6

Regulation of the Functional Na^+/I^- Symporter (NIS) Expression in Breast Cancer Cells

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1. Introduction

Iodide (I^-) is transported from bloodstream to lactating mammary gland tissue and it is secreted to mother's milk (Honour *et al*., 1952; Grosvenor, 1960; Eskin *et al*., 1974; Bakheet *et al*., 1998). I^- in milk is then used by the suckling newborn in thyroid hormone biosynthesis (Brown-Grant, 1957; 1961; Azizi and Smyth, 2009). As the supply of I^- to neonate comes exclusively from milk during first months of its life, I^- uptake by lactocytes and its accumulation in mother's milk is vital for proper development of the nervous system, skeletal muscles, and lungs at those early periods of life (Stanbury, 1992; Delange, 2000; Semba & Delange, 2001).

The activity of the sodium/I^- symporter (Na^+/I^- Symporter, or NIS) expressed in alveolar cells of lactating breast is essential for secretion of I^- to milk (Cho *et al*., 2000; Tazebay *et al*., 2000). In fact, by many clinicians and researchers, cellular I^- transport activity was mostly associated with the thyroid gland as the I^- uptake into thyroid follicular cells is the first step in enzymatic synthesis of iodide-containing thyroid hormones triiodothyronine (T_3), and thyroxine (T_4). Molecular investigations following the cloning of NIS gene by Dai *et al*. (1996) have proven that this symporter is not only a key enzyme in I^- uptake in thyroid, but it is also functional in extrathyroidal tissues such as lactating mammary glands, stomach, intestine, salivary glands, kidney, and placenta (Ajjan *et al*., 1998; Tazebay *et al*., 2000; Bidart *et al*., 2000; Mitchell *et al*., 2001; Spitzweg *et al*., 2001; Nicola *et al*., 2009). Therefore, together with its key role in thyrocytes, NIS seems to be the long seeked (Wolff & Maurey, 1961) major I^- transport protein in extrathyroidal tissues as well (De la Vieja *et al*., 2000). But, as one could expect, the expression and activity of NIS is differentially regulated in thyroid and in above mentioned non-thyroidal tissues (see below).

In human, NIS is encoded by the gene annotated as Solute Carrier 5A5 (SLC5A5) in NCBI databases (www.http://www.ncbi.nlm.nih.gov). Among its other members, SLC5A transporter family includes Na^+/glucose co-transporters 1 and 2, the Na^+/myoinositol transporter, and the Na^+/monocarboxylate transporter (Wright and Turk, 2004). The human NIS gene is located on chromosome 19, mapping to loci 19p13.2-p12 (Smanik *et al*., 1997). Previous extensive molecular studies carried out by Nancy Carrasco's group at the Albert Einstein College of Medicine have revealed that the transporter is a glycoprotein belonging to the Na^+/solute symporter family, and it is integrated to membrane by 13 hydrophobic

trans-membrane domains with N-terminus located extracellularly and C-terminus facing the cytosol (Dai *et al*., 1996; Levy *et al*., 1997; 1998). Rat NIS protein is composed of 618 amino acids, while its human homologue contains 643 residues with a relative molecular mass of about 75 kD to 110 kD depending on the glycosylation status of the protein in different tissues (Dai *et al*., 1996; Smanik *et al*., 1996; Tazebay *et al*., 2000).

In order to establish biophysical properties of the transporter, the rat thyroid Na^+/I^- symporter (NIS) was expressed by Eskandari *et al*. (1997) in *Xenopus laevis* oocytes and the symporter was characterized using electrophysiological, tracer uptake, and electron microscopic methods. In this work, I^- transport activity of NIS against its gradient was shown to couple the inward transport of Na^+, occurring in favor of its Na^+/K^+-ATPase-generated electrochemical gradient. Moreover, these authors have clearly shown that the transport activity was electrogenic, and the stoichiometry of cotransport by NIS was 2 Na^+ ions to 1 I^-. Interestingly, this work have also shown that other than I^-, the symporter was capable of transporting a wide variety of anions such as, ClO_3^-, SCN^-, $SeCN^-$, NO_3^-, Br^-, BF_4^-, IO_4^-, BrO_3^- (Eskandari *et al*., 1997). It also transports radioisotopes such as pertechnetate ($^{99m}TcO_4^-$; Tazebay *et al*. 2000, Van Sande *et al*. 2003), perrhenate ($^{188}ReO_4^-$; Dadachova *et al*. 2002) and astatine ($^{211}At^-$; Lindencrona *et al*. 2001). Binding sites of these substrates on NIS protein, and their molecular interactions during passage through the symporter is not currently known. However, a recent publication on extensive mutational analysis carried out by Antonio De la Vieja and his co-workers indicated that particularly amino acids Thr-351, Ser-353, Thr-354, Ser-356, Thr-357, Asn-360, and Asp-369, all of which either face the same side of the helix in trans-membrane segment IX or close to it at the membrane/cytosol interface (Asp-369) according to their current structural model, are involved in Na^+ binding or translocation activity by NIS (De la Vieja *et al*., 2007). In fact, T354P mutation in NIS was previously identified as an autosomal recessive mutation resulting in iodide transport defect (ITD), a rare disorder causing congenital hypothyroidism in human (Fujiwara *et al*., 1998).

In addition to its role in normal breast physiology during lactation, a very significant NIS upregulation as well as increased radioiodide transport activity was reported in large percentage of breast cancers and its metastasis (Tazebay *et al*., 2000; Wapnir *et al*., 2003; 2004; Upadhyay *et al*., 2003; Renier *et al*., 2009; 2010, and see below). These results indicate that NIS-mediated radionuclide imaging and therapy methods could potentially be used as alternative aproaches, or in combination with existing methodologies, towards a better management of the malignant breast disease, as it is routinely used against thyroid cancer and its metastasis for more than 60 years (Daniel & Haber, 2000; Welcsh & Mankoff, 2000; Spitzweg & Morris, 2002; Boelaert & Franklyn, 2003). Currently, breast cancer management involves adjuvant systemic therapies that are administered before or after mastectomy surgeries with the purpose of ablating micro-metastatic cells and improving disease free overall survival of the patient. In fact, it seems that neo-adjuvant and adjuvant therapies are rather successful, and they reduce recurrences by nearly 50% (Early Breast Cancer Trialist's Collaborative Group, 1998; Citron *et al*., 2003). The administration of cytotoxic agents used in breast cancer chemotherapy is usually by venal intervention, and it can cause significant side effects, such as loss of healthy blood cells, increased possibilities to get microbial infections, hair loss, tiredness, feeling sick, sore mouth, sore eyes, and diarrhea. Patients and families are looking forward for development of alternative strategies that target breast cancer cells with high specificity, that are less toxic to healthy tissues and with less side-effects. Because of previous, and decades long, clinical work in diagnosing and treating thyroid cancers by radioisotope substrates of NIS ($^{131}I^-$, $^{125}I^-$, $^{123}I^-$, or $^{99m}TcO_4^-$), use of NIS

