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The Na^+/I^- Symporter (NIS): Recent Advances

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The Na^+/I^- symporter (NIS) catalyzes the accumulation of iodide into thyroid cells, an essential step in the biosynthesis of thyroid hormones. As a result of the isolation of the rat NIS cDNA, steadfast advances in the study of NIS at the molecular level have resulted in the following accomplishments: generation of high-affinity anti-NIS antibodies, elucidation of NIS stoichiometry and specificity by electrophysiological analysis, biochemical and immunological experimental testing of the proposed NIS secondary structure model, monitoring the regulation of NIS protein expression by thyroid stimulating hormone and iodide, characterization of the rat NIS gene promoter, isolation of the cDNA clone encoding human NIS and subsequent determination of human NIS genomic organization, description of NIS mutations in patients with congenital lack of iodide transport, and the molecular identification of NIS in extrathyroidal tissues.

KEY WORDS: Thyroid; iodide; symporter; NIS.

INTRODUCTION

The study of iodide (I^-) transport dates back to 1915, when the ability of thyroid follicular cells to concentrate I^- was first reported (Marine and Feiss, 1915). More than 80 years later a cDNA that encodes the rat Na^+/I^- symporter (NIS) was isolated in our laboratory (Dai *et al.*, 1996a). NIS is a 618 amino acid plasma membrane glycoprotein that catalyzes the active transport of I^- into the thyroid follicular cells, a crucial step in the biosynthesis of the thyroid hormones T_3 and T_4 [triiodothyronine and thyroxine (or tetraiodothyronine) respectively]. The isolation of the NIS cDNA marked the beginning of the characterization of I^- transport at the molecular level. The pace of NIS research has since increased dramatically. Recent findings on NIS are having considerable impact on both the study of thyroid pathophysiology and the continued structure/function analysis of Na^+-coupled transporters. Interestingly, whereas I^- accumulation has long been known to also exist in a few other tissues besides the thyroid, new data show that NIS itself, rather than a different protein, catalyzes I^- transport in at least one extrathyroidal tissue, the lactating mammary gland. Considering these results, it is conceivable that NIS mediates I^- transport in the other I^--transporting tissues as well, namely salivary glands, choroid plexus, and gastric mucosa, thereby suggesting that the study of NIS alone probably encompasses all I^- active transport processes, even in tissues where a role for I^- has yet to be elucidated. The topic of I^- transport has previously been extensively reviewed in 1961 (Halmi), 1964 (Wolff), 1993 (Carrasco), 1996 (Dai *et al.*, 1996b), and 1997 (Levy and Carrasco). The present article focuses primarily on the most recent developments in the subject.

BRIEF SUMMARY OF THYROID PHYSIOLOGY

The characteristic microscopic morphology of the vertebrate thyroid consists of a series of follicles of various sizes, each of which is roughly a spherical structure consisting of a single layer of epithelial follic-

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ular cells or thyrocytes surrounding the colloid (Fig. 1A). The thyroid follicular cells accumulate I^-, and synthesize thyroglobulin (Tg) and thyroid hormones T_3 and T_4. These cells exhibit a basolateral end facing the basement membrane, the interstitial space and the capillary bed, and an apical end facing the colloid (Fig. 1B). The apical surface of the follicular cells contains numerous microvilli that result in a greatly expanded area of exposure to the colloid. This region is known as the cell/colloid interface.

Tg is the most abundant of the thyroid-specific proteins, a large (~660 kDa) homodimeric glycoprotein that serves as the molecular template for the synthesis of T_3 and T_4 at the cell/colloid interface, and as a storage substrate for hormones and I^-. Tg is by far the principal component of the colloid, where it is found at a concentration of >50 mg/ml (Werner and Ingbar, 1991; Degroot, 1995). The thyroid hormones are iodothyronines, i.e., the result of two coupled iodotyrosines (the structure of T_4 is shown in Fig. 1B). The basic events leading to the biosynthesis of these hormones may be briefly summarized as follows (Fig. 1B): I^- is actively accumulated across the basolateral plasma membrane of the thyroid follicular cells in a

