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Implications of Na^+/I^- Symporter Transport to the Plasma Membrane for Thyroid Hormonogenesis and Radioiodide Therapy

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Iodine is a crucial component of thyroid hormones; therefore, a key requirement for thyroid hormone biosynthesis is that iodide (I^-) be actively accumulated in the thyroid follicular cell. The ability of the thyroid epithelia to concentrate I^- is ultimately dependent on functional Na^+/I^- symporter (NIS) expression at the plasma membrane. Underscoring the significance of NIS for thyroid physiology, loss-of-function mutations in the NIS-coding *SLC5A5* gene cause an I^- transport defect, resulting in dyshormonogenic congenital hypothyroidism. Moreover, I^- accumulation in the thyroid cell constitutes the cornerstone for radioiodide ablation therapy for differentiated thyroid carcinoma. However, differentiated thyroid tumors often exhibit reduced (or even undetectable) I^- transport compared with normal thyroid tissue, and they are diagnosed as cold nodules on thyroid scintigraphy. Paradoxically, immunohistochemistry analysis revealed that cold thyroid nodules do not express NIS or express normal, or even higher NIS levels compared with adjacent normal tissue, but NIS is frequently intracellularly retained, suggesting the presence of posttranslational abnormalities in the transport of the protein to the plasma membrane. Ultimately, a thorough comprehension of the mechanisms that regulate NIS transport to the plasma membrane would have multiple implications for radioiodide therapy, opening the possibility to identify new molecular targets to treat radioiodide-refractory thyroid tumors. Therefore, in this review, we discuss the current knowledge regarding posttranslational mechanisms that regulate NIS transport to the plasma membrane under physiological and pathological conditions affecting the thyroid follicular cell, a topic of great interest in the thyroid cancer field.

Freeform/Key Words: Na^+/I^- symporter, I^- deficiency disorders, I^- transport defect, congenital hypothyroidism, differentiated thyroid cancer, radioiodine therapy

Active iodide (I^-) accumulation in the thyroid follicular cell constitutes the first step in the biosynthesis of the iodine-containing thyroid hormones [1]. Severe dietary iodine deficiency results in impaired thyroid hormone synthesis, leading to hypothyroidism and subsequently goiter and mental retardation in infants and children [2]. Na^+/I^- symporter (NIS), an integral plasma membrane glycoprotein located at the basolateral plasma membrane, efficiently

Abbreviations: AP, adaptor protein; ClO_4^-, perchlorate; I^-, iodide; GPI, glycosylphosphatidylinositol; ITD, I^- transport defect; NIS, Na^+/I^- symporter; PBF, pituitary tumor–transforming gene–binding factor; PIGU, phosphatidylinositol glycan anchor biosynthesis class U.

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mediates active I^- accumulation into the thyroid follicular cell [3]. Although NIS was initially thought to be a thyroid-specific protein, functional NIS protein expression is found in several other tissues, such as salivary glands, stomach, small intestine, lactating mammary gland, kidney, placenta, and ovary [4–11]. Because NIS mediates I^- transport in several tissues other than the thyroid, it may be considered to be a master regulator of iodine metabolism.

The human NIS-coding *SLC5A5* gene, located on chromosome 19p13.11, comprises 15 exons with an open reading frame of 1.929 nucleotides encoding a protein of 643 amino acids [12]. The experimentally tested secondary structure model for NIS shows a hydrophobic 13-transmembrane segment protein with an extracellular amino terminus and a large intracellular carboxyl terminus. NIS is *N*-glycosylated at three different asparagine residues (N225, N489, and N502) located in the third and sixth extracellular loops, turning NIS into a highly glycosylated plasma membrane protein [13]. In the thyroid, the electrophoretic pattern of NIS includes a partially glycosylated (~60-kDa) and a fully glycosylated (~90- to 100-kDa) polypeptide [14]. Partially glycosylated NIS corresponds to the polypeptide that has not reached the medial-Golgi compartment, whereas the fully glycosylated polypeptide corresponds to that located beyond medial-Golgi compartments (*e.g.*, *trans*-Golgi network, secretory vesicles) and at the plasma membrane. However, in nonpolarized thyroid epithelial cells, *N*-glycosylation is not critical for NIS intrinsic activity and plasma membrane expression, as demonstrated by substituting all glycosylated asparagines with glutamines [13, 15].

