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CHARACTERIZATION OF RET/PTC1 TRANSGENIC MICE AND DEVELOPMENT
OF CANCER THERAPY USING RADIOIODIDE ABLATION MEDIATED
BY NA+/I SYMPORTER IN CANCERS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy
in the Graduate School of The Ohio State University

By

Je-Yoel Cho, M. S.

*****

The Ohio State University

1999

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The ret/PTC1 transgenic mouse model, specifically the early cellular abnormalities induced by ret/PTC1 expression in embryological days was studied. In conjunction with these studies, a recently cloned transporter gene, Na^+/I^- symporter (NIS), which is responsible for I^- uptake activity in thyroidal and extrathyroidal tissues was used to develop radioiodide therapy for extrathyroidal cancers by either up-regulating or introducing hNIS to those tumors.

The early cellular abnormalities induced by ret/PTC1 oncogene in thyroid-targeted transgenic mice were characterized. The ret/PTC1 oncogene, a rearranged form of the RET proto-oncogene, has been reported to be associated with human papillary thyroid carcinomas. We have shown that targeted expression of ret/PTC1 in the thyroid gland leads to the development of thyroid carcinomas in transgenic mice with histologic and cytological similarities to human papillary thyroid carcinoma. To further investigate how RET/PTC1 expression contributes to the pathogenesis of papillary thyroid tumor, the time of tumor onset and the early phenotypic consequences of RET/PTC1 expression in thyrocytes were determined. All high copy transgenic mice developed bilateral thyroid tumors as early as 4 days of age. At embryological days 16-18, increased proliferation rate, distorted thyroid follicle formation, and reduced radioiodide concentrating activity were identified in transgenic embryos. The reduced radioiodide concentrating activity
was attributed to decreased expression of the sodium-iodide symporter. Our study showed that \textit{ret/PTC1} not only increased proliferation of thyrocytes, it also altered morphogenesis and differentiation. These findings provide a model for the role of \textit{ret/PTC1} in the formation of abnormal follicles with reduced iodide uptake ability observed in human papillary thyroid carcinoma.

Hormonal regulation of radioiodide uptake activity and \textit{Na}^+/\textit{I}^- symporter expression was studied in mammary glands. Radioiodide uptake activity, mediated by NIS, in thyroid has allowed radioiodide to ablate postsurgical remnants and to treat residual, recurrent, and metastatic thyroid cancers. The observation that radioiodide uptake activity is significantly increased in lactating breast has led us to hypothesize that patients with breast cancer could also benefit from radioiodide therapy. Mammary glands of lactating rats had the highest radioiodide accumulation and NIS expression level, when compared to that of virgin and pregnant rats as well as the involuting mammary gland. In lactating mammary gland, NIS is clustered on the basolateral membrane of alveolar cells as a less glycosylated form than NIS in thyroid. A selective oxytocin antagonist and bromocriptin had inhibitory effects on the radioiodide accumulation of lactating mammary gland. NIS was expressed in epithelial-enriched primarily cultured human normal breast cells and human breast cancer cells, and NIS mRNA level was increased by oxytocin and prolactin in a dose-dependent manner in histocultured human breast tumors. Further optimization of dosages in the combination of oxytocin and prolactin to stimulate NIS expression in breast tumors may prove to be sufficient to facilitate radioiodide therapy for patients with breast cancer.
In an attempt to develop hNIS gene transfer for radioiodide therapy for patients with brain tumors, we have constructed a recombinant adenovirus, rAd-CMV-hNIS#9, to express exogenous hNIS in U1240 and U1240Tag human glioma cells. U1240Tag differs from U1240 glioma cells in that it expresses the SV40 large T antigen oncoprotein. In both U1240 and U1240Tag cells, radioiodide uptake activity in the cells infected with rAd-CMV-hNIS#9 increases as the adenoviral multiplicity of infection increases. At multiplicity of infection 10, and at 24 hr post-infection, the infected U1240 cells had an 82-fold increase in radioiodide uptake activity, while the infected U1240Tag cells had a 25-fold increase in radioiodide uptake activity, compared to their corresponding non-infected cells. The protein expression profile of hNIS in infected cells is generally in agreement with the profile of their radioiodide uptake activity. Although the expressed hNIS#9 protein appeared to have a short half-life, hNIS#9 expression could be maintained by multiple infections in these cells. In addition, we showed that hNIS#9 can be expressed in a xenografted human glioma (U251) by intratumoral injection of rAd-CMV-hNIS#9. Taken together, our findings of hNIS gene transfer conferring radioiodide uptake activity in human glioma cells provide a basis for exploring further gene therapy strategies based on hNIS gene transfer followed by radioiodide treatment in patients with brain tumors.
Dedicated to Myong-Ja and my parents
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LIST OF ABBREVIATIONS

bp  base pair
Br-CT  bromocriptin
BrdU  bromo-deoxyuridine
cAMP  cyclic adenosine monophosphate
cDNA  complementary deoxyribonucleic acid
CMV  cytomegalovirus
DMEM  Dulbecco’s minimum essential medium
DNA  deoxyribonucleic acid
FRTL-5  Fischer rat thyroid cell line – 5
H & E  hematoxylin and eosin
hNIS  human sodium iodide symporter
kb  kilobase pair
MG(s)  mammary gland(s)
mNIS  mouse sodium iodide symporter
MOI  multiplicity of infection
mRNA  messenger ribonucleic acid
MW  molecular weight
nt  nucleotide
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<tr>
<td>OT</td>
<td>oxytocin</td>
</tr>
<tr>
<td>OTA</td>
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<td>OTR</td>
<td>oxytocin receptor</td>
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<td>PC(s)</td>
<td>papillary thyroid carcinoma(s)</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>ppm</td>
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<td>PRLR</td>
<td>prolactin receptor</td>
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<tr>
<td>rAd</td>
<td>recombinant adenovirus</td>
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<tr>
<td>RIA</td>
<td>radioiodide accumulation</td>
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<td>RIU</td>
<td>radioiodide uptake</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>rNIS</td>
<td>rat sodium iodide symporter</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription — polymerase chain reaction</td>
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<tr>
<td>Tg</td>
<td>thyroglobulin</td>
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<tr>
<td>TPO</td>
<td>thyroid peroxidase</td>
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<td>TSH</td>
<td>thyroid stimulating hormone</td>
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CHAPTER 1

INTRODUCTION

The ret Oncogene

A novel transforming gene was discovered by transfection of NIH/3T3 cells with human lymphoma DNA. The transforming gene consisted of two segments that were found only in the NIH/3T3 cells transfected with human lymphoma DNA but not in both normal human and primary lymphoma DNAs, suggesting that the novel transforming gene is a rearranged gene during transfection. Thus, the ret proto-oncogene was named rearranged during transfection (Takahashi et al., 1985). The ret proto-oncogene encodes for receptor type tyrosine-kinase proteins (Tahira et al., 1990; Takahashi and Cooper, 1987), which transduce signals for cell growth and differentiation. Receptor tyrosine kinases (RTKs) consist of a large extracellular domain, a single transmembrane domain, and an intracellular tyrosine-kinase domain (Itoh et al., 1989; Takahashi et al., 1988). The extracellular domain contains a juxtamembrane cysteine-rich region as well as a cadherin homology domain (Iwamoto et al., 1993). The ligands for RET were identified to be members of the glial-cell-line derived neurotrophic factor (GDNF) protein family, including GDNF (Durbec et al., 1996; Trupp et al., 1996), neurturin (NTN) (Buj-Bello
et al., 1997; Klein et al., 1997), artemin (Baloh et al., 1998) and persephin (Milbrandt et al., 1998).

Ligands for RET

Two homologous growth factors, GDNF and NTN, were found to be the ligands for RET. Both factors promote the survival of sympathetic, sensory and central nervous system neurons, and GDNF is required for the development of the enteric nervous system and kidney (Buj-Bello et al., 1997). GDNF and NTN mediate their actions through a multicomponent receptor system composed of ligand-binding glycosylphosphadidylinositol (GPI)-linked proteins. Binding of GDNF to their GPI-linked proteins forms a physical complex of GDNF, GDNFR-α and RET on the cell surface and induces tyrosine-phosphorylation of RET in the cytoplasm (Jing et al., 1996; Treanor et al., 1996). NTN also activates RET by forming the NTN, NTNR-α and RET complex (Buj-Bello et al., 1997; Klein et al., 1997). Recently, some other members of the GDNF protein family were also found to be ligands for RET. These include artemin (Baloh et al., 1998) and persephin (Milbrandt et al., 1998), which are also neurotrophic factors for a variety of neurons. Artemin and persephin also activate RET tyrosine kinase by forming GFRα3-RET and GFRα4-RET complexes, respectively (Baloh et al., 1998; Enokido et al., 1998). Similar to ret knockout mice, the most severe phenotypes of GDNF gene knockout mice include renal agenesis and the absence of enteric neurons (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). NTN knockout mice also have defects in the enteric nervous system including reduced myenteric plexus innervation as well as defects in the sensory and parasympathetic neurons (Heuckeroth et al., 1999).
Expression of *ret*

*ret* mRNA was found preferentially in neuroblastomas, pheochromocytomas and medullary thyroid carcinomas, all of which originate from neural crest cells (Ikeda et al., 1990; Santoro et al., 1990). The pattern of *ret* transcript (Pachnis et al., 1993) and RET protein (Tsuzuki et al., 1995) expression was analyzed during mouse and rat embryogenesis, respectively. The *ret* transcripts were detected beginning at day 8.5 of embryogenesis (E 8.5d), and were observed in a number of cell lineages in the developing peripheral and central nervous systems (sensory, autonomic and enteric ganglia), as well as in the excretory system (Wolffian duct and ureteric bud epithelium) (Pachnis et al., 1993). During rat embryogenesis, RET protein expression became detectable by E 11.5d in the developing peripheral and central nervous system as well as in the excretory system (Tsuzuki et al., 1995). After birth, neurons in the nervous system continued to express the RET protein at variable levels while no RET expression was observed in the kidney of adult rats. Outside the nervous system, the RET protein was found in the acinar cells of the salivary gland, the epithelial cells of the thymus, follicular dendritic cells of the spleen and lymph node, adrenal chromaffin cells and thyroid C cells in infant and adult rats (Tsuzuki et al., 1995). The importance of the *ret* proto-oncogene in the developing excretory and enteric nervous systems was confirmed by targeted mutation of *ret* in knockout mice. Mice homozygous for a targeted mutation in *ret* develop to term, but die soon after birth, showing renal agenesis or severe dysgenesis, and lacking enteric neurons throughout the digestive tract (Schuchardt et al., 1994). In early human embryos, *ret* mRNA is detected in the developing kidney (nephric duct,
mesonephric tubules, and ureteric bud), the presumptive enteric neuroblasts of the developing enteric nervous system, cranial ganglia (VII, VIII, IX and X) and in the presumptive motor neurons of the spinal cord (Attie-Bitach et al., 1998).

**Signal transduction by RET**

RET, like other tyrosine kinase receptors, mediates signaling through a variety of signaling pathways, most notably the Ras signaling pathway (Ras/Raf/MAPK pathway) and the phosphatidylinositol-3 (PI-3) kinase pathway. These pathways are activated through the interaction of adaptor proteins to tyrosine phosphorylated receptors (van Weering and Bos, 1998). The intracellular domain of RET consists of 14 tyrosine residues, and the long isoform of RET has two extra tyrosine residues at the C-terminus. Among those tyrosine residues, Tyr^{905}(P), Tyr^{1015}(P), and Tyr^{1062}(P) of RET were identified as the docking sites for Grb7/Grb10, PLCγ, and Shc/Enigma adaptor proteins, respectively (Borrello et al., 1994; Durick et al., 1996; Liu et al., 1996).

**RET and Human Disease**

Aberrant *ret* seems to be the causative gene for at least five different human diseases; Hirschsprung disease (HSCR), multiple endocrine neoplasia type 2A (MEN 2A), MEN 2B, familial medullary thyroid carcinoma (FMTC) and papillary thyroid carcinoma (PC). The germline mutations of *ret* are responsible for the development of HSCR and MEN 2A and 2B. HSCR is a common congenital malformation responsible for intestinal obstruction in neonates, and characterized by the absence of
parasympathetic intrinsic ganglion cells in the terminal hindgut. Mutations of the \textit{ret}
tyrosine kinase receptor have been identified in 50% and 15-20% of familial and sporadic
HSCR, respectively. These mutations include deletion, insertion, frameshift, nonsense,
and missense mutations dispersed throughout the \textit{ret} coding sequences (Pelet et al.,
1998). HSCR mutations represent loss of function mutations, and most of HSCR
mutations detected in the extracellular domain impair cell surface expression of the \textit{ret}
(Takahashi et al., 1998).

Point mutations in the juxtamembrane cysteine-rich region of \textit{ret} have been
associated with FMTC, a tumor that develops in the C-cells of the thyroid gland, and
MEN 2A disease (Mulligan et al., 1993). MEN 2A disease is a group of dominantly
inherited cancer syndromes with common features of medullary thyroid carcinoma,
pheochromocytoma and parathyroid hyperplasia. In contrast to HSCR mutations, MEN
2A mutations result in the constitutive activation of RET by germline point mutations in
\textit{ret}. MEN 2A mutations cause the replacement of one of five clustered cysteines by a
different amino acid, and thus induce disulfide-linked RET dimerization on the cell
surface (Asai et al., 1995; Eng et al., 1996; Santoro et al., 1995). A point mutation in
methionine 918 of the catalytic tyrosine kinase domain is known to activate RET without
dimerization, probably due to a conformational change of its catalytic core region
(Borrelllo et al., 1995; Takahashi et al., 1998). This Met 918 to Thr mutation in RET
induces the MEN 2B disease, a more aggressive form of MEN 2 disease, which is
characterized by ganglioneuromas and skeletal abnormalities, in addition to the common
features seen in MEN 2A disease (Hofstra et al., 1994). Interestingly, \textit{ret} mutations in
cysteines 618 and 620 were reported in several families who developed both MEN 2A
and HSCR (Mulligan et al., 1994). Finally, somatic rearrangements of the ret proto-oncogene, named ret/PTC oncogene, are frequently found in papillary thyroid carcinoma (PC).

The ret/PTC Oncogene

The ret proto-oncogene was found to be rearranged with a variety of irrelevant genes by either in vitro transfection artifact (ret, ret II, ret III) (Ishizaka et al., 1988; Kunieda et al., 1991; Takahashi et al., 1985) or paracentric inversion of chromosome in tumor cells in vivo (ret/PTC), as shown in Fig 1.1 (Grieco et al., 1990). Several different forms of rearranged ret/PTC oncogenes have been found to be associated with human papillary thyroid carcinomas (PCs). ret/PTC1 is derived from the fusion of the tyrosine kinase (TK) domain of the ret proto-oncogene with the 5'-terminal region of another gene called 'H4' (D10S170 locus). Thus a 354 bp-long coding sequence belonging to the H4 gene replaces the truncated transmembrane and extracellular domains of the ret proto-oncogene (Grieco et al., 1990). In ret/PTC2, the ret TK domain is fused to the 5’ portion of the regulatory subunit ‘RIα’ of the cAMP dependent protein kinase A (Bongarzone et al., 1993). In the case of ret/PTC3, the fusion of the ret TK domain occurred with the 5’-terminal sequence of the ele1 gene (Santoro et al., 1994; Smanik et al., 1995). ret/PTC3 rearrangements include the common form (PTC3r1), and two other subtypes of ele1/ret rearrangements that were identified in PCs of children after the Chernobyl nuclear reactor explosion (Klugbauer et al., 1998). One aberrant subtype (PTC3r2) is shorter by one 144
bp exon, and the second atypical form (PTC3r3) has an 18 bp shorter ele1 part than PTC3r1.

Transforming mechanism of the ret/PTC oncogene

In each ret rearrangement, there are common features that might be important for inducing transforming activity. The intracellular domain of RET, which has tyrosine kinase activity, is fused to the N-terminus of the activating genes, the products of which are capable of dimerization. Consequently, the RET oncogene products lose the ligand binding sites, and the expressions of ret/PTC chimeric oncogenes are driven by the promoter of N-terminal activating genes. The products of the chimeric ret/PTC oncogenes are translocated to the cytoplasm and display constitutive tyrosine-kinase activity in thyroid follicular cells. Taken together, constitutive dimerization of the ret/PTC chimeric oncogene products, mediated by the N-terminal sequences of H4, R1α, or ELE1, may provide the mechanism for the constitutive activation of the RET tyrosine kinase. (Lanzi et al., 1992; Tong et al., 1997).

It has been found that the ret/PTC1 oncogene alters the expression of the thyroid-differentiated phenotype in a rat thyroid epithelial cell-line, PC Cl 3, by at least two different mechanisms; i) down-regulation of the mRNA and protein expressions of Pax-8, and ii) impaired function of TTF-1 and Pax-8 thyroid transcription factors, which occurs at a posttranslational level (De Vita et al., 1998). It has been recently demonstrated that RET/PTC activation in primary cultures of human thyroid epithelial cells by ret/PTC retroviral constructs led to changes in nuclear envelope and chromatin structures, changes of which are characteristic for PC (Fischer et al., 1998). The signaling pathway
downstream of RET/PTC causing these changes does not appear to depend entirely on a RAS pathway.