transported radionuclides could offer novel prospective breast cancer treatment modalities with advantages such as, less toxicity to healthy tissues, easier administration procedures, and relatively less side-effects as compared to current chemotherapeutic drugs. However, as discussed in this chapter, a broader knowledge on molecular mechanisms that operate in modulation (upregulation and repression) of functional NIS expression in mammary gland is certainly required in order to achieve a better diagnostic and/or therapeutic effect on breast cancer by using radiolabelled substrates of the symporter.

In this chapter, first, I am going to focus on the biological function of NIS in normal mammary gland epithelia. Then, recent studies on levels of NIS expression in different histopathological types of breast tumours will be analyzed. Subsequently, I will introduce recent findings on regulatory interplays, and small molecular agents that modulate NIS gene expression and activity both in normal mammary gland during gestation and lactation, and in malignant transformation in breast tissue. Recent *in vivo* animal studies, as well as human clinical trials addressing uses of NIS substrate radionuclides for imaging purposes will be introduced in later parts of this review. I will conclude by reviewing current litterature that analyze potential future strategies for the development of NIS radionuclide transport activity based therapeutic and diagnostic methods that could lead to novel clinical approaches in breast cancer management.

2. Regulation of NIS expression during mammary gland development and in lactating breast

I^- accumulation in lactating mammary gland has been recognized for nearly 60 years (Honour *et al*., 1952; Brown-Grant, 1957; Grosvenor, 1960; Thorpe, 1976; Bakheet *et al*., 1998). In normal physiology, mammary gland accumulates I^- only at later stages of gestation or pregnancy, and during lactation. An adequate supply of I^- in mother's milk is a must for sufficient thyroid hormone synthesis, which is essential for healthy development of the newborn. With molecular cloning of NIS gene in 1996, and subsequent generation of high affinity antibodies specific for human and rodent NIS proteins, researchers acquired essential tools to investigate whether NIS could have any role in the uptake of I^- in lactating mammary glands. First the group of Spitzweg *et al*. (1998) by relatively simple RT-PCR methods, and later on groups of Cho *et al*. (2000), Tazebay *et al*. (2000), and Perron *et al*. (2001) with more sophisticated immunological and molecular biochemistry techniques have addressed expression of NIS in mammary glands of human or rodent experimental models. From results obtained in above mentioned studies, it is now clear that NIS is not expressed in non-lactating healthy mammary glands, and the expression of symporter rises above detectable limits in the mammary gland tissue only with the onset of gestation. Subsequently, NIS expression is up-regulated at transcription level concomitant to increased proliferation of lactocytes in the gland, and then, after delivery, its expression remain high exclusively in response to hormonal stimuli induced by suckling of nipples by newborn pups.

2.1 Regulation of NIS expression during gestation in mice

In developing mammary glands of healthy CD1(ICR)BR strain pregnant mice, NIS expression starts at mid-gestation, i.e. around days 10 or 11 in a full gestation period of 19 days (Tazebay *et al*., 2000), and it becomes maximal near the end of gestation (days 18-19)

before delivery (Cho *et al*., 2000; Tazebay *et al*., 2000). Delivery is not essential for an increased uptake of I^- to mammary glands during gestation, and accumulation of radio-labelled substrates of NIS in the gland is observable in pregnant mice (Tazebay *et al*., 2000). By using immuno-histochemical techniques, NIS protein was detected on the basolateral membranes of alveolar epithelial cells of lactating mammary glands (Cho *et al*., 2000; Tazebay *et al*., 2000). These cells take up I^- from the bloodstream and secrete it to milk for neonatal nutrition, leading to a concentration of I^- up to 15 fold as compared to the plasma I^- concentration (Nurnberger & Lippscomb, 1952; Thorpe, 1976). It is also important to note here that, about 20% of transported I^- were found to be organified (i.e., inorganic I^- conjugated to an organic molecule, or enzyme) by lactoperoxidases in the alveolar cells (Patrick, 2008).

Thyroid gland, mammary glands, salivary glands, and stomach are tissues where most intense NIS expression is observed (Tazebay *et al*., 2000; Riedel *et al*., 2001). Even though, these tissues express NIS at relatively high levels, the timing of expression and stimuli for its up or down-regulation are not the same. In thyroid, thyroid stimulating hormone (TSH) that is synthesized and released by thyrotrope cells located in the anterior pituitary gland, is the main regulator of thyroid cell proliferation, differentiation, and endocrine functions, including molecular regulation of NIS expression both at transcriptional and at post-translational levels (Levy *et al*., 1997; Riedel *et al*., 2001). The effects of TSH are primarily mediated through the activation of the cAMP cascade via the GTP-binding protein Gs. TSH stimulates iodide accumulation by positively regulating NIS expression at the protein and mRNA level via the cAMP pathway (Levy *et al*., 1997). Hypophysectomized rats with low circulating levels of TSH have a decreased protein expression of NIS, whereas a single injection of TSH leads to a robust increase in NIS expression. Rats maintained on an iodide-deficient diet or treated with propylthiouracil, an agent blocking iodide organification, have high concentrations of TSH, which correlates with a very significant increase in NIS protein expression (Levy *et al*., 1997). The reader is advised to refer wonderful reviews by Dohan *et al*. (2003), or by Kogai *et al*. (2006) among many others for detailed information on TSH dependent regulation of thyroid specific genes including NIS. Even though NIS expression is continuous in the thyroid throughout life, in mammary glands it is strictly a gestation/lactation specific event, independent of blood TSH levels (Eskin *et al*., 1974; Tazebay *et al*., 2000; Kogai *et al*., 2006).