NIS-mediated active I^- transport is electrogenic and relies on the driving force of the Na^+ gradient generated by the Na^+/K^+ ATPase to simultaneously transport one I^- and two Na^+ ions into the cells [16]. However, NIS also transports other anions, such as the environmental pollutant perchlorate (ClO_4^-), but with an electroneutral stoichiometry (one Na^+/one ClO_4^-) [17]. Statistical thermodynamics analysis revealed that in the absence of Na^+, NIS has a very low intrinsic affinity (estimated at 200 μM) for I^-; however, when two Na^+ ions bind to the transporter it significantly increases to ~20 μM. Therefore, at physiological Na^+ concentrations, ~80% of NIS molecules are occupied by two Na^+ ions, enabling them to transport I^- highly efficiently even when the physiological I^- concentration in the bloodstream is submicromolar [18]. When the first Na^+ binds to the transporter, the NIS affinity for the second Na^+ and for I^- increases significantly, indicating an allosteric interaction [18]. Scintillation proximity assays using radioactive Na^+ showed a strong cooperativity between the two Na^+ binding sites, which is lost when the Na^+-interacting residues S353 and T354 are replaced by alanine [19].

The crystal structure of NIS has not yet been determined with atomic resolution. However, Paroder-Belenitsky *et al.* [20] generated a rat NIS structural homology model based on the crystal structure of the Na^+/galactose symporter of *Vibrio parahaemolyticus*. The development of the NIS homology model contributed to our understanding of the relationship between the structure and function of the protein, allowing *in silico* simulations and biochemical experiments to investigate the mechanism of transport. In particular, Ferrandino *et al.* [21] identified residues involved in coordinating Na^+ at the Na2 binding site using molecular dynamics simulations. The simulations provided evidence for the role of residues S66, D191, Q194, and Q263, in addition to S353 and T354 [19, 22], in coordinating Na^+ at the Na2 site. Moreover, the NIS homology model allowed the identification of a putative I^--binding cavity equivalent in position to the galactose-binding site uncovered in the crystal structure of the *V.* parahaemolyticus Na^+/galactose transporter [23]. Significantly, the Cβ of nonglycine residues of different amino acid substitution at position G93 points toward the inside of the cavity, and its side chain may interact with NIS substrates (I^- or I^- and Na^+) during the transport cycle, as reflected by significant changes in I^- affinity and Na^+/ClO_4^- transport stoichiometry in certain G93 mutants [20]. Moreover, NIS homology model-based molecular dynamics simulation revealed several residues that may coordinate I^- (F67, Q72, Q94, M258, and S416) and Na^+ (F67, Q72, Q94, and L289) inside the putative I^--binding cavity during the transport cycle [21]. However, additional *in silico* simulations

and biochemical validation of the proposed I^- and Na^+ coordinating residues are required to provide further conclusions.