**Clinical significance of the ret/PTC oncogene**

The activation of all ret/PTC oncogenes appears to be restricted to the papillary histotype of thyroid carcinomas (Jhiang et al., 1992; Santoro et al., 1992). In fact, more than one hundred non-papillary thyroid tumors including follicular, anaplastic, medullary carcinomas, and benign adenomas were negative for ret/PTC rearrangements (Santoro et al., 1992). The unique involvement of ret/PTC in PC suggests that ret/PTC rearrangement is a specific genetic lesion that leads to the development of human PC. The prevalence of ret/PTC in spontaneous PCs ranges from 3% to 43% depending on geographic areas. Among the ret/PTC oncogenes, ret/PTC1 is the most commonly detected version, followed by ret/PTC3, and then ret/PTC2.

It has been proposed that ret/PTC oncogene activation represents an early event in thyroid carcinogenesis, since a significantly higher prevalence of ret/PTC (mainly ret/PTC1) rearrangements was observed in occult papillary thyroid micro-carcinomas than in clinically evident PCs (Sugg et al., 1998; Viglietto et al., 1995). It seems that, compared to non-ret-rearranged PCs, the sporadic PCs harbouring activating ret rearrangements occur in younger ages and occur frequently as slow growing, less aggressive papillary tumors that do not progress to more aggressive, less differentiated tumor phenotypes (Soares et al., 1998; Tallini et al., 1998).

**Radiation and ret/PTC**

8
Radiation exposure is a well-known risk factor for cancer development. Chromosomal rearrangements induced by radiation are most likely one of the principal genetic alterations resulting in malignant transformation. It has been demonstrated that X-irradiation of both thyroid and non-thyroid cell lines in vitro can induce the formation of ret rearrangement in these cells (Ito et al., 1993). Childhood papillary thyroid cancers following the Chernobyl nuclear reactor accident exhibit a high prevalence of ret rearrangements. The ret rearrangement was observed in approximately 60% of PCs of children from areas contaminated by the Chernobyl nuclear reactor accident (Fugazzola et al., 1995; Ito et al., 1994; Klugbauer et al., 1995). These studies also suggested that the radiation exposure may be a direct inducer of activating rearrangements in the ret gene. The most frequent form of ret rearrangement in these patients was ret/PTC3 in early studies (ret /PTC3: ret /PTC1 = 3:1). However, in later studies, more ret/PTC1 was frequently observed, resulting in a 1:1 ratio of ret/PTC3 to ret/PTC1 (Pisarchik et al., 1998; Pisarchik et al., 1998; Smida et al., 1999). These results suggest that ret/PTC3 may be typical for radiation-related childhood PC with short latency periods, whereas ret/PTC1 may be a marker for later-occurring PC in radiation-exposed adults and children. Interestingly, when the ret rearrangement was studied in human thyroid tumors from non-Chernobyl patients who had received external radiation for benign or malignant conditions, 66% to 84% of PCs had ret rearrangements with the most frequent form being ret/PTC1 rather than ret /PTC3. However, in 'spontaneous' tumors, only 15% of PCs exhibited ret rearrangements. (Bounacer et al., 1997; Learoyd et al., 1998). These studies suggested that external radiation could also cause ret/PTC rearrangements at
higher rates than in the general population, and the major form of ret rearrangements by medical external radiation still seemed to be ret/PTC1 instead of ret/PTC3.

**ret/PTC Transgenic Mice**

*In vitro* cell line experiments demonstrated the capability of the ret/PTC gene to alter the growth and differentiation of rat thyroid cells (Santoro et al., 1993). Also, ret/PTC expression was demonstrated to be uniquely restricted to the papillary histotype of thyroid neoplasia by several researchers (Grieco et al., 1990; Jhiang and Mazzaferri, 1994; Santoro et al., 1992). However it has been questioned whether ret/PTC oncogene activation is involved in the initiation or the progression of papillary thyroid carcinomas. To address this question, we have generated a transgenic mouse model of PC with targeted expression of ret/PTC1 in the thyroid gland using the bovine thyroglobulin (Tg) promoter (Jhiang et al., 1996). Transgenic mice develop thyroid carcinomas with considerable similarities to human PCs, particularly in the nuclear cytologic features and presence of local invasion. Thus this study indicated that ret/PTC is not only a biomarker related to PC, but it is also the specific genetic event leading to the development of PC. Chapter 2 further characterizes the ret/PTC1 transgenic mice. High copy transgenic mice were used to determine the time of tumor onset, identify cellular abnormalities preceding tumor development, analyze the expression of thyroid-specific markers, and examine the role of thyroxine (T4) supplementation in tumor progression. One of the findings was that radioiodide concentrating activity was substantially decreased in the thyroid tumors of ret/PTC1 transgenic mice. The effect was already observed in the embryological days
(E 18d). The reduced radioiodide concentrating activity was attributed to decreased expression of the sodium-iodide symporter (NIS). This study showed that RET/PTC1 not only increased proliferation of thyrocytes, but also altered morphogenesis and differentiation including NIS expression and function.

The Thyroid Gland

The thyroid gland is an unique endocrine organ producing the hormones thyroxine (T4) and triiodothyronine (T3), which are important for metabolism, postnatal development, and regulation of gene expression (Fig 1.2). Iodide (I) from the blood is transported across the basolateral membrane of thyroid follicular cells against its concentration gradient by an energy-dependent mechanism. The I is incorporated into thyroglobulin (Tg) in the apical membrane by an enzymatic reaction requiring thyroperoxidase (TPO). Further coupling is necessary to produce T4 and T3. Thyrotropin (TSH) is the main regulatory hormone that controls thyroid proliferation and function via its receptor (TSHR) on thyroid follicular cells. The TSH-TSHR signaling pathway increases thyroid hormone levels by up-regulating the expression and the function of NIS, TPO and Tg.

The Na+/I− Symporter (NIS)

Iodide uptake across the basolateral membrane of thyroid follicular cells is the initial and rate-limiting step in the production of T4 and T3. This iodide uptake activity
is mediated by NIS. Radioiodide concentrating activity in the thyroid has been used for effective radioiodide treatment for patients who have invasive, recurrent, and metastatic thyroid cancers after surgical intervention (Mazzaferri, 1996). Until recently, it was not possible to study the iodide uptake activity at the molecular level. A cDNA of rat NIS (rNIS) was cloned by functional screening of a cDNA library from a Fischer rat thyroid cell line (FRTL-5) in *Xenopus laevis* oocytes (Dai et al., 1996). NIS is an intrinsic membrane protein with 13 putative transmembrane domains (Levy et al., 1998). The rNIS protein is composed of 618 amino acids with a predicted molecular mass of approximately 65.2 kDa (Levy et al., 1997). Human NIS (hNIS) cDNA was also cloned (Smanik et al., 1996) and identified as an open reading frame of 1929 nucleotides encoding a protein of 643 amino acids (Fig 1.3) with 84% identity to the rNIS. The coding region of hNIS is interrupted by 14 introns and the chromosome location of the hNIS gene has been mapped to chromosome 19p (Smanik et al., 1997). In chapter 2, a portion of mouse NIS (mNIS) cDNA was cloned to study the expression of mNIS mRNA in the *ret/PTC1* transgenic mice. NIS protein exists as a highly glycosylated protein in the plasma membrane of cells. The rNIS protein migrates in SDS-PAGE gels as glycosylated proteins of higher molecular mass (approximately 80 to 110 kDa) than nonglycosylated rNIS, (approximately 50 to 55 kDa) (Levy et al., 1998; Paire et al., 1997). The half-life of rNIS protein in FRTL-5 cells was found to be ~4 days (Paire et al., 1997). In the case of hNIS, fully glycosylated hNIS proteins migrate as a molecular mass of ~90kDa, and nonglycosylated as a mass of ~60 kDa (Jhiang et al., 1998). It has been shown that, to a considerable extent, the function and stability of NIS seems to be
preserved in the partial or even total absence of N-linked glycosylation (Levy et al., 1998).

Electrophysiological studies revealed that NIS transports one $\Gamma$ ion with two $\text{Na}^+$ ions, and NIS was capable of transporting a wide variety of anions including $\text{ClO}_3^-$, $\text{SCN}^-$, $\text{NO}_3^-$ and $\text{IO}_4^-$. However, perchlorate ($\text{ClO}_4^-$), a known NIS inhibitor, was not transported (Eskandari et al., 1997; Yoshida et al., 1997).

NIS is expressed primarily in thyroid tissues, but variable degrees of NIS expression were observed in various tissues including stomach, salivary glands, and mammary glands, which is discussed in detail later.

Regulation of NIS

Comprehension of the regulation of hNIS gene expression is critical to understanding its effects on radioiodide uptake (RIU) activity of thyroid and thyroid cancers. As expected by previous RIU assays in which RIU activity was up-regulated by TSH, NIS mRNA and protein were elevated by TSH in cultured rat or human thyroid cells (Ajjan et al., 1998; Kogai et al., 1997; Saito et al., 1997). Being known as the primary downstream signaling molecule to TSH, cAMP (or its analogue) also induces the expression of NIS in thyroid cells. Forskolin, a protein kinase A (PKA) activator, which leads to increased cAMP, mimics the TSH or cAMP stimulatory effect on NIS expression (Kogai et al., 1997; Saito et al., 1997). As the function of adult thyroid gland is controlled by the opposite actions of TSH and iodide ($\Gamma$), NIS mRNA levels were reduced by low doses of $\Gamma$ for 48 hr in TSH-stimulated dog thyroid gland (Uyttersprot et al., 1997). As a normal, feedback, compensatory mechanism, thyroglobulin (Tg) counter-
regulates TSH-increased NIS transcription and other thyroid-specific gene transcriptions by suppressing the expression of the thyroid transcription factor (TTF)-1, TTF-2, and Pax-8 genes (Suzuki et al., 1998).

Transforming growth factor-β1 (TGF-β1) is known to affect thyroid function. TGF-β1 suppresses TSH-stimulated NIS mRNA and protein levels in FRTL-5 cells (Kawaguchi et al., 1997; Pekary and Hershman, 1998). Cytokines are known to have an important role in autoimmune hypothyroidism and Graves' disease, and cytokines also modulate RIU activity of thyroid follicular cells. Cytokines, such as interferon γ (IFNγ), interleukin-1α (IL-1α), tumor necrosis factor α (TNFα) and ceramide (a mediator of TNF actions) inhibit TSH-stimulated NIS mRNA levels in FRTL-5 cells (Ajjan et al., 1998; Ajjan et al., 1998; Pekary and Hershman, 1998). Interestingly, all-trans retinoic acid markedly increases NIS mRNA in the follicular thyroid carcinoma cell lines FTC-133 and FTC-238, but not in the anaplastic thyroid carcinoma cell lines HTh74 and C643. However, retinoic acid downregulates expression of NIS mRNA and suppresses the TSH-stimulated induction of NIS mRNA and RIU in non-transformed FRTL-5 cells (Schmutzler et al., 1997). Several reversible NIS inhibitors have also been found, which include econazole, 5-(N,N-hexamethylene) amiloride (HMA), and dysidenin (Vroye et al., 1998). As RIU activity can be reduced by aging, the NIS mRNA levels in aged FRTL-5 cells (>40 passages) were only 2% of those in young cells (<20 passages) (Pekary and Hershman, 1998).

NIS promoter analysis
Following NIS cloning, NIS promoter regions were analyzed by several researchers in order to elucidate the regulation of NIS gene expression. The transcription start sites for rNIS are clustered between −98 to −93 nucleotides (nt) relative to the ATG translation initiation site (Endo et al., 1997; Tong et al., 1997). Currently, tissue specificity of the NIS promoter is a controversial issue. Tong et al (1997) reported that the DNA regulatory elements within 8 kb of the 5' flanking region of rNIS are not sufficient to confer thyroid-selective transcription, whereas Endo et al (1997) reported that thyrocyte-selective nuclear factors bind to the -250 to -211 nt region of rNIS gene. In addition, TTF-1 seems to bind the region and activate the promoter activity of rNIS (Endo et al., 1997). It has also been reported that the paired-domain thyroid-specific transcription factor Pax8 binds to the upstream enhancer (-2264 to -2495 nt region) of the rNIS gene and participates in both thyroid-specific and cAMP-dependent transcription (Ohno et al., 1999). Ohmori et al (1998) also identified a TSH-responsive element (TRE) between −420 to −370 nt of the rNIS 5'-flanking region. They suggested that TSH/cAMP-induced up-regulation of NIS requires a novel thyroid transcription factor, which binds to this TRE and appears to be involved in TTF-1-mediated thyroid-specific NIS gene expression (Ohmori et al., 1998).

The transcription start site was mapped to −375 nt relative to the ATG site in the hNIS DNA regulatory element. The hNIS minimal promoter is localized to a region of 144 bp -519 to −376 nt, which contains a 90-bp stretch of DNA highly conserved between hNIS and rNIS. However, DNA regulatory elements in the 2 kb of the 5’-flanking region of hNIS were not sufficient to confer thyroid-selective transcription of hNIS gene, because the promoter was activated in non-thyroid cells as well as FRTL-5.
cells (Behr et al., 1998; Ryu et al., 1998). Another study demonstrated that the 1.2 kb portion of the 5'-flanking region of hNIS was active in the human thyroid cell line KAT-50, but not in non-thyroid cells, implying the presence of a tissue-selective promoter (Venkataraman et al., 1998). There might be some responsive elements to TSHR-cAMP-(TTF1 or Pax8) signaling in thyroid tissues, but the DNA regulatory elements of NIS may be responsible for signaling in extrathyroidal tissues. Thus, the spectrum of hNIS mRNA expression is found in a variety of tissues with different expression levels.

**NIS in Human Disease**

**NIS expression**

NIS is primarily expressed in thyroid tissue. In normal thyroid, hNIS expression is heterogeneous among different thyrocytes, and hNIS protein is localized in the basolateral membrane of thyroid follicular cells (Jhiang et al., 1998). As clinically proven by $^{99m}$TcO$_4^-$ or I$^-$ scanning, higher levels of NIS mRNA and protein are detected in Graves' thyroid tissue than in normal thyroid (Saito et al., 1997). Immunohistochemical (IHC) studies also revealed abundant, homogenous hNIS protein expression in Graves' thyroid tissue (Jhiang et al., 1998). The levels of hNIS mRNA and protein are greatly reduced, or even undetectable by IHC, in thyroid tumors including papillary thyroid carcinomas (PCs), compared to normal thyroid tissues (Caillou et al., 1998; Smanik et al., 1997). Thus, the reduced RIU activity of PCs is, at least in part, due to reduced hNIS mRNA or protein expression. One study, which contradicts reduced RIU activity in PC, reported that mRNA was 2.8-fold higher in PCs than in normal
thyroid tissues, and increased hNIS protein level was observed in ~40% of PCs (Saito et al., 1998). However, since NIS in the thyroid is regulated by TSH, which is elevated in patients undergoing radioiodide therapy, the sampling time and serum TSH levels in PC patients need to be considered.

**NIS mutations in thyroid disease**

Iodide transport defects (ITD) have been observed in some patients with congenital hypothyroidism. The cloning of hNIS gene has led several researchers to identify hNIS mutations in patients with ITD, whom are characterized by low or no RIU activity in both thyroid and salivary glands. Thus far, the most common hNIS mutation found in ITD patients in Japan is a homozygous T354P missense mutation (Fujiwara et al., 1998; Kosugi et al., 1998; Kosugi et al., 1998; Matsuda and Kosugi, 1997). This mutation occurs when adenine (A) at nucleotide 1060 is changed to cytosine (C) resulting in an amino acid replacement of Thr 354 to Pro in the middle of the ninth transmembrane domain. Although the phenotype of patients with this mutation is clinically heterogeneous, especially regarding goiter and hypothyroidism, all the patients with T354P homozygous mutations seem to have ITD. The loss of function in T354P NIS mutation is not due to a structural change of hNIS protein induced by Pro, but rather due to the absence of a hydroxyl group at the beta-carbon of the amino acid at position 354, suggesting the hydroxyl group in position 354 is important for NIS function (Levy et al., 1998). This T354P mutation appears to be a recurrent mutation and a major cause of ITD (Fujiwara et al., 1998). NIS mRNA and protein levels have been found to be significantly increased (>100-fold and ~10-fold, respectively) in the thyroid of these
patients, compared with the thyroid in normal individuals, possibly due to compensatory mechanisms.

