STUDIES ON ESTRADIOL DEPENDENT TRANSCRIPTIONAL REGULATION OF HUMAN SODIUM IODIDE SYMPORTER GENE IN MAMMARY GLANDS

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BY
NERİMAN TUBA GÜLBAĞCI
AUGUST 2002

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

____

Prof. Dr. Mehmet Öztürk

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

____

Assoc. Prof. Ediz Demirpençe

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

____

Assist. Prof. Uygar Tazebay

Approved for the Institute of Engineering and Science

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Director of Institute of Engineering and Science
Prof. Dr. Mehmet Baray

ABSTRACT

STUDIES ON ESTRADIOL DEPENDENT TRANSCRIPTIONAL REGULATION OF HUMAN SODIUM IODIDE SYMPORTER GENE IN MAMMARY GLANDS

Neriman Tuba Gülbağcı

M.S. in Molecular Biology and Genetics

Supervisor: Assist. Prof. H. Uygar Tazebay

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Sodium Iodide Symporter (NIS) is a transmembrane protein, which is expressed in thyroid, mammary gland (mg), stomach, and salivary gland. NIS's transcriptional regulation in terms of *cis*-and *trans*-acting elements in thyroid gland is widely studied. However, despite identification of NIS and studies on its hormonal regulation in mammary gland, *cis*-and *trans*-acting elements controlling the mgNIS gene in this tissue are not identified yet. From *in vivo* experiments, it was learned that estrogen has an up regulatory effect on mgNIS transcriptional regulation. In this study, it was shown that *in vitro*, estrogen (even in pharmacological concentrations) was not able to induce mgNIS in estrogen receptor positive (ER(+)) MCF-7 breast carcinoma cells, and it had no additive effect on retinoic acid (RA) in NIS up regulation when it was administered in physiological concentrations. In ER (-) MDA-MB-231 breast cancarcinoma cells, ERα might be insufficient to induce mgNIS transcription inspite of the fact that ERα was able to transactivate ERE elements. Interestingly, our study indicates that tamoxifen antagonist of ER, together with estrogen induces mgNIS transcription in MCF-7 cell lines in the absence of RA. This study clearly shows the presence of a yet unidentified link between mgNIS regulation and estrogen responsive mechanisms. Bearing in mind that tamoxifen is a powerful substance in treatment of ER(+) breast cancers, and that radioactive iodide is used in thyroid cancer diagnosis and treatment. This weak induction of mgNIS expression in response to tamoxifen may also have interesting novel applications in fight against breast cancer.

Key Words: NIS, Estrogen, ER-α, RA.

ÖZET

Meme Dokusunda Sodyum İyot Taşıyıcı Proteinin
Estradiyol ile Regülasyonu

Neriman Tuba Gülbağcı

M.S. Moleküler Biyoloji ve Genetik

Danışman: Assist. Prof. H. Uygar Tazebay

Ağustos 2002, 64 sayfa

Sodyum İyot Taşıyıcısı (NIS) tiroid bezi, meme dokusu, miğde ve tükrük bezlerinde sentezlenen bir hücre zarı proteinidir. NIS proteininin tiroid bezindeki transkripsiyonel kontrolünde rol alan *cis*- ve *trans*-etkin genetik elemanlar daha önce belirlenmiştir. Nevar ki meme dokusunda NIS'in transkripsiyonunun hangi hormonlar tarafından düzenlendiği belirlenmiş ancak *cis*- ve *trans*-etkin elemanlar henüz belirlenmemiştir. Farelerle yapılan *in vivo* deneylerde elde edilen sonuçlarda östrojenin memede NIS transkripsiyonel düzenlenmesinde indükleyici bir etkisi olduğu gösterilmişti. Bu çalışmada, (ER(+) östrojen reseptörü içeren MCF-7 meme kanser hücrelerinde yapılan araştırmalar, bu hücre hattında NIS transkripsiyonunu artırabilmek için retinoik asitin gerekli olduğunu ancak estradiolun bu artışa etkisi olmadığını göstermiştir. ER(-) MDA-MB-231 meme kanser hücrelerinde ERα nın varlığı ERE elemanları indüklemesine rağmen mgNIS transkripsiyonunu indükleyebilmek için yetersiz kalmıştır. İlginç bir sonuç olarak, çalışmamız ER antagonisti olan tamoksifenin MCF-7 hücrelerinde RA yokluğuna rağmen NIS transkripsiyonunu indüklediğini göstermiştir. Bu sonuç memede NIS regulasyonu ve östrojene dayalı kontrol mekanizmaları arasında bir ilişki olduğuna işaret etmektedir. ER(+) meme kanseri tedavisinde kullanılan etkin bir ilaç olan tamoksifenin bir de NIS genini memede artırması, tamoksifen tedavisine tabi tutulan tümörlerin radyoaktif iyot izotopları kullanılarak da gözlemlenmesini ve kontrol altında tutulmasını sağlayabilmesi açısından oldukça ilginç ve meme kanserinde tedaviye yönelik uygulamaları olabilecek bir sonuçtur.