1. I^- Transport Defects Cause Dyshormonogenic Congenital Hypothyroidism

Dyshormonogenic congenital hypothyroidism is caused by functional deficiency of thyroid hormone synthesis as a consequence of loss-of-function mutations in any of the genes involved in the biosynthesis of thyroid hormones [24]. Very recently, mutations in the *SLC26A7* gene were associated with thyroid dyshormonogenesis [25, 26]. Patients with abnormal SLC26A7 function showed preserved I^- accumulation but reduced I^- organification [25]; however, the role of SLC26A7 in intrathyroidal I^- metabolism physiology remains uncertain. Significantly, mutations in the *SLC5A5* gene cause an uncommon autosomal recessive condition known as I^- transport defect (ITD), a consequence of impaired I^- accumulation in the thyroid follicular cell [27]. An ITD is suspected when clinical or biochemical hypothyroidism is diagnosed in the presence of reduced to absent I^- accumulation in a eutopic thyroid gland, a reduced saliva-to-plasma I^- ratio, and normal to high serum thyroglobulin levels [28]. To date, 16 different loss-of-function *SLC5A5* gene mutants (-54C>T, V59E, G93R, R124H, Q267E, V270E, C272X, Y324L*fs*X12, Y348D, T354P, G395R, S509R*fs*X6, G543E, M143_Q323del, V287_G288del, and A439_P443del) have been identified in the homozygous or compound heterozygous state in patients with an ITD [27, 29]. However, genetic defects in other genes potentially required for functional NIS expression in thyrocytes have not been reported to cause ITDs. To date, the only protein known to facilitate efficient NIS-mediated I^- transport in thyroid cells is the constitutively active K^+ channel KCNQ1–KCNE2. Significantly, KCNE2 knockout mice developed hypothyroidism owing to decreased NIS-mediated I^- accumulation, but not abnormal NIS expression at the plasma membrane, in the thyroid follicular cell [30, 31].

Detailed evaluation of patients with different loss-of-function *SLC5A5* mutations has demonstrated a substantial clinical heterogeneity that seems to correlate with residual mutant NIS activity [32]. The resulting raise in TSH levels after a reduction in thyroid hormone synthesis may overcome a partial defect in mutant NIS activity by enhancing NIS expression, as demonstrated in patients carrying the missense mutant T354P NIS [33, 34]. In sharp contrast, patients harboring homozygous fully inactive NIS mutants developed hypothyroidism with significant clinical manifestations as a neonate [32]. Additionally, a marked clinical heterogeneity has been reported in patients harboring the same NIS mutant [33, 34]. The levels of dietary I^- intake significantly influence thyroid function in patients with an ITD, especially in those whose mutant NIS protein retains residual activity. Significantly, marked differences in hypothyroidism onset were noticed between siblings fed during infancy with breast milk produced by a lactating mother under high dietary I^- intake or regular artificial milk that contains lower levels of I^- [33]. Consistent with these findings, Ferrandino *et al.* [35] recently developed an NIS knockout mouse model that recapitulated the conditions of ITDs and provided evidence that high dietary concentrations of I^- make it possible for I^- to enter the thyroid follicular cells—even in the absence of functional NIS expression—through low-affinity mechanisms, thus facilitating partial thyroid hormone biosynthesis. On a different note, Mizokami *et al.* [36] demonstrated the importance of prophylactic iodine supplementation in healthy breast-fed newborns whose lactating mothers carry mutations in the *SLC5A5* gene, as NIS mediates I^- accumulation in breast milk, to prevent the development of hypothyroidism in the nursing newborn due to I^--deficient breast milk.

A thorough molecular characterization of several ITD-causing NIS mutants has provided significant insights into the mechanisms operating during the transport cycle and ion coordination [20, 22], the identification of specific residues or regions required for proper folding of the protein [15, 37, 38], and specific regions important for NIS transport to the plasma membrane, but not for its intrinsic activity, and potentially involved in the interaction with adaptor proteins (APs) required in the intracellular transport process [37, 39]. In particular,

the missense mutant R124H NIS does not mediate I^- accumulation in whole cells because the mutant protein is fully retained in the endoplasmic reticulum. Amino acid substitutions at position 124, located in the intracellular loop 2, revealed a key structural role for the δ-amino group of R124 or Q124 in NIS targeting to the plasma membrane. Indeed, an intramolecular interaction between the δ-amino group of R124 and the thiol group of C440, located in the intracellular loop 6, is essential for proper folding required for NIS sorting out through an endoplasmic reticulum quality-control system [37]. Moreover, V270E NIS mediates markedly reduced I^- uptake in whole cells because the transport of the protein to the plasma membrane is severely impaired. A negatively charged residue at position 270, located at the intracellular end of transmembrane segment 7, produces a profound change in the electrostatic potential surface of a positive patch in the intracellularly facing domain of the mutant NIS protein that may mask an uncharacterized sorting motif recognized by APs key for NIS transport to the plasma membrane [39].