Two other missense and loss-of-function mutations in NIS gene causing ITD were found in other Japanese patients. They are G93R and G543E located in the 3rd and 13th transmembrane domain of hNIS (Kosugi et al., 1998). It seems that none of these mutations act as a dominant-negative mutant, because the expression of one normal hNIS allele in patients with a heterozygous hNIS mutation is sufficient to maintain active RIU activity, resulting in euthyroidism. Two nonsense mutations in congenital hypothyroidism have also been identified. A homozygous change of the normal cytosine at nt 1163 to an adenine, resulting in a stop (TGA) codon at codon 272 in a Brazilian kindred, produces truncated NIS and undetectable RIU activity (Pohlenz et al., 1997). C to G transversion at nt 1146 in exon 6, resulting in Gln 267 to Glu substitution, produces an early stop codon and undetectable RIU activity (Pohlenz et al., 1998). For every mutation, the functional defects of the isolated mutant hNIS gene from patients have been confirmed by expressing the corresponding cDNA into non-thyroid cells such as COS-7 cells.

**NIS as an autoantigen**

Since other thyroid markers such as Tg, TPO and TSHR can present as autoantigens in patients with autoimmune thyroid disease, NIS has also been suspected as an autoantigen. Prior to the cloning of NIS, indirect evidence suggesting the presence of inhibitory anti-NIS autoantibody in patients with Hashimoto's thyroiditis was reported (Raspe et al., 1995). The detection of specific NIS autoantibodies was made possible by
the cloning of NIS. When the sera from patients with autoimmune thyroid disease were tested for immune reactivity against recombinant rNIS or rNIS peptide fragments, 44-84% of sera from patients with Graves' disease and 15-26% of sera from patients with Hashimoto's thyroiditis were positive for the presence of rNIS autoantibodies (Endo et al., 1996; Morris et al., 1997). The sera from patients with Hashimoto's thyroiditis that had positive NIS immune reactivity showed moderate inhibition in RIU activity (Endo et al., 1996). These studies suggest that patients with Graves' disease or Hashimoto's thyroiditis possess autoantibodies against NIS, and that anti-NIS autoantibodies might participate in the pathogenesis of Hashimoto's thyroiditis and modulate thyroid function in patients with the disease. However, the antigen used in these studies was rNIS peptides. Sequence differences exist between rNIS and hNIS. Therefore, these data need to be confirmed using hNIS antigen.

NIS in Extrathyroidal Tissues

Radioiodide accumulation (RIA) and hNIS expression are primarily detected in thyroid tissues. However, the thyroid is not the only organ that accumulates radioiodide. RIA has been demonstrated in various extrathyroidal tissues such as salivary gland, stomach, and lactating mammary gland. After hNIS cloning, hNIS mRNA or protein expression was studied in a variety of tissues. By a sensitive RT-PCR analysis, hNIS mRNA has been found in salivary gland, stomach, breast, extraocular muscle, pituitary gland, pancreas, testis, adrenal, heart, thymus, lung, ovary, and colon (Ajjan et al., 1998; Smanik et al., 1997; Spitzweg and Heufelder, 1997). The nucleotide sequences of hNIS
parotid salivary gland, mammary gland, and gastric mucosa was identical to that of hNIS cDNA isolated from thyroid (Spitzweg and Heufelder, 1997).

In salivary glands, hNIS protein is detected in the basolateral membranes of ductal cells, but not in acinar cells (Jhiang et al., 1998). As shown in chapter 3, rNIS protein was mainly detected in the basolateral membranes of alveolar cells as a less glycosylated glycoprotein than that of the thyroid, but not in ductal cells of lactating rat mammary gland (MG). This finding suggests that iodide is added to the milk in the alveolar epithelial cells of MG, yet the iodide is added to the saliva in the ductal cells of the salivary gland.

Exploring Radioiodide Therapy Using hNIS

Radioiodide (I-131) therapy is proven to be an effective treatment for patients with thyroid cancer to ablate post-surgical remnants and to treat residual, recurrent and metastatic disease after stimulation by elevated serum thyrotropin (TSH). Many studies have confirmed that radioiodide therapy significantly improves the prognosis for thyroid cancer patients (Mazzaferri, 1996).

It has been shown that human breast tumors have an increased RIA (Eskin, 1977), and thus breast cancers can be detected by radioiodide/\(^{99m}\)Tc scintigraphy (Cancroft and Goldsmith, 1973; Eskin et al., 1974). Furthermore, one study indicates that high iodide uptake may prove to be the most specific biochemical characteristic of hormone-dependent breast tumors when compared to hormone-independent tumors (Briand, 1983). The fact that RIA is significantly increased in lactating breast tissues (Bakheet and Hammami, 1994) indicates that RIA is subjected to hormonal control in breast tissues.
This leads to the hypothesis that breast cancer patients could also benefit from radioiodide therapy, provided that i) the hormonal factor(s) inducing RIA in lactating breast tissues can be identified, and ii) the identified hormonal factor(s) can be used to further increase RIA in breast cancers. Thus, chapter 3 investigates the hormonal regulation of NIS expression and RIA in the MG for the development of radioiodide therapy for breast cancer.

It is also speculated that radioiodide therapy can be applied to other tumors such as brain tumors, if hNIS can be efficiently and specifically delivered to tumor cells, resulting in radioiodide (I-131)-mediated killing. Viral vector-mediated gene delivery into mammalian cells is very efficient and thus is being used for gene therapy. Of the available vectors, recombinant adenovirus serotype 5 (Ad5) vectors are promising for gene therapy of gliomas due to their capacity to achieve high gene transfer efficiency in vivo upon intracranial administration (Davidson et al., 1993; Le Gal La Salle et al., 1993). Adenoviral particles are relatively stable and amenable to high purification and concentration (Huyghe et al., 1995). The expression level of the exogenous gene by recombinant adenoviral-mediated gene delivery can be easily controlled by changing multiplicity of infection (MOI). Thus, adenoviral-mediated hNIS gene delivery to glioma brain tumor cells was studied in chapter 4.
Fig 1.1. Schematic drawing of the structure of the ret proto-oncogene and the rearranged forms of ret activated during in vitro transfection experiments or in human papillary thyroid carcinomas.
Fig 1.2 Schematic drawing showing the various steps involved in the biosynthesis of thyroid hormones (Taurog, 1996).
Fig 1.3 Schematic drawing showing the structure of human Na\(^+\)/T symporter (hNIS).
CHAPTER 2

EARLY CELLULAR ABNORMALITIES INDUCED BY RET/PTC1 ONCOGENE IN THYROID-TARGETED TRANSGENIC MICE

INTRODUCTION

Differentiated thyroid tumors derived from follicular cells in humans are subdivided into papillary and follicular thyroid carcinomas. These two tumor types have differences in cytological nuclear features, etiology, clinical behavior, and oncogene involvement. In areas where dietary iodine is sufficient, papillary thyroid carcinoma (PC) accounts for about 80% of thyroid cancers. For patients who had previous radiation exposure, PC occurs frequently. A dramatic increase in PC was recently found in children who were exposed to radiation from the Chernobyl nuclear reactor accident. Among oncogenes studied, the ret/PTC oncogenes are uniquely restricted to PCs (Grieco et al., 1990; Jhiang and Mazzaferri, 1994; Santoro et al., 1992). The prevalence of ret/PTC in spontaneous PCs ranges from 3% to 43% depending on geographic areas. However, ret/PTC is detected in about 70% of Chernobyl-related thyroid tumors (Klugbauer et al., 1995).

The RET/PTC oncogenes are rearranged forms of the RET proto-oncogene (RET), which encodes a receptor tyrosine kinase. The ligands of RET have been recently
identified as glial cell line-derived neurotrophic factor GDNF (Lindsay and Yancopoulos, 1996; Vega et al., 1996) and neurturin (Buj-Bello et al., 1997; Klein et al., 1997), for which RET serves as a signaling component for the corresponding receptor complex. Three different activating genes, \( H4 \) (D10S170 locus), \( RIA \), and \( elel \) have been shown to rearrange with \( RET \) to form \( ret/PTC1 \), \( ret/PTC2 \), and \( ret/PTC3 \), respectively. In each case, the intracellular domain of \( RET \), which has tyrosine kinase activity, was fused to the N-terminus of the activating gene that is capable of dimerization. Consequently, the \( RET/PTC \) chimeric oncogenes are constitutively expressed and activated in thyroid follicular cells (Tong et al., 1997).

To investigate whether \( RET/PTC \) causes PC, we have generated a transgenic mouse model of PC with targeted expression of \( ret/PTC1 \) in the thyroid gland using the bovine thyroglobulin (Tg) promoter. Our studies focused on two transgenic lines, differing in the relative copy number of inserted transgene. All transgenic offspring from these two lines developed bilateral thyroid tumors with considerable similarities to human PCs, particularly in the nuclear cytological features and the presence of local invasion (Jhiang et al., 1996). Our results indicate that \( ret/PTC1 \) is a specific genetic lesion that leads to the development of PCs.

In this study, we focused our investigation on the high copy transgenic mice to determine the time of tumor onset, to identify the cellular abnormalities preceding tumor development, to analyze the expression of thyroid-specific markers, and to examine the role of thyroxine (T4) supplementation in tumor progression.
MATERIALS AND METHODS

Animals and tissue preparation

ret/PTC1 transgenic mice established in the FVB/N mouse strain were maintained on either a normal rodent chow (LM-485, Harlan Teklad, Madison, WI) or on a powdered diet containing 300 ppm L-thyroxine (T4 diet; TD-95053, Harlan Teklad, Madison, WI). Transgenic offspring were identified by polymerase chain reaction (PCR) using tail biopsies. For histopathological examination, tissues from various organs were prepared as described (Jhiang et al., 1996). For embryological studies, nontransgenic females were mated with transgenic males. The embryos were fixed for 3 h, and the Crown-Rump (CR) lengths as well as the limb structure was used to confirm the embryological ages of fetus (Kaufman, 1994).

Serum TSH and T4 levels

Serum was collected from the mice prior to euthanasia and frozen at -80°C until analysis. Serum TSH and T4 levels were determined by radioimmunoassay as previously described (Sagartz et al., 1997).

Thyroglobulin (Tg) and TSHR Immunochemistry

Tissue sections were deparaffinized and incubated with 3% H2O2 in methanol for 10 min to inactivate endogenous peroxidase. After blocking with 1.5% normal goat serum for 30 min, the tissues were incubated overnight with 1:500 dilution of anti-Tg polyclonal
antibody (Dako) or 1:1,000 dilution of anti-TSHR polyclonal antibody at 4°C. The slides were then incubated with 1:200 dilution of biotinylated goat anti-rabbit IgG for 1 hour, with peroxidase-conjugated ABC reagent for 30 min and then with diaminobenzidine DAB for 5 min. The sections were counterstained with hematoxylin. Negative control for the immunostaining was carried out by replacing the primary antibody with the same concentration of normal rabbit serum.

**Anti-BrdU staining**

Bromo-deoxyUridine (BrdU) (Sigma, St. Louis, MO) was injected intra-peritoneally (i.p.) into mice at 100 μg/g of body weight. One hour later, mice were sacrificed with CO₂. The thyroid glands and a section of intestine (as a positive control) were fixed in 10% formalin. Tissue slides were treated with 0.1% protease for 1 min and then 2N HCl for 1 h prior to inactivation of endogenous peroxidase. After blocking with 10% normal rabbit serum for 15 min, the tissues were incubated with 1:400 dilution of anti-BrdU monoclonal antibodies (Harlan Bioproducts for Science) for 1 h at RT and then 1:400 dilution of biotinylated rabbit anti-rat IgG (Dako, Carpinteria, CA) for 30 min to detect the nuclei of proliferating cells. Anti-BrdU staining was analyzed by a computer image analyzer (Roche Pathology Workstation, Elon College, NC) or by light microscope in several fields for quantification.

**¹²⁵Iodide autoradiography**

Na-¹²⁵I (Amersham, Arlington Heights, IL) was injected i.p. into mice at 0.3 μCi/g of body weight. Six hours later, mice were sacrificed with CO₂. Thyroid gland, liver, and
salivary gland were fixed overnight and 3 μm tissue sections were generated. Micro-
autoradiography emulsion was performed using LM1 emulsion solution (Amersham,
Arlington Heights, IL) in the darkroom. Slides were exposed at 4°C for 3 to 8 days.
Tissue sections were counterstained with hematoxylin for 1 min and then mounted with
Aqua-Poly/Mount (Polysciences Inc.).

Iodide uptake and organification

Mice were injected with Na-^{125}I and sacrificed as described above. Thyroid glands
were removed, weighed, and washed with ice-cold Hank’s balance salt solution. Thyroid
tissues were then minced and homogenized in 0.5 ml of ice-cold 0.1M NaOH. The
radioactivity of tissue homogenates was counted by a γ-counter, and this value was
referred as total iodide uptake of the thyroid. The thyroid homogenates were centrifuged
at 15,800 g, at 4°C, for 20 min to remove tissue debris, and the supernatants were
subjected to TCA (5% w/v) precipitation to measure the protein-bound ^{125}I to evaluate
iodide organification in vivo (Bachrach et al., 1985). Organified ^{125}I was expressed as
the percentage of total iodide uptake measured prior to TCA precipitation.

RNA preparation and RT-PCR

Total RNA, extracted from frozen thyroid tissues using TRIZOL reagent (Gibco-
BRL, Gaithersburg, MD), was reverse transcribed and used for PCR amplification using
the following primers: for ret/PTC1, TPC-4: GTC GGG GGG CAT TGT CAT CT and
KD-2: AGT TCT TCC GAG GGA ATT CC, which amplify a DNA fragment of 203 bp;
for mouse Na^{+}/I symporter (mNIS), mNIS-F1: GCT CAA TTC GCT GCT CAT CT and
mNIS-R1: AAG TCC ATC AGG TTG ATC CG, which amplify a DNA fragment of 420 bp; for mouse thyroperoxidase (mTPO), mTPO-F1: GAG CTC CGC CCT GCT TCC TGG CTG GA and mTPO-R2: GGT ACC ATG GTG ATG ATC TGG TGC, which amplify a DNA fragment of 202 bp; for mouse β2-microglobulin (mMG), mMG-F1: TGT CAG ATA TGT CCT TCA GC and mMG-R1: GAA GGT GAT GTG TAG ATT GC, which amplify a DNA fragment of 265 bp. The PCR cycling conditions were: 1 cycle at 94°C for 3 min, followed by 35 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 2 min, and followed by 1 cycle at 72°C for 8 min.

In situ hybridization

A 160 bp mNIS cDNA fragment was amplified by PCR using mouse thyroid cDNA as template with primers: mNIS-F2: GAG CTC GCT CAA TTC GCT GCT CAT AG and mNIS-R2: GGT ACC TCA CGA TGC CAG TAT ACA GC. This cDNA fragment was subcloned into pBluescript (Stratagene, La Jolla, CA). Sense or anti-sense riboprobes were synthesized by in vitro transcription using T7 or T3 RNA polymerase (Stratagene, La Jolla, CA) in the presence of ^35S-UTP. The thyroid glands of non-transgenic and transgenic littermates were fixed in 10% formalin overnight and 10μm tissue sections were generated. The tissue sections were post-fixed with 4% paraformaldehyde, treated with proteinase K and acetylated. Hybridization was carried out overnight at 55°C with 200 μl of hybridization mix containing the sense or anti-sense mNIS riboprobe (1 x 10^7 cpm/ml). The slides were then washed at 50°C in a solution containing Denhardt’s, 10 mM DTT and 50% formamide, treated with RNase to remove unhybridized and non-specifically bound probes, and washed again in the same washing solution at 50°C.
overnight. Autoradiography was performed as described above for $^{125}$Iodide autoradiography except a longer exposure time of 8 to 14 days.

**Statistical analysis**

Each value represents the mean value of at least three replicate measurements. Statistical evaluation of the results was performed with Student’s $t$ test and $P < 0.05$ was considered as significant.

**RESULTS**

*Early thyroid tumor onset in high copy transgenic mice*

The time of tumor onset was determined in both high copy and low copy transgenic mice. All high copy transgenic mice had bilateral thyroid tumors by 4 days of age, yet all low copy transgenic mice had bilateral thyroid tumors with similar phenotypes by 21 days of age. At 4 days of age, the thyroid glands in non-transgenic littermates contained round or ovoid thyroid follicles filled with colloid, and the nuclei of thyrocytes were round and regularly shaped, with a normal chromatin organization (Fig 2.1A & 2.1C). In contrast, high copy transgenic mice had bilateral thyroid carcinomas (Fig 2.1B) with regions containing papillary projections of neoplastic cells with high cellular density (Fig 2.1D). Nuclei were enlarged, highly vesicular, round to oval in shape, and contained occasional nuclear grooves and pseudoinclusions. Foci of spindle cells without formation of identifiable follicles were sometimes found within the thyroid tumors. Thyroid tumors
in transgenic mice tended to arise from the central portions of the thyroid lobes with peripheral areas frequently containing irregular, dilated follicles (Fig 2.1B). Interestingly, the thyrotropin receptor (TSHR) was expressed at a higher level in the central regions of the thyroid glands of both non-transgenic and transgenic mice as demonstrated by immunohistochemical staining (Fig 2.1E & 2.1F).

