Enhancement of sodium/iodide symporter expression in thyroid and breast cancer

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Abstract

The sodium/iodide symporter (NIS) mediates iodide uptake in the thyroid gland and lactating breast. NIS mRNA and protein expression are detected in most thyroid cancer specimens, although functional iodide uptake is usually reduced resulting in the characteristic finding of a 'cold' or non-functioning lesion on a radioiodine image. Iodide uptake after thyroid stimulating hormone (TSH) stimulation, however, is sufficient in most differentiated thyroid cancer to utilize β -emitting radioactive iodide for the treatment of residual and metastatic disease. Elevated serum TSH, achieved by thyroid hormone withdrawal in athyreotic patients or after recombinant human thyrotropin administration, directly stimulates NIS gene expression and/or NIS trafficking to the plasma membrane, increasing radioiodide uptake. Approximately 10–20% differentiated thyroid cancers, however, do not express the NIS gene despite TSH stimulation. These tumors are generally associated with a poor prognosis. Reduced NIS gene expression in thyroid cancer is likely due in part, to impaired trans-activation at the proximal promoter and/or the upstream enhancer. Basal NIS gene expression is detected in about 80% breast cancer specimens, but the fraction with functional iodide transport is relatively low. Lactogenic hormones and various nuclear hormone receptor ligands increase iodide uptake in breast cancer cells *in vitro*, but TSH has no effect. A wide range of 'differentiation' agents have been utilized to stimulate NIS expression in thyroid and breast cancer using *in vitro* and *in vivo* models, and a few have been used in clinical studies. Retinoic acid has been used to stimulate NIS expression in both thyroid and breast cancer. There are similarities and differences in NIS gene regulation and expression in thyroid and breast cancer. The various agents used to enhance NIS expression in thyroid and breast cancer will be reviewed with a focus on the mechanism of action. Agents that promote tumor differentiation, or directly stimulate NIS gene expression, may result in iodine concentration in 'scan-negative' thyroid cancer and some breast cancer.

Endocrine-Related Cancer (2006) 13 797–826

Introduction

The thyroid contains 70–90% total iodine in the body (9–10 mg; Riggs 1952). The thyroid gland must trap about 60 μ g iodine/day from the circulation to maintain adequate thyroid hormone production. The sodium/iodide symporter (NIS, or SLC5A5 on the NCBI database; <http://www.ncbi.nlm.nih.gov/>) is expressed on the basolateral membrane of thyroid follicular cells and mediates the accumulation of iodide from the bloodstream to thyroid follicles (Dai *et al.* 1996). NIS is a membrane-bound glycoprotein with 13 trans-membrane domains and belongs to the sodium/solute symporter family (Dohan *et al.* 2003). In normal

thyroid tissue, NIS transports two Na^+ and one I^- down the Na^+ ion gradient generated from the activity of Na^+/K^+ ATPase (Fig. 1). NIS actively transports iodide producing an iodine concentration gradient from the thyroid cell to extracellular fluid greater than 30:1. Ouabain, which inhibits the Na^+/K^+ ATPase, blocks thyroidal iodide uptake (Eggo *et al.* 1986, Carrasco 1993). Trapped iodide in the follicular cells is released into the lumen through the apical iodide transporter (AIT or SLC5A8; Rodriguez *et al.* 2002) and pendrin (SLC26A4, the product of *Pendred's syndrome* gene; Royaux *et al.* 2000, Yoshida *et al.* 2004, Fig. 1). Thyroglobulin (Tg) is localized at the apical membrane

Figure 1 Schematic representation of iodide transport in the thyroid gland.

outside the follicular cells, where internal tyrosyl residues are iodinated by thyroid peroxidase (TPO). The thyroid oxidases (ThOX1 and ThOX2; De Deken *et al.* 2000) produce oxidative conditions by generation of H_2O_2 , and are required for the normal function of TPO. Since iodide is bound to an organic compound, this process is known as ‘organification’ of iodide. Thyrotropin (TSH) increases the expression of genes involved in thyroid iodide metabolism and thyroid hormone synthesis, including NIS, Tg, and TPO (Dunn & Dunn 2001).

Radioiodide is widely used for the diagnosis and treatment of thyroid disease. Radioiodide is administered orally, absorbed in the stomach and small intestine, and concentrated in thyroid tissues through the action of NIS. Thyroid tissues in benign disease, such as Graves’ hyperthyroidism and colloid goiter, and in some differentiated thyroid cancer, organify the trapped radioiodide with Tg, prolonging the biological half-life of the radioiodide. Short half-life, low-energy isotopes, such as ^{123}I and technetium-pertechnetate ($^{99\text{m}}\text{TcO}_4^-$), are used to image functional thyroid tissue. Longer half-life, high-energy isotopes like ^{131}I are used therapeutically to destroy thyroid tissue in both hyperthyroidism and metastatic thyroid cancer after total thyroidectomy. TSH, a pituitary glycoprotein hormone, induces NIS expression in the thyroid (Kogai *et al.* 1997, Saito *et al.* 1997, Pekary & Hershman 1998). The influence of TSH on the stimulation of radioiodide uptake in metastatic thyroid cancer has been recognized, since radioiodine was first used in the 1950s. TSH stimulation is achieved by the cessation of thyroid supplementation after thyroidectomy or

exogenous administration of recombinant human TSH (rhTSH). More than 70% differentiated thyroid cancer concentrates radioiodine after TSH stimulation (Robbins *et al.* 1991, Schmutzler & Koehle 2000, Jarzab *et al.* 2003). Radioiodide treatment may fully destroy occult micro-carcinoma and is associated with a reduction in the rate of progression of metastatic tumor (Hershman *et al.* 1995, Schlumberger 1998). Extensive experience with ^{131}I treatment of thyroid cancer has demonstrated the importance of maximizing the magnitude of iodide uptake and prolonging the period of radioiodine tumor residence.

Most differentiated thyroid cancer has an excellent prognosis; survival rates are 93–98% in papillary cancer, and 85–92% in follicular cancer (Gilliland *et al.* 1997, Dean & Hay 2000). Many metastatic-differentiated thyroid carcinomas, as well as undifferentiated anaplastic cancer, however, do not concentrate sufficient ^{131}I for therapy (Maxon & Smith 1990). In these cases, other therapeutic options, such as surgical removal of metastases (Niederle *et al.* 1986), external radiation (Kim & Leeper 1983, Tubiana *et al.* 1985), or chemotherapy (Kim & Leeper 1983, Shimaoka *et al.* 1985, Ain *et al.* 1996), are utilized, but are largely unsuccessful (Tyler *et al.* 2000). The correlation between NIS expression in thyroid tumors and their ability to concentrate radioiodine has been confirmed (Caillou *et al.* 1998, Castro *et al.* 2001). NIS mRNA expression in papillary thyroid cancer with a poor prognosis is markedly decreased (Ward *et al.* 2003). The regulation of NIS expression in normal and malignant thyroid cells has been investigated extensively and the various agents that have been recognized to influence expression are summarized (Table 1).

NIS is expressed in extrathyroidal tissues, including; salivary gland, gastric mucosa, mammary gland, ciliary body of the eye, and the choroid plexus (Dohan *et al.* 2003). Modest iodide uptake is usually detected in the salivary gland, stomach, and intestines during whole body imaging with radioiodide. Lactating breast tissue concentrates a significant amount of iodide as a result of stimulation of NIS expression (Cho *et al.* 2000, Tazebay *et al.* 2000). The trapped iodide is secreted in milk and provides iodine for thyroid hormone synthesis to the developing infant (Welch & Mankoff 2000). The lactating breast can concentrate iodide to a similar degree as that seen in the thyroid, producing milk with an iodine concentration of 20–700 $\mu\text{g/l}$ (Simon *et al.* 2002b). NIS expression has been demonstrated in more than 80% breast cancer tissue, although the fraction of tumors that functionally concentrate iodine is likely to be much lower (Tazebay *et al.* 2000, Wapnir *et al.* 2003).

Table 1 NIS expression stimulator in thyroid

Agent	Pharmacology	Experimental system	Iodide uptake	NIS mRNA	NIS protein	Reference
Thyrotropin (TSH)	TSHR agonist	FRTL-5 rat cell line	X	X	X	Kogai <i>et al.</i> (1997)
		PCCI3 rat cell line	X	X		Trapasso <i>et al.</i> (1999)
		WRT rat cell line			X	Cass & Meinkoth (2000)
		Primary human thyroid cell culture (normal)	X	X	X	Saito <i>et al.</i> (1997), Ajjan <i>et al.</i> (1998)
		KAT50 human cell line	X	X		Venkataraman <i>et al.</i> (1998)
		Long-term culture of human thyroid (normal)	X	X	X	Kogai <i>et al.</i> (2000b)
hCG	TSHR agonist	Rat normal thyroid, <i>in vivo</i>			X	Levy <i>et al.</i> (1997)
		FRTL-5 rat cell line	X	X	X	Arturi <i>et al.</i> (2002)
Forskolin	Adenylyl cyclase activator	FRTL-5 rat cell line	X	X	X	Kogai <i>et al.</i> (1997)
		PCCI3 rat cell line		X		Trapasso <i>et al.</i> (1999)
		Primary human thyroid cell culture (normal)	X	X	X	Saito <i>et al.</i> (1997)
Adenosine	A ₁ receptor agonist	FRTL-5 rat cell line	X	X	X	Harii <i>et al.</i> (1999)
		Retinoic acid receptor (RAR) agonist		X		Schmutzler <i>et al.</i> (1997)
Troglitazone	PPAR- γ ligand	FTC-133, follicular cancer cell line		X		Park <i>et al.</i> (2005)
		TPC-1, papillary cancer cell line		X		
Depsipeptide	Histone deacetylase (HDAC) inhibitor	FTC-133 and FTC-236, follicular cancer cell lines	X	X		Kitazono <i>et al.</i> (2001)
		SW-1736 and KAT-4, anaplastic cancer cell lines	X	X		
		BHP 18-21v, papillary cancer cell line	X	X	X	Furuya <i>et al.</i> (2004b)
		BHP18-21v xenograft <i>in vivo</i>	X			
Trichostatin A	HDAC inhibitor	ARO, anaplastic cancer cell line	X	X	X	Zarnegar <i>et al.</i> (2002)
		TPC-1, papillary cancer cell lines		X		
		FTC-133, follicular cancer cell line		X		
		XTC-1, Hurthle-cell cancer cell line		X		Furuya <i>et al.</i> (2004b)
		BHP18-21v, papillary cancer cell line		X	X	
		ARO, anaplastic cancer cell line		X	X	
Valproic acid	HDAC inhibitor	NPA, papillary cancer cell line	X	X	X	Fortunati <i>et al.</i> (2004)
		ARO, anaplastic cancer cell line		X	X	
5-Azacytidine	Demethylation agent	NPA, KAT5, KAT10, papillary cancer cell lines	X	X		Venkataraman <i>et al.</i> (1999)
Clinical use						
hrTSH		Metastatic/recurrent cancer (50–90%)	X			Ladenson <i>et al.</i> (1997), Jarzab <i>et al.</i> (2003)
13- <i>Cis</i> RA	Pro-drug of α RA	Metastatic/recurrent cancer (0–42%)	X			Simon <i>et al.</i> (2002a)

X in column indicates significant induction by agent. TSHR, thyroid stimulating hormone receptor.