In my opinion, a research student who for the first time observes mammary gland development in rodents by animal dissections will certainly be very much surprised in seeing tremendous size differences in mammary glands of virgin and lactating females. In virgin mice/rats, the organ is composed of a thin sheet of tissue close to skin, hard to find and dissect without previous experience. However, in lactating rodents, it is difficult not to notice them, because by size they are comparable to largest internal organs of the animal. This makes mammary glands invaluable experimental models for organ development and morphogenesis. It is branching morphogenesis of mammary gland epithelia that leads to formation of buds and milk ducts, and that characterizes mammary gland growth. In this developmental process, mammary specific molecular networks interpret signals from local cytokines/growth factors in both the epithelial and stromal microenvironments, which is orchestrated by secreted ovarian and pituitary hormones (McNally & Martin, 2011). Because I^- transport and functional expression of NIS in the mammary gland is first observed at mid-pregnancy, and during lactation, both us and others have inquired possible roles of ovarian and pituitary lactogenic hormones in regulation of NIS in mammary glands. In two

independent studies, steroid hormones estrogen (17-β-estradiol) and progesterone, and two pituitary lactogenic hormones, prolactin and oxytocin were administered to female virgin animals either one by one, or in different combinations, and their effects on NIS upregulation were monitored (Cho *et al*., 2000; Tazebay *et al*., 2000). In the study carried out by Tazebay *et al*. (2000), together with normal virgin female mice, the authors have also used ovariectomized females in order to prevent possible interferences between administered and endogenous steroid levels. In these work, it has been demonstrated that estrogen and the two pituitary hormones oxytocin and prolactin have up-regulatory roles in mammary gland NIS expression. Interestingly, estradiol was previously shown to down-regulate NIS expression in FRTL-5 rat thyroid cells under culture conditions (Furlanetto *et al*., 1999). On the contrary, in mammary gland cell lines in culture, major receptor that interacts with estradiols, estrogen receptor-alpha (ER-α) was shown to be essential for basal expression of NIS in a ligand independent manner, and for its further induction in response to other non-estrogenic extracellular factors (Alotaibi *et al*., 2006; and see below). Moreover, recently a significant positive correlation was reported between human NIS and ER-α expressions in a cohort of malignant (n=75) or normal breast tissue (n=10) specimens (Ryan *et al*., 2011).

In fact, the work carried out by Alotaibi *et al*. (2006) could connect these two seemingly conflicting results, and bring possible mechanistic explanations on how estrogens and ER dependent molecular mechanisms (in the presence or absence of their ligands) might have both positive and negative regulatory roles on NIS expression. In this work, first a clear correlation between the transcription factor ER-α and activation of NIS expression was shown in human mammary epithelial cell models. Then, it has been demonstrated that ER-α physically interacts with a novel responsive element (ERE) in NIS gene promoter, occupying this position under conditions where NIS activation is pronounced. It is important to precise here once again that regulation of NIS by ER system has some very uncommon characteristics. Unliganded ER-α (Apo-ER) seems to be functional in basal expression of NIS, and it is shown to be essential to keep the system inducible by other ligands, namely by retinoids (see Part 5 below, for a detailed mechanistic analysis). Another interesting point is that mutations inserted to NIS ERE in a lusiferase reporter gene system have, in fact, increased transcription of the reporter in MCF-7 mammary tumor cells in culture conditions (Hani Alotaibi and U.H.T.; unpublished observations), instead of decreasing the expression of the reporter gene, as one would normally expect in classical ER regulated genes. These results might suggest the presence of a relatively complex and dynamic interplay between ER and other transcription factors (specific or general) operating on NIS gene promoter. Remarkably, the position of this novel functional NIS ERE sequence was precisely conserved in rodent and human genomes, and it was found to be localized only about 9 base pairs far from NIS gene TATA box element (Fig. 1; Alotaibi *et al*., 2006). The key point in this uncommon regulation of a gene (NIS) by ER may reside at this exceptionally close positioning between NIS ERE and TATA box elements. Speculatively, when concentrations of 17-β-estradiol raises above normal physiological levels (as used in cell culture conditions), over-activated ER-α might create steric effects by continuously disturbing or shielding interaction of general transcription factors with the TATA element of NIS gene, resulting in a negative or down-regulatory effect. According to this hypothesis, among other genetic and epigenetic factors, the amount of ER-α ligands would define the type of interactions (synergistic or antagonistic) and the outcome of interactions (up-regulatory or down-regulatory) between ERE bound ER-α and TATA bound general transcription factors. This might also provide some hints to understand why goiter is more common in females as

compared to males, or possible explanations related with molecular mechanisms leading to I^- transport deficiencies (ITD) in females, seen with a higher prevalence during or after pregnancy (Patrick, 2008).

Fig. 1. Functional NIS ERE sequence is located in close proximity of NIS TATA box element in the promoter region. Human NIS gene proximal promoter region sequence is partly shown. Numbers indicate positions of nucleotides relative to NIS start (ATG) codon. The transcription start site is indicated in blue letter (G), TATA box element, and novel ERE sequences are indicated in red, and in writing above these sequences. Two inverted repeats (half sites) of the NIS ERE element located in minus strand are underlined in red.

As mentioned above, NIS expression increases near the end of gestation, and mammary glands start actively transporting I^- before delivery (Tazebay *et al*., 2000). After delivery, the main physiological regulator of NIS expression is suckling of nipples. In fact, after delivery, in rodent models suckling regulates NIS expression in a reversible way: when pups are separated from the mother for 48 hours, steady-state NIS levels drop below detection levels by Western blots. Subsequently, when pups and mother are reunited for 24 hours, NIS accumulation reaches back to its maximal levels (Cho *et al*., 2000; Tazebay *et al*., 2000). Both prolactin and oxytocin, two pituitary hormones that were shown to have up-regulatory effects on NIS expression in breast were released simultaneously in response to suckling (Wakerley *et al*., 1978). This creates a wonderful regulatory mechanism preventing unnecessary active transport of I^- to mother's milk once grown-up pups stop suckling.