**Distorted thyroid follicles with increased proliferation rate at E16.5**

Cellular abnormalities preceding tumor development were investigated in high copy transgenic mice. In the mouse, the Tg promoter is known to be active from the embryological day 14.5 (E14.5) (Zannini et al., 1997), and the thyroid glands begin to form follicles at E14.5. At E16.5, while primitive and less defined follicles were found in the thyroid glands of nontransgenic littermates (Fig 2.2A), large and irregular thyroid follicles were identified in high copy transgenic embryos (Fig 2.2B). At E18, the thyroid glands of non-transgenic littermates had small, round, and definitive follicles filled with colloid (Fig 2.2C), while large distorted thyroid follicles with little colloid were found in transgenic embryos (Fig 2.2D). The proliferation rate of thyroid follicular cells at different stages was evaluated by measuring the percentage of cells labeled with BrdU. At E16.5, the BrdU index of transgenic thyroids was 15.74±0.67% (Fig 2.2B), as compared with non-transgenic thyroids which had a BrdU index of 9.09±0.70% (Fig 2.2A). In comparison with non-transgenic thyroids, which had very little cell turnover after 2-3 weeks of age, the BrdU index of transgenic thyroids was maintained at a higher level throughout life although it decreased progressively (Fig 2.3).
Reduced radioiodide concentrating activity as early as E18

In addition to developing early-onset bilateral thyroid carcinomas, all high copy transgenic mice had marked congenital hypothyroidism (Jhiang et al., 1996). Iodide, an essential component of thyroid hormones, is concentrated 20-40 fold with respect to the iodide concentration in plasma. To investigate the mechanisms underlying congenital hypothyroidism, radioiodide concentrating activity was studied in high copy transgenic embryos after intraperitoneal injection of Na$_{125}$I into pregnant dams. At E18, while radioiodine concentrating activity was evident in non-transgenic littersmates (Fig 2.2E), it was greatly reduced in high copy transgenic embryos, as only a few silver grains were observed to be scattered in small portions of thyroid follicles (Fig 2.2F). Radioiodine concentrating activity was reduced in the thyroid glands of high copy transgenic mice at all ages. A high copy transgenic mouse of 7 months of age, originally fed with a T4-supplemented diet and then switched to a normal diet 1 week before sacrifice, had only minimal radioiodine concentrating activity at the periphery of the thyroid gland (Fig 2.4B), whereas the thyroid glands of the age-matched non-transgenic mouse showed abundant radioiodide concentrating activity (Fig 2.4A).

The reduction of radioiodide concentrating activity in the thyroid glands of ret/PTC1 transgenic mice appeared to correlate with the expression levels of the ret/PTC1 transgene. The radioiodide concentrating activity in high copy transgenic mice was reduced to a greater extent than in the age-matched low copy transgenic mice (data not shown). Furthermore, the radioiodide concentrating activity was not detectable in the central portions of the thyroid lobes of transgenic mice, while it remained detectable at the edge of the thyroid glands. Since TSHR was expressed at a higher level in the central
portions of thyroids (see Fig 2.1F), ret/PTC1 would be expected to express at a higher level in the central portions of the thyroid lobes.

**Iodide uptake versus iodide organification**

The iodide concentrating activity in the thyroid glands is contributed by two distinct processes, active iodide uptake across the basolateral membrane of thyroid follicular cells and iodide organification to further sequester iodide in the form of colloid. In high copy transgenic mice, total iodide uptake activity in the thyroid glands was 30 fold lower than those of age-matched non-transgenic mice (Fig 2.5A), despite the fact that serum TSH level was greatly elevated in high copy transgenic mice due to congenital hypothyroidism (Fig 2.6B). Under T4-supplemented dietary conditions, when serum T4 and TSH levels in high copy transgenic mice were comparable to those of non-transgenic mice (Fig 2.6A & 2.6B), total iodide uptake activity in the thyroid glands of high copy transgenic mice was 7 fold lower than those of non-transgenic mice (Fig 2.5A). In non-transgenic mice, iodide uptake activity was decreased by T4 supplementation (Fig 2.5A), confirming that iodide uptake activity is TSH dependent. However, in high copy transgenic mice, there was no significant difference in iodide uptake activity between mice fed with normal diet and T4-supplemented diet (Fig 2.5A). This observation suggests that the stimulatory effect of TSH on iodide uptake appeared to be surpassed by the suppressive effect of increased expression of RET/PTC1 in high copy transgenic mice.

Once iodide is transported into thyroid follicular cells, it is incorporated into tyrosine residues in the Tg molecule by thyroperoxidase (TPO), a process referred as iodide organification. The percentage of protein-bound radioiodide in the thyroid glands was
measured to evaluate the efficiency of iodide organification. There was no significant difference in the efficiency of iodide organification between high copy transgenic mice and non-transgenic mice (Fig 2.5B, 89±3.0% versus 91±4.3%) nor between mice fed with normal diet and mice fed with thyroxine-supplemented diet (Fig 2.5B).

### Down-regulation of the Na\(^+\)/I symporter

Active iodide uptake activity in thyroid follicular cells is mainly mediated by the Na\(^+\)/I symporter (NIS) (Dai et al., 1996; Smanik et al., 1996). The NIS mRNA level was reduced in the thyroid glands of high copy transgenic mice, when compared with those of non-transgenic mice, as demonstrated by in situ hybridization (Fig 2.4C & 2.4D) and semi-quantitative RT-PCR analysis (Fig 2.5C, middle panel). In both transgenic and non-transgenic mice, the NIS mRNA level was lower in the thyroid glands of mice fed a T4-supplemented diet than mice fed a normal diet, indicating that NIS expression is up-regulated by elevated serum TSH level. For WT mice and PTC1+T4 mice, the total iodide uptake activity of thyroid glands (Fig 2.5A) is in agreement with the NIS expression level (Fig 2.5C). However, total iodide uptake activity of PTC1 mice appears to be lower than that of WT+T4 mice, despite the fact that the NIS mRNA expression level of PTC1 mice is higher than that of WT+T4 mice. This result suggests that, while NIS expression is stimulated by elevated TSH level in PTC1 mice, the increased expression of RET/PTC1 may inhibit NIS function at the post-transcriptional level.

As expected, ret/PTC1 was not expressed in the thyroid glands of non-transgenic mice, but it was expressed in the thyroid carcinomas of high copy transgenic mice (Fig 2.5C, top panel). ret/PTC1 expression was higher in the thyroid tumors of transgenic...
mice fed a normal diet than those of transgenic mice fed a T4-supplemented diet. Finally, the TPO mRNA level in the thyroid glands was comparable among all mice investigated (Fig 2.5C, bottom panel), which corroborates the observed equivalent efficiency of iodide organification. Therefore, ret/PTC1 appeared to have little effect, if any, on TPO expression and iodide organification.

Thyroglobulin expression and thyroxine suppression in tumor progression

In human thyroid tumors, continued expression of Tg enables physicians to measure patients’ serum Tg levels to monitor for the presence of residual and/or recurrent thyroid cancers in patients who have undergone total thyroidectomy. Similarly, in high copy transgenic mice, Tg expression was detected throughout tumor progression, from early stages (Fig 2.7A) to advanced tumors with foci containing a spindle cell population (Fig 2.7B). Thyroxine supplementation is often prescribed to patients who have unresectable or metastatic thyroid tumors, in order to suppress the growth of residual neoplastic tissues. Likewise, thyroid tumor development and progression was delayed in ret/PTC1 transgenic mice by administration of exogenous T4 (Fig 2.7C & 2.7D). Thyroid tumors from thyroxine-treated transgenic mice had less cellularity and were mostly composed of distended follicles filled with colloid (Fig 2.7D).
DISCUSSION

*ret/PTC1 and tumor onset*

The time of tumor onset in *ret/PTC1* transgenic mice appears to depend on the expression level of RET/PTC1. All high copy transgenic mice had earlier tumor onset than low copy transgenic mice. Furthermore, thyroid tumors in transgenic mice arose in the central portions of the thyroid lobes, where the TSHR is highly expressed. Since the bovine Tg promoter used to drive RET/PTC1 expression in transgenic mice is stimulated by TSH, RET/PTC1 expression is expected to be higher in cells with greater expression of TSHR. Taken together, the early tumor onset in high copy transgenic mice was contributed by high expression of RET/PTC1 in cells with high proliferation capacity and sustained elevation of serum TSH.

*ret/PTC1 and proliferation rate*

The proliferation rate of thyroid follicular cells in high copy transgenic mice appeared to be determined by the interplay among RET/PTC1 expression, serum TSH levels, and the intrinsic programming of declining proliferation capacity in mature thyroid cells. Thyroid follicular cells in high copy transgenic mice had a higher proliferation rate than cells in non-transgenic littermates at E16.5 (Fig 2.2A & 2.2B). Since the pituitary-thyroid axis in the fetus is not established until late gestation (Jokinen and Botts, 1994), the early onset of increased proliferation in the thyroids of high copy transgenic mice indicates that *ret/PTC1* stimulates proliferation in thyroid cells. Indeed, others have reported that RET/PTC expression stimulates proliferation in primary human thyrocytes.
(Bond et al., 1994). In high copy transgenic mice, serum TSH levels were greatly elevated due to congenital hypothyroidism. Therefore, the increased proliferation rate in the thyroid follicular cells of high copy transgenic mice may also result from TSH stimulation. However, despite the persistence of elevated TSH levels in high copy transgenic mice, the proliferation rate of thyroid follicular cells in transgenic mice declined gradually with age (Fig 2.3). This suggests that the intrinsic programming for declining proliferation capacity in mature thyroid cells antagonizes the stimulative effects of ret/PTC1 and TSH on proliferation rate.

**ret/PTC1 and thyroid follicle formation**

The signaling pathways leading to thyroid follicle formation during development appear to be disturbed by ret/PTC1-activated signaling pathways. Distorted follicle formation could be identified in the thyroid glands of high copy transgenic mice as early as E16.5. It has been reported that thyroid follicle formation in cultured primary porcine thyroid cells was promoted by TSH-cAMP mediated signaling pathways, but was blocked by tyrosine kinase mediated signaling pathways (Yap et al., 1997). Furthermore, aberrant tyrosine kinase activity, including chimeric oncoproteins of RET and TRK, as well as deregulation of MET, has been frequently found in human PCs (Bongarzone et al., 1996; Di Renzo et al., 1992; Santoro et al., 1995). In PCs, thyroid follicles are improperly or incompletely formed due to papillary infoldings. These findings suggest that tyrosine kinase-mediated signaling pathways have an adverse effect on the maintenance of thyroid follicular structure, and this may explain why aberrant tyrosine kinase activity is frequently associated with PCs.
**ret/PTC1 and iodide uptake activity**

The iodide uptake activity in thyroid follicular cells appears to be inhibited by RET/PTC1 expression. Our study suggests that the reduced iodide uptake in RET/PTC1 expressing cells is contributed at least in part by the decreased NIS expression, and/or by the inhibitory effect of ret/PTC1 on NIS at the post-transcriptional level. Indeed, our previous study showed that NIS expression was decreased in human PCs (Smanik et al., 1997). Since radioiodide has been used to detect and to treat recurrent and metastatic disease in patients with thyroid cancers, it will be of clinical significance to determine the molecular mechanisms underlying the loss of iodide uptake activity in thyroid cancers.

The reduced iodide uptake activity in thyroid follicular cells accounts, at least in part, for the phenotype of congenital hypothyroidism in high copy transgenic mice. Consequently, it also contributes to the early tumor onset in high copy transgenic mice by increasing serum TSH levels, which in turn leads to higher RET/PTC1 expression and higher proliferation rate in the thyroid follicular cells of high copy transgenic mice. Therefore, the initial expression level of RET/PTC1 in the thyroid glands of transgenic embryos plays an important role in determining tumor formation in ret/PTC1 transgenic mice.

**ret/PTC1 and papillary thyroid carcinoma**

It is noteworthy that ret/PTC1 is a somatic acquired event in human PCs whereas ret/PTC1 is predisposed as a germ-line genetic determinant in our transgenic mouse model. Furthermore, the expression level of RET/PTC1 in the thyroid tumors of high
copy ret/PTC1 transgenic mice may be higher than that in human PCs. Therefore, thyroid tumors developed in the ret/PTC1 transgenic mice are not completely representative of human PCs.

Nevertheless, we have shown that RET/PTC1 expression in the thyroid glands of transgenic mice leads to the development of thyroid tumors with many features similar to human PCs. Further characterization of the thyroid glands of ret/PTC1 transgenic mice demonstrated several distinctive cellular changes preceding tumor development, including an increased proliferation rate, abnormal thyroid follicle formation, and reduced iodide concentrating activity. These findings provide a model for the role of ret/PTC1 in the formation of abnormal follicles with reduced iodide uptake ability observed in human PCs. It will be of great interest to further investigate which signaling pathway(s) perturbed by RET/PTC1 expression are responsible for these distinctive cellular changes and which signaling pathway(s) are essential for RET/PTC1 to induce tumors with many characteristics of PCs.

In addition, our data indicates that thyroid tumor development and progression in ret/PTC1 transgenic mice is primarily determined by the expression levels of RET/PTC1 in thyrocytes. Signaling pathways perturbed by RET/PTC1 in thyrocytes could conceivably be overcome by other factors, if RET/PTC1 expression occurs at a low level. Therefore, both the intensity and duration of ret/PTC1 activation in proliferating thyroid follicular cells may be essential to confer thyroid tumor development in transgenic mice as well as in humans.
Fig 2.1. A - D: H & E staining of thyroid glands from non-transgenic (A & C) and transgenic mice (B & D) at 4 days of age. Non-transgenic mice showed round thyroid follicles with cells of dense nuclei. Transgenic mice had bilateral thyroid tumors with increased cellular density and irregularly shaped, hypochromatic nuclei. C & D (bars = 20 μm) are higher magnification of A & B (bars = 500 μm). E & F: Anti-TSHR immunostaining on the thyroid sections from non-transgenic (E) and transgenic (F) mice at 1 week of age. Note that TSHR expression is higher in the central portions of thyroids. (E & F: bars = 100 μm)
Fig 2.2.  **A & B:** Anti-BrdU immunostaining of thyroid glands from non-transgenic (A) and transgenic (B) embryos at E16.5 day. More cells were undergoing proliferation in the thyroid glands of the transgenic embryo compared to non-transgenic embryo. (A & B: bars = 100 μm)  **C & D:** Anti-thyroglobulin immunostaining of thyroid sections from non-transgenic (C) and transgenic (D) embryos at E18.0 day. In the transgenic embryo, there were large and distorted thyroid follicles with multiple layers of thyroid follicular cells and little colloid. (C & D: bars = 20 μm)  **E & F:** Radioiodide concentrating activity in the thyroid glands of non-transgenic (E) and transgenic (F) embryos at E18.0 day. The thyroid glands of high copy transgenic embryo had greatly reduced radioiodide concentrating activity with only occasional silver grains. (E & F: bars = 100 μm)
Fig 2.2.
Fig 2.3. BrdU labeling index of the thyroid glands of transgenic and non-transgenic mice at different ages. The ages of mice are indicated as (E) for embryological day and (wk) for week after birth.
Fig 2.4  A & B: Radioiodide concentrating activity in thyroid glands from non-transgenic (A) and transgenic (B) mice at 7 months of age. The radioiodide concentrating activity was reduced in the thyroid tumors of transgenic mice as compared with thyroid glands of non-transgenic mice. (A & B: bars = 1 mm)  C & D: In situ hybridization detecting mouse NIS mRNA with $^{35}$S-labeled riboprobe in thyroid glands from non-transgenic (C) and transgenic (D) mice at 1 month of age. The mRNA level of NIS was reduced in the thyroid glands of transgenic mice compared with those of non-transgenic mice. No background signal was detected when the sense riboprobe was used as control (data not shown). (C & D: bars = 50 μm)
Fig 2.5. **A & B:** Radioiodide uptake (A) and radioiodide organification (B) in the thyroid glands of transgenic and non-transgenic mice fed a normal diet or a T4-supplemented diet. WT: non-transgenic mice fed a normal diet (n=5). WT+T4: non-transgenic mice fed a T4-supplemented diet (n=4). PTC1: transgenic mice fed a normal diet (n=5). PTC1+ T4: transgenic mice fed a T4-supplemented diet (n=4). C: RT-PCR analysis for the expression of ret/PTC1, mouse sodium-iodide symporter (mNIS), and mouse thyroperoxidase (mTPO). A ubiquitously expressed gene, mouse β2-microglobulin (mβ2-MG), was included in each PCR reaction as an internal control. WT: pooled thyroid glands from four non-transgenic mice at 5 months of age fed a normal diet. WT+T4: pooled thyroid glands from four non-transgenic mice at 5 months of age fed a T4-supplemented diet. PTC1: thyroid gland from a transgenic mouse at 12 months of age fed a normal diet. PTC1+T4: thyroid gland from a transgenic mouse at 12 months of age fed a T4-supplemented diet.
Fig 2.6. A: Serum T4 levels of transgenic mice versus their age-matched non-transgenic littermates at different ages and fed different diets. Note that transgenic mice had lower serum T4 levels than non-transgenic mice. However, serum T4 levels in transgenic mice fed a T4-supplemented diet was comparable to those of non-transgenic mice. B: Serum TSH levels of transgenic mice versus their age-matched, non-transgenic littermates at different ages and fed different diets. Note that transgenic mice had elevated serum TSH levels, compared with non-transgenic mice. However, serum TSH levels in transgenic mice fed a T4-supplemented diet was comparable to those of non-transgenic mice.
Fig 2.7. A & B: Anti-thyroglobulin (Tg) immunostaining of the thyroid sections from transgenic mice of 1 day of age (A) and 7 months of age (B). Note that diffuse thyroglobulin expression is still noticeable in the thyroid carcinoma within foci of spindle shaped cells. (bars = 100 μm in A, 10 μm in B) C & D: H & E staining of thyroid glands from a transgenic mouse at 4 months of age fed a normal diet (E), and a transgenic mouse at 5 months of age fed a T4-supplemented diet (F). The transgenic mouse fed a T4-supplemented diet had cystic thyroid tumors with less cellularity, and the tissue was primarily composed of distended follicles filled with colloid. (E & F: bars = 1 mm)
CHAPTER 3

HORMONAL REGULATION OF RADIOIODIDE ACCUMULATION AND NA⁺/I⁻ SYMPORTER EXPRESSION IN MAMMARY GLANDS

INTRODUCTION

The iodide concentrating activity of thyroid tissues has allowed the use of radioiodide /⁹⁹mTc for scintigraphic imaging and as an adjunct to surgical therapy for ablation of postsurgical remnants and as a treatment of residual, recurrent, and metastatic disease. Many studies have demonstrated that radioiodide therapy significantly increases the disease-free interval and survival of patients with residual, recurrent, and metastatic thyroid tumors that retain iodide concentrating activity (Mazzaferri, 1996). For patients with thyroid cancers, iodide uptake activity in thyroid cancers is minimal unless it is intensively stimulated by thyrotropin (TSH). Therefore, prior to radioiodide therapy, patients' serum TSH level is elevated by thyroxine withdrawal or by exogenous TSH administration.

