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 radiodine 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.

Introduction

The thyroid contains 70–90% total iodine in the body (9–10 mg; Riggs 1952). The thyroid gland must trap about 60 μg 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.
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$_2$O$_2$, 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 ($^{99m}$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 & Koehrle 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 (Gillilland 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 chemotheraphy (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 (Welsh & 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 $\mu$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>
<thead>
<tr>
<th>Agent</th>
<th>Pharmacology</th>
<th>Experimental system</th>
<th>Iodide uptake</th>
<th>NIS mRNA</th>
<th>NIS protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyrotropin (TSH)</td>
<td>TSHR agonist</td>
<td>FRTL-5 rat cell line</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Kogai et al. (1997)</td>
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<tr>
<td></td>
<td></td>
<td>PCCl3 rat cell line</td>
<td>X</td>
<td>X</td>
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<td>Trappaso et al. (1999)</td>
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<td></td>
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<td>WRT rat cell line</td>
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<td>X</td>
<td>X</td>
<td>Cass &amp; Meinkoth (2000)</td>
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<td>Primary human thyroid cell culture (normal)</td>
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<td>X</td>
<td>Saito et al. (1997), Ajan et al. (1998)</td>
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<td></td>
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<td>KAT50 human cell line</td>
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<td>Venkataraman et al. (1998)</td>
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<td>Rat normal thyroid, <em>in vivo</em></td>
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<td>X</td>
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<tr>
<td>hCG</td>
<td>TSHR agonist</td>
<td>FRTL-5 rat cell line</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Levy et al. (1997)</td>
</tr>
<tr>
<td>Forskolin</td>
<td>Adenyl cyclase activator</td>
<td>FRTL-5 rat cell line</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Arturi et al. (2002)</td>
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<tr>
<td></td>
<td></td>
<td>PCCl3 rat cell line</td>
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<td>Kogai et al. (1997)</td>
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<tr>
<td></td>
<td></td>
<td>Primary human thyroid cell culture (normal)</td>
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<td>X</td>
<td></td>
<td>Trapasso et al. (1999)</td>
</tr>
<tr>
<td>Adenosine tRA</td>
<td>A&lt;sub&gt;1&lt;/sub&gt; receptor agonist</td>
<td>FRTL-5 rat cell line</td>
<td>X</td>
<td>X</td>
<td></td>
<td>Saito et al. (1997)</td>
</tr>
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<td></td>
<td>Retinoic acid receptor (RAR)</td>
<td>FTC-133 and FTC-238, follicular cancer cell lines</td>
<td>X</td>
<td>X</td>
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<tr>
<td>Troglitazone</td>
<td>PPAR-γ ligand</td>
<td>FTC-133, follicular cancer cell line</td>
<td>X</td>
<td>X</td>
<td></td>
<td>Park et al. (2005)</td>
</tr>
<tr>
<td>Depsipeptide</td>
<td>Histone deacetylase (HDAC)</td>
<td>FTC-133 and FTC-236, follicular cancer cell lines</td>
<td>X</td>
<td>X</td>
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<td></td>
<td>inhibitor</td>
<td>SW-1736 and KAT-4, anaplastic cancer cell lines</td>
<td>X</td>
<td>X</td>
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<tr>
<td>Trichostatin A</td>
<td>HDAC inhibitor</td>
<td>BHP 18-21v, papillary cancer cell line</td>
<td>X</td>
<td>X</td>
<td></td>
<td>Furuya et al. (2004b)</td>
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<tr>
<td></td>
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<td>BHP18-21v xenograft <em>in vivo</em></td>
<td>X</td>
<td>X</td>
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<tr>
<td>Valproic acid</td>
<td>HDAC inhibitor</td>
<td>ARO, anaplastic cancer cell line</td>
<td>X</td>
<td>X</td>
<td></td>
<td>Zarnegar et al. (2002)</td>
</tr>
<tr>
<td>5-Azacytidine</td>
<td>Demethylation agent</td>
<td>NPA, papillary cancer cell line</td>
<td>X</td>
<td>X</td>
<td></td>
<td>Fortunati et al. (2004)</td>
</tr>
<tr>
<td>Clinical use</td>
<td></td>
<td>ARO, anaplastic cancer cell line</td>
<td>X</td>
<td>X</td>
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<tr>
<td>hrTSH</td>
<td></td>
<td>Metastatic/recurrent cancer (50–90%)</td>
<td>X</td>
<td>X</td>
<td></td>
<td>Ladenson et al. (1997), Jarzab et al. (2003)</td>
</tr>
<tr>
<td>13-Cis RA</td>
<td>Pro-drug of tRA</td>
<td>Metastatic/recurrent cancer (0–42%)</td>
<td>X</td>
<td>X</td>
<td></td>
<td>Simon et al. (2002a)</td>
</tr>
</tbody>
</table>

X in column indicates significant induction by agent. TSHR, thyroid stimulating hormone receptor.
Uptake of $^{125}$I or $^{99m}$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.