2. Regulation of NIS Transport to the Plasma Membrane

NIS expression at the plasma membrane in the thyroid follicular cell is not only important for I^- accumulation required for thyroid hormone biosynthesis, but also constitutes the cornerstone for radioiodide therapy for hyperthyroidism and differentiated thyroid carcinoma [40, 41]. Despite the physiological and clinical relevance of NIS plasma membrane expression, little is known regarding the molecular mechanisms underlying NIS transport to the plasma membrane, a pursuit that could lead to new therapeutic interventions to increase the effectiveness of radioiodide therapy.

TSH constitutes the primary regulator of NIS expression in the thyroid follicular cell by not only stimulating NIS expression at the transcriptional level, but it is also required at posttranslational levels for targeting NIS to, and/or retaining it at, the plasma membrane. In FRTL-5 rat thyroid cells, immunofluorescence analysis demonstrated that after TSH withdrawal, NIS molecules located in the plasma membrane are redistributed to uncharacterized intracellular compartments [42]. Indeed, I^- uptake was evidenced in sealed membrane vesicles prepared from FRTL-5 cells that have lost the ability to accumulate I^- due to TSH deprivation, suggesting that those intracellularly retained NIS molecules are fully active [43]. Moreover, in FRTL-5 cells, TSH modulates the phosphorylation pattern of the NIS carboxyl terminus, which mainly occurs on serine residues [42]. Bioinformatics analyses predict that the NIS carboxyl terminus contains several phosphorylation consensus sequence motifs for protein kinases, including glycogen synthase kinase 3, protein kinase A, and protein kinase C. In particular, considering that phosphorylation has been reported to play a role in regulating the transport to the plasma membrane of different channels and transporters, as well as the activation of several protein kinases as mediators of TSH actions in thyroid cells, it is possible to speculate that phosphorylation might constitute a posttranslational modification involved in the NIS intracellular transport process. Significantly, Vadysirisack *et al.* [44] identified different intracellularly located, functionally relevant NIS phosphorylated residues by mass spectrometry in HEK-293 heterologously expressing rat NIS. Although biochemical data suggested that the phosphorylation status of S43 and S581 modulates the activity of the protein, whereas that of T577 may modulate its stability, none of the identified phosphorylated residues affects NIS transport to the plasma membrane. Thus, the molecular mechanism regulating TSH-stimulated NIS transport to, retention at, and removal from the plasma membrane remains unknown.

Regarding structural determinants that control NIS transport to the plasma membrane, new avenues were opened after the functional characterization of the ITD-causing NIS truncated and frame-shifted mutant S509R*fs*X6—reported in the literature as S515X NIS—missing transmembrane segment 13 and the carboxyl terminus of the protein. S509R*fs*X6 NIS is fully intracellularly retained [45], thus suggesting that the carboxyl terminus may contain crucial information for proper NIS transport to the plasma membrane. Recently, based on the NIS homology model, we reported that the intracellularly facing

human NIS carboxyl terminus comprises residues I546 to L643, and we provided biochemical evidence showing that the deletion of the carboxyl terminus rendered the mutant I546* NIS retained in the endoplasmic reticulum [46]. Moreover, given the role of the carboxyl terminus in NIS transport to the plasma membrane, we generated several NIS mutants missing internal regions of the carboxyl terminus, and its biochemical characterization revealed that the carboxyl terminus segment between residues I546 and K618, containing a putative tryptophan-acidic motif ($W^{565}D^{566}$), is required for NIS exit from the endoplasmic reticulum and subsequent transport to the plasma membrane in nonpolarized epithelial cells [46, 47]. Although the molecular mechanisms underlying NIS export from the endoplasmic reticulum in thyroid cells remain elusive, our experimental evidence supports that the carboxyl terminus contains crucial information for functional NIS plasma membrane expression [46].