3. NIS expression in human breast cancer

A high prevalence of NIS expression in human breast cancer combined with much lower prevalance in normal extratumoral tissues have been reported in many independent studies, and by using a variety molecular analysis methods. In a pioneering study, Tazebay *et al*. (2000) demonstrated a perchlorate inhibited NIS activity in experimental mammary adenocarcinomas in female transgenic mice carrying Murine Mammary Tumor Virus (MMTV)-promoter driven activated Ras oncogene or over-expressed Neu oncogene. These authors were able to follow in real-time the NIS specific accumulation of radiolabelled I^- isotopes, or another substrate of the symporter, $^{99m}TcO_4^-$, in mammary tumours of animals. These were also the first evidences regarding possible uses of radio-labelled substrates of NIS for imaging of non-thyroid tumours. Importantly, in the same study, also human breast tissue specimens were analyzed by immunobiochemical methods, and it was found that above 80% of invasive and ductal carcinoma *in situ* samples were expressing NIS, whereas only about 20% of non-cancerous samples adjacent to tumours were NIS-positive. Besides, none of the 8 normal specimens obtained by reductive mammoplasties were expressing NIS (Tazebay *et al*., 2000). In subsequent studies where larger cohorts and variety of molecular techniques were used, results were comparable to initial reports. Wapnir *et al*. (2003) analyzed NIS expression in a total of 202 human breast samples, and they reported a clear

NIS positivity in 76% of invasive breast carcinomas, 88% of ductal carcinomas *in situ*, and 80% of fibroadenoma samples. Only one-out-of eight (13%) normal breast samples analyzed in the same study were weakly expressing NIS. More recently, endogenous NIS expression were analyzed in a subtype of breast carcinomas known as triple-negative breast cancer (Renier *et al*., 2010). This clinical-pathological subtype with the worst prognosis among breast cancer subtypes is commonly defined by the absence of ER, progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) (Sorlie *et al*., 2003). In this study, Renier *et al*. (2010) demonstrated that 15 specimens out-of 23 were NIS positive (67% positivity), with 11/15 samples being strongly positive (47% of total), suggesting a potential use for NIS-dependent radio-ablative strategies against this aggressive tumour group. Independently, still another research group composed of Beyer and co-workers (2009) has analyzed NIS expression levels in 192 different breast cancer specimens found on tissue microarrays, with a particular focus on sub-cellular localization of NIS protein expressed in those samples. In this study, 72% of samples were found to express -at variable levels- the symporter, and in 28% of tissue samples NIS expression were reported to be null. Beyer *et al*. (2009) argued that according to their visual analysis of stained tissue sections only 27% of NIS-positive tumours the symporter had a cell surface (plasma membrane) localization, and in remaining samples NIS seemed to localize at a cytoplasmic compartment. This is in fact an important observation that could also bring some explanation to the discrepancy between high percentages of NIS positivity (around 80% overall) in breast tumours, whereas relatively low percentage of radionuclide uptake activity in patients (17-25%; see also below). Yet, it is arguable whether immuno-histochemical staining techniques and visual evaluations of stained tissue sections used as main analysis methods in the study of Beyer and co-workers (2009) could be taken as sufficient to comment on sub-cellular localization of a membrane protein. In future, precise cell science techniques with improved microscopy, and quantitative molecular methodologies would be essential in order to better establish quantities of NIS proteins found at variable sub-cellular localizations in breast cancer/normal samples coming from patients.

In a recent and elegantly designed study, both relative levels of NIS expression in breast cancer samples (n=75), in fibroadenomas (n=15), and normal human samples (n=10) coming from premenopausal and postmenopausal patients, as well as correlations between expressions of the symporter gene and that of several proven or putative regulators of NIS [ER-α, retinoic receptors alpha and beta (RAR-α and RAR-β), thyroid hormone receptors alpha and beta (THR-α and THR-β)] were assessed by quantitative molecular techniques by Ryan *et al*. (2011). Furthermore, these authors have tested results of their tissue analysis by treating two distinct mammary cancer cell culture models (ER/PR positive, HER2 negative T47D cells versus ER/PR negative, HER2 positive SK-Br-3 cells) with agonist of those regulators, and thus rigorously validated their findings in tissue analysis. Supporting previous *in vivo* and *in vitro* studies, expression of NIS, RAR-α, RAR-β, and ER-α were found to be higher in breast cancer samples as compared to normal adjacent tissues. Moreover, a significant positive correlation was detected between NIS and these three transcriptional regulators, the highest correlation being between NIS and retinoid inducible factor RAR-β (Ryan *et al*., 2011). These results were also tested by appropriate ligands used in mammary cell culture experiments, and they were largely validated (see part 5, below). Data obtained by Ryan and co-workers (2011) should certainly be taken into account when

deciding on how to better design novel approaches for increasing endogenous mammary cancer NIS expression in different neoplastic growth types by stimulation of appropriate nuclear receptors and other trans-acting regulatory factors.

NIS protein was also demonstrated to be detectable in soft tissue metastasis of breast cancer. Renier and co-workers (2010) have carried-out an immuno-histochemical analysis on 28 archival tumour samples obtained from breast cancer brain metastasis (BCBM). In this analysis, they have studied NIS positivity in BCBM tissues, and correlated it with ER, PR, and HER2/Neu status of specimens. 21/28 cases were reported to be expressing NIS. Among those, 9 were from triple-negative for ER/PR/HER2, and 12 were ER/PR-negative, but HER2 positive. ER/PR-positively stained group of brain metastatic samples did not express NIS protein. Again, these authors argued that only about 24% of NIS positive samples had plasma membrane staining. These studies provided invaluable preliminary results on differences of NIS protein localization in different patient samples, and future scientific studies addressing sub-cellular localization of NIS in BCBM tissues by using appropriate cell biology methods would be essential for a precise characterization of cellular localization of NIS in metastatic tissues. Yet, this study documents about 75% NIS positivity in BCBM, and certainly stands-out with its important implications on possible uses of NIS activity based diagnostic/therapeutic methods in breast cancer soft tissue metastasis cases. Functional NIS expression was also shown by *in vivo* scintigraphic imaging methods in human breast cancers and its soft tissue metastasis. Recent advances in methodologies and approaches concerning uses of radio-labelled NIS substrates in imaging and diagnosis of malignant tissue are described in the next section below.