Uptake of ^{125}I or $^{99\text{m}}\text{Tc}$ in breast cancer has been confirmed by imaging studies (Moon *et al.* 2001, Upadhyay *et al.* 2003, Wapnir *et al.* 2004). The NIS in breast cancer cells can be induced *in vitro* by lactogenic hormones (Cho *et al.* 2000, Arturi *et al.* 2005), insulin (Arturi *et al.* 2005), and some nuclear receptor ligands, such as retinoids, peroxisome proliferator-activated receptor- γ (PPAR- γ) ligands, and glucocorticoids (Kogai *et al.* 2000b, 2005, Tanosaki *et al.* 2003, Table 2). The induction of iodide uptake and NIS expression has been confirmed in some mouse breast cancer models (Kogai *et al.* 2004). Selective cytotoxicity of ^{131}I has been demonstrated after *in vitro* retinoid treatment (Kogai *et al.* 2000b, 2005). Agents that stimulate NIS expression in breast cancer sufficient to concentrate radioiodide have been considered as a source of potential therapy for some differentiated breast cancer (Daniels & Haber 2000, Boelaert & Franklyn 2003).

In this review, we will describe the recent findings describing the mechanism of NIS gene regulation in normal and malignant, thyroid and breast. Agents utilized to directly stimulate NIS gene expression or promote differentiation in thyroid and breast cancer will be discussed. The rapidly expanding field of NIS as a gene therapy tool targeted to a range of malignancies has been previously reviewed (Spitzweg & Morris 2002), and will be discussed only briefly. The review will focus on agents that promote endogenous NIS expression.

TSH regulation of NIS and other iodide transporters in normal thyroid tissue

The thyroid follicle has a two-step process of iodide transport to accumulate iodide into the lumen, and three transporters, NIS, AIT (SLC5A8; Rodriguez *et al.* 2002), and pendrin (SLC26A4; Royaux *et al.* 2000), mediate the process. NIS on the basolateral membrane of thyroid follicular cells mediates iodide accumulation into the cells from the bloodstream. AIT and pendrin are expressed on the apical membrane (Bidart *et al.* 2000, Royaux *et al.* 2000, Rodriguez *et al.* 2002), and transport the trapped iodide to the lumen (Yoshida *et al.* 2002, 2004). NIS has a very high affinity for iodide (K_m 20–40 μM ; Weiss *et al.* 1984b, Mandell *et al.* 1999, Kogai *et al.* 2000b), so that thyroid cells can concentrate iodide from the bloodstream up to 2 mM (Yoshida *et al.* 2002). Pendrin requires a relatively high concentration of iodide (more than 1 mM) in the cytoplasm to function as an iodide transporter (Yoshida *et al.* 2004). Iodide efflux by pendrin to the lumen, therefore, likely depends on functional NIS expression.

TSH stimulation of NIS mRNA and protein expression are mediated by the cAMP pathway in rodent cell lines, Fisher rat thyroid cell line (FRTL-5 cells (Weiss *et al.* 1984a, Kogai *et al.* 1997), PC Cl3 immortalized rat thyroid cells (Trapasso *et al.* 1999), and human primary thyroid cells (Saito *et al.* 1997, Kogai *et al.* 2000a). The upregulation of NIS in response to TSH is at both the transcriptional and the post-translational levels. TSH stimulates the NIS promoter and NIS upstream enhancer (NUE; Endo *et al.* 1997, Ohmori *et al.* 1998, Ohno *et al.* 1999, Taki *et al.* 2002), increases the half-life of the NIS protein, and stimulates the trafficking of the NIS to the plasma membrane (Riedel *et al.* 2001). Direct stimulation of the TSH receptor in Graves' disease by antibody (Saito *et al.* 1997, Caillou *et al.* 1998, Jhiang *et al.* 1998, Castro *et al.* 1999, Lazar *et al.* 1999), constitutive activating mutations of the TSH receptor in a hyperfunctioning thyroid adenoma (Mian *et al.* 2001), or the weak agonist human chorionic gonadotropin (hCG; Hershman 1999, Arturi *et al.* 2002), activate the cAMP pathway and result in marked NIS expression. In contrast, the expression of AIT is not increased in hyperfunctioning thyroid adenomas or Graves' disease (Lacroix *et al.* 2004, Porra *et al.* 2005). The expression of pendrin protein is increased in hyperfunctioning thyroid tissues, possibly due to post-transcriptional upregulation by TSHR signaling (Bidart *et al.* 2000, Mian *et al.* 2001). Iodide transport in the thyroid gland is regulated by TSH, primarily through the stimulation of NIS expression.

Transcriptional regulation of NIS by TSH in normal thyroid cells

Recent progress from sequencing of human and rat genomes has allowed comparison of the NIS coding and regulatory gene sequence from various species. Since TSH stimulates NIS expression in both human and rodent thyroid cells, the regulatory region(s) for NIS induction by TSH was expected to be in sequences common to human and rat NIS genes. The human NIS gene maps to 19p13.2-p12 (Smanik *et al.* 1997) and contains 14 introns in 22 116 bases as measured from the first to the last exon. The rat NIS gene is markedly smaller in size compared with human (9260 bases), although the number of exons is the same and the size of mRNA is similar. The next gene upstream of NIS on the human genomic sequence, ribosomal protein L18a (RPL18a), is located 8657 bases upstream from the first exon of NIS, while the distance on the rat genome is only 2130 bases (Fig. 2A). The similarity of the 5'-flanking region (from the NIS-coding sequence to the RPL18a-coding sequence) between human and rat is only 11.8%.

Table 2 NIS expression stimulator in breast tissues

Agent	Experimental system	Iodide uptake ^a	NIS mRNA	NIS protein	References	Note
Oxytocin	Rat normal breast, <i>in vivo</i>	X		X	Cho <i>et al.</i> (2000), Tazebay <i>et al.</i> (2000)	E ₂ required
Prolactin	Cancer primary culture (3-D)		X		Cho <i>et al.</i> (2000)	
	Rat normal breast, <i>in vivo</i>	X		X	Cho <i>et al.</i> (2000), Tazebay <i>et al.</i> (2000)	E ₂ required
	Mouse breast explant	X		X	Rillema <i>et al.</i> (2000)	
	Cancer primary culture (3D)		X		Cho <i>et al.</i> (2000)	
Estradiol	MCF-7, ER + cancer cell line	~10 ^b	X	X	Arturi <i>et al.</i> (2005)	
	Rat normal breast, <i>in vivo</i>	X		X	Tazebay <i>et al.</i> (2000)	
8-Bromo-cAMP, cholera toxin	MCF-7, ER + cancer cell line	~3.3 ^b	X		Knostman <i>et al.</i> (2004), Arturi <i>et al.</i> (2005) ^b	
hCG	MCF-7, ER + cancer cell line	~3.0 ^c			Knostman <i>et al.</i> (2004)	
Prostaglandin E ₂	MCF-7, ER + cancer cell line	~2.3 ^c			Knostman <i>et al.</i> (2004)	
Insulin/insulin-like growth factor (IGF)-I/IGF-II	MCF-7, ER + cancer cell line	11–14 ^b	X		Arturi <i>et al.</i> (2005)	
All- <i>trans</i> RA (tRA)	MCF-7, ER + cancer cell line	~9.2 ^d	X	X	Kogai <i>et al.</i> (2000b, 2005)	
	MCF-7 xenograft <i>in vivo</i>	X	X	X	Kogai <i>et al.</i> (2004)	
	MMTV-PyVT <i>in vivo</i>	X	X		Kogai <i>et al.</i> (2004)	
	MCF-7, ER + cancer cell line	12–18 ^e	X		Kogai <i>et al.</i> (2005)	Synergistic effect with RA
AGN190168 (RAR β/γ ligand)	MCF-7, ER + cancer cell line	~9.3 ^f	X		Kogai <i>et al.</i> (2005)	Long duration
AGN194433 (RAR γ agonist)	MCF-7, ER + cancer cell line	~4.0 ^f			Kogai <i>et al.</i> (2005)	
AGN197496, 195183 (retinoic acid receptor α ligands)	MCF-7, ER + cancer cell line	~3.0 ^g , ~3.3 ^f	X		Tanosaki <i>et al.</i> (2003) ^g , Kogai <i>et al.</i> (2005) ^f	
AGN195203, 194204, 196060 (retinoid X receptor ligands)	MCF-7, ER + cancer cell line	~2.5 ^g , ~6.0 ^f	X		Tanosaki <i>et al.</i> (2003) ^g , Kogai <i>et al.</i> (2005) ^f	
9- <i>Cis</i> RA	MCF-7, ER + cancer cell line	~14 ^g , ~9.0 ^d	X		Tanosaki <i>et al.</i> (2003) ^g , Kogai <i>et al.</i> (2005) ^d	
	T47D, ER + cancer cell line		X		Tanosaki <i>et al.</i> (2003)	
	BT474, ER + cancer cell line		X		Tanosaki <i>et al.</i> (2003)	
	MCF-7, ER + cancer cell line	~9.6 ^h	X		Tanosaki <i>et al.</i> (2003)	Synergistic effect with RA

X in column indicates significant induction by agent.

^aFold induction compared to without treatment is shown for data from MCF-7 cells.

^bThe induction at 12 h. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 0.2% fetal bovine serum (FBS).

^cThe induction at 24 h (PGE₂) or 48 h (hCG). Cells were maintained in DMEM: F12, 50: 50 with 10% FBS.

^dThe duration is 36–72 h. Cells were maintained in Minimum essential medium (MEM) with 10% FBS or serum replacement.

^eThe duration is 2–4 days with 10⁻⁷ M tRA or 2–5 days with 10⁻⁶ M AG190168. Cells were maintained in MEM with 10% FBS or serum replacement.

^fThe induction at 48 h. Cells were maintained in MEM with 10% FBS or serum replacement.

^gThe induction at 24 h.

^hThe induction at 24 h with 10⁻⁷ M 9-*cis* RA.

Figure 2 Comparison of the human and rat sodium/iodide symporter (NIS) gene. (A) Three homologous regions (more than 60% homology) in the 5'-flanking region are shown. (B) Comparison of the NIS upstream enhancer (NUE) sequences of human and rodents. A Pax-8 element (PB) is missing on the human NUE.