Thyroid is not the only tissue that shows radioiodide accumulation (RIA). RIA has been demonstrated in various extrathyroidal tissues such as lactating mammary gland,
salivary gland and gastric mucosa. It has been shown that breast atypia and malignancy have an increased RIA in human (Eskin, 1977), and that breast cancers can be detected by radioiodide/99mTc scintigraphy (Cancroft and Goldsmith, 1973; Eskin et al., 1974). Furthermore, one study indicates that high iodide uptake may prove to be the most specific biochemical characteristic of hormone-dependent breast tumors when compared to the hormone-independent tumors (Briand, 1983). The fact that RIA is significantly increased in lactating breast tissues (Bakheet and Hammami, 1994) also indicates that RIA is subjected to hormonal control in breast tissues. Taken together, we hypothesize that breast cancer patients could also benefit from radioiodide therapy, provided that (a) the hormonal factor(s) inducing RIA in lactating breast tissues can be identified, and (b) the identified hormonal factor(s) can be used to further increase RIA in breast cancers.

Na+/T symporter (NIS) is the molecule that mediates iodide uptake activity into target cells. Molecular cloning of NIS (Dai et al., 1996; Smanik et al., 1996) has made it possible to investigate hormonal control of NIS expression and thus RIA in breast tissues. Since RIA in breasts is at its maximum when women are undergoing active lactation, hormones involved in active lactation are the most likely candidates to induce NIS expression in breasts. Prolactin (PRL) is the most important hormone for lactogenesis, and oxytocin (OT) is the essential hormone to maintain active lactation. An increased expression of prolactin receptor (PRLR) has been documented in some human breast tumors and human breast cancer cell-lines (Reynolds et al., 1997; Shiu, 1979; Touraine et al., 1998). Although oxytocin receptor (OTR) is expressed in myoepithelial cells in normal breast tissues, it has been reported to be expressed in human breast tumors (Bussolati et al., 1996; Ito et al., 1996; Sapino et al., 1998). Therefore, in this study, we
will investigate the hormonal effects of PRL and OT on RIA and NIS expression in breast tissues.

MATERIALS AND METHODS

Animal model

Sprague Dawley rats and Balb/c mice were purchased from Harlan Sprague Dawley and housed in the animal vivarium facility at the Ohio State University. For oxytocin antagonist treatment, lactating rats were intraperitoneally (i. p.) injected with a selective oxytocin antagonist, des Gly-NH$_2$,d(CH$_2$)$_5$[D-Tyr$^2$,Thr$^4$]OVT (OTA) (Manning et al., 1995) at a dosage of 3μg in 300 μl per injection, twice a day, for 6.5 days. Lactating mice were i. p. injected with OTA at a dosage of 0.6μg in 60 μl per injection, twice a day for 5.5 days. For Bromocriptin (Br-CT) (Sigma, St. Louis, MO) treatment, lactating rats were i. p. injected with Br-CT at a dosage of 0.6 mg in 500 μl per injection, twice a day for 8.5 days. Lactating mice i. p. injected with Br-CT at a dosage of 0.12 mg in100 μl per injection, twice a day for 7.5 days. Control animals were injected with the same volume of saline.

In vivo Tc-99m scintigraphy

Rats were anesthetized with inhalation of isoflurane, and then 1.5 mCi of $^{99m}$Tc-pertechnetate ($^{99m}$TcO$_4$) in 0.2 ml volume was administered via the tail vein injection. Rats were placed in a prone position on a gamma camera (QRS Systems, Nuclear
Rats were placed in a prone position on a gamma camera (QRS Systems, Nuclear Medicine Diagnostic Systems, San Antonio, Texas) with 140 keV-high resolution collimator interfaced to a Macintosh computer-based nuclear medicine imaging system (Nuclear MAC 2.9, Scientific Imaging, Littleton, Co). The images were obtained 20 min post-injection of $^{99m}$TcO$_4^-$ with 2 min acquisition time.

**Radioiodide accumulation (RIA) assay for in vivo animal**

NIS functional activity in MG was assessed by measuring radioiodide accumulation (RIA), the sum total of iodide influx and efflux. $^{125}$I was i.p. administrated to rats/mice at 0.3 μCi (in 0.2 ml of PBS) per gram body weight. Rats/mice were sacrificed one our later, and tissues including axillary and inguinal mammary glands were collected to measure radioactivity using a γ-counter (Packard Instruments, Downers Grove, IL). The data were presented as a fold increase of radioactivity, compared to spleen by the following formula: (counts of MG/mg)/(counts of spleen/mg).

**Western blot analysis**

Western blot analysis was performed as previously described (Jhiang et al., 1998) with some modifications. Frozen tissues were pounded in a vinyl bag, homogenized, and briefly sonicated (20 sec) in a lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin). The lysates were centrifuged at 12,000 xg for 20 min at 4 °C. The supernatants (100 or 200 μg/lane) were solubilized for 30 min at 37 °C in the same volume of reducing sample buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 20% glycerol) and
subjected to 7.5% SDS-PAGE. The proteins were transferred onto nitrocellulose filter, and the filter was blocked with 5% nonfat dry milk in TBST buffer (10mM Tris-HCl (pH8.0), 150 mM NaCl, 0.1% Tween 20) at 4°C overnight. Western blot analysis was then performed by incubating the filter with an anti-rNIS peptide polyclonal antibody pAb 716 (1:5,000) (Paire et al., 1997) in TBST containing 5% nonfat dry milk for 1.5 hr at room temperature, followed by incubation with peroxidase (HRP)-conjugated donkey anti-rabbit IgG (1:4,000) for 1 hr at room temperature. The filter was then incubated with the ECL detection reagent (Amersham, Arlington Heights, IL) for 1 min and exposed to an X-ray film.

For deglycosylation of the proteins, 100 or 200 µg of proteins were denatured in the denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) for 30 min at 37°C. Denatured proteins were treated with 1 µl (500 U) of Peptide: N-glycosidase F (PNGase F) (New England Biolabs) in 50 mM sodium phosphate buffer (pH7.5) containing 1% NP-40 at 37°C for 1 hr. The deglycosylation reaction was quenched by adding the same volume of reducing sample buffer.

**Immunohistochemical (IHC) staining of mammary glands (MGs)**

Mammary glands were isolated and fixed in 10% formalin overnight. Immunohistochemical staining was performed to detect rNIS proteins on the paraffin-embedded MG sections (5 µm) as reported previously (Jhiang et al., 1998) with some modifications. The tissue sections were incubated with 3% H₂O₂ in methanol for 5 min to inactivate endogenous peroxidase, and then subjected to antigen retrieval in 10 mM citric acid buffer (pH 6.0) at 94°C for 30 min. The tissue sections were incubated with a
rat NIS polyclonal antibody pAb 716 at a 1:3,000 dilution at room temperature for 1 hr, followed by Avidin and Biotin (Dako) blocking for 10 min each. The sections were then incubated with 1:200 dilution of biotinylated goat anti-rabbit IgG (Vector Lab. Inc., Burlingame, CA) for 20 min, streptavidin peroxidase for 10 min, and DAB substrate chromogen for 5 min. Counterstaining was performed using Hematoxylin.

Primary human breast cell culture

Human breast tissue specimen was procured from the OSU Cancer Hospital through the Tissue Procurement Program at the OSU Comprehensive Cancer Center and Cooperative Human Tissue Network. Normal breast tissues were obtained from patients undergoing reductive mammoplasty. Tissue specimen was minced into 3-5 mm³ pieces, digested for 18 hr at 37°C in 5% CO₂ with 0.01% collagenase (Gibco BRL, Gaithersburg, MD) in DMEM/F12 (Sigma, St. Louis, MO) medium supplemented with 5% fetal calf serum (FCS) (Atlanta Biologicals, GA) and antibiotic-antimycotic (100 unit/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B) (Gibco BRL, Gaithersburg, MD). The cell suspension generated was centrifuged at 800 x g for 5 min to separate enriched populations of epithelial cells (pellet), adipose stromal cells (immediately below the floating layer of fat), and fibroblasts (supernatant). After careful collection of the adipose stromal cells, fibroblasts were collected by re-centrifugation (1,000 x g for 10 min) of the supernatant. The original pellet containing epithelial cells were resuspended and allowed to settle by gravity, and then the supernatant and sediment were separated into different tubes. This process was repeated 5 times with epithelial cells and fibroblasts being pooled into respective fractions.
Cells were cultured as previously described (Soule and McGrath, 1986; Zhang et al., 1998). Briefly, epithelial cells were maintained in low-calcium DMEM/F12 (0.04mM CaCl₂) supplemented with Chelex-100 (Bio-Rad Laboratories, Richmond, CA)-treated horse serum (at a final concentration of 5%), 10μg/ml insulin (Sigma, St. Louis, MO), 0.02μg/ml epidermal growth factor (Gibco BRL, Gaithersburg, MD), 0.5μg/ml hydrocortisone (Sigma, St. Louis, MO), 0.1μg/ml cholera enterotoxin (Gibco BRL, Gaithersburg, MD) and antibiotic-antimycotic. The epithelial cells were allowed to grow as monolayer in 10ml medium in 75cm² culture flask at 37°C in a humidified atmosphere of 95% air-5%CO₂. Fibroblasts were cultured in phenol red-free DMEM/F12 (1.05mM CaCl₂) with 5%FCS (final concentration) and antibiotic-antimycotic. Adipose stromal cells were cultured at the same condition as fibroblasts. The medium was changed every 48 hr and when cells reached 80% confluency, they were trypsinized for further propagation.

Three-dimensional (3-D) histocultures

The specialized collagen gel was purchased from Health Design Industries (Rochester, NY) and was manufactured from pigskin. The dehydrated collagen gel was pre-soaked in DMEM (Gibco BRL, Gaithersburg, MD) with penicillin (10 units/ml), and streptomycin (10 μg/ml) at 37 °C for 2 hrs. Then, after cutting the collagen gel into 12 pieces, each piece was placed in 6 well tissue-culture plates containing DMEM with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD), 0.1 mM nonessential amino acids, and the antibiotics penicillin (10 units/ml) and streptomycin (10 μg/ml). The gels were soaked in this medium with at least one day before use.
Immediately after surgery or biopsy, tumors in RPMI 1640 medium with penicillin/streptomycin were brought to the laboratory. Fat and necrotic tissues were cut away, and the remaining tumor tissue was minced with double blades into ~1 mm³ pieces. About ten of these tumor pieces were placed on each collagen surface. Medium was added until the upper part of the gel was reached but not covered (4 ml) and was changed every day when hormones (OT or hPRL) were added in the media.

**RNA preparation and RT-PCR**

RNA preparation and RT-PCR were performed as previously described (Cho et al., 1999). Briefly, total RNA, extracted from primary breast cells or frozen 3-D histocultured breast tumors using TRIZOL reagent (Gibco BRL, Gaithersburg, MD), was reverse transcribed and the cDNA was used for PCR amplification using the following primers: for human Na⁺/T symporter (hNIS), hNIS-F₂: CCG GAT CAA CCT CAT GGA CT, and hNIS-R₂: CCT GAG GGT GCC ACT GTA AG, which amplify a DNA fragment of 396 bp; for human prolactin receptor (hPRLR), hPRLR-F₁: CTG TGG ATT AAA TGG TCT CC, and hPRLR-R₁: TGC AGG TCA CCA TGC TAT AG, which amplify a DNA fragment of 361 bp; for human β₂-microglobulin (hβ₂-MG), hβ₂-MG₅: GTG GAG CAT TCA GAC TTG TCT TTC AGC A, and hβ₂-MG₃: TTC ACT CAA TCC AAA TGC GGC ATC TTC, which amplify a DNA fragment of 201 bp. The PCR cycling conditions were: 1 cycle at 94°C for 3 min, followed by 35 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 2 min, and followed by 1 cycle at 72°C for 8 min.
RESULTS

Radioiodide accumulation (RIA) activity is maximal in lactating mammary glands (MGs) 

$^{99m}$TcO$_4^-$ whole-body scintigraphy was performed in live female rats to examine the difference of RIA in MG of virgin, pregnant, and lactating rats, as well as in the involuting MG of rats which had their pups removed at 4th day post-delivery for 42 hrs (Fig 3.1A). In virgin rats, RIA was not detectable in the MG, while it was observed in the stomach, thyroid, and bladder (data not shown). In late pregnancy (P-18d), RIA in the MG becomes visible by $^{99m}$TcO$_4^-$ whole-body scintigraphy (Fig 3.1A). In the lactating MG at 1 day post-delivery (L-1d), RIA was evident and was further increased in the lactating MG at 4 days post-delivery (L-4d). However, RIA in involuting MG was decreased substantially when the pups were removed from dams for 42 hrs (I-42h).

A more sensitive and quantitative RIA assay, $^{125}$I $\gamma$-counts in isolated tissues, revealed that RIA in the MG of virgin rat has $2.3\pm0.9$ fold increase over that of spleen (as a control). In the lactating MG at 16 days post-delivery, RIA was $35\pm0.4$ fold increase over that of spleen (Fig 3.1B). In the involuting MG, RIA was only $5.47\pm0.53$ fold increase over that of spleen (Fig 3.1B).

NIS expression is increased in lactating MG

Western blot analysis indicates that two bands of molecular weight of $\sim 65$ kDa and $\sim 75$ kDa were detected by an anti-rNIS antibody in lactating rat MG (Fig 3.2A). The sizes of rNIS protein in MG were distinct from that of the rNIS protein in thyroid ($\sim 90$ kDa).
kDa). However, when post-nuclear lysates of MG and thyroid were treated with PNGase F prior to Western blot analysis, the rNIS proteins detected in both MG and thyroid had a molecular weight of ~60 kDa. This result suggests that rNIS glycoproteins in MG have a less degree of glycosylation, compared to the rNIS glycoproteins in thyroid. Furthermore, it appears that the expression level of rNIS in lactating MG was lower than that of thyroid glands, when equal amount of total protein (100 μg/lane) was loaded on SDS-PAGE (Fig 3.2A). In contrast, rNIS protein was not detectable in MGs of virgin, late pregnancy, and the involuting MG (Fig 3.2B) when 200 μg of total protein was loaded in each lane.

Cellular localization and tissue distribution of rNIS protein was examined in MG by immunohistochemical staining. In the MG of virgin rats, immunostaining was detected in the apical membrane of the alveolar cells (Fig 3.3A). In the MG of late pregnant (P19) rat, immunostaining was faintly detected in the apical membrane of the alveolar cells (Fig 3.3B). In the lactating MG, immunostaining was strongly detected in the basolateral membrane of the alveolar cells (Fig 3.3C) but not in the ductal cells. However, immunostaining was not detected in the involuting MG (Fig 3.3D). When the primary anti-rNIS antibody was substituted with a rabbit IgG, no immunostaining was observed in the MG of virgin, pregnant, and lactating rats as well as in the involuting MG of rats (data not shown).