4. Human clinical trials on selective targeting of breast cancer by radionuclide substrates of NIS

Specific targeting of tumours with anti-cancer agents is one of the major research areas in cancer biology. Selective targeting of thyroid cancer and its metastasis by radioisotopes of NIS substrates has been successfully developed by nuclear medicine and effectively used as a diagnostic/therapeutic method in fight with malignant thyroid disease. Immediately after the discovery that lactating mammary gland concentrates I^- by the same transport mechanism operating in thyroid, and that the same symporter (NIS) is expressed in more than %80 human of breast cancer cases, there started translational medicine approaches for testing effectiveness of NIS activity based methodologies to diagnose and eradicate malignant breast disease in different groups of patients (Moon *et al*., 2001; and also see the clinical trial sponsored by Stanford University, entitled: Scintigraphic Assessment of I^- Transport in Metastatic Breast Cancer and Evaluation of Ablative Therapy: Radioiodide Imaging Study; clinicaltrials.gov identifier number NCT00185809, http://www.clinicaltrials.gov). Optimistic results from several studies on uses and effectiveness of radioiodide based therapeutic techniques in non-thyroid cancers that externally received NIS gene by viral delivery methods (Spitzweg *et al*., 2000; Faivre *et al*., 2004; Dwyer *et al*., 2006) gave further hopes to researchers who try to develop NIS based diagnostic/therapeutic tools for breast cancer management.

Several groups have assessed whether NIS substrates were accumulated by breast tumours in human subjects, at high enough concentrations, durations and specificities in order to permit imaging or therapy of malignant tissues by these substances. Moon *et al*. (2001) have

investigated the correlation between $^{99m}TcO_4^-$ uptakes and NIS mRNA expressions in breast cancer patients. Among 25 patients studied, $^{99m}TcO_4^-$ scintigraphy revealed positive uptake in 4 patients. There were a positive correlation between $^{99m}TcO_4^-$ uptake and NIS gene mRNA expression levels in tumours, and positive uptake tumours expressed higher levels of NIS as compared to negative uptake breast tumours (Moon *et al*., 2001). This was an important result indicating that a substrate of NIS, $^{99m}TcO_4^-$, could actually be used in imaging of breast tumours. However, even though in all previous tissue analysis studies NIS positivity in breast cancer samples were reported to be around 80%, in the study carried by Moon et al. (2001) only in 17% of breast cancer patients tumours were visible by $^{99m}TcO_4^-$ imaging. In another study, Upadhyay *et al.* (2003) investigated $^{99m}TcO_4^-$ transport capacities of tumours in a study comprised of 12 female patients (age 33-58 years) with infiltrating ductal carcinomas confirmed by fine needle aspiration cytology (FNAC) and subsequent histopathological analysis. Out of these 12 patients, samples obtained from 5 patients were analyzed by molecular techniques investigating NIS expression at transcriptional and translational levels, and scintigraphic imaging were performed in 4 out-of 5 patients prior to mastectomy. These authors reported both high NIS expression levels in all 4 tumours analyzed by scintigraphy, and high ability to concentrate $^{99m}TcO_4^-$ in tumour tissues. Because of the small sample size used in this study, it is very hard to comment on ratios related with NIS positivity versus radionuclide uptake positivity. Nevertheless, the study indicated possibilities of using $^{99m}TcO_4^-$ for imaging purposes in breast cancer.

So far, only the study of Wapnir *et al*. (2004) addressed whether NIS expressing metastatic breast tumours could be detected by using radioiodides ($^{123}I^-$ and $^{131}I^-$) or $^{99m}TcO_4^-$ in scintigraphic analysis. These authors investigated NIS activity in BCBM tissues of 27 women by administering either $^{99m}TcO_4^-$ or $^{123}I^-$ followed by a whole body scintigraphic analysis. I^- uptake was noted only in 25% of NIS expressing BCBM tissues (2 out-of 8). Apparently, nearly 30% of breast cancer related deaths demonstrate central nervous system involvement on autopsy, and the treatment of BCBM is particularly challenging because many anticancer drugs have limited access to the central nervous system (Renier *et al*., 2010). Therefore, systemic agents capable of crossing the blood-brain barrier, such as I^- or $^{99m}TcO_4^-$, could especially be important if they are effective against NIS positive breast cancer subtypes that metastasize to the brain.

Importantly, in the investigations carried-out by Wapnir *et al.* (2004), these authors also succeded to suppress thyroidal uptake of radiolabelled tracers by administering thyroxine (T_3) and methimazole (an inhibitor of I^- organification in thyroid) to patients under clinical trials. Suppression of thyroidal uptake in this study by a hormone regiment have in fact answered present discussions on whether it would be possible to protect healthy thyrocytes (which take-up and organify I^-) in case radioiodide based therapies are used to treat non-thyroidal tumours. If thyroidal cells were compromised during administration of radio-labelled NIS substrates, then the patients would need to depend on thyroid hormone medications for the rest of their lives. This is of course not preferable, and it was important to find ways of successfully protecting healthy thyroids of patients under clinical investigations. It seems in future clinical trials, integrity of thyroid cells could be protected by inhibiting thyroidal activity of NIS with hormone treatment regiments similar to the ones used in this study, while the non-thyroid origin tumour is targeted by cytotoxic NIS substrates. There is in fact a pilot radioiodide based breast cancer imaging clinical trial

currently carried-out under sponsorship of Stanford University at U.S. where a combination of T_3/methimazole regiment would be used for protection of thyrocytes (Pilot Study to Determine Radioiodide Accumulation and Dosimetry in Breast Cancers Using 124I PET/CT; clinicaltrials.gov identifier number: NCT00725946; http://www.clinicaltrials.gov).

5. Modulation of NIS expression in breast cancer cell models and in xenograft tumors in mice

NIS expression is present in about 80% of breast cancers suggesting that breast cancer patients may benefit from NIS-mediated radioiodide imaging and/or therapeutic methods. Nevertheless, only 17-25% of NIS positive breast tumors had detectable radionuclide transport activity as assessed by scintigraphic imaging (Moon *et al*., 2001; Upadhyay *et al*., 2003; Wapnir *et al*., 2004). Currently, there is a clear need to identify possible mechanisms underlying this discrepancy between NIS expression and NIS-mediated radionuclide uptake activity. Previous studies (reviewed above) based on analysis of breast cancer samples suggested that, in addition to NIS mRNA and protein expression, post-translational modification(s) of the symporter and appropriate membrane targeting are likely to be important for the substrate transport activity of NIS. Differential levels of NIS expression may account for variable cell surface NIS levels among breast cancer samples, and particular hormone combinations may be needed to upregulate expression and activity of the symporter at higher levels for successful future theraputic or diagnostic applications involving NIS activity.

