obtained from ligand treated (as indicated with "+" signs on each lane) and untreated cells, then electrophoresed and blotted to immunoblot membrane. Subsequently, the membrane was treated with anti-human-ERα, anti-human-RARα, and anti-human-calnexin antibodies, respectively. Calnexin expression was used as loading control. (C) Total RNA was extracted from pellets collected as above, and total cDNA was prepared and submitted to the RT-PCR analysis using NIS, pS2, and GAPDH gene specific primers. GAPDH expression was used as an internal control.

MDA-66 cells the expression of NIS was remarkably increased, and it was not responsive to treatments with E2, tRA, or E2 together with tRA (Fig. 3C).

Taken together, these results indicated that ERα activity together with the intrinsic tRA-responsiveness was not sufficient for tRA responsive NIS expression in this ERα− mammary cell model (Fig. 3). Concerning the differences between cell lines, we must point out that, when compared to MCF-7, MDA-MB-231 (or MDA-66) cells are less differentiated, and it is known that the differentiation stage of breast cancer cell lines strongly affects the transcriptional activity of nuclear receptors [18]. In parallel to this, our results suggest that in addition to functionally expressed RARα and ERα, (an)other so far unidentified factor(s) are (is) essential for ligand-responsive expression of this gene in MDA-MB-231. On the other hand, in ovariectomized animals, even when administered alone, E2 was shown to significantly upregulate NIS expression [1]. This indicated that, additional factors that are present in hormonal and cellular microenvironment of mammary gland cells were needed for E2 responsive expression of NIS in isolated cells cultured in vitro.

Our results demonstrate a role of unliganded ERα (or apo-ERα) in regulating expression of NIS gene (Fig. 3). In general, unliganded nuclear receptors are considered to be transcriptionally unproductive or even repressive. Upon ligand binding, receptor-associated co-repressors are exchanged for co-activators, resulting in the activation of transcription. However, a growing body of evidence indicates that this model is too simple and not adequate to explain the dynamic pattern of transcriptional regulation [20]. It has previously been shown that liganded ERα is able to interact with a selective repressor protein illustrating an unpredictable mode of action for liganded receptors [21]. On the other hand, in a recent report, the activator function of apo-ERα was also demonstrated [7]. In this report, it was shown that apo-ERα recruits several histone acetyl transferases and a histone methyl transferase, destabilizing nucleosomes positioned around apo-ERα binding site in pS2 gene promoter region. An important suggestion of data gathered by Métivier et al. [7] was that apo-ERα, by binding to its cognate sequences, induces a chromatin environment that is permissive for transcription to occur. It is conceivable that similar mechanisms operate on NIS gene promoter, and apo-ERα is essential for holding the NIS gene at a transcriptionally competent state. This would suggest the absence of transcriptionally competent NIS gene loci in ERα− mammary cell lines, and thus explain the lack of both uninduced and tRA-responsive NIS expression in these cells (Fig. 1C).

Identification of a novel, non-canonical ERE in NIS promoter region

To evaluate the possibility of a direct regulation of NIS gene by ERα, we have first carried out an in silico analysis using the Dragon ERE finder program [22]. We searched for possible EREs in a 3 kb region upstream of the transcription start site in human NIS. As a result of this analysis we identified only one putative ERE sequence