*RIA in lactating MG is decreased by treatment of an OTA or Br-CT.*

To investigate whether OT or PRL hormone has any regulatory effect on RIA in lactating MG, lactating rats and mice were treated with either a selective OTA, des Gly-
NH₂d(CH₂)₅[D-Tyr², Thr⁴]OVT (Manning et al., 1995), or bromocriptin (Br-CT), an inhibitor of PRL release. ¹²⁵I γ-counts on the lactating rat MG showed that RIA was decreased 38% in the OTA-treated rats and was decreased 29% in Br-CT-treated rats, when compared to saline-injected lactating rats (Fig 3.4). Similarly, RIA was decreased ~31% in the MGs of both OTA-treated mice and Br-CT-treated mice (data not shown). These data suggest that OT and PRL may exert some positive regulation on the RIA in lactating MG. In both rats and mice studied, it is worthwhile to note that the pups were still suckling in both OTA-treated group and Br-CT-treated group.

The histology of MG in OTA-treated rats (Fig 3.5B) or Br-CT-treated rats (Fig 3.5C) does not appear to be different from that of saline-injected lactating rats (Fig 3.5A). Furthermore, immunostaining of rNIS protein in the MGs of OTA-treated rats or Br-CT-treated rats does not appear to be different from that of saline-injected lactating rats (Fig 3.5D). However, occasionally, we found areas with minimal or no immunostaining in the alveolar cells of the MG in OTA-treated rats (Fig 3.5E) and of the MG in Br-CT-treated rats (Fig 3.5F). Indeed, Western blot analysis of these MGs showed that the expression levels of rNIS protein in the MGs of OTA or Br-CT treated rat were not obviously different from that of saline-injected lactating rats (Fig 3.6).

*The expression of hNIS is detected in epithelial-enriched primarily cultured normal breast cells and in human breast cancer cell-lines*

The mRNA level of hNIS was examined in the primarily cultured human breast cells enriched in epithelium, fibroblast or adipocyte. RT-PCR analysis showed that hNIS
expression was only detected in the epithelial-enriched human breast cells (Fig 3.7). The presence of hNIS expression in epithelial cells is further supported by the findings that hNIS expression was detected in established human breast cancer cell-lines, MCF-7 cells (Fig 3.7) and T47D cells (data not shown). Interestingly, a higher expression level of hPRLR was observed in MCF-7 cells which also showed a higher expression level of hNIS when compared to epithelial-enriched human breast cells (Fig 3.7).

OT and hPRL increase hNIS mRNA level in 3-D histocultures of human breast cancer

To investigate whether OT or hPRL has any direct effect on hNIS expression in human breast tumors, we examined the mRNA level of hNIS in 3-D histocultures of tumor tissues treated with OT or hPRL. The hNIS mRNA was barely detected by RT-PCR analysis in a breast carcinoma from a 45 years-old woman (Fig 3.8). However, the mRNA levels of hNIS were increased in a dose-dependent manner in the histocultures of this breast carcinoma treated with OT at concentrations of 5 and 50 μU/ml or with hPRL at concentrations of 1 and 10 ng/ml (Fig 3.8). Without any hormonal treatment, the hNIS mRNA level in the histocultures of this breast carcinoma appears to be similar to that of the original tumor tissues. Similar results showing an increase of hNIS mRNA upon the treatments with OT or hPRL were also seen in several breast tumor tissues from other patients (data not shown). These data indicate that hNIS expression in human breast tumor can be stimulated directly by either OT or hPRL.
DISCUSSION

Functional development of mammary gland proceeds in distinct stages, which are defined basically by the hormonal status of the animal (Hennighausen and Robinson, 1998). The mammary anlage is established during fetal development, ductal elongation and branching is obtained primarily after the onset of puberty, alveolar proliferation occurs during pregnancy, and functional differentiation is accomplished with parturition and lactation. Continuous suckling is required to maintain lactation for extended alveolar cell survival. When pups are removed from dams, mammary gland undergoes involution through two distinct stages: (a) induction of program cell death in alveolar cells, and (b) irreversible remodeling of the lobular-alveolar structure (Li et al., 1997). In mice, lactation can be restored to dams whose pups are removed for 48 hr, but can not be resumed in dams whose pups are removed for 72 hr (Li et al., 1997).

In this study, we investigated RIA and NIS expression in the MGs of virgin, pregnant, and lactating rats, as well as the MG undergoing involution. The expression level of NIS is generally correlated with RIA in rat MG, which is maximal during lactation. This finding suggests that NIS expression in MG is induced by hormones that are essential to maintain lactation. It is interesting to note that rNIS expression drops significantly in the MG undergoing involution when pups were only removed from dams for 42-44 hrs. This finding indicates a quick turn-over of rNIS in the MG undergoing involution. We showed that the rNIS protein in lactating MG is less glycosylated than
the rNIS protein in thyroid. It has been reported that the degree of glycosylation of a same molecule can be different among different cell types (Dorner and Kaufman, 1990), and that the less glycosylated rNIS may have a short half-life. It would be interesting to investigate whether the half-life of NIS protein in MG is different from that of NIS protein in thyroid.

As expected, immunostaining of rNIS in the lactating rat MG was detected in the basolateral membrane of the alveolar cells. Therefore, iodide is transported from blood to alveolar cells in the MG, and then moves to the lumen of the alveolar where the milk is accumulated to be secreted. In contrast to our previous report that hNIS was detected in ductal cells but not in acinar cells of the human salivary gland (Jhiang et al., 1998), rNIS was mainly found in alveolar cells but not in ductal cells of rat MG (data not shown). This finding suggests that iodide is added to the milk in the alveolar epithelial cells of MG, yet the iodide is added to the saliva in the ductal cells of salivary gland.

Surprisingly, immunostaining of rNIS in virgin MG was detected in the apical membrane, instead of basolateral membrane, in the alveolar cells. Since rNIS was not detected in virgin MG by Western blot analysis, it is difficult to conclude that the immunostaining of the apical membrane in virgin MG is truly the molecule of rNIS. It will be worthwhile to examine NIS expression by Western blot analysis using apical membrane fractions of isolated epithelial cell of virgin MG.

The findings that RIA in the lactating MG was decreased ~30% by the treatment of a selective OTA (Manning et al., 1995) or Br-CT are significant, since the administration of both drugs was not optimized. Our data showed that the expression level of rNIS in lactating MG was not significantly decreased by the treatment with an
OTA or Br-CT. This discrepancy may be explained partially by the presence of post-translational effects of the OTA and Br-CT on rNIS function. Alternatively, a modest decrease of rNIS may not be detected by our Western blot analysis and immunohistochemical staining.

It has been previously demonstrated by RT-PCR analysis that hNIS mRNA is expressed in the human breast tissues (Ajjan et al., 1998; Smanik et al., 1997; Spitzweg and Heufelder, 1997). In this study, we further showed that hNIS is expressed in the epithelium-enriched primary human breast cell cultures and in human breast cancer cells. It has been reported that different breast tumor cell-lines, including MCF-7 and T47D cells, had more PRLR molecules when compared to one normal breast cell-line (Shiu, 1979). Interestingly, a higher expression of hNIS was also seen in MCF-7 and T47D cells. Since PRL-PRLR pathway is necessary for breast cells to proliferate and differentiate, it is likely that higher expression of hNIS in MCF-7 cells is partially contributed by the increased expression of PRLR and the stimulation of its downstream signal transduction pathways. Clinically, it has been reported that some breast tumors have an increased RIA (Eskin, 1977) and can be detected by Tc-99m-pertechnetate scintigraphy (Cancroft and Goldsmith, 1973), suggesting that some breast tumors do have increased expression levels of hNIS.

We showed that PRL and OT increase hNIS mRNA level in 3-D histocultures of human breast cancer in a dose-dependent manner. It has been reported that PRL stimulates iodide accumulation in cultured MG from mid-pregnant mouse (Rillema and Yu, 1996). Furthermore the expressions of PRL and PRLR has been reported in human breast carcinomas (Reynolds et al., 1997), suggesting that an autocrine/paracrine loop for
the PRL-PRLR complexes is present within human breast tissues. Since the expression level of PRLR is increased in the human breast tumors compared to normal breast tissues (Shiu, 1979; Touraine et al., 1998), the mechanism(s) underlying the increase of RIA in breast tumors (Briand, 1983; Eskin et al., 1974) may be partially contributed by the stimulation of NIS expression by PRL-PRLR mediated signal transduction pathways. The facts that OT increases hNIS mRNA levels in human breast tumors and that OTR was found to be expressed in 50-90% of the breast cancers derived from glandular or ductal epithelium (Bussolati et al., 1996; Ito et al., 1996; Sapino et al., 1998) suggests that OT is a good candidate for patients with breast cancer to induce NIS expression to facilitate radioiodide therapy.

Before an optimal regimen of hormonal stimulation of hNIS expression can be developed to facilitate radioiodide therapy in patients with breast cancer, a thorough understanding of the hormonal regulation of NIS expression in breast tissue is desired. Furthermore, a potential limitation for the use of radioiodide therapy in breast cancer is the less efficiency of iodide organification in breast tissues, compared to thyroid tissues. Although breast tissue is known to exert iodide organification activity, the regulation of iodide organification in breast tissues is poorly understood. It is possible to increase the efficiency of iodide organification in breast tissues, provided that the regulatory mechanism(s) underlying iodide organification in breast tissues is further investigated.

Similar to the patients with thyroid cancers, we do not expect every breast cancer patient will benefit from radioiodide therapy following hormonal stimulation of hNIS expression and thus RIA. However, based on the success of radioiodide therapy in many patients with thyroid cancers, we believe that a substantial number of patients with breast
cancer will benefit from radioiodide therapy upon stimulation of hNIS expression. Finally, it is possible to protect thyroid glands from radioiodide ablation during radioiodide therapy of breast cancers. Since hNIS expression and RIU activity in thyroid tissues is TSH-dependent, thyroxin supplementation can be used to suppress serum TSH level to minimize RIU activity in thyroid.
Fig 3.1. (A) $^{99m}$TcO$_4^-$ whole body scintigraphies in rats. Note that the $^{99m}$TcO$_4^-$ concentrating activity was barely detected in the mammary glands (MGs) of late pregnancy (P-18d), was evident in lactating MG (L-1d, L-4d), and was decreased in the involuting MG of rat which had their pups removed for 42 hrs (I-42h). (B) $^{125}$I $\gamma$-counts in the rat MGs. Rats were i.p. injected with $^{125}$I (0.3 $\mu$Ci/g body weight) prior to sacrifice. MG of lactating rats (16 day post-delivery) had a 35±0.4 fold increase over the spleen, whereas MG of virgin rats (11 week-old) and MG of involution had a 2.3±0.9 and a 5.47±0.5 fold increase over the spleen, respectively. Arrowheads indicate mammary glands; S, stomach; B, Bladder; T, thyroid.
Fig 3.2. (A) Western blot analysis for rNIS expression in the MGs of lactating rat and rat thyroid using cell lysates. Note the two smaller weaker bands (**) of ~65 and ~70 kDa in the lactating MG compared to the ~90 kDa band (**) in thyroid. After PNGase F treatment, bands of same ~ 60 kDa molecular weight (*) were detected in both MG and thyroid. (B) Western blot analysis for rNIS expression in the MG of 11 week-old virgin (V-11w), late pregnant (P-19d), and lactating rats at 10 days post-delivery (L-10d), as well as in the involuting MG of rats which had their pups removed for 44 hrs (I-44h). Same amount of cell lysates (200 µg/lane) was loaded on each lane. The rNIS protein was detectable only in the lactating MG (L-10d).
Fig 3.3. Immunohistochemical staining for rNIS expression on rat MG sections. (A) MG of 11 week-old virgin rat, (B) MG of late pregnant rat (P-19 days), (C) MG of lactating rat (L-16 days), (D) involuting MG (I-44 hr). Note the intense immunostaining of rNIS on the basolateral membrane of alveolar epithelial cells of lactating MG (C). The apical membrane staining of rNIS in the virgin rat (A) is under investigation. X 140.
Fig 3.4. \(^{125}\text{I}\) \(\gamma\)-count of the MGs in lactating rats treated with saline, OTA, a selective oxytocin antagonist, or Br-CT, an inhibitor of PRL release. Note that MG of lactating rats under OTA treatment for 6.5 days had a 38% drop of RIA, and that under Br-CT treatment for 8.5 days had 29% drop of RIA, compared to saline-treated control.
Fig 3.5. H & E staining (A–C) for morphology and IHC staining (D–F) for rNIS expression in MG of lactating rats injected with saline (A & D), OTA (B & E), or Br-CT (C & F). The majority regions of each group showed similar cellular morphology as shown in (A–C). IHC staining showed majority regions of each group had evident basolateral membrane staining on the alveolar epithelial cells of MG (D). However, occasionally, some areas of MG under OTA or Br-CT treatment showed the absence or minimal staining of rNIS (E & F). X 140.
Fig 3.6. Western blot analysis for rNIS expression in the MGs of rats treated with saline, OTA a selective oxytocin antagonist, or Br-CT an inhibitor for PRL release. Same amount of cell lysates (200 μg/lane) was loaded in each lane. Note the similar amount of rNIS protein was detected in the lactating MGs among different experimental groups (the lower ~65 kDa band was not visible in this experiment).
Fig 3.7. RT-PCR analysis of hNIS in the differentially cultured primary human breast cells and MCF-7 breast cancer cell line. Breast tissues were obtained from a normal woman who had breast reduction surgery. Cells were separated by centrifuge and were grown in the media containing different concentrations of Ca\(^{2+}\) to acquire either epithelial (E)-enriched or fibroblast (F)-enriched or adipocyte (A)-enriched breast cells. RNAs were prepared from each of the cell populations and RT-PCR was performed as described in the Methods section. The hNIS mRNA was detected in the epithelial (E)-enriched breast cells and higher expression was detected in the MCF-7 cells that had also higher hPRLR mRNA level. hNIS, human Na\(^{+}/T\) symporter; hPRLR, human prolactin receptor; \(\beta_2\)-MG, human \(\beta_2\)-microglobulin as an internal control.
Fig 3.8. RT-PCR analysis of hNIS in the 3D-histocultured, hormone-treated human breast carcinoma. A breast carcinoma obtained from a 45 year-old patient by surgical biopsy was minced and cultured on collagen gels. Tissues were grown for 2 days in the media without any hormonal treatment, and then OT or hPRL was treated for 38 hrs. RNAs were prepared from the tissues and RT-PCR was performed as described in the Methods section. Note that NIS mRNA level was increased by OT (5 and 50 μU/ml) and hPRL (1 and 10 ng/ml) in a dose-dependent manner.
CHAPTER 4

EXPRESSION AND ACTIVITY OF HUMAN NA^+/T SYMPORTER IN HUMAN GLIOMA CELLS BY ADENOVIRUS-MEDIATED GENE DELIVERY

INTRODUCTION

Gliomas are the most frequent primary brain tumors, which affect 15,000 to 17,000 Americans every year (Kurpad et al., 1995). Despite clinical advances of surgery, radiation therapy and chemotherapy, these malignant tumors are almost always fatal with a median survival rate of less than a year (Fine, 1995; Mahaley et al., 1989; Shapiro and Shapiro, 1998). This poor prognosis is related to the fact that malignant glioma cells aggressively infiltrate into normal brain tissues, making total removal of the tumor impossible, thus the tumors have high relapse rates (Kaba and Kyritsis, 1997; Uhm et al., 1997). Since gliomas are highly resistant to conventional therapies, there is a need to explore novel and innovative approaches for their treatments.

For patients with thyroid cancer, the iodide concentrating activity of the thyroid tissues has allowed the use of radioiodide as an effective treatment. Since more than half of residual, recurrent or metastasized thyroid tumors can concentrate radioiodide when the tumor cells are stimulated by elevated serum thyrotropin (TSH), radioiodide (I-131)
has been used to ablate post-surgical remnants and to treat residual, recurrent and metastatic disease. Many studies have confirmed that radioiodide therapy significantly improves the prognosis of the thyroid cancer patient (Mazzaferri, 1996).

Viral vector-mediated gene delivery into mammalian cells is very efficient and thus is being used for gene therapy. Of the available vectors, recombinant adenovirus serotype 5 (Ad5) vectors are promising for gene therapy of gliomas due to their capacity to achieve high efficiency in vivo gene transfer upon intracranial administration (Davidson et al., 1993; Le Gal La Salle et al., 1993). Adenoviral particles are relatively stable and amenable to high purification and concentration (Huyghe et al., 1995). The expression level of the exogenous gene by recombinant adenovirus-mediated gene delivery can be easily controlled by changing multiplicity of infection (MOI).