The basal promoter of the human NIS gene (−475 to −393) has a strong similarity (72%) with the rat promoter (−196 to −114; Fig. 2A). The human sequence between −790 and −728 also has some similarity (63.5%) with the rat sequence between −422 and −361, although no functional regulatory elements have been characterized. The NUE is a strong TSH-responsive enhancer, located between −9470 to −9046 on the human NIS (Taki *et al.* 2002) and −2611 to −2230 on the rat NIS (Ohno *et al.* 1999), with about 70% homology between these species.

These regulatory regions contain putative *cis*-elements for thyroid-selective transcription factors, Pax-8 (a paired domain containing transcription factor) and thyroid transcription factor-1 (TTF-1 or Nkx-2.1, a homeo-domain containing transcription factor), both of which are required for thyroid development and differentiation (De Felice & Di Lauro 2004). The NIS gene promoter/enhancer is regulated by these *trans*-acting factors although with some variation among species (Endo *et al.* 1997, Ohmori *et al.* 1998, Ohno *et al.* 1999, Taki *et al.* 2002). The NUE requires Pax-8 and cAMP-responsive element binding protein (CREB) for its full activity (Ohno *et al.* 1999, Taki *et al.* 2002). CREB is one of the basic-leucine zipper (B-ZIP)

transcription factor, containing a leucine zipper domain, which mediates DNA binding and dimerization to form homodimers or heterodimers with other B-ZIP proteins (Vinson *et al.* 2002). A participation of other B-ZIP proteins, such as c-Fos, c-Jun, and the activating transcription factor-2 (ATF-2), may play a role in NUE activation (Chun *et al.* 2004).

Regulation of the NIS proximal promoter

The core promoter region of the NIS gene contains a TATA-like motif (AATAAAT) and a GC box (CCCGCCCC). Binding of Sp-1 and an 'Sp-1-like' protein to the GC-box has been demonstrated, and is required for full activity of the NIS basal promoter (Xu *et al.* 2002). The rat NIS proximal promoter contains two *cis*-elements for thyroid-specific transcription factors, TTF-1 between −245 and −230 (Endo *et al.* 1997) and NIS TSH-responsive factor-1 (NTF-1) around −405 (Ohmori *et al.* 1998). TSH/cAMP-induced upregulation of the rat NIS gene expression requires NTF-1, which also contributes to TTF-1-mediated thyroid-specific NIS gene expression (Ohmori *et al.* 1998). The binding of NTF-1 to the *cis*-element is diminished by an oxidizing agent, diamide, and restored by the reducing agent dithiothreitol, suggesting oxidation/reduction (redox)

state regulation of NTF-1 (Ohmori *et al.* 1998). The human NIS 5'-flanking region contains two putative NTF-1 sites with a consensus sequence, GNNCGGANG, located –558 to –550 (one base mismatch) and –439 to –430 (two base mismatch; Kogai *et al.* 2001).

Characterization of the NUE

The NUE responds strongly to TSH and cAMP stimulation in thyroid cells (Ohno *et al.* 1999, Schmitt *et al.* 2002, Taki *et al.* 2002, Lin *et al.* 2004). In the rodent NIS gene, the NUE contains two Pax-8 elements (PA and PB, see Fig. 2B). A cAMP-responsive element (CRE)-like sequence (TGACGCA) is located between the two Pax-8 elements (Ohno *et al.* 1999, Lin *et al.* 2004). In the human NUE, one of the Pax-8 elements downstream of the CRE is missing (Schmitt *et al.* 2002, Taki *et al.* 2002), reducing sequence similarity between human and rodent to about 70% (Fig. 2B). Our mutagenesis study of these elements indicated that both the Pax-8 element and the CRE-like sequence, but not the TTF-1 element, are required for NUE activity (Taki *et al.* 2002). TSH and cAMP agonists significantly activate the NUE through the Pax-8 element and the CRE-like sequence in human and rodent cells (Ohno *et al.* 1999, Taki *et al.* 2002).

Redox state regulation of the NIS promoter

The redox state regulates a number of cellular responses by modifying the status of redox-sensitive cysteine residues on signal transduction molecules and transcription factors. Some NIS gene regulatory factors, such as Pax-8 (Puppin *et al.* 2004), rat TSH-responsive factor NTF-1 (Ohmori *et al.* 1998), and p38-mitogen-activated protein kinase (MAPK; Pomerance *et al.* 2000), are regulated by redox state. TSH stimulates the reduction of Pax-8 and binding to the *cis*-element in thyroid cells (Kambe *et al.* 1996). TSH increases the expression of redox factor-1 (Ref-1, also called apurinic apyrimidinic endonuclease, APE; Asai *et al.* 1997), a nuclear enzyme mediating reduction of transcription factors (Nakamura *et al.* 1997, Rothwell *et al.* 1997), as well as the translocation of Ref-1 into the nucleus (Tell *et al.* 2000). Ref-1 stimulates Pax-8 DNA binding in thyroid cells (Tell *et al.* 1998a,b).

Signal transduction of the NUE in thyroid

TSH stimulates the NUE in thyroid cells through the cAMP pathway (Ohno *et al.* 1999, Schmitt *et al.* 2002, Taki *et al.* 2002, Lin *et al.* 2004). TSHR is a guanine nucleotide-binding protein (G-protein) coupled receptor and activates adenylyl cyclase through stimulatory G protein (Gs protein) to generate cAMP.

An adenylyl cyclase agonist, forskolin, increases cAMP accumulation and activates the NUE in thyroid cells, mimicking the TSHR stimulation (Ohno *et al.* 1999, Taki *et al.* 2002). cAMP activates both protein kinase-A (PKA)-dependent pathways and PKA-independent pathways in thyroid cells. These pathways include PKA-CREB, APE/Ref-1-Pax-8, and two of three major MAPK pathways, the extracellular signal-regulated kinase (ERK) MAPK pathway and the p38 MAPK pathway (Fig. 3).

Chronic TSH stimulation of FRTL-5 cells down-regulates a catalytic subunit of PKA and leads to a lack of response of the CRE to further cAMP stimulation (Armstrong *et al.* 1995). Treatment with forskolin after chronic TSH stimulation, therefore, determines whether a cAMP pathway to a CRE is dependent on PKA or not. We have observed that forskolin stimulates the NUE even after chronic TSH stimulation without endogenous PKA, while overexpression of exogenous PKA increases the NUE activity without cAMP stimulation by forskolin (Taki *et al.* 2002). These results indicate that both PKA-dependent and -independent pathways are involved in the stimulation of NUE.

Members of the Ras superfamily (Rho family) of small guanosine triphosphate (GTP)-binding proteins are involved in the regulation of cell growth, differentiation, cytoskeletal reorganization, and protein kinase activation. Rap1 (Tsygankova *et al.* 2001) and Rac1 (Pomerance *et al.* 2000), Ras family members, have been reported mediators of TSH-stimulated NIS expression in thyroid cells.

In mammalian cells, guanine-nucleotide-exchange factors (GEFs) positively regulate small GTP-binding proteins in response to a variety of signals. GEFs catalyze the dissociation of GDP from the inactive GTP-binding proteins. GTP can then bind and induce structural changes that allow interaction with effectors. Some GEFs bind to cAMP and are directly activated by cAMP (cAMP-GEFs; Kawasaki *et al.* 1998, de Rooij *et al.* 2000). Some investigators have reported that TSH upregulates Rap1 in a PKA-independent manner in thyroid cells (Dremier *et al.* 2000, Iacovelli *et al.* 2001, Tsygankova *et al.* 2001). Rap1 is one of the effectors of cAMP-GEFs, suggesting the possibility that TSH/cAMP regulates Rap1 through cAMP-GEFs. On the other hand, PKA activates Rap1 by phosphorylation in mammalian cells (Hata *et al.* 1991, Altschuler *et al.* 1995). The Rap1, therefore, is regulated by both cAMP-GEFs and PKA, and this dual regulation likely brings about the PKA-dependent and -independent pathways in thyroid cells.

Rap1 may contribute to TSH induction of NIS expression in FRTL-5 rat thyroid cells (Tsygankova

Figure 3 TSH signal transduction to the NIS gene in thyroid cells. CHOP, CCAAT/enhancer binding protein (C/EBP) homologous protein.

et al. 2001), likely through the v-raf murine sarcoma viral oncogene homolog B1 (BRAF) MAP kinase (MEK)1/2–ERK1/2 MAPK pathway (Iacovelli *et al.* 2001, Taki *et al.* 2002, Fig. 3). Dominant-negative mutant of Rap1A partially (50%) inhibits the cAMP-induced NUE activity (Chun *et al.* 2004). TSH stimulates the MEK1/2–ERK1/2 MAPK pathway in FRTL-5 cells through Rap1 and BRAF without PKA activation (Iacovelli *et al.* 2001). An inhibitor of MEK1/2, PD98059, partially (43%) inhibits human NUE activation by forskolin (Taki *et al.* 2002). In contrast, the activation of the MEK–ERK pathway in response to TSH is not observed in primary dog thyrocytes (Vandeput *et al.* 2003). The regulation of

NUE by MEK–ERK may vary among different species or cell lines.

Phosphatidylinositol 3-kinase (PI3K) phosphorylates the inositol ring of phosphatidylinositol and related compounds at the 3'-position. These products serve as second messenger-signaling molecules for regulating cell growth and differentiation. The TSH/cAMP stimulation of cell proliferation with insulin-like growth factor-I (IGF-I) is dependent on a small GTP-binding protein Ras and the PI3K in thyroid cells (Cass & Meinkoth 2000, Coulonval *et al.* 2000, Saito *et al.* 2001). Although TSH activates PI3K signaling, PI3K downregulates NIS expression in thyroid cells (Cass & Meinkoth 2000, Garcia &

Santisteban 2002). Expression of a Ras mutant that selectively stimulates PI3K markedly decreases the TSH-induced NIS expression in Wistar rat thyroid (WRT) cells (Cass & Meinkoth 2000). IGF-I inhibits the cAMP-induced NIS expression in FRTL-5 cells through PI3K activation (Garcia & Santisteban 2002). An evaluation of the rat NIS gene regulatory sequence has indicated that the region between –1947 and –1152 is responsible for the inhibitory effect of IGF-I (Garcia & Santisteban 2002).