(Fig. 4A), albeit it was a novel sequence which was not previously described as an ERE [22]. This putative ERE (5′-CG-GGTCA-CCG-CGACT-CC-3′) was located 9 bp upstream of NIS TATA element (Fig. 4A). This new element had the characteristic head-to-head inverted repeat sequences with high homology to the ERE consensus, and it was similar to previously established EREs (Fig. 4B). Significantly, this putative NIS ERE sequence and its position vis-à-vis TATA element was also conserved in rat and mouse genomes (Fig. 4C). In order to establish the transcriptional activation potential of this novel element in response to E2, we have constructed a reporter vector containing two tandem copies of putative NIS ERE sequence; followed by the E1b TATA element and the luciferase reporter gene, pNIS2XERE (see Materials and methods). We also constructed a similar vector containing two copies of ERE sequence which was previously shown to bind ERα and lead to E2-responsive upregulation of the pS2 gene, and named this vector as pPS2XERE. We then transiently transfected MCF-7, MDA-MB-231, and MDA-66 mammary cell lines with these reporter vectors and studied E2-dependent luciferase activity. In these experiments, both pS2 ERE and putative NIS ERE showed significantly activated luciferase expression in response to E2 in both MCF-7 and in MDA-66 (Fig. 5A). About 5-fold stimulation by NIS ERE was obtained in MCF-7 cells in response to E2 treatment, whereas, under same conditions, pS2 ERE stimulated reporter gene expression about 3-fold. A similar result is obtained in MDA-66 cells, where NIS ERE-stimulated expression of the reporter was 2.4-fold, whereas pS2 ERE-dependent stimulation was 3.4-fold. We also noticed that, in MCF-7 cells, in terms of potency, NIS ERE-driven reporter gene expression was five times stronger than that of pS2. These results indicated that NIS ERE has the potential to mediate E2-dependent transcription. In fact, such a close localization of TATA and ERE elements is very unusual considering that all previously characterized ERE elements were shown to be localized at relatively distant positions to transcription start sites in corresponding genes (although varying remarkably between +23,088 and −2687 [23]). However, it has also been known for long time that the response element preferences and DNA binding properties of nuclear receptors cannot be simply attributed to classical spacing,