Human Na\(^+\)/T symporter (hNIS) is the molecule that mediates the active iodide uptake from blood to thyroid follicular cells in humans. Our previous study showed that active iodide uptake can be induced in non-thyroid cells in vitro by expressing exogenous hNIS (Smanik et al., 1996). In an attempt to develop hNIS gene transfer for radioiodide therapy for patients with brain tumors, we have constructed a replication-defective recombinant adenovirus, rAd-CMV-hNIS#9, to express exogenous hNIS in U1240 and U1240Tag human glioma cells. In this paper, we demonstrated that exogenous hNIS can be expressed and function in human glioma cells by adenovirus-mediated gene delivery in vitro. We also demonstrated that hNIS#9 can be expressed in a xenografted human glioma tumor by intratumoral injection of rAd-CMV-hNIS#9. Thus, our study provides a basis for exploring further gene therapy strategies based on hNIS gene transfer followed by radioiodide treatment in patients with brain tumors.
MATERIALS AND METHODS

Cell culture

The U1240 and U251 glioma cell lines derived from human brain glioblastomas were obtained from Dr. B. Westermark at University of Uppsala, Uppsala, Sweden. The cells were maintained in Eagle's MEM media supplemented with 10% calf serum (for U1240) or 10% fetal bovine serum (for U251) (Gibco-BRL), 10 units/ml penicillin, and 10 μg/ml streptomycin. The U1240Tag cell line differs from U1240 cells in that U1240Tag cells are stable transfectants of U1240 cells expressing SV40 large T antigen oncoprotein. Thus, U1240Tag glioma cells were maintained in media also containing 100 μg/ml Geneticin (G418). An E1A-transformed human embryonic kidney cell line, 293 cells, was maintained in E-MEM media (Quality Biological Inc.) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine.

Generation of the rAd-CMV-hNIS#9 recombinant adenovirus

The hNIS#9 cDNA clone, encoding amino acids 1-612 of hNIS (Smanik et al., 1996), was used to generate the rAd-CMV-hNIS#9 recombinant adenovirus vector (Fig 4.1). The hNIS#9 cDNA fragment in the pcDNA3 vector, along with the CMV promoter and bovine growth hormone (BGH) poly-A signal, were subcloned into the E1-deleted shuttle plasmid pΔE1sp1A (Microbix Biosystems Inc.), which retains the Ad5 packaging signal sequence (ψ). The resulted pΔE1-CMV-hNIS#9 plasmid and the Ad5 helper vector pBHGE3 (Microbix Biosystems Inc.) were then co-transfected into low passage (<
P13) 293 cells in 100 mm dishes via a calcium phosphate method. Infectious rAd-CMV-
hNIS#9 recombinant adenovirus was produced by homologous recombination in which
the \( \psi \) sequence and the CMV-hNIS#9-poly-A DNA fragment were rescued into the E1-
deleted region (1.0-9.8 m.u.) of the Ad 5 genome (Fig 4.1).

The culture medium of the 293 cells showing the complete cytopathic effect was
collected and centrifuged at 1000 xg for 15 min. The pooled supernatants were stored at
-80 °C as primary viral stock. Before the viral plaque was purified, the function of the
rAd-CMV-hNIS#9 was confirmed by performing RIU assay in infected Cos-7 cells. The
rAd-CMV-hNIS#9 was propagated in 293 cells, further purified by gradient and
isopycnic CsCl ultracentrifugation banding and dialysis. The virus stocks (1.46x10^{10}
plaque-forming units/ml) were stored at -80 °C before use.

*Adenovirus-mediated LacZ expression in vitro*

To evaluate the infectivity of adenovirus in the U1240 and U1240Tag cells, cells
plated in 100mm dishes were infected with rAd-CMV-LacZ at MOI 10 or 50. Cells were
washed with cold PBS 48 hr after viral infection and fixed with 2%
paraformaldehyde/0.2% glutaraldehyde for 15 min. To detect the expression of \( \beta \)-
galactosidase, the cells were stained with 1mg/ml X-gal overnight at 37 °C. After
staining, the results were photographed under an inverted microscope (Nikon/Diaphot) at
100x magnification.

*Radioiodide uptake (RIU) assay*
Glioma cells (2x10^5 cells/well) were seeded in 24 well plates 24 hr before rAd-CMV-hNIS#9 infection. At various times of post-infection, cells were incubated for 30 min at 37°C with growth media containing 2.0 μCi of Na^125I and 5-10 μM of NaI carrier. The medium was aspirated and cells were quickly washed twice with ice-cold Hank's Balanced Salt Solution (HBSS). Cells were then lysed by incubation with 95% ethanol. The radioactivity of the cell lysate was measured by γ-counter (Packard Instruments). Experiments were performed in triplicate. The RIU activity of the infected cells was expressed in the fold increase compared to that of mock infected parental cells.

**Western blot analysis**

Western blot analysis was performed as previously described (Jhiang et al., 1998) with some modifications. U1240 or U1240Tag cells (2x10^6 cells) were plated in 100-mm dishes 24 hr prior to infection. The cells were incubated with rAd-CMV-hNIS#9 in DMEM with 2% FBS (without antibiotics) for 1.5 hr at 37 °C. Infected glioma cells were harvested and homogenized in a homogenizing buffer (10 mM Tris-HCl (pH 7.5), 5 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 50 μg/ml leupeptin) containing 0.25 M sucrose. The lysates were centrifuged at 700 xg for 10 min at 4 °C. The supernatants were further centrifuged at 200,000 xg for 90 min at 4 °C to obtain membrane fractions, which were resuspended in homogenizing buffer without sucrose and kept at -80°C until use.

The membrane fractions (20 μg/lane) were solubilized for 30 min at 37 °C in the same volume of reducing sample buffer (0.125 M Tris-HCl (pH6.8), 4% SDS, 10% β-mercaptoethanol, 20% glycerol) and subjected to 4-20% gradient SDS-PAGE. The proteins were transferred onto nitrocellulose filter, and the filter was blocked with 5 %
nonfat dry milk in TBST buffer (10mM Tris-HCl (pH8.0), 150 mM NaCl, 0.1 % Tween 20) at 4 °C overnight. Then, Western blot analysis was performed by incubating the filter with affinity-purified hNIS antibody (1:3,000) in TBST containing 5% nonfat dry milk for 1 hr at room temperature, followed by incubation with peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:3,000) for 1 hr at room temperature. The filter was then incubated with the ECL detection reagent (Amersham) for 1 min and exposed to an X-ray film. For the quantification of the protein expression, the blot was analyzed with Lumi-Imager (Bio-Rad).

Iodide organification

Glioma cells (1 x 10^6 cells) were plated on 100 mm dishes 24 hr prior to rAd-CMV-hNIS infection at MOI 10. Two days later, the cells were incubated with Na^{125}I, 10 μCi per plate (1 μCi/ml) for 2 hr. The organification procedure was done as described previously (Cho et al., 1999). Briefly, the glioma cells were solubilized in 1 ml of ice-cold 0.1M NaOH. The radioactivity of the cells was counted by a γ-counter, and this value was referred as total iodide uptake of the cells. The cells were then subjected to TCA (5% w/v) precipitation to measure the protein-bound ^{125}I to evaluate iodide organification. Organified ^{125}I was expressed as the percentage of total iodide uptake measured prior to TCA precipitation.

Radioiodide accumulation (RIA) assay for in vivo animal

U251 glioma cells at log-phase were harvested by trypsinization and washed with HBSS without Mg^{2+} and Ca^{2+}. 1 x 10^6 cells in 250 μl of complete
media plus 250 μl of Matrigel (Collaborative Biomedical Products, Bedford, MA) were immediately subcutaneously injected into 4 week-old nude mice. When the tumor had grown to approximately 10 mm in diameter 5-6 months after cell injection, rAd-CMV-hNIS#9 was injected into 6 spots per tumor (1.2 x 10^9 pfu total). As a control, saline (100 μl) was injected into a tumor on the opposite flank of the same animal. Forty-three hours later, NIS functional activity in xenografted glioma tumors was assessed by measuring radiiodide accumulation (RIA), the sum total of iodide influx and efflux following ^125^I injection. ^125^I was intraperitoneally administrated to mice at 0.3 μCi per gram body weight (in 0.15 ml of PBS). Mice were sacrificed 1 hr later, and tissues including tumors were collected in 10% formalin to measure radioactivity using a γ-counter (Packard Instruments, Downers Grove, IL). The data is presented as a fold increase in radioactivity of respective tissues, compared to spleen by the following formula: (Counts of tissue "X"/mg of tissue "X")/(Counts of spleen/mg of spleen). The tumor was fixed in 10% formalin overnight. Paraffin-embedded tissue sections (5 μm) were used for immunohistochemical staining.

**Immunohistochemical staining of xenografted tumor**

Immunohistochemical staining was performed to detect hNIS proteins in the neuroblastoma tumor sections as published previously (Jhiang et al., 1998) with some modifications. The tissue sections were incubated with 3 % H_2O_2 in methanol for 5 min to inactivate endogenous peroxidase, and then subjected to antigen retrieval in 10 mM citric acid buffer (pH 6.0) at 94 °C for 30 min.
tissue sections were incubated with the purified hNIS antibody at a 1:3,000 dilution at room temperature for 1 hr, followed by Avidin and Biotin (Dako) blocking for 10 min each. The sections were then incubated with 1:200 dilution of biotinylated goat anti-rabbit IgG (Vector Lab. Inc., Burlingame, CA) for 20 min, streptavidin peroxidase for 10 min, and DAB substrate chromogen for 5 min. Counterstaining was performed using Hematoxylin.
RESULTS

Radioiodide uptake activity in glioma cells infected by rAd-CMV-hNIS#9

In order to demonstrate the function of hNIS#9 in the glioma cell lines after rAd-CMV-hNIS#9 infection, U1240 and U1240Tag cells were infected with rAd-CMV-hNIS#9 at MOI of 1, 5, and 10. The functional activity of hNIS#9 in the infected glioma cells was analyzed by RIU assay. In both infected U1240 and U1240Tag cells, RIU activity increased as the adenoviral MOI increased. At any given dosage (MOI 1, 5, 10), RIU activities in the infected U1240 cells were higher than those in the infected U1240Tag cells (Fig 4.2A). The RIU activities appear to be maximal at 24 hr post-infection in both infected cell lines. At 24 hr post-infection of MOI 10, the infected U1240 cells had an 82-fold increase in RIU activity, while the infected U1240Tag cells had a 25-fold increase in RIU activity, compared to their corresponding non-infected cells (Fig 4.2B). Basal RIU activities in non-infected U1240 cells and U1240Tag cells were about 2,000 and 4,000 cpm per well, respectively.

Correlation of infectivity with RIU activity

To investigate whether the higher RIU activity found in rAd-CMV-hNIS#9 infected U1240 cells versus U1240Tag cells is due to the difference in efficiency of adenovirus infection in these two cell lines, we compared the adenoviral infectivity in U1240 and U1240Tag cells using the β-galactosidase-expressing recombinant adenovirus (rAd-CMV-LacZ). As shown in Fig 4.3A & Fig 4.3B, at MOI 10 of rAd-CMV-LacZ infection, 40-50% of U1240 cells were stained with X-gal while only about 10% of
U1240Tag cells stained with X-gal. The difference of infectivity between these two cell lines is in proportion to the difference of RIU activities between these two cell lines following infection with rAd-CMV-hNIS#9 at MOI 10 (see Fig 4.2A). At MOI 50, the percentage of infected U1240Tag cells increases to 80-90%, while the percentage of infected U1240 cells is close to 100% (Fig 4.3C & Fig 4.3D). These data suggest that the higher RIU activity observed in rAd-CMV-hNIS#9 infected U1240 cells than in infected U1240Tag cells is likely due to a higher efficiency of adenovirus infection in U1240 cells than U1240Tag cells.

**Correlation of hNIS#9 protein expression with RIU activity**

To investigate whether the expression levels of hNIS#9 protein correlate with RIU activity in infected U1240 and U1240Tag cells, the expression levels of hNIS#9 protein were determined by Western blot analysis in cells infected with rAd-CMV-hNIS#9 at MOI 0, 1, 5, and 10. As shown in Fig 4.4, in both U1240 and U1240Tag cells, the expression levels of hNIS#9 protein increased as the MOI of rAd-CMV-hNIS#9 increased. The expression levels of hNIS#9 protein in the infected cells (Fig 4.4) are in general agreement with the RIU activities of the infected cells (see Fig 4.2A). It is believed that the 90 kDa band represents fully glycosylated hNIS#9 protein, while the 65 kDa band is under-glycosylated hNIS#9 protein (Jhiang et al., 1998).

**Maintenance of hNIS#9 protein expression by multiple infections of rAd-CMV-hNIS#9**

The temporal expression profile of hNIS#9 protein was studied by infecting the glioma cells with rAd-CMV-hNIS#9 at an MOI of 10, and then harvesting membrane
fractions of the infected cells at 12, 24, 48 and 72 hrs post-infection for Western blot analysis. In both U1240 and U1240Tag cells, the highest protein expression levels were observed 24 hrs post-infection (Fig 4.5A), which is in accordance with the time of maximal RIU activity observed in infected cells (see Fig 4.2B). The half-life of hNIS#9 protein expressed in both U1240 and U1240Tag cells is estimated to be less than 24 hours (Fig 4.5A). However, the expression level of hNIS#9 protein can be maintained by multiple infections of rAd-CMV-hNIS#9 (Fig 4.5B). U1240 cells were initially infected with rAd-CMV-hNIS#9 at MOI of 20 on day 0, and re-infected at MOI of 10 on day 2 and day 4. The cells were collected from day 2 to day 6. For comparison, the cells exposed to a single initial viral infection were collected at day 6. As shown in Fig 4.5B, the expression of hNIS#9 was maintained for 6 days by multiple infections in these cells, although the expressed hNIS#9 appeared to have a short half-life.

Iodide organification rate in U1240 and U1240Tag cells

As radioiodide concentrating activity in thyroid tissues is not only contributed by active iodide uptake but also by iodide organification, we investigated the efficiency of iodide organification in the infected glioma cells. Our in vitro iodide organification experiment showed that 5-6% of iodide was organified in infected U1240 and U1240Tag cells (Fig 4.6). As a comparison, 10-12% of radioiodide was organified in cultured FRTL-5 rat thyroid cells.
Intratumoral injection of rAd-CMV-hNIS#9 for hNIS function and hNIS protein expression in vivo

We used xenografted human U251 glioma tumors in nude mice to test whether hNIS can be expressed and function in vivo by intratumoral injection of rAd-CMV-hNIS#9. As shown in Fig 4.7, tumors injected with rAd-CMV-hNIS#9 showed as high as 28-fold increase over spleen control in RIU activity at 44 hrs after rAd-CMV-hNIS#9 injection, whereas the RIU activity in the tumors injected with saline was not different than that of spleen. The tumor piece that was injected into 3 locations with rAd-CMV-hNIS#9 (hNIS-T1) showed higher RIU activity than the tumors injected into 1 location (hNIS-T3) (Fig 4.7). When RIU activity was calibrated per mg of tissue, a portion of the tumor (hNIS-T1) infected with rAd-CMV-hNIS#9 had RIU activity higher than that of the stomach, a significant source of RIU in vivo, but lower than that of thyroid. The tumors were then isolated and subjected to immunohistochemical staining using an anti-NIS antibody. The hNIS protein was expressed in the xenografted human glioma by intratumoral injection of rAd-CMV-hNIS#9 (Fig 4.8B to 4.8D), whereas the tumors injected with saline showed no immunostaining (Fig 4.8A). Furthermore, hNIS protein expression was shown to be adenovirus dose-dependent as more cells showed hNIS protein expression in the tumor piece injected with more rAd-CMV-hNIS#9 (Fig 4.8B) than the tumor piece injected with less rAd-CMV-hNIS#9 (Fig 4.8C). Fig 4.8D shows tumor cells expressing hNIS near needle penetration site.
Gliomas are the most common brain tumors in adults and the prognosis for the patient with a malignant glioma is dismal. Patients who are treated with conventional therapies have a median survival time of only 12 months (Delattre and Uchuya, 1996). Novel therapeutic approaches, such as gene therapy protocols with cytokine-transduced cells (Sobol et al., 1995) or using the herpes simplex virus thymidine kinase gene (HSV-TK) in combination with ganciclovir (GCV) (Culver et al., 1992; Culver et al., 1994), have been reported. In addition, recombinant adenovirus-mediated p53 tumor-suppressor gene delivery (Kock et al., 1996), or recombinant retrovirus-mediated *Escherichia coli* cytosine deaminase (CD) / HSV-1 TK fusion gene delivery for enhanced metabolic suicide and radiosensitization (Rogulski et al., 1997), were also implemented.