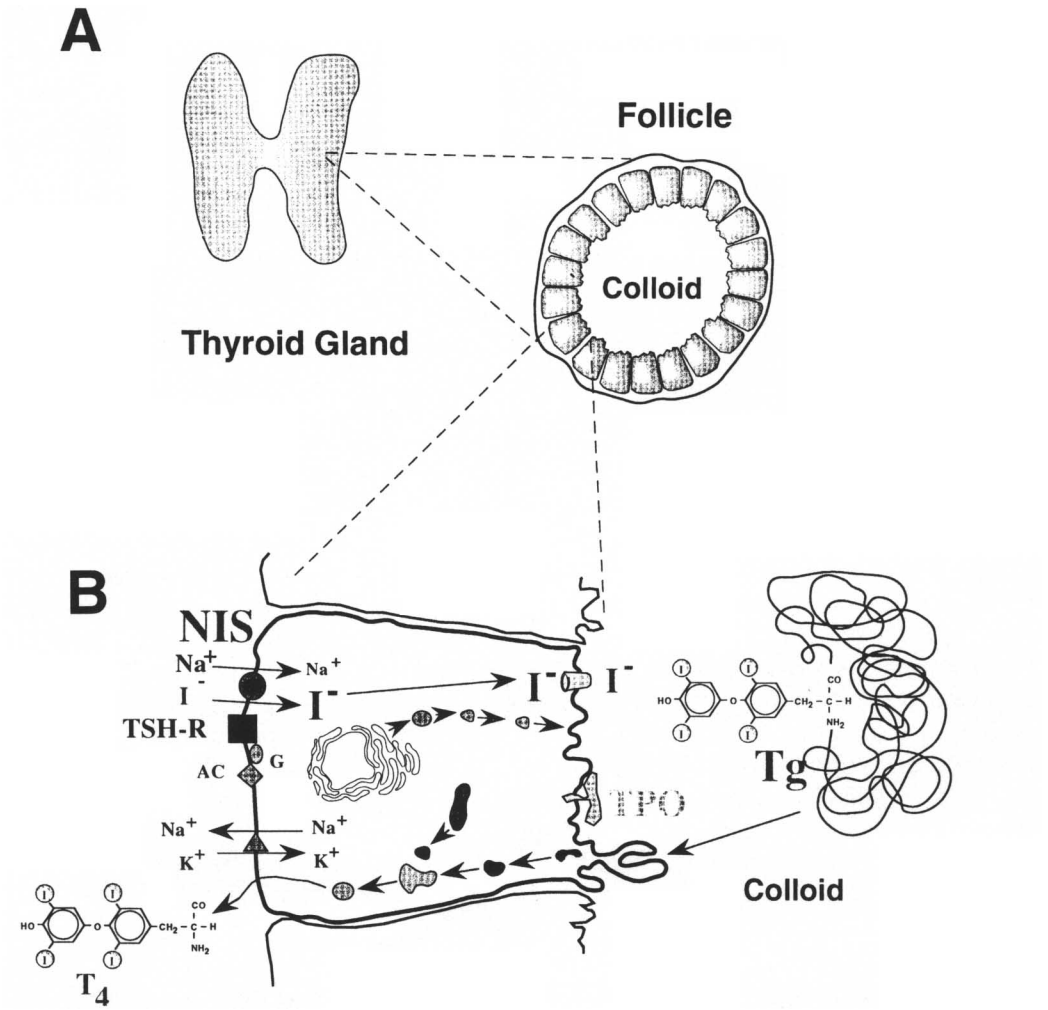

Fig. 1. (A) Schematic representation of the thyroid gland and a thyroid follicle. The thyroid follicle is comprised of a layer of epithelial cells which surrounds the colloid. (B) Schematic representation of the biosynthetic pathway of thyroid hormones T_3 and T_4 in the follicular cell. The basolateral end of the cell is shown on the left side of the figure, and the apical end on the right. (●) Active accumulation of I^-, mediated by the Na^+/I^- symporter (NIS); (▲) Na^+/K^+ ATPase; (■) TSH receptor; (◆) adenylate cyclase; (G) G protein; (⬭) I^- efflux towards the colloid; (TPO) TPO-catalyzed organification of I^- (←) endocytosis of iodinated Tg, followed by phagolysosomal hydrolysis of endocytosed iodinated Tg, and release of T_3 and T_4.

process catalyzed by NIS, and passively translocated across the apical membrane into the colloid via a putative I^- channel. NIS-catalyzed I^- accumulation is a Na^+-dependent active transport process that couples the energy released by the inward "downhill" translocation of Na^+ down its electrochemical gradient to driving the simultaneous inward "uphill" translocation of I^- against its electrochemical gradient (Carrasco, 1993). The Na^+ gradient acting as the driving force for I^- accumulation is generated by the Na^+/K^+ ATPase. Na^+/I^- symport activity in the thyroid is characteristically blocked by the competitive inhibitors perchlorate and thiocyanate (Carrasco, 1993).

Accumulated I^- that has reached the cell/colloid interface is oxidized and incorporated into some tyrosyl residues within the Tg molecule in a reaction catalyzed by thyroid peroxidase (TPO), leading to the subsequent coupling of iodotyrosine residues. This incorporation of I^- into organic molecules is called "I^- organification," a reaction pharmacologically blocked by such antithyroid agents as 6-*n*-propyl-2-thiouracil (PTU) and 1-methyl-2-mercaptoimidazole (MMI). Iodinated Tg is stored extracellularly in the colloid. In response to demand for thyroid hormones, phagolysosomal hydrolysis of endocytosed iodinated Tg ensues. T_3 and T_4 are secreted into the bloodstream, and nonsecreted iodotyrosines are metabolized to tyrosine and I^-, a reaction catalyzed by the microsomal enzyme iodotyrosine dehalogenase. This process facilitates reutilization of the remaining I^- (Carrasco, 1993). All steps in the thyroid hormone biosynthetic pathway, including NIS-catalyzed I^- accumulation, are stimulated by thyroid stimulating hormone (TSH) from the pituitary. The effect of TSH results from binding of the hormone to the TSH receptor. Unlike hormone biosynthesis in other endocrine glands, hormone production in the thyroid occurs to a large extent in the colloid, an extracellular compartment. T_3 and T_4 play essential roles in regulating intermediary metabolism in virtually all tissues and in maturation of the nervous system, skeletal muscle, and lungs in the developing fetus and the newborn (Werner and Ingbar, 1991; Degroot, 1995).

At the most fundamental level, the immediate functional significance of NIS lies in that no thyroid hormone biosynthesis is possible in the absence of I^-. Since I^- is relatively scarce in the environment and thus in the diet, without NIS not enough I^- would reach the thyroid at average daily I^- intake levels to keep a person euthyroid. Indeed, even with fully functional NIS, nearly one billion people throughout the world are at risk of suffering from endemic iodine deficiency disorders (IDD) often associated with hypothyroidism as a result of insufficient dietary supply of iodine (Stubbe *et al.*, 1986). In clinical endocrinology practice, NIS activity is a key attribute of the thyroid gland for diagnostic, prognostic, and therapeutic purposes. Thus, NIS provides the molecular basis for the thyroidal radioiodide uptake test and for thyroid scintigraphy, two thyroid function tests of considerable value as diagnostic aids in a variety of thyroid pathological conditions (Werner and Ingbar, 1991; Degroot, 1995). For example, the possible existence of thyroid cancer must be ruled out whenever a thyroid nodule is detected. Thyroid nodules that are determined by scintigraphy to accumulate I^- equally or more efficiently than the normal surrounding tissue are generally benign, while most thyroid cancers display markedly reduced I^- accumulation activity relative to healthy tissue (Werner and Ingbar, 1991; Degroot, 1995). Therefore, it is likely that thyroid cancer can have a deleterious effect on the function of NIS. Still, NIS is sufficiently active in some thyroid cancers and metastases to render them amenable to detection and even destruction with radioiodine (Werner and Ingbar, 1991; Degroot, 1995). Conversely, large doses of radiation reaching the gland via NIS in the form of iodine isotopes can cause thyroid cancer. The most dramatic example of this is the alarming rise in the incidence of thyroid cancer cases in children in Ukraine and Belarus in the wake of the 1986 Chernobyl power plant accident (Likhtarev *et al.*, 1995; Abbott and Barker, 1996; Balter, 1995). In this instance, ^{131}I in the nuclear fallout was ingested largely through milk and concentrated in the thyroid via NIS. The same pattern, even if on a much smaller scale, occurred in the United States as a result of nuclear blasts at the Nevada Test Site near Las Vegas in the 1950s, which may have caused as many as 10,000 to 75,000 cases of thyroid cancer throughout the country (Wald, 1997; Brooke, 1997).