Although heterologous rat NIS expression in the epithelial cell line MDCK—a cell model that recapitulates a polarized epithelial monolayer and preserves the native polarity of several heterologously expressed thyroid proteins [48, 49]—is largely targeted to the basolateral plasma membrane, Dohan *et al.* [17] developed a rat NIS mutant missing the last 43 amino acids (T575* NIS) that exhibited equivalent I^- transport properties to wild-type rat NIS, but when heterologously expressed into polarized MDCK cells, it is targeted to the apical plasma membrane. Although T575* NIS was developed to study NIS-mediated polarized ClO_4^- transport, these findings indirectly suggest that the region comprised between amino acids T575 (the residue equivalent to V580 in human NIS) and L618 in the rat NIS sequence (the rat ortholog has 618 amino acids) carries essential determinants for basolateral sorting. Therefore, considering that the segment between amino acids V580 and K618 (the segment between amino acids K618 and Q639 is dispensable for NIS basolateral expression) would be critical for human NIS basolateral sorting, we focused our studies on elucidating the role of these amino acids. Significantly, we uncovered a highly conserved basolateral sorting motif consisting of an acidic cluster followed by a single leucine (EExxxL) between amino acids 578 and 583 of human NIS [46]. Disruption of the carboxyl-terminal monoleucine-based sorting motif causes human and rat NIS to be missorted to the apical plasma membrane in polarized MDCK cells, indicating that this sorting motif, which is highly conserved across species, constitutes a sorting signal exclusively required for basolateral NIS expression. Interestingly, similar observations were evidenced in polarized FRT rat thyroid cells that, although do not express NIS endogenously, constitute the only thyroid follicular cell line that forms polarized epithelial monolayers [46].

Leucine-based sorting motifs are frequently recognized by heterotetrameric clathrin AP complexes that link clathrin to the cargo in clathrin-coated vesicles that carry proteins to different destinations within the cell; in particular, the AP-1A and AP-1B hemicomplex γ-σ1 recognizes basolateral [D/E]xxxL[L/I] leucine–based sorting motifs. Therefore, considering that AP-1B expression is epithelial cell specific and differs from the ubiquitous AP-1A by the medium subunit μ1B, our studies in polarized μ1B knocked-down MDCK cells heterologously expressing human NIS demonstrated that the AP-1B complex is required for NIS sorting exclusively to the basolateral plasma membrane [46]. Moreover, computer simulations support a direct recognition of the monoleucine-based basolateral sorting motif by the AP-1 γ-σ1 hemicomplex [46]. Taken together, our results strongly suggest that AP-1B participates in the recognition of the carboxyl terminus–located monoleucine-based motif, thus sorting NIS to the basolateral plasma membrane.

Bioinformatics assessing the amino acid sequence encoding the NIS carboxyl terminus revealed the presence of several conserved sorting motifs that, in other plasma membrane proteins, are involved in their transport to the plasma membrane [50]. In particular, NIS contains a putative class I PDZ-binding motif [S/T]-X-Φ_{COOH} located at the carboxyl-terminal edge of the protein. PDZ-binding motifs are recognized by PDZ domain–containing proteins that by working as scaffold or APs participate at various levels in membrane protein transport and sorting to the plasma membrane [51]. However, it seems unlikely that the PDZ-binding motif is the determinant for NIS expression at the plasma membrane, as the addition of an epitope tag at the carboxyl terminus that masks the carboxylate group required for the

recognition of the PDZ motif does not impair NIS expression at the plasma membrane [52, 53]. Significantly, the recognition of the PDZ-binding motif of NIS by the PDZ domain–containing leukemia-associated RhoA guanine exchange factor promotes cell invasion and migration in intracellularly NIS-expressing cancer cells [54, 55].