Results from different laboratories have revealed that both the expression of mammary gland NIS gene at the transcriptional level, and the post-translational trafficking/localization of NIS to plasma membrane are tightly regulated in a cellular context and an extracellular stimuli dependent way (Tazebay *et al*., 2000; Riedel *et al*., 2001; Kogai *et al*., 2004; 2008; Dentice *et al*., 2004; Alotaibi *et al*., 2006; 2010; Willhauck *et al*., 2011). The list of hormones that were shown to have an upregulatory effect on NIS expression in a variety of model mammary gland cell lines and in animal experiments (xenografted and normal) include lactogenic hormones (prolactin and oxytocin), steroids (estrogens), retinoids, dexamethasone (Dex), and carbamazepine.

In initial animal experiments, lactogenic hormones prolactin and oxytocin were proven to upregulate NIS expression in the mammary tissue (see above, Cho *et al*., 2000; Tazebay *et al*., 2000). Both the two hormones were tested for their upregulatory activities by Cho *et al.* (2000) on human breast cancer cultures kept on collagen gels. In this experimental set-up, individual prolactin (500ng/ml) and oxytocin (100 ng/ml) treatments have positively affected NIS gene regulation by inducing it at the transcriptional level, but a combination of the two hormones failed to do so. A significant induction of NIS gene by prolactin was also reported by Arturi *et al.* (2005) in a mammary cancer cell line, MCF-7. This is a cell line derived from mammary adenocarcinoma metastatic to a pleural effusion of lung, and it is one of the most common ER-positive breast cancer cell line models. This cell line, together with MDA-MB-231 cells which are ER-negative cell models, is used in most of the studies addressing upregulatory effects of a variety of substances on NIS gene regulation (for instance see, Alotaibi *et al*., 2006; Arturi *et al*., 2005; Kogai *et al*., 2006). Using MCF-7 cells, both our group and Kogai *et al.* (2005) have tested the effects of 17-β-estradiol (at 10 nM concentration; one of the most potent estrogens) on NIS gene induction, and neither of the

two groups have seen a positive regulatory effect of this estrogen on the induction of the gene when introduced to cell lines in culture (Alotaibi *et al*., 2006). However, independent of the presence of ligand, ER plays a very critical regulatory role on NIS expression in a number of mammary gland cell models (see above, and also Alotaibi *et al*., 2006). The fact that some ER-negative tumours have upregulated NIS expression (Tazebay *et al*., 2000; Ryan *et al*., 2011) indicate that ER is not absolutely essential for inducibility of NIS in breast tumours, and in tumor milieu *in vivo* other ER-independent molecular mechanisms may operate making NIS induction possible. Recently, in response to 17-β-estradiol administration to ER-negative and ER-positive mammary cancer model cell lines, Ryan and co-workers (2011) have observed a 3 to 6-fold increased NIS expression in T47D cells (ER-positive), and about 5-fold increase in Sk-BR-3 cells (ER-negative). Because they observed estradiol inducible NIS expression in Sk-BR-3 mammary cell line which is an ER-negative cell model, they argued that similar to some breast tumours, ER may not be essential for inducibility of NIS in this cell line model as well, and additional mechanisms which bypass ER dependent regulatory components for activation of NIS gene may be operating in those cells.

Retinoids have a robust effect on functional NIS induction in human MCF-7 cells (Kogai *et al*., 2000; 2004; 2005; 2008). These compounds are active metabolites of vitamin A, and they have been used in animal models and humans as differentiation agents for various types of cancers including breast cancer (Mark *et al*., 2006). The typical retinoid pathway involves the ligand-activated nuclear receptors belonging to retinoic acid receptor family (RAR) and retinoid-X-receptor family (RXR). RAR and RXR form heterodimers, and bind corresponding RA or RXR response elements (RARs) at *cis*-acting regulatory DNA sites and activate gene transcription (Mark *et al*., 2006). All-*trans* retinoic acid (tRA; at 1μM) stimulates NIS induction up to 10-fold above the baseline in ER-positive MCF-7 cells, but it can not induce expression of the symporter in ER-negative model, MDA-MB-231 (Kogai *et al*., 2000; 2004), and this is because ER-positivity is absolutely essential for retinoid responsive upregulation of NIS virtually in all breast cancer cell line models (Alotaibi *et al*., 2006), except Sk-BR-3 (Ryan *et al*., 2011). In fact, Kogai *et al*. (2005) analyzed the isoform specificity of retinoid receptors for the upregulation of NIS gene expression by using isoform selective retinoid receptor ligands. They reported that a RAR-β/γ agonist (AGN190168) is a more potent inducer of NIS than a RAR-α agonist (AGN195183) or a RAR-γ agonist (AGN194433), indicating a central role to RAR-β in upregulation of NIS by retinoids. In line with these results, Ryan *et al*. (2011) have reported a robust positive correlation between NIS upregulation and RAR-β gene expression in human breast tissue samples (n=100) that they have analyzed by quantitative real-time PCR methods.

Even though, RAR and RXR were known to upregulate NIS expression since a while, *cis*-acting elements directly interacting with these nuclear receptors were only identified recently (Alotaibi *et al*., 2010). This delay in identification of RA responsive elements (RARE) that activate NIS transcription could be due to uncommon positioning of those *cis*-acting sites in multiple intronic positions in NIS gene. In our recent work, we have described that a functional RARE is present in the first intron of human NIS gene, and it seems there are at least six others in other introns of this gene (Alotaibi *et al*., 2010). In fact, initially we found three putative RAREs in the first intron intron. The first was a perfect DR2-type consensus RARE sequence (AGGTCAggAGTTCA), the second was a DR10-type sequence [AGGTGG(n10)AGGTCA], and the third was again a DR2-type sequence identical to the