ISOLATION OF A cDNA CLONE ENCODING RAT NIS

In 1989, Vilijn and Carrasco expressed perchlorate-sensitive NIS activity in *Xenopus laevis* oocytes by microinjection of poly A^+ RNA isolated from FRTL-5 cells, a highly functional rat thyroid cell line (Vilijn and Carrasco, 1989). Thus the oocyte system was shown to be of potential value for the possible

expression cloning of the cDNA that encodes NIS, particularly in the absence of oligonucleotides based on protein sequence data or anti-NIS antibodies (Abs). Whereas this expression cloning strategy proved lengthy, it was eventually successful. By means of functional screening in *X. laevis* oocytes of a cDNA library from FRTL-5 cells, we isolated a cDNA encoding NIS in our laboratory (Dai *et al.*, 1996a). The I^- concentration gradient generated in oocytes microinjected with transcript from the single cDNA encoding NIS was >30-fold, i.e., virtually identical to that observed in the thyroid gland *in vivo* (Carrasco, 1993). This signal corresponded to a >700-fold increase in perchlorate-sensitive NIS activity over background. NIS activity rates in oocytes displayed saturation kinetics. The apparent K_m for I^- was 36 μM, a value consistent with the range of values reported for FRTL-5 cells (Dai *et al.*, 1996a,b). COS cells transfected with the NIS cDNA exhibited perchlorate-sensitive NIS activity, in contrast to control cells (Dai *et al.*, 1996a). The report includes the complete nucleotide sequence of the cloned NIS cDNA and the deduced amino acid sequence, showing that NIS is a protein of 618 amino acids (relative molecular mass 65,196).

MOLECULAR CHARACTERIZATION AND SECONDARY STRUCTURE MODEL OF NIS

A NIS secondary structure model was initially proposed upon isolation of the cDNA that encodes NIS, on the basis of the secondary structure algorithms (Dai *et al.*, 1996a). This original model predicted an intrinsic membrane protein with 12 putative transmembrane domains and both the NH_2 and COOH termini on the cytoplasmic side of the membrane (Fig. 2A) (Dai *et al.*, 1996a,b). Three putative *N*-linked glycosylation consensus sites were identified, at positions 225, 485, and 497, with the first predicted to face intracellularly and the other two extracellularly. However, subsequent experimental testing of NIS has led to revisions of this model. A major development in the characterization of the NIS molecule was the generation of anti-NIS Abs. Levy *et al.* have reported the generation of a high-affinity (K_d~100 pM) site-directed polyclonal anti-NIS Ab against the carboxy terminus, which immunoreacts with a ~87 kDa polypeptide from FRTL-5 cells (Levy *et al.*, 1997a). Significantly, no immunoreactivity was detected with polypeptides from FRT cells, a line of well-differentiated rat thyroid-derived cells that exhibit no NIS activity. Using their anti-NIS Ab, they confirmed the predicted cytosolic-side location of the carboxy terminus by indirect immunofluorescence experiments in permeabilized FRTL-5 cells. They also showed that treatment of membrane fractions from three systems expressing NIS (FRTL-5 cells, *X. laevis* oocytes, and COS cells) with peptidyl *N*-glycanase F converted the mature NIS polypeptide into a nonglycosylated NIS ~50 kDa species, the same relative molecular weight exhibited by NIS expressed in *E. coli*.

Subsequently and independently, another site-directed Ab against the same carboxy terminus domain of NIS was generated, which similarly immunoreacts with an ~80–90 kDa glycosylated NIS protein from FRTL-5 cells, and this Ab was used to explore the regulation of NIS by TSH in FRTL-5 cells (Paire *et al.*, 1997). These authors observed that tunicamycin, an inhibitor of the synthesis of *N*-linked oligosaccharides, prevented both the synthesis of mature NIS and the TSH-dependent reinduction of NIS activity in FRTL-5 cells. On this basis, they suggested that *N*-linked glycosylation of NIS is essential for NIS biosynthesis, correct folding, and stability. However, conclusive evidence showing that neither partial nor total lack of *N*-linked glycosylation impairs activity, stability, or targeting of NIS has recently been obtained (Levy *et al.*, 1997b). Using site-directed mutagenesis, these authors substituted both separately and simultaneously the Asn residues (amino acids 225, 485, and 497) in all three putative *N*-linked glycosylation consensus sequences of NIS with Gln, and assessed the effects of the mutations on function, targeting, and stability of NIS in COS cells. All mutants were active and displayed 50–100% of wild-type NIS activity, including the completely nonglycosylated triple mutant, which migrated as a ~50 kDa NIS polypeptide. They observed that the half-life of nonglycosylated NIS was similar to wild-type NIS and that the K_m value for I^- (~30 μM) in triple mutant NIS was virtually identical to wild-type NIS. These findings demonstrate that, to a considerable extent, function, and stability of NIS are present even in the total absence of *N*-linked glycosylation.

In their study of *N*-linked glycosylation of NIS, Levy *et al.* demonstrated that the putative *N*-linked glycosylation site at N225, which had originally been predicted to face intracellularly, is indeed glycosylated. Therefore, it is now clear that the hydrophilic loop that contains this sequence faces the extracellular milieu rather than the cytosol. Thus, these authors