Recently, Darrouzet *et al.* [50] reported the first systematic evaluation of potential NIS intracellularly located sorting motifs presumably involved in the transport of the protein to the plasma membrane. The authors identified an internal noncanonical PDZ-binding motif comprising residues 118 to 121, located in the intracellular loop 2, which plays a crucial role in NIS transport to the plasma membrane. The substitution L121A disrupted the mentioned PDZ-binding motif leading to complete retention of the mutant NIS protein in the endoplasmic reticulum, as revealed by its electrophoretic pattern on immunoblot analysis [50]. However, a remaining open question is to determine whether the mutant L121A prevents proper folding of the protein and then the quality control system retains it in the endoplasmic reticulum or, alternatively, the recognition of this PDZ-binding motif is required to export NIS from the endoplasmic reticulum.

3. The Molecular Basis for Radioiodide Therapy of Differentiated Thyroid Carcinoma

For >75 years, the ability of thyroid cells to accumulate I^- has constituted the molecular basis for the diagnosis and treatment of differentiated thyroid carcinoma [56]. Radioiodide therapy used to ablate thyroid cancer metastases and remnants after thyroidectomy has been the most successful targeted internal radiation therapy ever designed. Retrospective studies have demonstrated that the ability of tumor cells to accumulate I^- is the best indicator of disease-free survival [57–59]. Currently, TSH-stimulated radioiodide adjuvant therapy is routinely recommended after total thyroidectomy for high-risk differentiated thyroid carcinomas [60]. However, differentiated thyroid tumors often exhibit reduced (or even undetectable) I^- transport compared with normal thyroid tissue, and they are diagnosed as cold nodules on thyroid scintigraphy. Despite this reduction, >70% of differentiated thyroid carcinomas accumulate I^- to some extent, which is still sufficient to achieve appropriate radioiodide accumulation for treatment [61]. Unfortunately, 30% to 50% of metastases from differentiated thyroid tumors completely lose their ability to accumulate I^-, causing them to become refractory to radioiodide therapy [62]. Therefore, in these cases, other therapeutic alternatives should be considered [63]. Loss of I^- accumulation is associated with poor prognosis; patients with thyroid cancer metastases that accumulate I^- showed a survival rate at 10 years of ~56%, whereas survival is drastically reduced to ~10% in patients with radioiodide refractory metastases [58].

Radioiodide therapy effectivity is ultimately dependent on functional NIS expression at the plasma membrane of tumor cells, as deficient radioiodide accumulation is the major cause of treatment failure [62]. However, NIS gene expression is frequently downregulated in thyroid cancer. The Cancer Genome Atlas study of nearly 500 papillary thyroid carcinomas, the most common form of differentiated thyroid cancer, revealed that NIS gene expression is lower than in normal thyroid tissue [64]. Moreover, the study demonstrated that NIS gene expression is significantly higher in carcinomas showing a RAS-like phenotype, having relatively higher thyroid differentiation scores than in those with a BRAF-like phenotype [64]. Indeed, NIS gene expression is totally silenced in poorly differentiated and anaplastic thyroid carcinomas [65]. Although several transcriptional and posttranscriptional mechanisms have been postulated to explain a repression of NIS gene expression in thyroid tumors, because of the thrust of this review, these mechanisms are not reviewed [66, 67]. Paradoxically, several immunohistochemical analyses (using different antihuman NIS antibodies) showed that NIS is frequently expressed at different levels in differentiated thyroid carcinomas compared with adjacent normal tissue. Recently, Tavares *et al.* [68] reported a comprehensive bibliographic revision of different studies assessing NIS protein expression by immunohistochemistry in thyroid carcinomas. Surprisingly, NIS expression was mainly

located in intracellular compartments, suggesting the presence of plasma membrane transport abnormalities [68–75]. Significantly, Peyrottes *et al.* [76] have questioned that the real significance of NIS intracellular staining is due to nonspecific binding of the antibodies, a topic that remains to be clarified. Interestingly, intracellular NIS retention in differentiated thyroid carcinomas has been pointed out as a reason for the decreased radioiodide accumulation in tumor cells, as impaired NIS transport to the plasma membrane would hamper its activity. Significantly, NIS mutations have not been identified in thyroid tumors [77], so it cannot be structural defects that retain NIS intracellularly in these tumors, which stands in contrast to the situation in some patients with an ITD [15, 37]. Therefore, the paradoxical observations of reduced I^- uptake and intracellularly retained NIS protein expression highlight the importance of elucidating the posttranslational mechanisms that regulate NIS expression at the plasma membrane under physiological and pathological conditions.