TSH and cAMP activate another MAPK pathway, MKK3/6–p38 MAPK, through a small GTP-binding protein Rac1 (Pomerance *et al.* 2000). An inhibitor of the p38 MAPK, SB203580, significantly decreases the cAMP-induced NIS mRNA expression in FRTL-5 cells (Pomerance *et al.* 2000). The TSH/cAMP stimulates the p38-MAPK phosphorylation PKA-dependently, but not PKC or PI3K (Pomerance *et al.* 2000). TSH stimulates the phosphorylation of MKK3/6, p38-MAPK, and ATF-2, a substrate of the p38 (Pomerance *et al.* 2000). The ATF-2 has been reported to bind the CRE-like sequence in the rat NUE (Chun *et al.* 2004). Reactive oxygen species is involved in the activation of p38-MAPK by cAMP (Pomerance *et al.* 2000), indicating redox regulation of the p38-MAPK pathway in thyroid cells. Another substrate of p38-MAPK, CCAAT/enhancer-binding homologous protein (CHOP), is also involved in the TSH stimulation of the rat NUE (Pomerance *et al.* 2003), although binding of CHOP to the NUE has not been confirmed.

Some B-ZIP transcription factors, CREB1, ATF1, and/or the cAMP-response element modulator (CREM), bind to the CRE-like regulatory sequence in the human NUE. These B-ZIP factors form homodimers or heterodimers with other B-ZIP proteins using a leucine zipper (B-ZIP) domain and then bind to these *cis*-elements on the target gene (Vinson *et al.* 2002). The cAMP-activated PKA phosphorylates the CREB to regulate cAMP-responsive genes, such as Tg, TPO, TSHR (Nguyen *et al.* 2000), and NIS (Chun *et al.* 2004). Our study has indicated the requirement of the CRE-like element in the human NUE for the cAMP-induced NUE activity, and the binding of CREB1, ATF1, and/or CREM to the CRE-like element (Taki *et al.* 2002).

Thyroid-specific genes Tg (Civitareale *et al.* 1989, Sinclair *et al.* 1990, Donda *et al.* 1991, Zannini *et al.* 1992, Berg *et al.* 1996, Espinoza *et al.* 2001) and TPO (Mizuno *et al.* 1991, Francis-Lang *et al.* 1992, Zannini *et al.* 1992, Miccadei *et al.* 2002) require both Pax-8 and TTF-1 for full gene expression. Recent studies have demonstrated the critical role of Pax-8 in TSH regulation of NIS through the NUE (Ohno *et al.* 1999, Taki *et al.* 2002). The role of TTF-1, however, in

human NIS gene expression, is likely less important. Overexpression of exogenous TTF-1 significantly increases the expression of Tg and TPO, but not NIS, in human thyroid cancer cells (Furuya *et al.* 2004b). TTF-1 is not recruited to the human NIS proximal promoter (Kogai *et al.* 2001) and does not stimulate promoter activity (Schmitt *et al.* 2001).

Post-translational regulation of NIS by TSH

TSH stimulates NIS expression at the post-translational level in FRTL-5 rat thyroid cells (Riedel *et al.* 2001). TSH stimulates NIS trafficking to the membrane and prolongs the half-life of NIS protein from 3 to 5 days (Riedel *et al.* 2001). Although TSH in the medium is required to maintain FRTL-5 cells (Ambesi-Impiombato *et al.* 1980), they can survive without TSH for up to 10 days (Kogai *et al.* 1997). The removal of TSH markedly reduces iodide uptake, NIS mRNA, and NIS protein expression in these cells (Kogai *et al.* 1997, Paire *et al.* 1997, Riedel *et al.* 2001). The addition of TSH restores NIS expression with a time lag between the induction of iodide uptake and NIS protein expression (Kogai *et al.* 1997). TSH induces NIS protein in 36 h to ~80% maximum, while the iodide uptake reaches only ~25% maximum at 36 h (Kogai *et al.* 1997). Immunocytochemistry in FRTL-5 cells shows an intense staining of NIS on plasma membrane with continuous TSH treatment, while intracellular staining is observed 3 days after withdrawal of TSH (Riedel *et al.* 2001).

The NIS protein in FRTL-5 cells is randomly distributed on the plasma membrane and does not exhibit cell polarity (Paire *et al.* 1997). In contrast, in normal thyroid gland, NIS protein is expressed on the basolateral membrane, but not on the apical side facing the lumen (Paire *et al.* 1997, Caillou *et al.* 1998, Jhiang *et al.* 1998, Castro *et al.* 1999). We developed a culture system for primary human thyroid cells, which survives for up to 3 months (Curcio *et al.* 1994, Kogai *et al.* 2000a). The primary cells in monolayer constitutively expressed the thyroid-specific genes, TTF-1, Pax-8, Tg, TPO, and NIS (Curcio *et al.* 1994, Perrella *et al.* 1997). TSH treatment stimulates cAMP production (Curcio *et al.* 1994) as well as the expression of NIS mRNA and protein (Kogai *et al.* 2000a). No significant induction of iodide uptake, however, was observed in the monolayer cells, even after TSH stimulation (Kogai *et al.* 2000a). Specific culture conditions promote the formation of three-dimensional ‘follicle-like’ structures with a periodic acid schiff (PAS)-positive colloid filled lumen. In contrast to monolayers, TSH stimulation of the

follicles results in significant induction of iodide uptake (Kogai et al. 2000a). These results indicate that cell polarity is required for the expression of the functional NIS in the human primary thyroid cells, which likely promotes NIS translocation to the basolateral membrane. The importance of thyroid follicle structure for NIS induction has been replicated in primary porcine thyroid preparations (Bernier-Vallentin et al. 2006).

A role for NIS glycosylation and phosphorylation in the regulation of NIS trafficking to the membrane has been proposed (Dohan et al. 2003). The cytoplasmic carboxyl-terminal domain of NIS contains a PDZ target motif and a dileucine motif tail that are important for protein–protein interactions. A recent study reported that the deletion of these motifs prevented the transport and insertion of NIS protein into the plasma membrane (Dohan et al. 2005). Those motifs are likely to be involved in the post-translational regulation of NIS by TSH.

NIS expression in thyroid cancer

The NIS expression in the primary tumor of differentiated thyroid cancer has been studied extensively, although findings have differed. Several investigators reported reduced expressions of NIS mRNA and protein in papillary and follicular thyroid cancer (Smanik et al. 1997, Arturi et al. 1998, Caillou et al. 1998, Ryu et al. 1998, Lazar et al. 1999, Ringel et al. 2001). Increased NIS expression, however, has been reported in papillary cancer (Saito et al. 1998, Dohan et al. 2001, Wapnir et al. 2003). An immunohistochemical analysis of NIS with an affinity-purified high-sensitivity anti-human NIS polyclonal antibody has shown strong positive staining in ~68% of the 72 cases of papillary cancer using conventional whole tissue sections (Wapnir et al. 2003).

NIS expression in the primary tumor of differentiated thyroid cancer and iodide uptake in recurrent or metastatic cancer, are correlated (Castro et al. 2001, Min et al. 2001). Positive NIS protein staining in primary intrathyroidal tumor was reported in 86% case of papillary cancer (Castro et al. 2001). The subsequent whole body scan with ^{131}I , following the endogenous TSH stimulation achieved by thyroxine withdrawal, shows positive iodide uptake in metastatic tumor in 90% patients with NIS-positive primary tumors (Castro et al. 2001). The expression of TSHR is usually retained in differentiated thyroid cancers (Brabant et al. 1991), except for insular carcinoma (Gerard et al. 2003), although significant cytoplasmic distribution has been observed (Mizukami et al. 1994, Gerard et al. 2003). A study with primary culture of

papillary thyroid cancer cells has shown increased iodide uptake after TSH treatment (Saito et al. 1998), consistent with the data from clinical specimens.

In normal thyroid tissue, NIS protein is expressed on the basolateral membrane, even when the serum TSH level is in the normal range, while the NIS protein in differentiated thyroid cancer is expressed predominantly in the cytosol (Saito et al. 1998, Dohan et al. 2001, Wapnir et al. 2003). The loss of tissue polarity is a characteristic change seen in epithelial tumors (Fish & Molitoris 1994). NIS-trafficking and correct polarity, therefore, are likely impaired in thyroid cancer. Our study with the three-dimensional culture of primary thyroid cells indicates the importance of cell polarity in the full expression of functional NIS (Kogai et al. 2000a). Restoration and/or stimulation of the trafficking of NIS in thyroid cancer are likely to increase the efficacy of ^{131}I therapy.

Transcriptional regulation of NIS in thyroid cancer

Some thyroid papillary cancer cells express reduced NIS mRNA, likely due to the reduced activity of the NIS promoter (Kogai et al. 2001, Puppini et al. 2004). The human NIS gene region –596 to –415 has reduced promoter expression in thyroid cancer cells compared with FRTL-5 cells (Kogai et al. 2001). Binding of unknown nuclear factor(s) to the region –596 to –415 is decreased or absent in the BHP 2–7 cells compared with FRTL-5 cells (Kogai et al. 2001).

An anti-oxidative stress nuclear factor, Ref-1, is related to the upregulation of Pax-8 by TSH in thyroid cells, stimulating the human NIS regulatory sequence (up to –2.4 kb of 5'-flanking region) activity with Pax-8 or an ubiquitous transcription factor early growth response (Egr)-1 in HeLa cells (Puppini et al. 2004). Impaired translocation of Ref-1 to the nuclei has been reported in papillary and anaplastic thyroid cancer cell lines as well as thyroid cancer tissues (Russo et al. 2001). The reduced NIS promoter activity in some thyroid cancer cells, therefore, may be due to the reduced Ref-1 localization in the nuclei (Puppini et al. 2004).

The potent enhancer NUE requires Pax-8 binding for the full activity (Ohno et al. 1999, Taki et al. 2002). Endogenous Pax-8 expression is markedly reduced in 70% differentiated thyroid cancer, especially in aggressive disease (Fabbro et al. 1994, Puglisi et al. 2000).

Papillary thyroid cancer (20–85% with geographic variation) has frequent somatic rearrangements of the RET receptor (RET/PTC; Santoro et al. 1992, 2002, Chua et al. 2000, Nikiforov 2002), leading to a constitutive activation of the RET tyrosine kinase.

The auto-activated RET receptor stimulates cell proliferation and motility through the ERK–MAPK pathway (Melillo *et al.* 2005). One of the most common RET/PTC rearrangement, RET/PTC1, consists of the intracellular portion of RET with the tyrosine kinase domain fused to H4, a ubiquitous gene of unknown function. The RET/PTC1 expression impairs the activity of Pax-8, which is required for the full activation of the NIS (Ohno *et al.* 1999, Taki *et al.* 2002), in PC Cl3 rat thyroid cells (De Vita *et al.* 1998). Reduced expression of NIS has been reported in a PC Cl3 constitutively expressing exogenous RET/PTC1 (Trapasso *et al.* 1999, Venkateswaran *et al.* 2004). In transgenic mice of thyroid cancer model with thyroid-targeted RET/PTC1 expression, iodide uptake is decreased in thyroid glands (Jhiang *et al.* 1996). In RET/PTC1-expressing PC Cl3 rat thyroid cells, there is reduced localization of PKA to the nucleus (Venkateswaran *et al.* 2004). Forskolin and substitution of exogenous PKA restore the NIS expression in the RET/PTC1-expressing cells, suggesting interference of RET/PTC1 with PKA-dependent signaling. On the other hand, our study has indicated a partial interruption of the NIS by the blocking of MEK–ERK pathway in FRTL-5 rat thyroid cells (Taki *et al.* 2002). Activating mutations of BRAF (Kimura *et al.* 2003) and an activating rearrangement of the BRAF with A-kinase anchor protein 9 (AKAP9-BRAF; Ciampi *et al.* 2005) have been reported recently in some papillary thyroid cancer. Crosstalk between the ERK pathway and signaling to NIS gene expression remains to be further investigated.