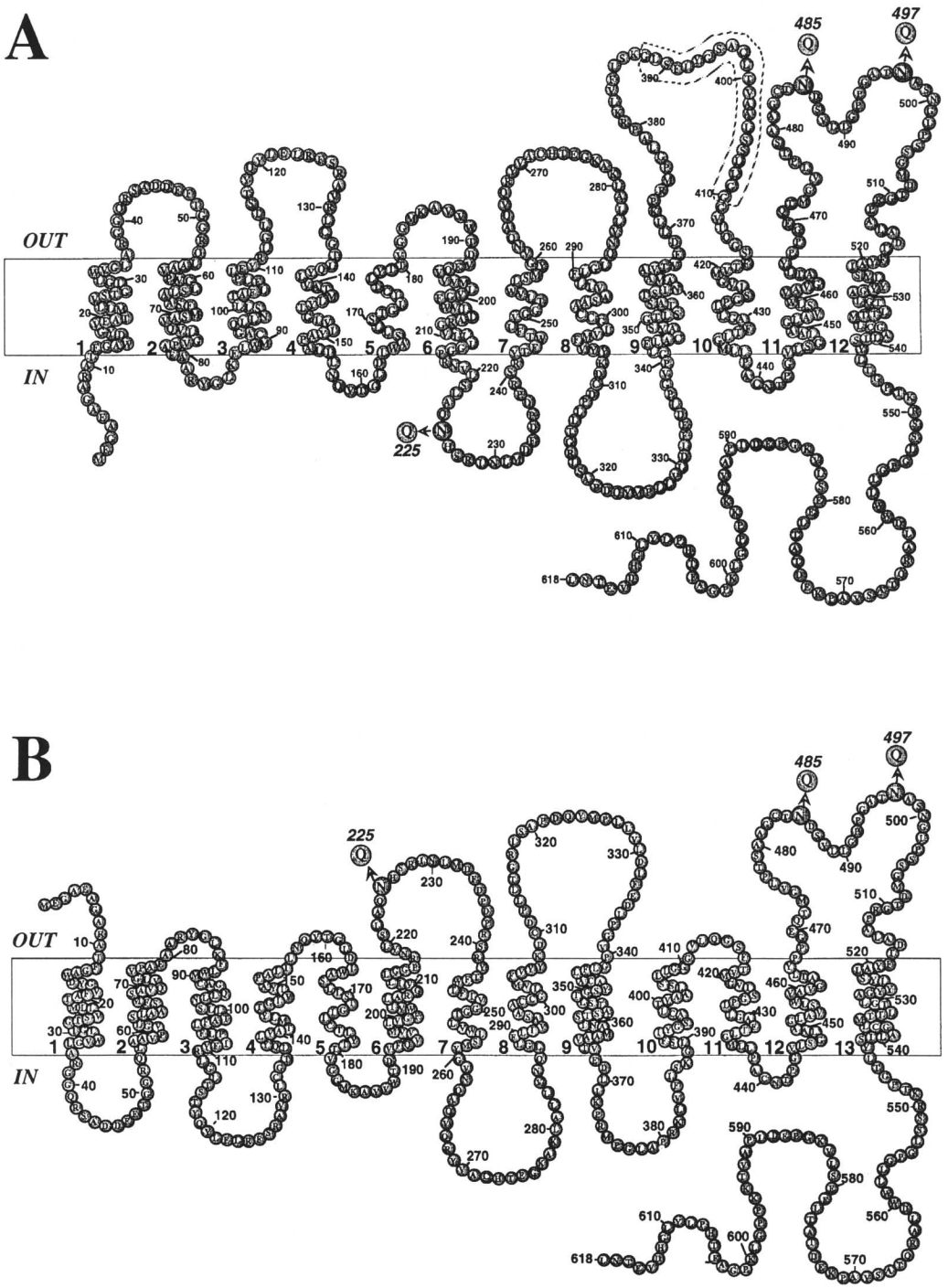

Fig. 2. (A) Original NIS secondary structure model. Original NIS secondary structure model with 12 putative transmembrane helices (2). Both the NH_2 and COOH termini are proposed to face intracellularly. Two putative *N*-linked glycosylation consensus sequences are indicated with bold **Q**'s at positions 485 and 497. The third *N*-linked glycosylation sequence at position 225 is located in the third predicted intracellular loop. (B) Revised NIS secondary structure model. Current NIS secondary structure model with 13 putative transmembrane helices. Both the hydrophilic loop containing N225 and the NH_2 terminus face extracellularly. All three *N*-linked glycosylation consensus sequences are indicated with bold **Q**'s at positions 225, 485, and 497. Amino acid residues 389–410 form a new transmembrane domain (helix X) between helices 9 and 10 of the original model (see dotted line, panel A).

currently propose a 13-helix model to be the most likely secondary structure for NIS (Fig. 2B). In contrast to the original model, in the current one both the NH_2 terminus and the hydrophilic loop containing N225 are predicted to be on the extracellular side. This model is consistent with recent findings reported on Na^+/glucose cotransporter (SGLT1) using *N*-linked glycosylation scanning mutagenesis (Turk *et al.*, 1996), which have led to a new model for SGLT1 that also places the NH_2 terminus on the extracellular face of the membrane.

ELECTROPHYSIOLOGICAL ANALYSIS OF NIS: MECHANISM, STOICHIOMETRY, AND SPECIFICITY

The mechanism, stoichiometry, and specificity of NIS by means of electrophysiological, tracer uptake, and electron microscopic methods in *X. laevis* oocytes expressing NIS have been thoroughly examined (Eskandari *et al.*, 1997) using the two-microelectrode voltage clamp technique. These studies showed that an inward steady-state current (i.e., a net influx of positive charge) is generated in NIS-expressing oocytes upon addition of I^- to the bathing medium, leading to depolarization of the membrane. Since the recorded current is attributable to NIS activity, this observation confirms that NIS activity is electrogenic. Simultaneous measurements of tracer fluxes and currents revealed that 2 Na^+ ions are transported with one anion, demonstrating unequivocally a 2:1 Na^+/I^- stoichiometry. Therefore, the observed inward steady-state current is due to a net influx of Na^+ ions (Fig. 3).

Similar steady-state inward currents were generated by a wide variety of anions in addition to I^- (including ClO_3^-, SCN^-, $SeCN^-$, NO_3^-, Br^-, BF_4^-, IO_4^-, and BrO_3^-), indicating that these anions are also transported by NIS. However, perchlorate (ClO_4^-), the most widely characterized inhibitor of thyroidal I^- uptake, surprisingly did not generate a current, strongly suggesting that it is not transported. Similarly, it has been reported that ClO_4^- did not induce an inward current in Chinese hamster ovary (CHO) cells stably expressing NIS (Yoshida *et al.*, 1997), as measured using the whole-cell patch clamp technique. The most likely interpretation of these observations is that ClO_4^- is not transported by NIS, although the unlikely possibility that ClO_4^- is translocated by NIS on a 1:1 Na^+/ClO_4^- stoichiometry cannot be ruled out. In conclusion, ClO_4^- is a potent inhibitor of NIS acting as a blocker, not as a substrate.