Considering the high prevalence of $BRAF^{V600E}$-positive radioiodide-refractory metastatic papillary carcinomas, Riesco-Eizaguirre *et al.* [78] investigated NIS expression by immunohistochemistry in a cohort of 60 papillary carcinomas, and they reported a significant reduction of NIS expression and impaired transport to the plasma membrane in tumors harboring the oncogene $BRAF^{V600E}$. Furthermore, *in vitro* experiments demonstrated that ectopic $BRAF^{V600E}$ expression in PCCl3 thyroid cells induces a sharp redistribution of NIS expression from the plasma membrane to uncharacterized intracellular compartments, thus reducing I^- accumulation, followed by a gradual decrease in NIS transcriptional expression involving TSH-independent processes. Mechanistically, the oncogenic transformation induced by $BRAF^{V600E}$ might either repress APs involved in NIS transport to the plasma membrane or, alternatively, induce the expression of APs that remove NIS from the cell surface, thus leading to its intracellular retention.

To date, the pituitary tumor–transforming gene–binding factor (PBF) has been characterized as the only NIS-interacting protein that may be involved in defective NIS plasma membrane expression in thyroid cancer. Smith *et al.* [79] reported that ectopic PBF overexpression posttranslationally represses I^- uptake by binding to NIS and leading to its internalization into clathrin-coated CD63-positive late endosomes. Moreover, the proto-oncogene tyrosine kinase Src-mediated PBF phosphorylation at tyrosine 174 is required for its physical interaction with NIS. Significantly, abrogation of Src kinase activity restores NIS expression at the plasma membrane and I^- accumulation in human thyroid cancer cells [80].

Recently, Amit *et al.* [81] provided evidence of a reduced expression of phosphatidylinositol glycan anchor biosynthesis class U (PIGU), a subunit of membrane-bound glycosylphosphatidylinositol (GPI) transamidase complex that catalyzes the addition of a GPI anchor to substrate proteins in the endoplasmic reticulum, in papillary thyroid carcinoma. Significantly, PIGU overexpression restored NIS expression at the plasma membrane and I^- accumulation, allowing radioiodide therapy, in the human well-differentiated thyroid carcinoma cell line K1 carrying the oncogene $BRAF^{V600E}$ [81]. Although functional PIGU expression appears to participate in a posttranslational mechanism necessary for NIS transport to the plasma membrane, the absence of a consensus sequence for GPI anchoring rules out that NIS is a GPI-anchored protein. Thus, a defect in the GPI transamidase complex might cause either a deficient transport of membrane proteins to the plasma membrane, whose sorting into specific secretory vesicles depends on GPI-anchored proteins, or a deficiency in a key, still unidentified GPI-anchored protein necessary for proper NIS transport to the plasma membrane.

Recent progress in understanding the molecular mechanisms that repress functional NIS expression has brought about possibilities of new therapeutic approaches, which may decrease the dose of radioiodide as well as expand the application of radioiodide therapy to radioiodide-refractory thyroid cancers. Indeed, emerging therapies, still in the clinical phase of study, using small-molecule inhibitors (*i.e.*, dabrafenib and selumetinib) have shown promising effects enhancing radioiodide accumulation in radioiodide-refractory differentiated thyroid cancer metastasis [82, 83]. Although single-agent therapy has shown poor long-term responses, dabrafenib and selumetinib treatment overcome radioiodide resistance, thus

allowing subsequent radioiodide ablation protocols. Future phase 3 studies evaluating the clinical benefit from the combination of dabrafenib or selumetinib and radioiodide therapy in larger cohorts of patients and, perhaps, in particular subgroups of patients according to the oncogenic driver event are eagerly awaited. Moreover, the identification of novel small-molecule inhibitors exhibiting stronger and sustained inhibition of MAPK/ERK signaling may provide novel strategies to enhance radioiodide accumulation in radioiodide-refractory differentiated thyroid cancer metastasis [84].