Promotion of NIS expression in thyroid cancer

To achieve the maximum iodide uptake in metastatic-differentiated thyroid cancer, TSH stimulation is required. This is achieved either by withdrawal of thyroid hormone replacement after a total thyroidectomy or by administration of rhTSH (Dow *et al.* 1997). The efficacy of rhTSH is generally equivalent to thyroid hormone withdrawal in the detection of thyroid cancer (Jarzab *et al.* 2003).

Some differentiated thyroid cancers (approximately 10–20%), however, do not concentrate radioiodide, even after TSH stimulation (Robbins *et al.* 1991, Schmutzler & Koehle 2000). TSH unresponsiveness of NIS induction is unlikely to be due to the absence of TSHR. Almost all differentiated thyroid cancer expresses the TSHR protein (Brabant *et al.* 1991, Mizukami *et al.* 1994, Gerard *et al.* 2003). Reduced expression of TSHR, however, is associated with a poor prognosis in papillary thyroid cancer (Tanaka

et al. 1997). Failure of signal transduction and/or transcription factors required for NIS gene expression is likely to be responsible for the lack of iodide accumulation in aggressive differentiated thyroid cancer. Defects in NIS protein trafficking and membrane insertion may also play a role. Recent studies have demonstrated the potential for NIS induction in aggressive thyroid cancer by re-differentiation agents, such as nuclear receptor ligands and inhibitors of epigenetic modifications.

NIS induction by nuclear hormone receptor ligands

Retinoic acid (RA), a vitamin A derivative, plays a pivotal role in development, differentiation, and cell growth. RA action is mediated through two families of nuclear receptors, retinoic acid receptors (RARs), and retinoid X receptors (RXRs). RA induces re-differentiation and apoptosis in cancer cells (Hong & Itri 1994). In thyroid cancer cells, RA induces type-I 5'-deiodinase (Schreck *et al.* 1994) and NIS (Schmutzler *et al.* 1997). Treatment for 24 h with all-*trans* RA (*t*RA; 10^{-6} M) markedly increased NIS mRNA expression in two follicular thyroid cancer cell lines, FTC-133 and FTC-238 (Schmutzler *et al.* 1997). Treatment of FRTL-5 rat thyroid cells with *t*RA, however, downregulates NIS mRNA (Schmutzler *et al.* 1997). These findings suggest differential regulation of NIS expression by RA in normal and malignant thyroid tissues.

Based on the findings of the RA induction of 're-differentiation' in thyroid cancer cell lines, clinical trials have been conducted to evaluate the efficacy of RA for improving radioiodide uptake in recurrent/metastatic thyroid cancer (Simon *et al.* 1996, 1998, 2002a, Grunwald *et al.* 1998, Koerber *et al.* 1999, Gruning *et al.* 2003). In most of these studies, treatment with 13-*cis* RA has been used. 13-*cis* RA is isomerized to *t*RA and/or 9-*cis* RA in tissues, and activates RAR and/or RXR (Blaner 2001), with less toxicity (Hixson *et al.* 1979) and a longer half-life (Brazzell *et al.* 1983), compared with *t*RA. These clinical studies have shown that 20–42% aggressive differentiated thyroid cancer responds to RA treatment by an increase in radioiodide uptake (Simon *et al.* 1996, 1998, 2002a, Grunwald *et al.* 1998, Koerber *et al.* 1999, Gruning *et al.* 2003, Coelho *et al.* 2004). In a study of 50 patients with advanced invasive or metastatic thyroid cancer and negative iodide scans, an oral dose of 13-*cis* RA (1.5 mg/kg) was given for 5 weeks (Simon *et al.* 2002a). After 13-*cis* RA treatment, 13 patients had a marked increase in radioiodide uptake in the invasive or metastatic tumor, and eight patients had a modest

increase in radioiodide uptake (Simon *et al.* 2002a). Reduced tumor volume was observed in seven of the 21 cases with functional NIS expression after the treatment with 80–270 mCi ^{131}I following the 13-*cis* RA treatment (Simon *et al.* 2002a). In some published reports with a small number (5–25) of cases, a marked increase in iodide uptake has been shown in follicular cancer, but not in papillary cancer (Grunwald *et al.* 1998, Gruning *et al.* 2003, Coelho *et al.* 2004). The studies, however, have not been randomized prospective studies of matched groups that would be necessary to confirm an effect of RA treatment.

The first demonstration of *in vitro* NIS induction was in follicular thyroid cancer cell lines (Schmutzler *et al.* 1997). Differential response of some thyroid cancer cell lines to RA has been described; cell lines expressing both RAR β and RXR γ demonstrate significant growth suppression with RA, whereas cell lines lacking these isoforms do not respond to RA (Haugen *et al.* 2004). Differential expression of RAR isoform may be important to predict the NIS induction in thyroid cancer with RA treatment, as well as histological difference. NIS expression and iodide uptake are increased in some breast cancer cells (Kogai *et al.* 2000b, 2004). The duration of the NIS expression with maximum function, however, is only a few days during the *in vivo* RA treatment (Kogai *et al.* 2004). In addition, a large systemic dose (160 mg/kg) is required to maximize the uptake in the mouse models (Kogai *et al.* 2004). Further evaluations are likely to be required in aggressive thyroid cancer to adjust the dose and duration of RA, as well as relationship among the histology, the tumor RAR isoform expression profile, and the response to RA.

Stimulation of another nuclear receptor, PPAR- γ , increases NIS expression in some thyroid cancer cell lines *in vitro*. Troglitazone, a PPAR- γ ligand, has been reported to increase the NIS mRNA significantly in the FTC-133 follicular thyroid cancer cell line and the TPC-1 papillary thyroid cancer cell line, but not in a Hurthle-cell cancer cell line (Park *et al.* 2005). Since troglitazone inhibits cell proliferation and induces apoptosis in some papillary thyroid cancer cell lines *in vitro* and *in vivo* (Ohta *et al.* 2001), a combination of troglitazone and radioiodide therapy might provide a synergistic inhibitory effect on some thyroid cancers.

Alteration of chromatin structure with histone deacetylase (HDAC) inhibitor

Epigenetic modifications, such as histone deacetylation and DNA hypermethylation, are commonly detected in human cancer cells, relevant to de-differentiation and

proliferation. Alteration of these epigenetic changes has been a target for re-differentiation in cancer cells.

Histone acetyltransferases and HDACs affect the acetylation status of histones, influencing gene expression (Marks *et al.* 2001). Inhibitors of HDACs induce growth arrest, differentiation, and/or apoptosis in many cancer cells (Marks *et al.* 2001). Some HDAC inhibitors, such as depsipeptide (FR901228) and valproic acid, have been reported to increase NIS expression in thyroid cancer cell lines (Kitazono *et al.* 2001, Zarnegar *et al.* 2002, Fortunati *et al.* 2004, Furuya *et al.* 2004b).

Depsipeptide significantly induces NIS mRNA and iodide uptake in follicular thyroid cancer cell lines (FTC-133 and FTC-236) and two anaplastic cancer cell lines (SW-1736 and KAT-4) at a low concentration (1 ng/ml) *in vitro* (Kitazono *et al.* 2001). Pharmacokinetics of the depsipeptide in patients have indicated that levels of more than 500 ng/ml are achieved without significant toxicity, promising to obtain the NIS-inducible concentration in patients (Kitazono *et al.* 2001). Another group has tried depsipeptide in a papillary thyroid cancer cells (BHP 18-21v) and an anaplastic cancer cell line (ARO), and found that 3–10 ng/ml of depsipeptide induces the NIS mRNA, protein, and iodide uptake, as well as Tg and TPO in association with iodide organification (Furuya *et al.* 2004b). The expression of TTF-1, but not Pax-8, is increased by depsipeptide in both cell lines (Furuya *et al.* 2004b). Since the overexpression of exogenous TTF-1 induces Tg and TPO (Furuya *et al.* 2004a), TTF-1 is likely to be responsible for the induction of iodide organification and decreased iodide efflux by the treatment of depsipeptide. The *in vivo* effect of depsipeptide on the iodide uptake has been confirmed in a BHP 18–21v xenograft model (Furuya *et al.* 2004b).

Another HDAC inhibitor, trichostatin A (TSA), also induces NIS mRNA in some papillary cancer cell lines (Zarnegar *et al.* 2002, Furuya *et al.* 2004b), a follicular cancer cell line (Zarnegar *et al.* 2002), an anaplastic cancer cell line (Furuya *et al.* 2004b), and a Hurthle-cell cancer cell line (Zarnegar *et al.* 2002). The induction of iodide uptake, however, has not yet been confirmed in thyroid cancer cells treated by TSA (Kogai *et al.* 2001).

Recently, the anticonvulsant valproic acid, acting as a HDAC inhibitor (Marks *et al.* 2001), has been shown to induce NIS expression in a papillary cancer cell line, NPA, and the anaplastic cancer cell line, ARO (Fortunati *et al.* 2004), although the induction is relatively modest. HDAC inhibitors, especially

depsipeptide, have a potential to increase radioiodide uptake in some aggressive thyroid cancer tumors.

Effects of hypermethylation on NIS promoter activity

Expression of some tissue-specific genes is regulated by cytidine methylation in a CpG dinucleotide sequence on regulatory sequences near the transcription start site (Antequera *et al.* 1990). The prevalence of abnormal methylation pattern of selected genes in thyroid tumors is high (Matsuo *et al.* 1993). The human NIS gene has three CpG-rich regions around the translation start site, the core promoter region (about 100 bp from the transcription start site), the 5'-untranslated region, and the coding region of the first exon (Venkataraman *et al.* 1999). The demethylation agent, 5-azacytidine, restores NIS mRNA expression and iodide uptake in three papillary cancer cell lines, NPA, KAT-5, and KAT-10, but not in two follicular cancer cell lines, MRO and WRO (Venkataraman *et al.* 1999). A correlation has been observed between the successful restoration of NIS expression by 5-azacytidine and demethylation of the 5'-untranslated region (Venkataraman *et al.* 1999). On the other hand, their evaluation of methylation status in thyroid cancer tumor specimen has revealed no significant correlation between the methylation status of these CpG-rich regions and NIS mRNA expression in thyroid cancer tumor samples (Venkataraman *et al.* 1999). The hypermethylation of the NIS 5'-untranslated region could be one of the factors contributing to reduced NIS expression in some thyroid cancers. The demethylation agent, 5-azacytidine, has the potential to restore radioiodide uptake in some thyroid cancer tumors.