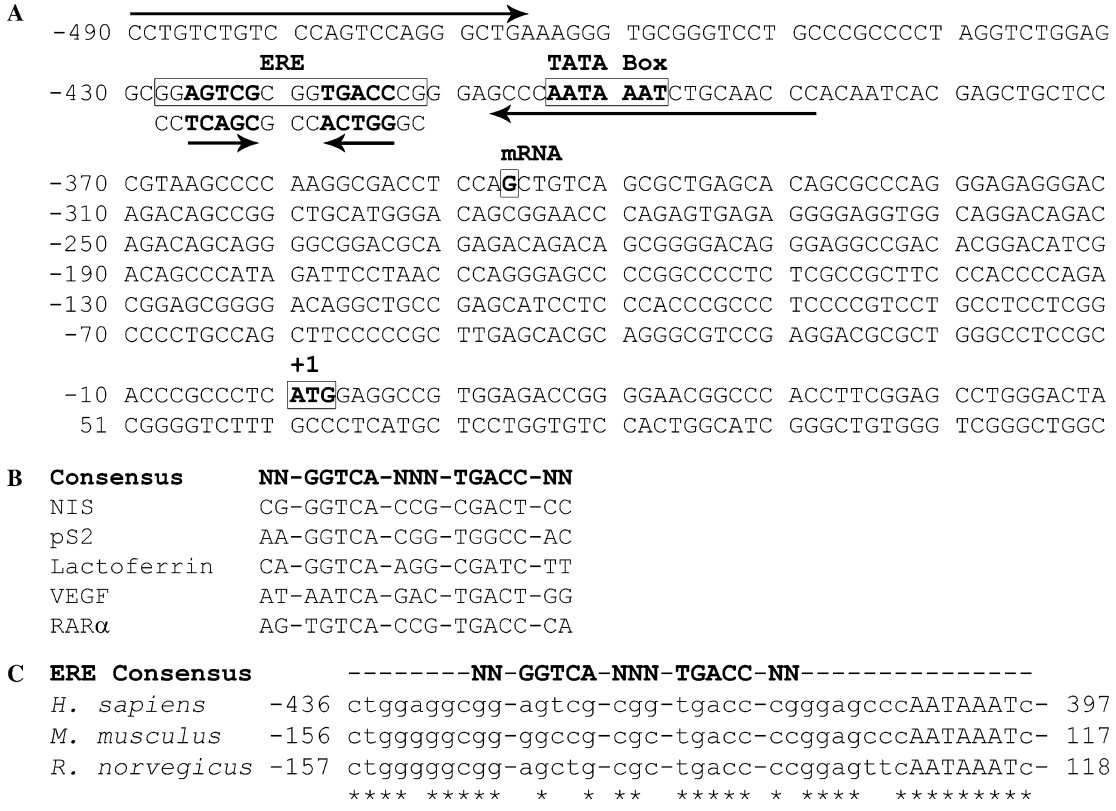

Fig. 4. A putative novel ERE sequence is located in close proximity of NIS TATA box element in the promoter region. (A) Human NIS gene proximal promoter region sequence is shown. The first codon (ATG) of NIS, transcription start site, TATA box element, and novel ERE sequences are indicated in boxes. Two inverted repeats of the ERE element located at the minus strand are shown by short inverted arrows. The position of primers used for the PCR amplification described in Fig. 5B is also indicated by long arrows. (B) Comparison of previously established ERE sequences that were found in several ER regulated genes, the putative NIS ERE sequence, and the consensus sequence. (C) Comparison of NIS putative ERE sequence in human, mouse, and rat genomes. Putative NIS EREs that were identified in all three genomes located in close proximity of TATA box elements, and they fit to functional ERE consensus sequence. NIS gene TATA element region is indicated by uppercase letters. (*) Signs indicate identical bases in human, mouse and rat sequences.

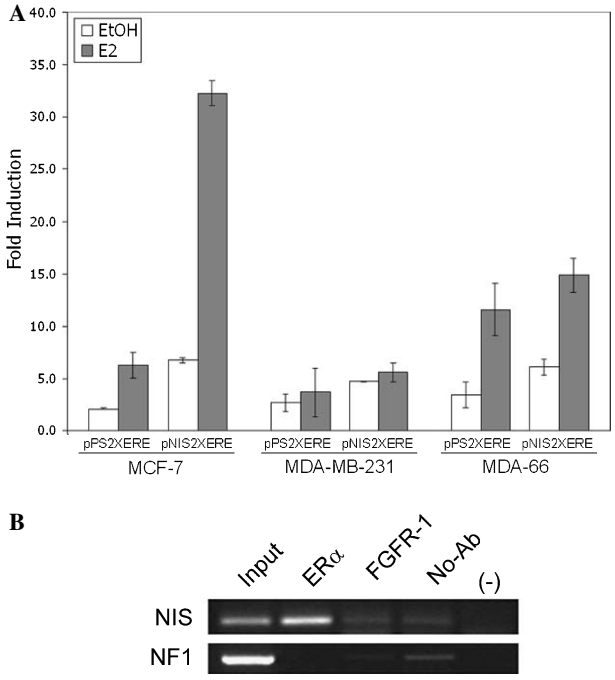

Fig. 5. Direct functional interaction between ERα and the NIS ERE sequence. (A) MCF-7, MDA-MB-231, and MDA-66 cells were transiently transfected with reporter vectors (pGL3 based) containing the luciferase gene under control of E1b TATA element and two tandem repeats of either pS2 gene ERE sequence (pPS2XERE) or NIS gene ERE sequence (pNIS2XERE). Transfected cells were treated either with 10 nM E2 (3 h) or with vehicle (ethanol; 10 μl in 10 ml culture medium). Then, luciferase activities were measured, and they were corrected using *Renilla* (phRL-TK) transfection efficiency control. Fold induction was calculated by normalizing luciferase values with those obtained from the empty vector. Data represent the average of four independent experiments. (B) MCF-7 cells grown in sf-DMEM and treated with 10 nM E2 were used for ChIP analysis using ERα specific antibody. DNA isolated from immunocomplexes was used as a template for PCR amplification using NIS promoter specific primers (indicated as long arrows in Fig. 4A), or unrelated intronic primers corresponding to *NF1* gene exon 22. Lanes: input, the input DNA used for ChIP analysis; ERα, estrogen receptor-α precipitated DNA; FGFR-1, fibroblast growth factor receptor-1 precipitated DNA; No-Ab, DNA precipitated with protein A–Sepharose beads only (background control); and (−), negative PCR without template DNA.