4. Perspectives and Future Directions

Radioiodide accumulation in the thyroid tissue has been exploited in clinical medicine to diagnose, treat, and follow up thyroid pathologies for several decades before the mechanism mediating I^- accumulation was characterized at the molecular level. Since the cloning of NIS, substantial progress has been made in understanding not only the mechanisms underlying ion coordination during the transport cycle, its transport to the plasma membrane, and its transcriptional repression in thyroid cancer, but also broadening NIS application to imaging and therapeutic procedures for various nonthyroid diseases [85, 86]. Although we have learned much about NIS, in the sections below, we pose major questions that remain to be investigated.

A. What Are the Mechanisms Underlying NIS Basolateral or Apical Plasma Membrane Sorting in Different Tissues Under Physiological Conditions?

Considering different tissue-specific I^- handling requirements (*i.e.*, absorption, accumulation, or secretion), NIS expression in different tissues also displays different basolateral-to-apical localization patterns. The analysis of NIS cDNA nucleotide sequence in different tissues (*i.e.*, thyroid, salivary gland, lactating mammary gland, and stomach) yielded full identity [87]. Therefore, factors other than the NIS sequence, such as posttranslational modifications or differential tissue expression of specific APs that decode common sorting signals located in the NIS carboxyl terminus, may regulate NIS polarized transport to the plasma membrane. In agreement, Schreiner *et al.* [88] showed that the absence of the epithelial-specific basolateral clathrin-adaptor AP-1B in renal proximal tubule epithelial cells determines that many cognate basolateral plasma membrane proteins are expressed in the apical membrane, thus optimizing the reabsorption of nutrients in the kidney. Currently, our knowledge regarding sorting motifs and APs involved in NIS export from the endoplasmic reticulum, polarized transport to the plasma membrane, and retention at the plasma membrane in the thyroid follicular cells remains partially understood, and in other tissues uncertain.

B. What Are the Mechanisms Underlying NIS Intracellular Retention in Thyroid Cancer?

The efficacy of radioiodide therapy is directly related to the therapeutic dose of radiation delivered to tumor cells, which is ultimately dependent on functional NIS expression at the plasma membrane [62]. From a therapeutic perspective, improving NIS-mediated radioiodide therapy for thyroid cancer is a priority for developing strategies aimed at enhancing NIS plasma membrane expression, not only to stimulate NIS transcription, as NIS defective transport to the cell surface may cause radioiodide treatment failure. Importantly, therapeutic interventions allowing more NIS molecules to reach the plasma membrane of tumor cells would dramatically improve radioiodide therapy efficacy and lead to the use of lower radioiodide doses, thus minimizing side effects, or to the use of radioiodide as an adjuvant with kinase inhibitors targeting oncogene activity or oncogene-activated signaling pathways to recover NIS transcriptional expression.

Recently, Amit *et al.* [81] demonstrated that functional PIGU expression in K1 cells is transcriptionally repressed in response to $BRAF^{V600E}$-triggered MAPK/ERK signaling, as

chemical inhibition of MEK/ERK signaling restores PIGU protein expression. Direct biochemical evidence indicating that inhibition of MEK/ERK signaling restores I^- accumulation in thyroid cancer cell lines relies on functional PIGU expression has not been established. However, these data uncover a novel posttranslational mechanism involved in radioiodide resistance, whose better understanding may lead to develop novel strategies to restore radioiodide accumulation in thyroid cancer cells.

5. Search Strategies

We searched MEDLINE for English language articles and references of relevant articles published between 1996 and 2018 using the search terms "Na^+/I^- symporter or sodium iodide symporter," "congenital hypothyroidism," "iodide transport defect," "basolateral or apical sorting in epithelial cells," "thyroid cancer," "radioiodine therapy", and "radioiodine-refractory thyroid cancer."

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