Thyroid cancer models

Concentration of radioiodine in response to TSH stimulation is observed in 70% of metastatic thyroid cancers (Robbins *et al.* 1991). However, only a few normal thyroid cell lines (Weiss *et al.* 1984b, Berlingieri *et al.* 1993, Venkataraman *et al.* 1998) and thyroid cancer cell lines (Ohta *et al.* 1996, 1997, Kogai *et al.* 2001) express endogenous NIS in response to TSH stimulation. Most of these thyroid cell lines have de-differentiated and lost expression of TSH-R and TTF-1, which are expressed in most differentiated thyroid cancers. Several transgenic mouse models of thyroid cancers have been developed with thyroid-targeted expression of oncogenes, including SV40-large T antigen (Ledent *et al.* 1991), human papilloma virus (HPV)-E7 oncogene (Ledent *et al.* 1995, Coppee *et al.* 1996), RET/PTC1 (Jhiang *et al.* 1996, Santoro *et al.* 1996), RET/PTC3 (Powell *et al.* 1998), and TRK-T1 (Russell *et al.* 2000).

These transgenic mice may provide better models to evaluate the regulation of NIS in thyroid cancer.

NIS expression in normal breast tissue

Iodide accumulation in the lactating breast has been recognized for more than 50 years (Honour *et al.* 1952, Brown-Grant 1957, Grosvenor 1960, Eskin *et al.* 1974, Thorpe 1976, Strum 1978, Bakheet *et al.* 1998, Perros *et al.* 2003). Once the NIS gene was cloned and available for study, a correlation between NIS expression and iodide uptake in lactating mammary glands was demonstrated (Cho *et al.* 2000, Tazebay *et al.* 2000). NIS is expressed on the basolateral membrane of alveolar cells in mammary glands (Spitzweg *et al.* 1998) and is markedly induced during lactation (Cho *et al.* 2000, Tazebay *et al.* 2000). The alveolar cells concentrate iodide in milk, 6 to 15-fold relative to the plasma iodide concentration (Thorpe 1976). Part of the trapped iodide (~20%) is organified by peroxidases in the alveolar cells and lumens adjacent to the alveolar cells (Strum 1978, Etling & Gehin-Fouque 1984, Shah *et al.* 1986).

Radioiodide therapy and imaging for thyroid diseases are contraindicated in breast-feeding patients. After thyroid imaging with ¹³¹I or ¹²³I, cessation of breast feeding is recommended until breast milk radioactivity levels are at a safe level (Stabin & Breitz 2000). Since the therapeutic administration of ¹³¹I for thyroid cancer (150 mCi) delivers approximately 2 Gy (200 rad) to the mammary glands, it is recommended that breast feeding should be discontinued (Stabin & Breitz 2000).

No correlation between thyroid uptake and breast uptake has been reported, suggesting differential regulation of iodide uptake in the thyroid and mammary glands (Eskin *et al.* 1974). Recent reports have demonstrated that fetoplacental estrogen and two pituitary hormones, oxytocin and prolactin, play an important role in the induction of NIS in the lactating mammary glands (Cho *et al.* 2000, Tazebay *et al.* 2000). Estradiol produces a modest induction of NIS in mammary glands from ovariectomized mice (Tazebay *et al.* 2000). In contrast, estradiol decreases the NIS expression in FRTL-5 rat thyroid cells (Furlanetto *et al.* 1999). The treatment of mice with the combination of oxytocin, prolactin, and estradiol markedly induces the NIS in mammary glands, while each hormone alone is not sufficient for NIS induction (Tazebay *et al.* 2000). Basal levels of these three hormones are significantly increased in late pregnancy and the lactogenic hormones, prolactin and oxytocin, are still elevated during the first few months of the *post-partum* period. The surge of oxytocin during

lactation is likely to be important for the maximum induction of NIS in mammary glands.

There is a particular concern regarding the impact of ^{131}I treatment for thyroid cancer in the *post-partum* period. Bromocriptine, which inhibits the secretion of prolactin, partially inhibits the iodide uptake in the lactating mammary glands in rats (Cho *et al.* 2000). Breast uptake of ^{131}I during the treatment of thyroid cancer in the *post-partum* period may increase the risk of breast cancer (Preston *et al.* 2002, Zheng *et al.* 2002). Cessation of breast feeding and the administration of bromocriptine have been reported to reduce ^{131}I uptake in breast tissues (Hsiao *et al.* 2004).

NIS activity is the primary regulator of iodide accumulation in the lactating breast, although other transporters may make a small contribution (Shennan 2001). A sulfate/iodide exchanger that is inhibited by 4,4'-diisothiocyanatostilbene 2,2'-disulfonic acid has been identified in rat mammary gland explants (Shennan 2001). Increased pendrin expression has also been reported in the lactating mammary gland (Rillema & Hill 2003). Since NIS is expressed on the basolateral membrane in the lactating mammary glands, other transporters, like pendrin, may mediate release of the trapped iodide into the lumen.

NIS expression and iodide uptake in breast cancer

It has been recognized for over 30 years from case reports and small clinical series that radioiodide and $^{99\text{m}}\text{TcO}_4^-$ are concentrated in some breast cancer, but not in normal non-lactating breast tissue (Cancroft & Goldsmith 1973, Eskin *et al.* 1974, Lyttle *et al.* 1979). Robust iodide uptake in estrogen-dependent breast cancer has been observed in rodent breast cancer models, with a magnitude of 5–7-fold, compared with plasma iodide concentration (Thorpe 1976, Briand 1983). Recently, the existence of NIS mRNA (Kilbane *et al.* 2000, Moon *et al.* 2001, Upadhyay *et al.* 2003) and protein expression (Tazebay *et al.* 2000, Rudnicka *et al.* 2003, Upadhyay *et al.* 2003, Wapnir *et al.* 2003, 2004) has been confirmed in human breast cancer tissues.

Immunohistochemical analysis of NIS protein expression in 169 cases of breast cancer from pathological archives identified positive staining of NIS in 88% ductal carcinoma *in situ* (strongly positive in 53%), and 76% invasive carcinoma (strongly positive in 40%; Wapnir *et al.* 2003). The NIS protein is expressed predominantly in the intracellular space, while NIS is on the basolateral membrane in lactating mammary glands (Wapnir

et al. 2003). NIS trafficking to the plasma membrane may be impaired in some breast cancer, as it is in some differentiated thyroid cancers.

The correlation of $^{99\text{m}}\text{TcO}_4^-$ uptake and NIS mRNA expression in 25 patients with primary breast tumors, however, showed that only 4 out of 25 tumors with NIS mRNA expression had functional uptake (Moon *et al.* 2001). A disparity in NIS mRNA expression and iodide uptake has also been reported in some studies of thyroid cancer. These observations in breast cancer suggest that, in addition to NIS mRNA expression, NIS protein synthesis, modification, and membrane targeting are likely to be important in conferring iodide uptake.

There is very limited data on NIS expression and iodide uptake in metastatic breast tumors. Evaluation of nine cases of metastatic breast cancer showed three of them positive for NIS protein, and two of the three NIS-positive tumors concentrated ^{123}I (Wapnir *et al.* 2004). The incidence of NIS protein positive tumors in metastatic disease is likely to be smaller than that in primary tumors, as differentiated function is often reduced in metastatic tumors (Wapnir *et al.* 2003). In thyroid cancer, NIS protein expression in the primary tumor is correlated with iodide uptake in metastatic and recurrent tumor, but only after TSH stimulation. Hormonal stimulation of NIS expression in breast cancer metastases is also likely to be important to achieve sufficient uptake for therapy.

NIS expression has been demonstrated in several transgenic mouse models of breast cancer (Tazebay *et al.* 2000, Knostman *et al.* 2004). In a study of transgenic mouse models that develop breast cancer, NIS expression in the tumors, as assessed by immunohistochemical staining, was present in 8 of the 14 models studied (Knostman *et al.* 2004). Strong NIS staining was observed in four models: two that overexpress the oncogenes HER2/neu or polyoma middle T antigen (PyVT) in mammary glands, one that overexpresses cyclooxygenase-2 in mammary glands, and one that overexpresses hCG in a variety of tissues leading to a high circulating level of hCG (Rulli *et al.* 2003). Functional uptake of $^{99\text{m}}\text{TcO}_4^-$ was reported in two transgenic models with overexpression of Ras or HER2/neu oncogenes, although the uptake was modest (about 1.6-fold compared with the background; Tazebay *et al.* 2000). HER2/neu amplification/overexpression was found in 20–30% human breast cancer (Ross and Fletcher 1998) and was associated with a more aggressive clinical course and decreased survival time compared with tumors with normal levels of HER2/neu (Slamon *et al.* 1989). Based on the findings in mouse models, HER2/neu overexpression in human

breast cancer may indicate greater likelihood of enhanced NIS expression and the potential for radioiodide therapy.

Iodide organification and iodide retention in thyroid and breast cancers

Iodide taken into the thyroid gland is organified, a process that promotes iodide retention by binding to thyroglobulin (Tg). The perchlorate discharge test was previously used clinically to identify organification defects in children, characterized by excessive loss of tracer radioiodine from the thyroid gland after administration of perchlorate. In normal thyroid glands, binding of iodide to tyrosyl residues on Tg is catalyzed by TPO, and this procedure requires H₂O₂, produced by ThOX1 and ThOX2. The TPO and ThOXs are on the apical membrane and mediate the organification of iodide released through AIT and/or pendrin with the Tg accumulated in the lumen.

The extent of iodide organification in thyroid cancer has been characterized in only a few studies. In some differentiated thyroid cancer, the expressions of TPO (Czarnocka *et al.* 2001, Gerard *et al.* 2003) and pendrin (Gerard *et al.* 2003) are reduced. An immunohistochemical analysis indicated that the expressions of TPO and pendrin are markedly decreased in papillary cancer; only 1.5% cells stained for TPO compared with 92% in normal tissue and 0% for pendrin compared with 55% in normal tissue (Gerard *et al.* 2003). The expression of TPO in a study of follicular cancer showed reduced expression to 26% cells, although pendrin is more widely expressed (64%; Gerard *et al.* 2003). ThOXs are expressed in almost every cancer. The distribution of TPO and ThOXs in these differentiated cancers is predominantly cytoplasmic (Gerard *et al.* 2003), demonstrating that the polarity for the iodide metabolism has been lost in these cancer tissues. Indeed, marked reduction of the TPO activity has been observed in papillary cancer tissues (Takamatsu *et al.* 1992). Absence of organification in metastatic thyroid cancer has been reported, even in those that concentrate radioiodide (Valenta 1966). Iodide organification in thyroid cancer is unlikely to be as efficient as the process in the normal thyroid.