Eskandari *et al.* (1997) observed also that in response to step voltage changes, NIS exhibited current transients that relaxed with a time constant of 8–14 ms, and that presteady-state charge movements (integral of the current transients) versus voltage relations obeyed a Boltzmann distribution. These charge movements are attributed to the conformational changes of the empty transporter within the membrane electric field. These authors determined that the turnover rate of NIS is $\sim 36\ s^{-1}$, and reported that expression of NIS in oocytes led to a ~2.5-fold increase in the density of plasma membrane protoplasmic face intramembrane particles, as ascertained by freeze-fracture electron microscopy. This is the first direct electron microscopy visualization of ostensible NIS molecules present in the oocyte plasma membrane. Moreover, on the basis of their kinetic results, these authors proposed an ordered simultaneous transport mechanism in which Na^+ binds to NIS before I^-, i.e., whereas transport of both ions is simultaneous, binding is ordered and sequential (Fig. 3). The combined data from electrophysiological measurements and freeze-fracture electron microscopy suggest that NIS may be multimeric in its functional form.

REGULATION OF NIS

The analysis of the regulatory roles played by TSH and I^- on NIS function have only recently started to be explored at the molecular level. It has been shown in rats that NIS protein expression is upregulated by TSH *in vivo* (Levy *et al.*, 1997a). NIS upregulation was observed in rats with increased TSH circulating levels caused either by propyl thiouracil (PTU) treatment or an I^- deficient diet. Conversely, NIS protein expression was decreased in hypophysectomized rats, which exhibit markedly lower TSH levels. A single injection of TSH to hypophysectomized rats reinduced the expression of NIS protein back to basal levels. Consistent with these findings is the observation that the expression of NIS mRNA in dog thyroid is dramatically upregulated by goitrogenic treatment (Uyttersprot *et al.*, 1997). These authors also reported in *in vivo* studies that low doses of I^- inhibited the expression of both TPO and NIS mRNAs in dog thyroid. They studied the effects of low doses of I^- (0.3 mg KI/10 kg dog) on thyroid-specific gene expression in hyperstimulated thyroids from dogs treated with goitrogens

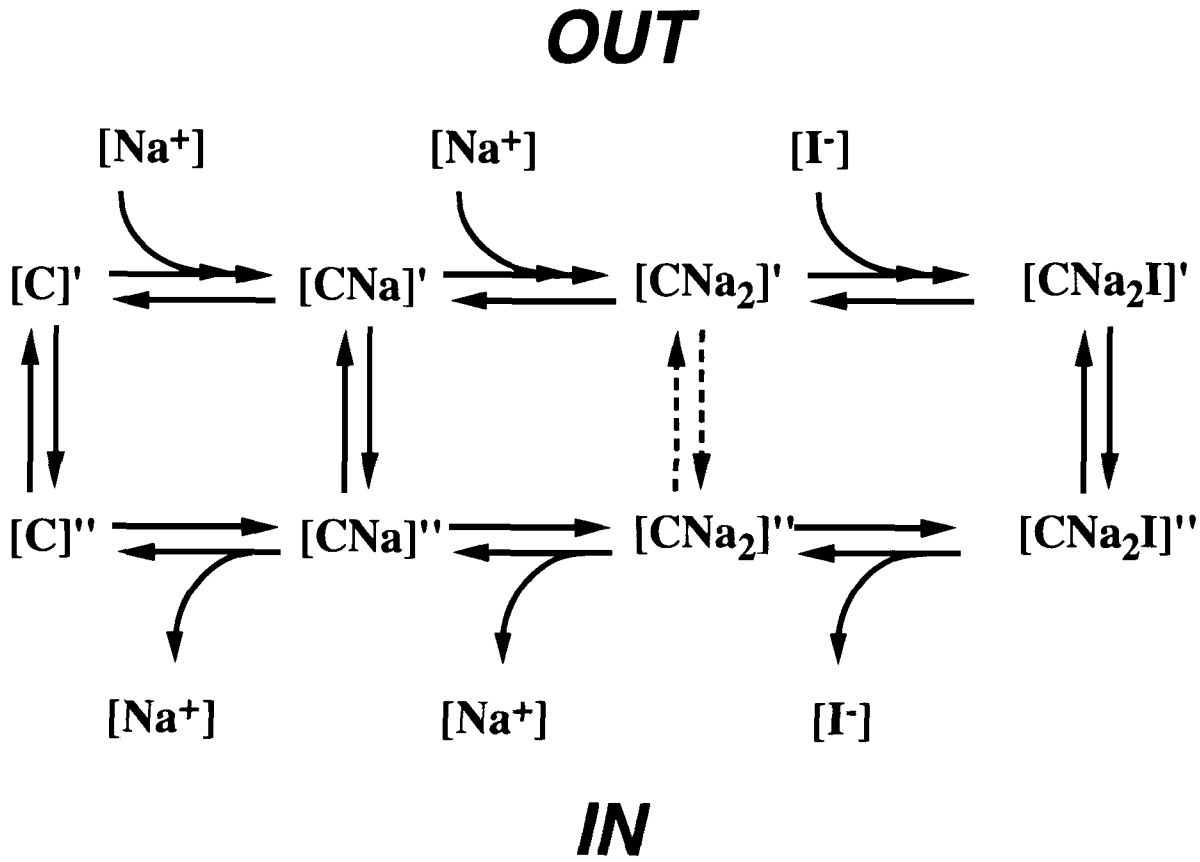

Fig. 3. Schematic representation of Na^+/I^- symport. As shown in this scheme, first one Na^+ ion binds to NIS which in the absence of substrate is able to cross the membrane via NIS in a Na^+ uniport mode ($CNa' \rightarrow CNa''$; C, carrier). Release of Na^+ into the cytoplasm is followed by the return of the empty binding site to complete the pathway ($C'' \rightarrow C'$). The kinetic data suggest that Na^+ binds to NIS before I^-. In the presence of I^- the complex CNa_2I' is formed which undergoes a conformational change to expose the bound I^- and 2 Na^+ ions to the interior of the cell ($CNa_2I' \rightarrow CNa_2I''$). Both Na^+ ions and I^- are released into the cytoplasmic compartment, and the empty carrier undergoes another conformational change to expose the binding sites to the external solution again. Charge movement data suggest that the Na^+ binding dissociation does not contribute greatly to the total observed charge. Thus, it is proposed that NIS charge movements arise primarily from conformational changes of the empty carrier ($C' \rightarrow C''$). For more details see reference 21.