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ABBREVIATIONS

APS	amonium persulfate
bisacrylamide	N, N, methylene bis-acrylamide
bp	base pairs
c-terminus	carboxyl terminus
cDNA	complementary deoxyribonucleic acid
kb	kilobasepairs
kD	kilo daltons
DMSO	Dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
EDTA	diaminoethane tetra-acetic acid
ERα	estrogen receptor alfa
ERβ	estrogen receptor beta
EtBr	ethidium bromide
I-	iodide
MCS	multiple cloning site
ml	milliliter
mg	milligram
N-terminus	amino terminus
NIS	sodium iodide symporter
MW	molecular weight
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Prl	prolactin
PrlR	prolactin receptor
RA	retinoic acid
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute

RXR	retinoid x receptor
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
TAE	tris-acetic acid-EDTA
TE	tris-EDTA
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane
Tris	tris (hydroxymethyl)-methylamine
TSH	thyroid stimulating hormone
UV	ultraviolet

1.INTRODUCTION

1.1. Iodide Transport

The metabolism of iodide (I^-) is mostly associated with the thyroid gland, because I^- is an essential constituent of the thyroid hormones triiodothyronine (T3), and thyroxine (T4). Under physiological conditions, most of the ingested dietary I^- is accumulated in the thyroid by means of a highly specific active I^- transport mechanism (De la Vieja *et al*., 2000). This transport mechanism is an important cellular adaptation to accumulate iodine, an environmentally scarce element. Iodine constitutes just 4-10% of the litosphere; its main sources on the planet are Chilean nitrate deposits, California oil well brines, and sea water (Carrasco *et al.* 1993).

I^- transport into the thyrocytes is the first and rate-limiting step in the biosynthesis of the thyroid hormones. These hormones are of major significance for the intermediary metabolism of virtually all tissues. Moreover, they play an essential role in the growth and maturation of the skeletal muscles and nervous system and lungs of the fetus and the newborn (Stubbe *et al*. 1986).

Besides the thyroid, iodide is known to accumulate in other organs or tissues such as the salivary glands, gastric mucosa, lactating mammary gland, choroid plexus, and the ciliary body of the eye (Carrasco *et al*. 1993). Striking similarities between the iodide transporting systems such as thiocyanate and perchlorate inhibition, Na^+ dependency, identical specificity, and similar kinetic parameters for iodide transport have been found in all these tissues. This, even before the identification of the iodide transporters in these tissues, led researchers to think that these transport systems may be very similar or identical (Vulsma *et al*., 1991). However, there are also important differences between these transport systems: 1) non-thyroid I^- transporting tissues do not have the ability to organify the accumulated I^-; 2) Thyroid Stimulating Hormone (TSH) exerts no regulatory role on non-thyroid tissue iodide accumulation; 3) unlike thyroid, salivary glands and gastric mucosa concentrate thiocyanate (Carrasco *et al.* 1993).

Functional significance of iodide transport in non-thyroidal tissues was studied by various groups. An active I^- translocating mechanism has been demonstrated in the choroid plexus in vitro, and has been suggested to be

physiologically responsible for the extraction of I^- out of the cerebrospinal fluid (Carrasco *et al.* 1993). Similarly, the ciliary body of the eye has been shown to translocate I^- out of the aqueous humor in rabbits (Carrasco *et al.* 1993). The existence of an active I^- transport system in the ducts of the salivary glands results in a higher concentration of I^- in saliva as compared to plasma. Similarly, I^- is actively transported from plasma to gastric secretions by an I^- translocating system (Spitzweg *et al*., 1998). Also, lactating mammary gland is capable of taking up iodide and concentrating it in milk (Carrasco *et al.* 1993; Tazebay *et al*., 2000). This supplies I^- to the newborn. Remarkably, non-lactating breast tissue does not accumulate I^- (Tazebay *et al*., 2000). During both the intrauterine and extrauterine stages of development, I^- is used for the biosynthesis of thyroid hormones by the infant's thyroid gland, which becomes active by the 12th week of gestation in humans (Fisher *et al.* 1990**)**

The gene encoding for the Na^+/I^- Symporter (NIS) of the rat thyroid was cloned by Dai *et al*. in 1996. Subsequently, Smanik *et al.* has cloned the human counterpart of this gene by a PCR based method, and identified it's chromosomal location. The gene is located on chromosome 19p12 and composed of 15 exons and 14 introns (Smanik *et al*., 1996). Lack of functional mutations in this gene is found in 37 numbers of families (Matsuda *et al.* 1997). This type of mutations in this gene lead to a genetic disorder known as iodide transport disorder (ITD), which is inability of the thyroid to maintain an iodide concentration difference between the plasma and the thyroid.