first one (AGGTCAggAGTTCA), and it was overlapping with the second half-site of the previous putative RARE. By using a series of luciferase reporter constructs containing different fragments of this intronic region, as well as *in vitro* mutagenized versions of these putative RARE sequences, we have demonstrated that the third RARE which is located at +1222 relative to the start codon of NIS was a functional RARE. In MCF-7 cells transfected with a luciferase reporter construct containing this element, tRA treatment resulted in an increase of about 4-fold when compared to cells treated with DMSO vector. We also investigated the potential of this intronic RARE to activate transcription through the native NIS minimal promoter. For this purpose, we constructed reporter plasmids containing the minimal NIS promoter (218 bp of NIS upstream region between -260 bp and +42 bp relative to transcription start site, and capable of initiating transcription as assessed by previous functional reporter assays. As anticipated, the intronic element significantly activated NIS promoter in response to tRA when positioned either at the 5′ or at 3′ of the reporter gene, in agreement with the function of an enhancer. Furthermore, by electromobility shift assays and chromatin immunoprecipitation (ChIP) methods, we have shown that both RARs and RXR interacts with this RARE in response to tRA (Alotaibi *et al*., 2010). Altogether these findings clearly indicated that the DR2-2 element that is present in the first intron of NIS is an important RARE in regulation of human NIS gene. Similar RARE sequences were also present in introns 5, 7, 8, 12, 13 and 14 of human NIS gene (some having identical sequences with RARE found in intron 1). All these sequences interact with RARs and RXR, and eventually with the RNA Polymerase II (PolII) in ChIP assays at different time points in response to tRA administration to cells (Fig. 2). Earlier work by others, and results obtained by Alotaibi *et al*. (2010) clearly indicate that molecular mechanisms governing NIS expression involve a number of classical and intronic elements that regulate temporal and spatial expression, and they are likely to be more complex than previously anticipated. Future experiments will show whether the activities of intronic enhancers lead also to a tissue or cell-type-specific regulation of NIS.

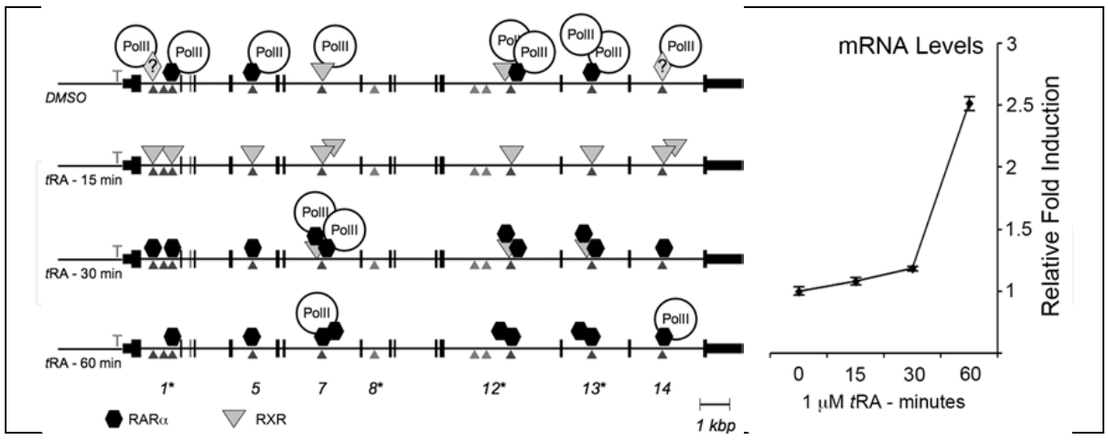

Fig. 2. Nuclear receptors and RNA Pol-II interact with NIS intronic elements in a dynamic manner during the initiation of transcription. MCF-7 cells grown in steroid-free and phenol-red-free DMEM were treated either with DMSO (time 0) or with tRA for 15, 30 and 60 min and used for ChIP analysis using RARa and RXR and RNA Pol-II specific antibodies. mRNA was isolated from a fraction of the cells used for this analysis, NIS expression was normalized to the levels of GAPDH, and presented as relative fold induction compared to DMSO-treated samples. A schematic representation of ChIP data depicting the events of

transcription initiation of NIS in response to tRA stimulation is shown. Numbers indicate introns studied, asterisks above numbers indicate RA responsive elements with identical DNA sequences; AGGTCAnnAGTTCA. Parallelograms with question mark represent unidentified interacting proteins. The figure is reproduced from Alotaibi *et al*., 2010, Intronic Elements in the NIS Gene Interact with Retinoic Acid Receptors and Mediate Initiation of Transcription. *Nucleic Acids Research*, Vol. 38 (10), pages 3172-3185. Reprinted with permission from The Oxford University Press © 2010.

In vivo studies with MCF-7 xenograft tumours in immunodeficient (CD1 nu/nu) mice have indicated that systemic tRA treatment increases radioiodide ($^{125}I^-$) accumulation capacities of xenografts about 15 fold above background, and this level is sufficient for selective imaging of tumours with $^{125}I^-$. Administration of dexamethasone (Dex), and carbamazepine (CBZ) together with tRA increased both iodide uptake by NIS, and NIS mRNA expression even more. In response to Dex and CBZ administration, tRA induced I^- uptake via NIS have increased about 12 fold in xenograft tumours in experimental animals, clearly indicating that the combination of an RAR isoform selective ligand and Dex and Cbz has a potential to provide a more effective induction of NIS, and it provides an important improvement for the effective use of symporter in theraputic/diagnostic medical approaches (Kogai *et al*., 2005; Willhauck *et al*., 2008; 2011).

6. Future strategies for development of NIS activity based diagnostic and therapeutic methods

Detection of radioiodide transport in thyroid cancer tissues at the beginning of 1940's marked the beginning of nuclear medicine as a discipline in medical sciences, by allowing the use of radioiodide and other selective radionuclide substrates of this symporter (for instance, $^{99m}TcO_4^-$) in successful management of thyroid cancers and their metastasis. Similarly, recent detection of functional NIS expression and radioiodide accumulation in breast cancer tissues provide important opportunities for development of novel adjuvant therapies and/or detection methods based on breast cancer specific NIS activity. Routine and successful use of radioiodide in hospitals to evaluate and cure thyroid cancer cases for more than 60 years, make it an even more attractive substance to be worked on for developing future innovative methodologies for implementation to non-thyroidal cancers. Furthermore, as I described in the previous section, a number of substances (especially retinoids and glucocorticoids) modulating NIS expression and enhancing radioiodide transport activities in mammary cancer cell models and xenografted animals were already developed, and these advances in basic research are currently waiting to be brought to "bed side" by translational medicine approaches.