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

X in column indicates significant induction by agent.

*Fold induction compared to without treatment is shown for data from MCF-7 cells.

<sup>a</sup>The induction at 12 h. Cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) with 0.2% fetal bovine serum (FBS).

<sup>b</sup>The induction at 24 h (PGE<sub>2</sub>) or 48 h (hCG). Cells were maintained in DMEM: F12, 50: 50 with 10% FBS.

<sup>c</sup>The induction at 24 h (PGE<sub>2</sub>) or 48 h (hCG). Cells were maintained in MEM with 10% FBS or serum replacement.

<sup>d</sup>The induction at 48 h. Cells were maintained in MEM with 10% FBS or serum replacement.

<sup>e</sup>The induction at 48 h. Cells were maintained in MEM with 10% FBS or serum replacement.

<sup>f</sup>The induction at 24 h with 10<sup>−7</sup> M 9-cis RA.
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 downregulates 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 et al. 2002).
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 &

Figure 3 TSH signal transduction to the NIS gene in thyroid cells. CHOP, CCAAT/enhancer binding protein (C/EBP) homologous protein.
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-1 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 −147 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 NUE. These B-ZIP factors form homodimers or bind to the CRE-like regulatory sequence in the human and/or the cAMP-response element modulator (CREM) (2003), although binding of CHOP to the NUE has not been confirmed.

The corepressor 2, a substrate of the p38 (Pomerance et al. 2000), 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. 2004). 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-Impimbiato 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 3 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
folicles 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-Valentin 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, Puppin 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 BPH 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 (Puppin 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 (Puppin 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 rearrangements, 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 NUE (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 NUE 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 & Koehrle 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 (tRA; 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 tRA, 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, Gruning et al. 1998, 2002, 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 tRA 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 tRA. 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, Gruning 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 RARβ and RXRγ 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 specimens 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 oncoproteins, 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, Grovenor 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'-disothiocyanatostilbene 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 $^{99m}$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 $^{99m}$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 $^{131}$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 $^{99m}$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 H$_2$O$_2$, 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 without iodide organification (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 uptake was observed in MCF-7 cells treated with prolactin or oxytocin, and the combination of these hormones did not produce an additive effect. The results of these experiments support the potential use of lactogenic hormones as therapeutic agents for the treatment of breast cancer.

Figure 4: Iodide uptake and NIS expression in all-trans RA (tRA)-stimulated MCF-7 cells. (A) Iodide uptake in all-trans RA (tRA)-stimulated MCF-7 cells. The cells were treated with 10⁻⁶ M tRA for 48 h. *P < 0.001 compared to 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–Ca2+ 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). tRA (10⁻⁶ M) markedly induces iodide uptake,
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 tRA treatment, and only faint staining of NIS in the absence of tRA (Fig. 4). In contrast, ER-negative breast cancer cell line MDA-MB 231 shows no iodide uptake and NIS protein expression even after tRA treatment.

Significant inductions of iodide uptake and NIS mRNA have been observed with isomers of tRA, 9-cis RA, and 13-cis RA (Kogai et al. 2000b, 2005, Tanosaki et al. 2003). tRA 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. tRA 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 tRA 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 RARβ/γ agonist (AGN190168) is a more potent inducer of functional NIS expression than AGN195183 (RARα agonist), AGN194433 (RARγ agonist), and AGN194204 (pan-RXR ligand), suggesting a central role of RARβ in NIS induction by retinoids (Kogai et al. 2005).

tRA induces NIS gene expression partially at the transcriptional level (Kogai et al. 2000a). Our study with RAR-selective ligands has suggested that signaling through RARβ 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[T/A][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 tRA 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 tRA 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 (T Kogai, Y Kanamoto & G A Brent, unpublished observations). Interestingly, the DR-5, but not the DR-2, responds to the tRA 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 tRA 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 tRA. RT-PCR of NIS was performed after 2 days treatment with the same dose of tRA. *, 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/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 μCi/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 rRA 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 rRA increases NIS protein expression, but does not stimulate NIS trafficking to the membrane (Fig. 6). The effect of rRA on the expression of functional NIS and its mRNA has been confirmed in another *in vivo* model, the transgenic mice overexpressing the PyVT oncoprotein 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 rRA has been observed in other organs expressing NIS, including thyroid, stomach, kidney, and normal mammary glands, the rRA 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 (Lytte 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 rRA for the induction of iodide uptake to $\sim 7\%$ (Fig. 7A). The *in vivo* systemic dose of rRA for the maximum induction of NIS is quite high (more than 100 times the clinical dose for acute promyeloctytic 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 rRA ($10^{-7}$ M) with Dex actually achieves cytotoxicity of $^{131}$I *in vitro* clonogenic assay as well as $10^{-6}$ M rRA (Fig. 7B). Interestingly, duration of the iodide uptake and NIS mRNA with the AGN190168 (RAR$\beta/\gamma$) is significantly longer than that with rRA, 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-$\gamma$ 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.

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