In this study, we introduce a new strategy of gene therapy to attack brain tumors. In the thyroid, TSH-stimulated hNIS expression provides the mechanism for thyroid tumor cells to concentrate radioiodide. Thus, radioiodide therapy is an effective and well-established treatment for the ablation of residual, recurrent and metastasized thyroid tumors after thyroidectomy. Molecular cloning of hNIS (Smanik et al., 1996) along with the established radioiodide therapy protocol for thyroid cancers prompted us to explore the possible application of radioiodide ablation for non-thyroid tumors, such as gliomas, after efficient gene delivery of hNIS. Thus, we have generated a replication-defective recombinant adenovirus containing hNIS#9 by homologous recombination in the E1A-transformed 293 cells. We have shown, for the first time, that recombinant adenoviral-
mediated delivery of the hNIS gene into human glioma cell lines results in dose-dependent expression and activity of hNIS. Glioma cells do not express endogenous hNIS and do not have detectable RIU activity. However, when exogenous hNIS expression was introduced by recombinant adenoviral mediated gene transfer, the infected glioma cells showed significant iodide uptake activity.

Our data showed that the efficiency of recombinant adenoviral infection is higher in U1240 cells than U1240Tag cells, stable transfectants of U1240 cells that express the SV40 large T antigen oncoprotein. It is known that the adenovirus entry is dependent upon several critical steps including virus attachment to cellular receptors called CAR (Bergelson et al., 1997), internalization via endocytosis, endosome escape and transport of virion DNA to the cell nucleus (Svensson and Persson, 1984). Therefore, it is likely that one or more of these processes are interfered with by the expression of SV40 large T antigen oncoprotein or other factors in U1240Tag cells. This suggests that the efficiency of adenoviral infection in vivo may not only vary among different tissue types but may also vary among different tumor cells in a given tumor with heterogeneity.

The hNIS#9 protein expressed in U1240 and U1240Tag cells appears to have a short half-life of less than 24 hours, which differs from the published half-life of rat NIS of 4 days in FRTL-5 rat thyroid cells (Paire et al., 1997). Interestingly, our transient transfection data revealed that Cos-7 cells expressing the full-length hNIS (FLhNIS) had about 3 times higher RIU activity than Cos-7 cells expressing hNIS#9 (data not shown). Since the protein encoded by hNIS#9 lacks the last 31 amino acids of hNIS, it is likely that hNIS#9 protein may have a shorter half-life than FLhNIS protein. Currently, we are constructing recombinant adenovirus containing FLhNIS cDNA (rAd-CMV-FLhNIS)
with the hope that higher and longer RIU activity can be accomplished in target cells upon gene delivery.

Once iodide is taken up into the cell, it passively leaks out of the cell unless the iodide is organified into cellular proteins. This organification process increases the effectiveness of radioiodide ablation therapy by allowing these cells to retain radioiodide long enough to kill the cells. Our data showed that only 5-6% of radioiodide is organified in cultured glioma cells infected by rAd-CMV-hNIS#9, compared to 10-12% in TSH-stimulated FRTL-5 rat thyroid cells. The iodide organification process in thyroid cells is mediated by the thyroperoxidase (TPO) enzyme that is not expressed in glioma cells. For maximal radioiodide ablation activity, it is desirable to introduce the TPO enzyme into glioma cells expressing exogenous hNIS to prolong the retention time of radioiodide. Currently, we are in the process of constructing the recombinant adenovirus, rAd-CMV-hTPO, in an attempt to increase the radioiodide organification rate.

During the preparation of this manuscript, Mandell et al reported that retroviral mediated gene transfer of rat NIS (rNIS) in four tumor cell lines confers radioiodide concentrating activity (Mandell et al., 1999). They showed that the cells expressing rNIS were selectively killed by accumulated radioiodide using in vitro clonogenic assays (56-69% of rNIS-transduced cells versus 10-17% of parental cells). Furthermore, they showed that rNIS-transduced human A375 melanoma xenografts established in vivo in athymic nude mice could be imaged using a gamma camera after administration of radioiodide (Mandell et al., 1999). In our study, we performed intra-tumoral injection of rAd-CMV-hNIS#9 to mimic in vivo gene delivery of hNIS, and we demonstrated that exogenous hNIS could be expressed in a xenografted SK-N-SH human neuroblastoma by
intratumoral injection of rAd-CMV-hNIS#9. Although our study differs from their study in many ways, such as adenoviral-mediated gene transfer of hNIS versus retroviral-mediated gene transfer of rNIS, both studies indicate that radioiodide can be used to treat patients with non-thyroidal tumors upon efficient delivery of hNIS.

It is conceivable that intracellular radioiodide (I-131) ablation of gliomas after hNIS gene delivery will have a greater tumor-killing effect, compared to that of external radiation therapy. However, specific delivery of hNIS into gliomas remains a major challenge. Recently, a vector re-targeting strategy for gliomas was reported, based on the fact that epidermal growth factor receptor (EGFR) is highly expressed in the majority of gliomas, but not in normal and mitotically quiescent neural tissues. (Miller et al., 1998). In order to target adenoviral gene transfer specifically to glioma cells, a bispecific antibody conjugate was used to ablate binding to fiber receptors and retarget binding to the EGFR. This bispecific antibody-mediated re-targeting strategy may be useful for our adenoviral-mediated hNIS gene transfer to glioma tumor cells.

Finally, it is desirable to protect the thyroid gland from radioiodide ablation when radioiodide is used to ablate non-thyroid tumors expressing exogenous hNIS. Since hNIS expression and RIU activity in thyroid tissues is TSH-dependent, we are exploring the strategy of using thyroxin supplementation to suppress serum TSH level so that RIU activity in thyroid tissues can be minimized. Taken together, our data provide the mechanism for a radioiodide ablation protocol in glioma cells after recombinant adenoviral-mediated gene delivery of hNIS. Our findings of hNIS gene transfer conferring RIU activity in human glioma cells provide a basis for exploring further gene
therapy strategies based on hNIS gene transfer followed by radioiodide treatment in patients with brain tumors.
Fig 4.1. The genomic structure of the replication-deficient recombinant adenovirus rAd-CMV-hNIS#9. The recombinant adenoviral vector, rAd-CMV-hNIS#9, was constructed by insertion of the hNIS#9 coding sequences along with CMV promoter (CMVp) and BGH polyadenylation signal (poly(A)) in the E1A- and E1B-deleted region of Ad5 (1.0-9.8 m.u.). ψ, Ad5 packaging signal sequence; ITR, inverted terminal repeat; m. u, map unit.
Fig 4.2. Radi iodide uptake (RIU) assay of U1240 and U1240Tag cells upon rAd-CMV-
hNIS#9 infection. (A) Cells (2x10^5) were seeded on 24-well plates and infected with rAd-
CMV-hNIS#9 at MOI of 0, 1, 5, and 10. RIU assay was performed 48 hrs after infection.
(B) The cells were infected with rAd-CMV-hNIS#9 at MOI of 10. RIU assays were
performed at 12, 24, 36, 48, and 72 hrs after infection. Data is presented as fold increase
compared to cpm of non-infected parental cells. MOI, multiplicity of infection.
Fig 4.3. Photomicrographs illustrating X-gal staining of glioma cells cultured on Petri-dishes. U1240 or U1240Tag cells were infected with rAd-CMV-LacZ at MOI of 10 or 50 and were stained by X-gal 48 hrs later. At MOI of 10, U1240 cells showed higher infectivity (40-50% cells stained) than U1240Tag cells (about 10% cells stained). At MOI of 50, the percentage of infected U1240Tag cells increased to 80-90%, while almost 100% of U1240 cells were infected. MOI, multiplicity of infection.
Fig 4.4. Western blot analysis and hNIS protein quantification from glioma cells infected with rAd-CMV-hNIS#9. Cellular membrane fractions were prepared and loaded on a 4-20% gradient SDS-PAGE gel (20 µg/lane). The hNIS#9 protein was detected using a polyclonal antibody against hNIS. Signal was quantified by a Lumi-imager. The expression levels of hNIS#9 increased as the MOI of rAd-CMV-hNIS#9 increased in a dose-dependent manner. At a given MOI, the expression level of hNIS#9 protein in infected U1240 cells was higher than infected U1240Tag cells. A. U., arbitrary unit (set 100 for strongest signal lane).
Fig 4.5. Western blot analysis and hNIS protein quantification showing the time-course of hNIS protein expression, and the effects of multiple infection, in the glioma cells infected with rAd-CMV-hNIS#9. (A) The temporal profiles of hNIS expression correlated with the temporal profiles of RIU activity (see Fig 4.2B). The half-life of hNIS#9 protein in the infected glioma cells appeared to be less than 24 hrs. (B) The U1240 cells were initially infected with rAd-CMV-hNIS#9 at MOI of 20 at day 0, and re-infected at MOI of 10 at day 2 and day 4. The cells were collected at day 2 (single infection), day 3 and day 4 (double infection), day 5 and day 6 (triple infection). At day 6, the expression of hNIS#9 protein was maintained by multiple infections (lane 6), while the hNIS#9 protein was not detectable in cells exposed to a single infection (lane 6s).
Fig 4.5
Fig 4.6. Iodide organification rate is expressed as TCA precipitable portion (%) of total iodide uptake measured prior to TCA precipitation. Minimal iodide organification rates (5-6%) were found in U1240 and U1240Tag cell lines. The iodide organification rate of FRTL-5 cells (10-12%) was also determined as a comparison. 6H, iodide organification assay was performed with FRTL-5 cells maintained in media containing TSH hormone (6H). 5H → 6H, iodide organification assay was performed with FRTL-5 cells which were initially maintained in media without TSH (5H), then switched to media with TSH (6H) for 2 days.
Fig 4.7. $^{125}$I $\gamma$-counts in the tissues from nude mouse xenografted with human U251 glioma cells. The tumor on the left flank was injected with saline (T1, T2 and T3), while the tumor on the right flank was injected with rAd-CMV-hNIS#9 (hNIS-T1 (3 spots, approximately $6 \times 10^5$ pfu), hNIS-T2 (2 spots, approximately $4 \times 10^5$ pfu) and hNIS T3 (1 spot, approximately $2 \times 10^5$ pfu)). Forty-three hrs after recombinant adenoviral injection, the mouse was injected with $^{125}$I (0.3mCi/gram body weight) and allowed to incorporate the nuclide for 1 hr. The collected tissues were subjected to $^{125}$I $\gamma$-count analysis. The data is presented as a fold increase of radioactivity, compared to spleen by the following formula: (Counts of tissue "X"/mg of tissue "X")/(counts of spleen/mg of spleen).
Fig 4.8. A xenografted U251 human glioma tissue section showing hNIS#9 expression after intratumoral injection of rAd-CMV-hNIS#9. The recombinant adenovirus was injected into several locations in the U251 tumor (1.2 x 10^9 pfu total) in a nude mouse. The tumor pieces used here are from the tumors used for RIU assay in Fig 6. The paraffin-embedded tissue sections were stained with a polyclonal antibody against hNIS protein (seen as brown color). (A) Tumor piece injected with saline. (B) Tumor piece injected with rAd-CMV-hNIS#9 in 3 spots. (C) Tumor piece injected with rAd-CMV-hNIS#9 in 1 spot. (D) Tumor piece injected with rAd-CMV-hNIS#9 showing the immunostaining near the needle penetration site. (bar = 5 μm).
CHAPTER 5

CONCLUSION AND FUTURE RESEARCH DIRECTIONS

In the ret/PTC1 transgenic mouse study described in chapter 2, it has shown that ret/PTC1 expression at embryological days 16-18 increased proliferation rate, distorted thyroid follicle formation, and reduced radioiodide concentrating activity. This study suggests that ret/PTC1 oncogene expression can alter thyroid morphogenesis resulting in the papillary histotype of thyroid cancer, and alter thyroid differentiation such as greatly reduced radioiodide concentrating activity. A more detailed dissection of these alterations, along with ret/PTC1 oncogene expression profile in earlier embryological stages of these ret/PTC1 transgenic mice, will provide better understanding about the tumorigenic mechanism of ret/PTC1 oncogene. Meanwhile, since in vivo animal models are restricted by their inability to immediately study the direct effects of ret/PTC1 on thyroid cellular changes, we are trying to introduce ret/PTC1 directly into porcine primary thyroid cell culture using recombinant adenovirus (Ad)-mediated gene delivery. The porcine primary thyroid cell culture system that shows follicular structure has been established. Using several Ad-ret/PTC1 mutants, each of which abolishes binding sites for Grb7/Grb10, PLCγ, or Shc/Enigma adoptor molecules, we can identify which signaling pathway is important for altered thyroid follicular morphogenesis and reduction
of NIS expression and radioiodide concentrating activity in thyroid cells. These findings will provide useful information for the role of ret/PTC1 in the formation of abnormal follicles with reduced iodide uptake ability observed in human papillary thyroid carcinoma.

Thus, we have been trying to produce a recombinant adenovirus, Ad-CMV-ret/PTC1. However, the recombinant virus could not be produced in more than 10 trials with either a mammalian homologous recombination system or a bacterial homologous recombination system, although all control-plasmid adenoviruses and two other non-ret/PTC1 adenoviruses were produced using same methods at the same times. Furthermore, when the ret/PTC1 was in anti-sense orientation in shuttle plasmid, the recombinant adenovirus was successfully generated. Taken together, these suggest that the ret/PTC1 oncogene expression may interfere with recombinant adenovirus production. RET/PTC1 is expressed prior (at 2 – 3 days after transfection) to the production of recombinant adenovirus particle which takes 7-12 days. Studying this inhibitory mechanism of RET/PTC1 for recombinant adenovirus production will be of great interest to understand the relationship between tyrosine kinase activity and the production of adenovirus particles. We can employ some specific blocking drugs for RET/PTC activity to prove this hypothesis. Again, using mutant Ad-ret/PTC1, we can identify which pathway is important for the inhibitory action of RET/PTC1 on the production of adenovirus.

In chapter 3, it has been showed that a selective oxytocin antagonist and bromocriptin, an inhibitor of PRL release, had inhibitory effects on the RIA of lactating
MG. In addition, NIS mRNA levels were increased by oxytocin and prolactin in a dose-dependent manner in histocultured human breast tumors. These findings, along with the published reports showing that hormone-dependent human breast tumors have an increased RIA (Briand, 1983; Eskin, 1977), suggest that radioiodide therapy may be useful to treat some patients with breast cancer. However, before clinical trials can be initiated, we need to optimize the dosages and the combinations of various hormones including OT and PRL to stimulate hNIS expression and thus RIA in breast tumors at the level sufficient for radioiodide therapy. After the optimization in histocultures in vitro, the optimal concentrations needs to be tested and reevaluated in vivo using animal models bearing breast tumors. There are several animal models that can be used for our future study. It is known that virgin rats develop breast tumors about 20 weeks after a single i.p. injection of N-Methyl-N-nitrosourea (MNU) (Guzman et al., 1999). Cats naturally develop breast carcinomas. Several transgenic mice with breast tumors are also available. In these animal models, we can evaluate NIS expression, RIU activity, and tumor-killing effect of radioiodide (1-131) upon hormonal stimulation of NIS.

In chapter 4, it has been demonstrated that exogenous hNIS can be expressed and function in human glioma cells by adenovirus-mediated gene delivery in vitro. It was also demonstrated that hNIS#9 can be expressed in a xenografted human neuroblastoma tumor by intratumoral injection of rAd-CMV-hNIS#9. These findings of hNIS gene delivery conferring RIU activity in human glioma cells provide a basis for exploring further cancer gene therapy strategies based on hNIS gene transfer followed by radioiodide ablation in patients with brain tumors.
The hNIS#9, a truncated form of hNIS, appears to have a short half-life. Therefore, for higher and longer RIU activity upon gene delivery, a recombinant adenovirus containing full-length hNIS (FLhNIS) cDNA (rAd-CMV-FLhNIS) has been also constructed by a bacterial homologous recombination system. Preliminary experiments showed that FLhNIS in rAd-CMV-FLhNIS seems to be more stable than hNIS#9. For maximal radioiodide ablation activity, it may be desirable to introduce the hTPO enzyme into glioma cells expressing exogenous hNIS to prolong the retention time of radioiodide. The recombinant adenovirus, rAd-CMV-hTPO, has been also constructed in an attempt to increase the radioiodide organification rate. COS-7 cells infected with the rAd-CMV-hTPO expressed hTPO and had hTPO enzymatic activity. Further studies will be performed to evaluate the rAd-CMV-FLhNIS in combination with rAd-CMV-hTPO in intracranially-xenografted gliomas. For the specific targeting of the adenoviral genes to gliomas, we will utilize the bispecific antibody conjugate to ablate rAd-CMV-FLhNIS and rAd-CMV-hTPO binding to fiber receptors and retarget binding to the EGFR.
LIST OF REFERENCES


oncogene in thyroid cancers of children from areas contaminated by Chernobyl accident [letter]. Lancet 344, 259.


product of two oncogenic rearranged forms of the RET proto-oncogene in papillary thyroid carcinomas. Oncogene 7, 2189-2194.


RET/PTC3; a novel rearranged version of the RETproto-oncogene in a human thyroid papillary carcinoma. Oncogene 9, 509-516.