The role of iodide organification in the treatment of thyroid cancer has not been established. Radioiodide therapy of thyroid cancer is usually carried out with elevated serum TSH. TSH increases the expression of TPO and Tg (Gerard *et al.* 1989, Nagayama *et al.* 1989), and synergistic *trans*-activation by TTF-1 and Pax-8 is required for the maximum induction of these

genes in normal thyroid cells (Miccadei *et al.* 2002). The expression of Pax-8, however, is markedly reduced in about 70% of thyroid cancers (Fabbro *et al.* 1994, Puglisi *et al.* 2000). The iodide organification activity, therefore, is likely to be reduced in the majority of differentiated cancer, even after TSH stimulation. On the other hand, iodide organification and the biological half-life of radioiodide residence in tumors are significantly increased by the overexpression of TTF-1 in aggressive differentiated thyroid cancer cells, which express endogenous Pax-8, *in vitro* and *in vivo* (Furuya *et al.* 2004a). Iodide organification may have an important role in radioiodide therapy for some thyroid cancers with endogenous TTF-1 and Pax-8 expressions.

The importance of iodide organification has been studied in several tumor models. NIS has been used for gene therapy in various xenograft models of thyroidal and non-thyroidal cancer cell lines with subsequent ¹³¹I treatment. The efficacy of radioiodide, however, has varied among tumor types. Some have achieved tumor shrinkage after the administration of ¹³¹I (Spitzweg *et al.* 2000, 2001, Cho *et al.* 2002, Dingli *et al.* 2003, Faivre *et al.* 2004), while some reported no shrinkage or insufficient uptake for a therapeutic absorbed dose (Shimura *et al.* 1997, Smit *et al.* 2002, Haberkorn *et al.* 2003, 2004). Long-term retention of ¹³¹I in the tumor, however, is likely to be important for effective treatment and can be achieved even in non-thyroidal cancer tissues without iodide organification (Dingli *et al.* 2004, Faivre *et al.* 2004). In an *in vitro* study of cells constitutively expressing exogenous NIS, but with no iodide organification activity, there was a positive correlation between the number of cells expressing NIS and the duration of iodide retention (Dingli *et al.* 2004). The addition of perchlorate significantly increases iodide efflux rate from these cells (Dingli *et al.* 2004). Since perchlorate inhibits the iodide uptake via NIS, these results suggest that re-uptake of iodide contributes to the retention of radioiodide in the cells (Dingli *et al.* 2004). Studies of a chemically induced rat liver cancer model has indicated a rapid efflux of radioiodide after the administration of perchlorate in NIS-expressing tumor cells (Faivre *et al.* 2004). They observed a long half-life of ¹³¹I in the tumors, attributable to re-uptake via the high hepatic blood flow (Faivre *et al.* 2004). These data suggest that long retention of radioiodide can be achieved, even without iodide organification, if sufficient NIS is expressed in the tumor, and the tumor is fed with sufficient blood flow. Since metastatic tumors generally have an abundant blood supply, such tumors with sufficient NIS expression could be good candidates for radioiodide therapy.

In the mammary gland, iodination of tyrosyl residues on caseins and other milk proteins correlates with peroxidase activity (Strum 1978, Shah *et al.* 1986). Iodide organification may occur even in inactive breast tissues (Strum *et al.* 1983). In contrast, iodide organification activity has not been observed in breast cancer. In GRS/A (GR) mice, which spontaneously develop breast adenocarcinomas, iodide uptake, and peroxidase activity in estrogen-dependent tumor are ten times greater than those in estrogen-independent tumor (Lyttle *et al.* 1979). The trapped iodide, however, is not organified in the tumor and no correlation has been reported between iodide uptake and peroxidase activity (Lyttle *et al.* 1979). An *in vitro* study showed no iodide organification in MCF-7 human breast cancer cells (Kogai *et al.* 2000b). Promotion of NIS expression in breast cancer, therefore, is important for greater efficacy of radioiodide therapy due to prolonged retention in the tumor.

Promotion of the NIS expression in breast cancer

The MCF-7 cell line, derived from adenocarcinoma metastatic to a pleural effusion, is the most commonly used breast cancer cell line model with endogenous estrogen receptor (ER) expression. MCF-7 cells express endogenous NIS, have modest iodide uptake, and have

been widely used to study the hormonal regulation of iodide uptake and the NIS expression (Kogai *et al.* 2000b, 2004, 2005, Tanosaki *et al.* 2003, Knostman *et al.* 2004, Arturi *et al.* 2005). As shown in Table 2, several hormones and synthetic receptor ligands have been reported to increase iodide uptake and NIS mRNA expression in MCF-7 cells. Other cell lines and primary culture have also been utilized to demonstrate NIS induction (Cho *et al.* 2000, Tanosaki *et al.* 2003).

Effects of lactogenic hormones on NIS expression in breast cancer cells

NIS expression in lactating mammary glands is stimulated by prolactin, oxytocin, and estrogen (Tazebay *et al.* 2000). These hormones have been tested for their ability to induce NIS expression and iodide uptake in breast cancer cells (Cho *et al.* 2000, Kogai *et al.* 2000b, 2005, Arturi *et al.* 2005). Prolactin and oxytocin treatment markedly induced NIS mRNA in human breast cancer tissues cultured primarily on collagen gel, while the combination of these hormones did not produce an additive effect (Cho *et al.* 2000). The response to these hormones varied among the tumor specimens, but prolactin significantly induced NIS mRNA in all samples tested, and oxytocin in three of the four specimens. A significant induction in iodide

Figure 4 Iodide uptake and NIS expression in all-*trans* RA (tRA)-stimulated MCF-7 cells. (A) Iodide uptake in three lots of MCF-7 cells from ATCC (Manassas, VA, USA). The cells were treated with 10^{-6} M tRA for 48 h. *, $P < 0.001$, comparison with the untreated group ($n=3$). (B and C) Immunocytochemical staining with anti-human NIS antibody. Cells were treated with 0 (B) or 10^{-6} M (C) of tRA for 48 h. These figures are reproduced from Kogai T, Kanamoto Y, Che H, Taki K, Moatamed F, Schultz JJ & Brent GA 2004 Systemic retinoic acid treatment induces sodium/iodide symporter expression and radioiodide uptake in mouse breast cancer models. *Cancer Research* 64 415–422. Reprinted with permission from the American Association for Cancer Research.

uptake and NIS mRNA by prolactin was reported in the MCF-7 breast cancer cell line (Arturi *et al.* 2005). The duration of the prolactin induction, however, was relatively short with maximum iodide uptake at 12 h, but reduced at 24 h. This is in agreement with our study showing no significant induction of prolactin treatment on NIS expression in MCF-7 cells at 48 h (Kogai *et al.* 2000b). Estradiol does not significantly influence iodide uptake in the MCF-7 cells (Kogai *et al.* 2005), as is seen in normal breast tissue (Tazebay *et al.* 2000).

The mechanism of lactogenic hormone action on NIS gene expression has been partially characterized. Oxytocin receptor is a G-protein coupled receptor, and activates the cAMP/PKA pathway and/or the inositol triphosphate– Ca^{2+} pathway (Thibonnier *et al.* 1998). The contribution of cAMP and PI3K to NIS expression has been suggested by the findings in some transgenic mouse models of breast cancer (Knostman *et al.* 2004).

HER2/neu and PyVT, overexpressed in some models, have a tyrosine kinase domain and activate PI3K. Prostaglandins, whose production is stimulated by cyclooxygenase-2, and hCG increase cAMP accumulation by activation of prostaglandin and luteinizing hormone (LH) receptors respectively. Induction of NIS is seen with cAMP stimulation as well as a result of overexpression of PI3K in MCF-7 breast cancer cells *in vitro* (Knostman *et al.* 2004). The PI3K and cAMP pathways, therefore, are critical for NIS expression in these transgenic models (Knostman *et al.* 2004).

Effects of retinoids on the NIS expression in breast cancer cells

Retinoids have a robust effect inducing functional NIS in MCF-7 cells (Kogai *et al.* 2000b, 2005, Tanosaki *et al.* 2003). *t*RA (10^{-6} M) markedly induces iodide uptake,

Figure 5 Iodide uptake in MCF-7 xenograft tumor. (A) Time-course study with 160 mg/kg per day of systemic *t*RA treatment. (B) Effects of various dose of *t*RA (5-days treatment) on the iodide uptake *in vivo*. *, $P=0.01$; $P=0.02$, comparisons with tumours from the untreated group ($n=3-6$). (C) Imaging of the MCF-7 xenograft tumors with ^{125}I . Systemic *t*RA treatment (160 mg/kg per day for 5 days) visualizes the tumors 2 h after the administration of ^{125}I . These figures are reproduced from Kogai T, Kanamoto Y, Che H, Taki K, Moatamed F, Schultz JJ & Brent GA 2004 Systemic retinoic acid treatment induces sodium/iodide symporter expression and radiiodide uptake in mouse breast cancer models. *Cancer Research* 64 415–422. Reprinted with permission from the American Association for Cancer Research.

up to 10-fold above the baseline, in three lots of MCF-7 cells (Fig. 4), although clonal variation in the hormone response of MCF-7 cells has been reported (Seibert et al. 1983). Our immunocytochemical analysis has demonstrated marked intracellular expression of NIS after *t*RA treatment, and only faint staining of NIS in the absence of *t*RA (Fig. 4). In contrast, ER-negative breast cancer cell line MDA-MB 231 shows no iodide uptake and NIS protein expression even after *t*RA treatment.

Significant inductions of iodide uptake and NIS mRNA have been observed with isomers of *t*RA, 9-*cis* RA, and 13-*cis* RA (Kogai et al. 2000b, 2005, Tanosaki et al. 2003). *t*RA and 9-*cis* RA stimulate formation of heterodimers of the RAR and the RXR, and the RAR–RXR complex binds to its *cis*-element (retinoic acid response element, RARE) on a target gene to stimulate or suppress the transcription. *t*RA is a ‘pan-retinoid’ ligand to all RAR isomers, α , β , and γ , but not RXRs (Allenby et al. 1993, Agarwal et al. 1996, Idres et al. 2002). 9-*cis* RA binds to a broader spectrum of retinoid receptors, RARs and RXRs, with almost equivalent affinity (Vuligonda et al. 2001, Idres et al. 2002). Although 13-*cis* RA has a low affinity for RARs and RXRs (Agarwal et al. 1996), it stimulates RARs, likely after isomerization to *t*RA and 9-*cis* RA (Blaner 2001).