localization or orientation rules [24]. The significant difference in magnitude of gene activation by NIS ERE in MCF-7 and MDA-66 cell lines may reflect differences in these two cell lines in terms of molecular components associated with ER activity. As expected, in our ERα− cell model, MDA-MB-231, neither NIS ERE, nor pS2 ERE has led to E2-dependent regulation of the luciferase reporter gene (Fig. 5A). We concluded that this non-canonical ERE sequence located in NIS promoter can potentially act as a *cis*-acting element and respond to E2 in a proper cellular context.

To establish whether endogenous ERα can occupy the novel ERE in NIS promoter in vivo, we carried out ChIP experiments in MCF-7 cells. In the presence of ERα antibodies, the NIS promoter was precipitated from formaldehyde cross-linked total cell lysates (Fig. 5B, lane 2). In contrast, neither control Fibroblast Growth Factor receptor (FGFR-1) antibodies, nor antibody uncoated protein-A–Sepharose beads precipitated the NIS promoter above background levels (Fig 5B, lanes 3 and 4, respectively). As expected, ERα antibody was unable to precipitate an unrelated DNA fragment corresponding to NF1 gene exon 22 (Fig. 5B, lane 2). These data therefore demonstrate that in MCF-7 cells endogenous ERα binds to the NIS gene promoter in vivo, thereby suggesting that at least part of the regulatory effects of ERα on NIS expression were due to a direct interaction between the receptor and NIS promoter (Fig. 5B). Taken together with E2-responsive transcriptional activation of luciferase reporter via this ERE sequence in transfected MCF-7 cells, our ChIP results provide very strong evidence in support of the functionality of this site in vivo. These results indicate that apo-ERα and tRA-activated factors functionally interact in NIS regulation in breast cancer cell models such as MCF-7 and MDA-66.

In mammary gland physiology, transport of I^- via NIS is observed after mid-gestation and during lactation [1]. Therefore, expression of NIS should be considered as one of the latest events in mammary gland development because it takes place in fully differentiated mammary epithelial lactocytes. So far, transcriptional molecular elements and ligands that were hitherto shown to regulate NIS expression were identified in studies that were either carried out in experimental animals or in a rather differentiated mammary cell line, MCF-7 [1,3,5,6,25–31]. Related with this, a particularity of our study is that we used a dedifferentiated tumor cell line such as MDA-MB-231 [18] in establishing the role of apo-ERα as a factor that activates NIS expression. Therefore, to our opinion, a less obvious but interesting implication of our data is the very early role of apo-ERα in activation of NIS transcription. Close localization of ERE and the TATA element in NIS promoter (Fig. 4) might provide an additional hint towards this possible early role of apo-ERα in initiating transcription in this promoter context. Future work will be needed for accurately establishing the position of apo-ERα in the sequence of molecular interactions leading to NIS regulation in mammary gland lactocytes.

Radioiodide ($^{131}I^-$ or $^{123}I^-$) and pertechnetate ($^{99m}TcO_4^-$) transport activity of NIS has successfully been used in detection, treatment, and follow-up of thyroid cancers [32]. In addition, the upregulatory effect of tRA on thyroid NIS expression was also previously established, and several clinical trials assessing RA redifferentiation therapy in dedifferentiated thyroid tumors and their metastasis were also previously started [33–35]. However, the potential of similar methods based on NIS activity in breast tumor cells still remains to be fully assessed. To our opinion, establishing molecular determinants, mechanisms, and ligands that have a role in NIS regulation is essential for successful implementation of possible NIS activity based novel methods for the management of malignant breast diseases.

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