(PTU and $NaClO_4$) and deprived of I^-. As the TSH level is not modified for up to 48 h after I^- administration, the first 24–48 h of treatment constitute a window during which the direct effects of I^- can be studied *in vivo* under TSH stimulation. Remarkably, during this period I^- did not alter the expression of Tg or TSH receptor (TSHr) mRNA. In contrast, TPO and NIS mRNA expression decreased 48 h after I^- administration. The low doses of I^- (which neither caused necrosis nor inhibited overall thyroid function) resulted in decreased cell proliferation and re-established the synthesis of thyroid hormones. These observations explain the well-known adaptation to the Wolff–Chaikoff effect observed *in vivo*, in which hours after treatment with I^- there is an inhibition of thyroidal I^- accumulation (Wolff, 1969).

The availability of NIS cDNA and anti-NIS Abs has made it possible to study the mechanism by which TSH regulates NIS mRNA and NIS protein levels in FRTL-5 cells. Re-addition of TSH to TSH(−) cells (7 days) significantly increased NIS mRNA, reaching a maximum (~6-fold) after 24 h (Kogai *et al.*, 1997). Forskolin and $(Bu)_2$cAMP mimicked this stimulatory effect on both the I^- transport activity and mRNA levels. Immunoblot analysis demonstrated that NIS protein is present in TSH(−) FRTL-5 cells, even when I^- transport activity is absent in these cells. NIS expression in TSH (−) cells was predicted from an earlier

finding that I^- transport activity is present in membrane vesicles from TSH (−) FRTL-5 cells (Kaminsky *et al.*, 1994). Re-addition of TSH increased NIS protein levels, reaching a maximum (~3-fold) at 72 h (Kogai *et al.*, 1997); this parallels the time course of I^- transport activity observed in re-addition of TSH to TSH(−) cells, in contrast to the delayed time course reported (Paire *et al.*, 1997). From these observations, it was concluded that stimulation of I^- transport activity by TSH in FRTL-5 cells is partly due to a rapid increase in NIS gene expression, followed by a slower increase in NIS protein synthesis (Kogai *et al.*, 1997). However, the coexistence of high amounts of NIS protein and absence of I^- transport activity in TSH(−) FRTL-5 cells suggests that this activity is controlled by other factor(s).

Upon either removal of TSH or addition of cycloheximide, I^- transport activity declined more rapidly than NIS. The half-life of NIS was estimated to be 4 days, on the basis of their cycloheximide data (Paire *et al.*, 1997). This is consistent with results from *in vivo* ^{35}S[Cys/Met] labeling pulse chase analysis indicating that NIS has an extremely long half-life in FRTL-5 cells (~4–5 days; Levy *et al.*, 1997a).

REGULATION OF THE rNIS GENE

It has long been established that thyroidal I^- transport is stimulated by TSH via the cAMP pathway. To explore whether any agents that increase the level of cAMP in thyrocytes, such as TSH or forskolin, regulate transcription of rNIS mRNA, the 5′-flanking region of rNIS gene has been isolated and characterized (Ohno *et al.*, 1997). This region revealed the presence of a constitutive promoter, contained within 0.56 kb upstream of the translation start, and of a cell-type specific enhancer, located between nucleotides −2945 to −2264. This enhancer is also capable of responding to cAMP in a cell-type specific manner. To respond to cAMP, the enhancer requires *de novo* protein synthesis. Notably, neither protein kinase A (PKA) nor CREB is indispensable for the transcriptional activity of rNIS, an intriguing observation that suggests that this regulatory element responds to a novel cAMP sensitive pathway. This is completely different from that observed in promoters of other genes encoding thyroid specific proteins, such as thyroglobulin, TPO, and TSH receptor, where most of the relevant regulatory elements necessary to obtain cell-type specific transcription are located proximal to the transcription start site and are largely cAMP independent. Ohno *et al.* also observed the presence of a negative regulatory element between −2264 and −564.

Recently, the presence of a NIS constitutive promoter contained within −291 to −135 bp was reported (Endo *et al.*, 1997). These authors determined that the sequence −240 to −237 bp, is important for TTF-1 (thyroid transcription factor 1) binding, but TTF-1 had only a modest effect on rNIS transcription activity. These authors did not find a CRE consensus or CRE-like sequence downstream of nucleotide −2264.

In contrast, Tong *et al.* concluded that the DNA regulatory elements within 8 kb the 5′-flanking region of rNIS are not sufficient to confer thyroid-selective transcription (Tong *et al.*, 1997). These results are at variance with those reported by Ohno *et al.* and Endo *et al.*

ISOLATION OF THE cDNA ENCODING HUMAN NIS (hNIS) AND ELUCIDATION OF THE GENOMIC ORGANIZATION OF hNIS

The cDNA encoding hNIS was identified on the expectation that hNIS would be highly homologous to rNIS. Using primers to the cDNA rNIS sequence a cDNA fragment of hNIS was amplified from human papillary thyroid carcinoma tissue by PCR. This cDNA fragment was utilized to screen a human thyroid cDNA library, and a single cDNA clone encoding hNIS was isolated (Smanik *et al.*. 1996). The nucleotide sequence of hNIS revealed an open reading frame of 1929 nucleotides which encodes a protein of 643 amino acids. hNIS exhibits 84% amino acid identity and 93% similarity to rNIS, differing only on account of two insertions not present in rNIS: a five amino acid insertion between the last two hydrophobic domains (amino acids 485–488 and 499) and a 20 amino acid insertion in the carboxy terminus (amino acids 618–637). Subsequently, the expression, exon–intron organization, and chromosome mapping of hNIS were elucidated (Smanik *et al.*, 1997). In general agreement with known patterns of I^- transport activity, hNIS expression was markedly reduced, albeit with considerable variability, in thyroid tumors. Fifteen exons encoding hNIS were found to be interrupted by 14 introns, and the hNIS gene was mapped to chromosome 19p (Smanik *et al.*, 1997).