A number of clinical studies have already addressed use of radioiodide in scintigraphic detection of breast tumours, and breast cancer metastasis *in vivo* (see above). Even though data coming from these studies have led to optimistic conclusions on prospective uses of $^{131}I^-$ radioablation techniques on at least some breast cancers and their metastasis, to our knowledge, a clinical trial subjecting breast cancer patients to radioiodide based ablation studies has not yet taken place.

As presented through-out the text above, there are three main problems that have to be solved for effective and routine use of radio-iodide (or other cytotoxic NIS substrates) in management breast cancers: 1) discrepancies in percentages of NIS expressing tumour

tissues (around 80%) versus percentages of cancers taking up radio-iodide (17-25%); 2) problems associated with low or insufficient cumulative I^- uptakes in breast cancers (0.00131% ID/ml; Renier *et al*., 2009), making it difficult to achieve a therapeutic effect; and 3) reported accumulation of radio-iodide in some benign fibroadenoma of breast (Berger *et al*., 2006), which brings problems of achieving sufficient malignant tissue specificity in diagnosing and targeting breast cancers with NIS substrate radionuclides. A fourth issue may be protection of healthy thyroid from cytotoxicity of agents transported by NIS in imaging or treatment of non-thyroidal tumours, as thyrocytes very robustly and constitutively express functional NIS (for a review see Kogai *et al*., 2006). In fact, this fourth point was addressed in studies carried out by Wapnir and co-workers (2004), and it has been shown that uptake of solutes to thyroid via NIS could selectively be down regulated by administering a combination of thyroid hormones and I^- organification inhibitor methimazole to imaged patients. Therefore, it seems in future studies this final issue could be at least easily solved with some optimization of hormone/methimazole combinations. However, solutions for the remaining three problems should be rigorously addressed in future clinical, translational, and basic research studies.

Regarding which subtype of breast cancer tissue responds best to, and therefore more suitable for, imaging/treatment with NIS substrate agents, the work of Ryan *et al*. (2011) indicated that cancers expressing RARs and ERs are amenable to radioiodine based imaging techniques. However, it has been shown by their study that the group of tissues that express RARs, ERs, and finally NIS at maximal levels were benign fibroadenoma samples. This suggests that together with radioiodine scintigraphic analysis of the mammary, additional analysis methods would be essential for differentiating radio-iodide transporting benign tissue from the malignant breast cancer. In future prospective approaches, once the possibility of fibroadenoma of the breast is eliminated, radio-iodide uptake in malignant cancerous tissue of the breast could be upregulated by hormone combinations (retinoids, dex, and cbz) as previously suggested by independent works of Kogai *et al*. (2004), and Willhauck *et al*. (2011). That could perhaps solve the problem of discrepancy between NIS expression and NIS-positive tumour radio-iodide uptake, leading to a robust expression of functional NIS in malignant breast tissue, which could then permit use of $^{131}I^-$ dependent ablation strategies for curing the disease. In the meantime, healthy thyroid of the patients could be protected from ablative effects of radionuclides by administering the hormone regiment containing thyroxine and methimazole, as suggested by Wapnir *et al*. (2004).

The beta-emitter radionuclide Rhenium-188-perrhenate ($^{188}RheO_4^-$), and the alpha emitter Astatine-211 (^{211}As) have shorter half lives (17 hours and 10,5 hours, respectively) as compared 8 days half-life of the beta emitter $^{131}I^-$ (Dadachova and Carrasco, 2004). $^{188}RheO_4^-$ have also previously been shown to be effective on NIS expressing mammary cancers of transgenic mice bearing polyoma-middle-T oncoprotein, as this radiosope have significantly inhibited growth of these experimental tumours (Dadachova *et al*., 2005). Therefore, in future studies, a variety of radionuclides that are transported by NIS could be selected to be used in these possible novel applications targeting malignant breast cancer cells with least possible side-effects, while having high specificity, improved efficiency, and remarkable safety.

7. Conclusion

Breast cancer management involves chemotherapeutic procedures before or subsequent to surgery with the aim of ablating micro-metastatic cells and improving disease free overall

survival of the patient. The administration of cytotoxic agents used in breast cancer chemotherapy is usually by venal intervention, and it can cause significant pain and side effects. Therefore, development and introduction of alternative strategies that target breast cancer cells with high specificity, less toxicity to healthy tissues, and with less overall side-effects is important. Because of previous, and decades long, clinical work in diagnosing and treating thyroid cancers by radioisotope substrates of NIS, use of NIS transported radionuclides could offer novel prospective breast cancer treatment modalities with advantages such as easier administration procedures, and relatively less side-effects as compared to current chemotherapeutic drugs.

Recent scientific studies on molecular biology and regulation of NIS expression in breast cancer models have very clearly shown the important potential of NIS activity based novel radiotherapy techniques in management of malignant breast disease. A number of lactogenic hormones, estradiol, retinoids, and glucocorticoids were shown to enhance radioiodide transport in breast cancer cells, as well as in *in vivo* models. Increased understanding of molecular mechanisms that upregulate NIS expression in response to these hormones and ligands, and enhance the activity of the symporter in breast tissue as well as in breast cancer metastasis will be essential for the development and improvement of methods that will involve use of NIS activity and cytotoxic substrates for imaging and therapeutic purposes in breast cancer clinics.

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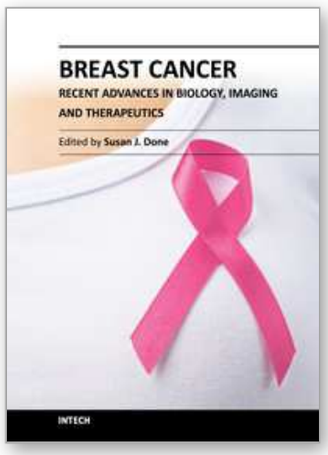

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In recent years it has become clear that breast cancer is not a single disease but rather that the term encompasses a number of molecularly distinct tumors arising from the epithelial cells of the breast. There is an urgent need to better understand these distinct subtypes and develop tailored diagnostic approaches and treatments appropriate to each. This book considers breast cancer from many novel and exciting perspectives. New insights into the basic biology of breast cancer are discussed together with high throughput approaches to molecular profiling. Innovative strategies for diagnosis and imaging are presented as well as emerging perspectives on breast cancer treatment. Each of the topics in this volume is addressed by respected experts in their fields and it is hoped that readers will be stimulated and challenged by the contents.

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