Differential regulation of gene expression by activation of each RAR isomer has been reported. To elucidate the isoform specificity of retinoid receptors for the upregulation of NIS gene expression, we have

utilized isoform-selective retinoid receptor ligands in MCF-7 cells. An RAR β/γ agonist (AGN190168) is a more potent inducer of functional NIS expression than AGN195183 (RAR α agonist), AGN194433 (RAR γ agonist), and AGN194204 (pan-RXR ligand), suggesting a central role of RAR β in NIS induction by retinoids (Kogai et al. 2005).

*t*RA induces NIS gene expression partially at the transcriptional level (Kogai et al. 2000a). Our study with RAR-selective ligands has suggested that signaling through RAR β is important for the RA induction of NIS mRNA (Kogai et al. 2005). The consensus sequence of RARE contains two of the core motifs, 5'-PuG[G/T][T/A]CA-3', directly repeating with a spacer of two or five bases (DR-2 or DR-5). Sequence inspection of the human NIS gene revealed two consensus DR-2 elements (AGGTCaggAGTTCA) in the first intron. These putative DR-2 elements, however, do not respond to *t*RA in MCF-7 cells (T Kogai, Y Kanamoto & G A Brent, unpublished observations). Interestingly, the DR-5, but not the DR-2, responds to the *t*RA stimulation in MCF-7 cells (Kogai et al. 2003). Recently, the cardiac homeobox transcription factor, Nkx-2.5, was shown to be induced by RA in MCF-7 cells, and involved in the RA induction of NIS, using the rat proximal promoter in MCF-7 cells (Dentice et al. 2004). These data suggest an indirect regulation of the NIS gene by RA through Nkx-2.5, but not through the RARE on the NIS gene.

Figure 6 Induction of NIS protein (A, B, and C) and mRNA (D) expression by systemic *t*RA treatment in MCF-7 xenograft tumors. Western blot analysis (A) and immunohistochemical analysis (B and C) were performed after 5-days treatment with or without 160 mg/kg per day of *t*RA. RT-PCR of NIS was performed after 2 days treatment with the same dose of *t*RA. *, $P=0.005$ when compared with the untreated group. **, $P=0.02$; ***, $P=0.008$, compared with the group at day 2 ($n=3$). These figures are reproduced from Kogai T, Kanamoto Y, Che H, Taki K, Moatamed F, Schultz JJ & Brent GA 2004 Systemic retinoic acid treatment induces sodium/iodide symporter expression and radioiodide uptake in mouse breast cancer models. *Cancer Research* **64** 415–422. Reprinted with permission from the American Association for Cancer Research.

Figure 7 The effects of dexamethasone (Dex) on the retinoid-induced iodide uptake in MCF-7 cells *in vitro*. (A) Various dose combinations of Dex and tRA were tested to induce iodide uptake in MCF-7 cells. Dex (10^{-7} M) enhances the uptake with 10^{-7} M tRA, resulting in more uptake than that with 10^{-6} M tRA only. *, $P < 0.02$; **, $P < 0.001$ for comparison as shown ($n = 3$). ***, significant difference ($P < 0.0001$) in two-factor factorial ANOVA test among the four groups treated with 0 or 10^{-7} M tRA and/or 10^{-7} M Dex. (B and C) Effects of long-term treatment with retinoids and Dex on iodide uptake (B) and NIS mRNA (C) in MCF-7 cells. Duration and magnitude of the NIS induction are markedly increased by the addition of Dex, especially in combination with an RAR β/γ ligand AGN190168. *, $P < 0.05$; **, $P < 0.01$, compared with the group without Dex at the same time point ($n = 3-4$). ***, $P < 0.05$ compared with the group of maximum value (at Day 2 of iodine uptake) and 12 or 24 h in NIS mRNA in the same treatment. (D) Selective cytotoxicity of ^{131}I in MCF-7 cells after the treatment with tRA and Dex. MCF-7 cells were treated with the indicated concentration of tRA and Dex for 48 h, and then incubated with $60 \mu\text{Ci/ml}$ of ^{131}I for 6 h. To estimate cell survival, clonogenic assay was carried out. The addition of Dex (10^{-7} M) significantly increases the cytotoxic effect of ^{131}I after the treatment with 10^{-7} M tRA. *, $P < 0.001$, when compared with the group of unstimulated MCF-7 cells. These figures are reproduced from 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 (NIS) gene expression by nuclear receptor ligands in MCF-7 breast cancer cells. *Endocrinology* **146** 3059–3069. Reprinted with permission from The Endocrine Society. Copyright 2005 The Endocrine Society.

Our *in vivo* studies with MCF-7 xenograft tumors in immunodeficient mice have demonstrated that induction of iodide uptake in the tumor by systemic *t*RA treatment increases iodine concentration up to 15-fold above the background (Fig. 5A and B). This level is sufficient to visualize the tumors in imaging studies with ^{125}I (Fig. 5C; Kogai et al. 2004). A modest amount of NIS protein expression is observed, predominantly in the intracellular space, and *t*RA increases NIS protein expression, but does not stimulate NIS trafficking to the membrane (Fig. 6). The effect of *t*RA on the expression of functional NIS and its mRNA has been confirmed in another *in vivo* model, the transgenic mice overexpressing the PyVT oncogene in breast tissue (Kogai et al. 2004).

Greater and more sustained NIS induction is likely to be necessary for effective radioiodide therapy. Although the maximum induction of iodide uptake and expression are seen within 5 days, reduction in expression occurs within the next 2 days after the maximum induction (Fig. 5A and 6D). A large dose of RA (160 mg/kg per day) is required to induce NIS in these mouse models (Fig. 5B), although no significant toxicity was detected during the 5 days of treatment in mice (Kogai et al. 2004). The relative short duration of NIS induction and the potential for toxicity with high-dose RA need to be improved to achieve an adequate therapeutic response.

There are a number of approaches to block the uptake of high-dose radioiodide into the thyroid. Systemic thyroid hormone treatment has been successful in humans (Wapnir et al. 2004) and rodents (Spitzweg et al. 2001, Cho et al. 2002, Furuya et al. 2004a, Kogai et al. 2004). In our imaging study, the MCF-7 xenograft model mice were treated with thyroxine for 12 days and the thyroid gland was not visualized (Fig. 5C; Kogai et al. 2004). Since no significant NIS induction by *t*RA has been observed in other organs expressing NIS, including thyroid, stomach, kidney, and normal mammary glands, the *t*RA effect appears selective for the breast tumor (Kogai et al. 2004). Prior thyroid ablation by radioiodide or surgical removal followed by levothyroxine replacement could also be utilized.

Effects of dexamethasone (Dex) on the RA-induced NIS expression

Breast cancer has reduced iodide organification activity (Lyttle et al. 1979, Kogai et al. 2000b), greater and more sustained induction of NIS is important to achieve the prolonged iodide retention, as suggested by the kinetics study of iodide efflux (Dingli et al. 2004). Insulin, IGFs,

and prolactin stimulate iodide uptake in MCF-7 breast cancer cells *in vitro*, but the duration is about 12 h, followed by rapid reduction in the next 12 h (Arturi et al. 2005). Prostaglandin E_2 and hCG also induce NIS in the MCF-7 cells, likely via cAMP, but with relatively modest iodide uptake (2–3-fold induction; Knostman et al. 2004). Our *in vivo* study with MCF-7 xenografts has shown a marked induction of iodide uptake (about 15-fold induction) with systemic retinoid treatment for 5 days, but followed by the rapid reduction in the next 2 days (Kogai et al. 2004).

We have recently found that Dex significantly increases the RA-induced iodide uptake (about 12-fold of that before the treatment) and NIS mRNA, partially by stabilizing the NIS mRNA, and prolongs the induction of iodide uptake (Kogai et al. 2005). The addition of Dex (10^{-7} M) reduces the median effective concentration (EC_{50}) of *t*RA for the induction of iodide uptake to $\sim 7\%$ (Fig. 7A). The *in vivo* systemic dose of *t*RA for the maximum induction of NIS is quite high (more than 100 times the clinical dose for acute promyelocytic leukemia). The combination treatment with Dex, therefore, has a potential to reduce the dose of RA, resulting an expected low toxicity *in vivo*. The reduced dose of *t*RA (10^{-7} M) with Dex actually achieves cytotoxicity of ^{131}I *in vitro* clonogenic assay as well as 10^{-6} M *t*RA (Fig. 7B). Interestingly, duration of the iodide uptake and NIS mRNA with the AGN190168 ($\text{RAR}\beta/\gamma$) is significantly longer than that with *t*RA, especially in combination with Dex (Fig. 7C and D). Isoform-selective retinoid agonists may have a more specific action, and reduced risk of adverse effects, especially if lower doses could be used (Chandraratna 1998). The combination of an RAR isoform-selective ligand and Dex has a potential to provide a more effective induction of radioiodide uptake in some breast cancer likely with less toxicity.

Conclusions

The mechanism of TSH stimulation of NIS gene expression in the thyroid has been extensively studied and many of the factors involved in the signal transduction pathways have been identified. The regulation of NIS expression in thyroid cancer, however, has been much more difficult to characterize. Differential expression of several nuclear factors involved in NIS gene expression has been reported, comparing normal thyroid cells and aggressive thyroid cancer cells. These studies, however, have not consistently shown a requirement for a specific combination of transcription factors. Differentiation agents, such as RA, PPAR- γ ligand, HDAC inhibitors,

and de-methylation agents, have shown a modest effect inducing NIS expression in some thyroid cancer cell lines. None of these agents, however, has been shown to consistently enhance radioiodide uptake in human clinical studies. NIS protein is located on the basolateral membrane in normal thyroid and lactating mammary glands, while intracellular expression of NIS predominates in thyroid and breast cancer. TSH stimulates not only NIS gene expression, but also the translocation of NIS to the plasma membrane in the normal thyroid gland. Agents that promote post-translational stimulation of NIS should be investigated to increase the efficacy of radioiodide therapy in thyroid and breast cancers. Three-dimensional primary culture of surgical specimens, followed by immunocytochemical analysis and iodide uptake assay, will be useful to evaluate the regulation of NIS translocation.

Recent studies promoting NIS expression in breast cancer models have demonstrated the potential utility of radioiodide therapy. Lactogenic hormones, cAMP stimulators, and retinoids, especially with glucocorticoid enhancement, have been shown to increase iodide uptake in breast cancer cells and in *in vivo* models. Further studies of the molecular mechanisms that underlie NIS stimulation by these hormones, as well as adjustment of dose and duration of these treatments to maximize the functional NIS expression, will be necessary to utilize radioiodide therapy in clinical breast cancer treatment.

Acknowledgements

We express our appreciation to Drs Jerome Hershman and Toshimasa Onaya for their seminal contributions to studies of NIS expression and their encouragement in our work. This review was supported by NIH RO1 CA089364 and VA merit review funds to GAB. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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