IMPACT OF NIS RESEARCH ON THYROID DISEASE

Congenital lack of I^- transport is a rare condition most often diagnosed in patients who exhibit coexistence of goiter with congenital hypothyroidism, low or absent thyroidal uptake of radioiodine, and little or no I^- uptake by the salivary glands and gastric mucosa (Wolff, 1983; Leger *et al.*, 1987). The availability of the NIS cDNA has made it possible for the first time to examine the molecular basis of this condition. A homozygous missense mutation in a hypothyroid patient with an I^- transport defect was identified (Fujiwara *et al.*, 1997). They found Pro instead of Thr at position 354. Transfection of the mutant T354P NIS cDNA into HEK-293 cells (human embryonic kidney cells) did not elicit any I^- uptake activity. Hence, on this basis, the authors proposed that T354P probably disrupts, through a structural change, the 9th putative transmembrane helix of NIS, where the mutation is located. However, such a hypothesis remained to be tested. More recently it was reported that a patient diagnosed 23 years ago with an I^- transport defect had a loss of function mutation in the NIS gene (Matsuda and Kosugi, 1997). Strikingly, this mutation is exactly the same as that in Fujiwara's report, i.e., T354P. The patient was euthyroid, apparently on account of a very high iodine dietary intake, but he exhibited greatly increased NIS mRNA levels in his thyroid, and a slightly increased level of TSH at the time of the open thyroid biopsy. Hence, these authors proposed that the observed increase in NIS transcription may reflect a mechanism that compensates for low NIS activity, possibly related in this case to transcriptional regulation of NIS by I^- itself. This patient was born from a consanguineous marriage, and his daughter was heterozygous for the mutation but had no abnormal phenotype, suggesting the recessive nature of the disease. It is of considerable interest to compare these two cases. First, it is significant that they illustrate the variety of phenotypes that can be found for this condition, as one of the patients was hypothyroid and the other largely euthyroid. Second, whereas Fujiwara *et al.* observed no I^- transport activity when they transfected the cDNA bearing the T354P mutation into HEK-293 cells, Matsuda *et al.* reported a low but measurable and potentially significant level of activity (0.4% with respect to wild-type NIS). This discrepancy clearly calls for further study. Moreover, both patients come from the same region in Japan, and thus the possibility that they may share a common ancestor deserves to be explored.

Given the undeniable significance of T354P NIS as a spontaneously-occurring mutation, we sought to determine whether T354P NIS is a nonfunctional but stable polypeptide properly targeted to the plasma membrane, or a fully or partially functional protein that is retained in intracellular organelles as a result of the mutation (Levy *et al.*, unpublished observations). The latter is the case with some mutations of CFTR in cystic fibrosis (Cheng *et al.*, 1990) and of SGLT1 in glucose-galactose malabsorption (Martin *et al.*, 1996). We generated T354P by site-directed mutagenesis and transfected mutant and wild-type NIS cDNA into COS cells, which were then assayed for I^- uptake activity. In addition, anti-NIS Abs were used to determine the levels of NIS expression. COS cells transfected with T354P NIS exhibited no I^- transport activity, and were functionally indistinguishable from untransfected COS cells. In contrast, COS cells expressing wild-type NIS accumulated ~30 pmol I^-/μg DNA, and this uptake was inhibitable by perchlorate. These data are consistent with the reported lack of I^- uptake in both HEK cells and COS cells expressing human T354P NIS cDNA (Fujiwara *et al.*, 1997; Matsuda and Kosugi, 1997). To assess whether the absence of I^- uptake activity in T354P-transfected cells was due to lack of mutant NIS protein expression, an immunoblot analysis using anti-NIS Ab was carried out with membranes from COS cells expressing T354P. The levels of NIS were indistinguishable between COS cells expressing T354P and wild-type NIS. As indicated above, the hypothesis proposed by Fujiwara *et al.* that the presence of this proline induces a kink in the 9th putative transmembrane helix is only one possibility, which calls for experimental verification. Thus, to determine whether the structural change in the ninth α-helix produced by Pro 354 caused an inactive NIS, the effect of various other substitutions at position 354 was explored. The effect on NIS function of substituting Thr with Ala, an α-helix stabilizing amino acid was compared, with that of substituting Thr with Gly, an α-helix breaker. Both mutants yielded expressed but nonfunctional NIS proteins, indicating that the absence of Thr itself is a major factor in the function loss, rather than the effect on the α-helix (Fig. 4). (Levy *et al.* 1998).

Even more recently a different NIS mutation causing congenital lack of I^- accumulation in a 36 year old Brazilian man was reported (Pohlenz *et al.*, 1997). In this case the authors sequenced the patient's NIS

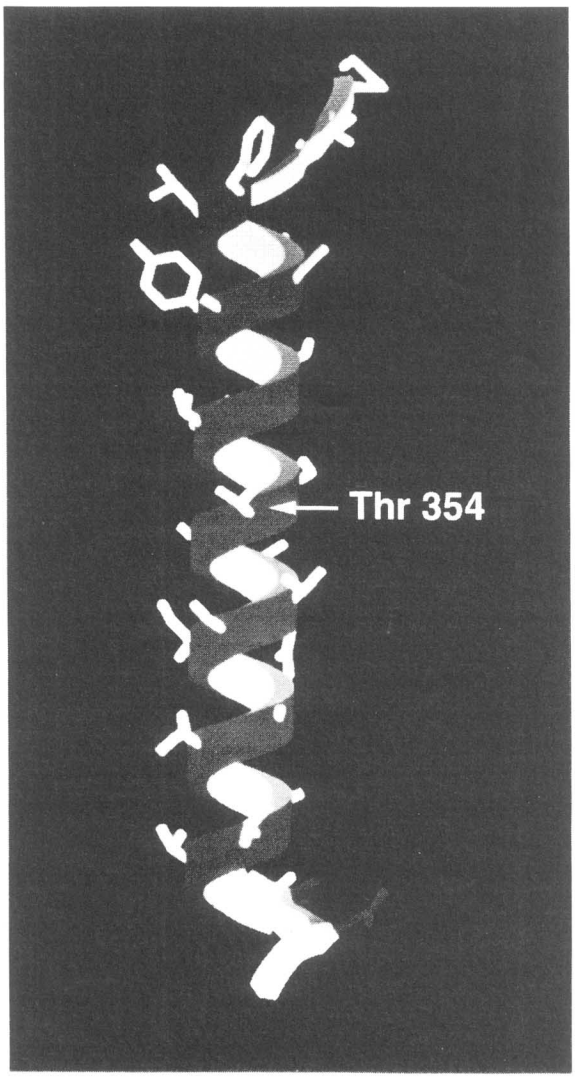

Fig. 4. Detail of putative NIS transmembrane domain nine.

cDNA derived from thyroidal mRNA, and found a homozygous substitution of the normal cytosine in nucleotide 1163 with an adenine, leading to a stop at codon 272. They determined that, not surprisingly, this mutation results in a nonfunctional NIS species.

EXTRATHYROIDAL EXPRESSION OF NIS: I^- TRANSPORT IN THE LACTATING MAMMARY GLAND

Several observations in the literature suggest that extrathyroidal I^- transport may be catalyzed by plasma membrane proteins that are very similar, if not identical, to thyroid NIS (Halmi, 1961; Wolff, 1964; Carrasco, 1993). As indicated above, patients suffering from congenital lack of thyroid I^- accumulation also lack the ability to transport I^- in gastric mucosa and salivary glands (Carrasco, 1993; Wolff, 1983; Leger *et al.*, 1987), pointing to the possibility of a genetic link between thyroid NIS and the gastric and salivary I^- transporters. Moreover, both thyroidal and extrathyroidal I^- accumulation are inhibitable by perchlorate and generate I^- concentration gradients of similar magnitude (~40-fold under steady-state conditions) (Carrasco, 1993). These data were therefore suggestive that anti-NIS Abs raised against thyroid NIS might crossreact with one or more of the extrathyroidal I^- transporters, and that anti-NIS Abs might be valuable probes for the characterization of these proteins.

A special functional link exists between I^- transport activity in the thyroid and in lactating mammary gland. I^- accumulated in lactating mammary gland and secreted into milk is used by the newborn for thyroid hormone biosynthesis. An adequate supply of I^- for sufficient production of thyroid hormones is essential for proper development of the newborn's nervous system, skeletal muscle, and lungs (Stubbe *et al.*, 1986). Iodine deficiency at this early stage in life results in severe mental retardation and in some cases dwarfism (Stubbe *et al.*, 1986). By analogy with thyroid, I^- transport in the mammary gland I^- is believed to be actively translocated from the bloodstream into the cytoplasm of the epithelial cells, from where I^- is probably secreted into milk via a facilitated diffusion channel. In our laboratory we have for the first time identified NIS extrathyroidally in the plasma membrane of mammary epithelial cells from lactating mammary gland (Levy *et al.*, unpublished observations). In addition, it was found that the expression and activity of mammary gland NIS (mg-NIS) are regulated by lactogenic stimuli, and that mg-NIS and thyroid NIS are subjected to different post-translational processing in their respective tissues.

Immunoblot analyses were performed to assess whether anti-NIS Ab would react with a mammary gland membrane protein. Strikingly, immunoreactivity against a single broad ~75 kDa polypeptide was observed in rat lactating mammary gland membranes, but not in membranes from nonlactating mammary gland or from lung, muscle, or heart, all tissues that do not transport I^-. This immunoreactive polypeptide is mg-NIS. The difference in electrophoretic mobilities between mg-NIS (~75 kDa) and thyroid NIS (~90

kDa) was investigated and found to be due to differences in their post-translational modifications. Membrane proteins from thyroid and lactating mammary gland were treated with *N*-glycosidase F, an enzyme that removes *N*-linked carbohydrates, and were probed with anti-NIS Ab. Under these conditions, anti-NIS Ab recognized a ~50 kDa polypeptide in membranes from either thyroid or lactating mammary gland. Significantly, both nonglycosylated NIS in thyroid and NIS expressed in *E. coli* exhibit an identical electrophoretic mobility (i.e., ~50 kDa). These results demonstrate that the ~75 kDa and ~50 kDa immunoreactive polypeptides detected in lactating mammary gland correspond to glycosylated and nonglycosylated mg-NIS, respectively.

Subsequently, we investigated whether a correlation existed between I^- accumulation activity and mg-NIS expression in the various physiological stages of the mammary gland (Levy *et al.*, unpublished observations). As determined by immunoblot analysis, we found that mg-NIS was absent in nubile and pregnant mammary gland, but clearly present in lactating mammary gland. To ascertain whether weaning had an effect on mg-NIS expression, mother rats were removed from their litters. Remarkably, 24 h after weaning, mg-NIS expression was significantly decreased, and after 48 h it was barely detectable. Furthermore, mg-NIS expression was reversible upon re-establishment of nursing. These results indicate that mg-NIS expression is clearly upregulated during active nursing, rapidly down-regulated upon cessation of it, and moreover exquisitely regulated in a reversible manner by suckling. These findings confirm further that anti-NIS Ab is a highly valuable probe for the identification and monitoring of mg-NIS as well as thyroid NIS.

CONCLUDING REMARKS

As this article shows, there has been remarkable progress in NIS research in recent months. Advances in the characterization of NIS and in the study of its structure/function relations has already led to increased understanding of thyroid disease at the molecular level, as in the case of the identified NIS mutations that cause congenital I^- trapping defects. It is clear that the proposed NIS secondary structure model will continue to be experimentally probed, thus conceivably leading to identification of the Na^+ and I^- binding sites and to elucidation of the domains and specific residues involved in transport activity. In addition, the first firm steps have been taken toward elucidating extrathyroidal I^- transport processes, providing evidence that these are mediated by virtually the same NIS molecule, albeit subjected to different post-translational processing. This development, in turn, has opened up new vistas on the issue of hormonal regulation of NIS. Whereas the expression and activity of thyroid NIS is primarily regulated by TSH, lactogenic hormones regulate the expression and activity of mg-NIS. Considering the role played by NIS in the treatment of hyperthyroidism and thyroid cancer metastases, it is likely that new information on NIS will prove useful in the development of novel therapeutic strategies for both thyroid and nonthyroid cancers.

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