In 1998, Spitzweg *et al*. made RT-PCR, and Northern blot analysis with several extrathyroidal human tissues with human thyroid NIS cDNA. They detected NIS mRNA by Northern blot, primarily in salivary glands, pituitary gland, pancreas, testis, mammary gland, gastric mucosa, prostate, and ovary. Spitzweg *et al*. also sequenced hNIS cDNA which was cloned from gastric mucosa, parotid gland, and mammary gland, and were able to show that all hNIS cDNA from different extrathyroidal tissues were identical to hNIS cDNA identified in thyroid (Spitzweg *et al*., 1998).

The protein encoded by the rat NIS gene is analysed both by computer and by biochemical and immunological experimental methods (Levy *et al.* 1998). I^- transport to thyroid cells is catalyzed by the sodium/iodide (Na^+/I^-) symporter (NIS),

a 618 amino acid 13 putative transmembrane glycoprotein located in the basolateral plasma membrane of the thyroid follicular cells (Levy *et al.* 1998).

NIS activity is Na-dependent and electrogenic, and the stoichiometry of co-transport is $2Na^{+}:1I^{-}$. From the kinetics of transporter as a function of external Na^{+} and substrate concentration, it is suggested that Na^{+} binds to the transporter first, and then the I^{-} binds. A variety of anions are transported by NIS with varying affinities. These are: I^{-}, $ClO3^{-}$, SCN^{-}, $SeCN^{-}$, NO_3^{-}, Br^{-}, BF_4^{-}, IO_4^{-}, BrO_3^{-}. From electrophysiological measurements and freeze fracture electron microscopy experiments, it is suggested that NIS may be multimeric in its functional form (Eskandari *et al*. 1998)

1.1.1. The Role of Na^{+}/I^{-} Symporter in Fight Against Thyroid Cancer

As a result of NIS activity in thyroid, radioactive iodide treatment is an effective thyroid cancer therapy with minimal side effects. It was first used for thyroid cancer treatment in 1941 and became widely available after the second World War as a consequence of atomic energy research, leading to the birth of nuclear medicine (Sawin and Becker, *et al.* 1996). Among the most important clinical thyroid function tests *in vivo* is the thyroidal radioiodide uptake test (RAIU). The degree of accumulation of I^{-}, as revealed by scans of the gland is used as an aid in the differential diagnosis of thyroid nodules. The possible existence of thyroid cancer is ruled out whenever a "hot" nodule is detected, since thyroid nodules that accumulate I- equally or more than the surrounding tissue are generally benign. However, the detection of a non I^{-} accumulating (a "cold" nodule) increases the likelihood of thyroid cancer (Carrasco *et al.* 1993). The isotope ^{123}I has a short half-life of 13 hours and delivers a low dose of radiation in γ-range that is optimal for thyroid imaging. Another isotope, ^{131}I, is primarily a β-emitter that has longer half-life (8 days) and can deliver large amounts of radiation to thyroid tumor cells with little damage to surrounding tissues (Daniels and Haber, *et al.* 2000). Therefore, it is commonly used for radioactive ablation of benign overactive thyroid and of locally invasive or metastatic thyroid cancer.

To treat the cold nodules in thyroid, first these nodules are taken by surgery, and then RA is given to patient to induce dedifferentiation process in differentiated cold nodule cells. After this RA treatment patients are given radioactive iodide to get rid off residual cancer cells.

1.2. Iodide Transport in Mammary Gland

1.2.1. Mammary Gland as an Organ

Mammary gland development is one of the most fascinating and puzzling biological phenomena. Interestingly, the mammary gland seems to be the only organ that is not fully developed at birth (Tobon and Salazar *et al*. 1974). Although immaturity at birth can be assumed in most systems, no other organ presents such dramatic changes in size, shape, and function as does the breast, during growth, puberty, pregnancy, and lactation (Tobon and Salazar *et al*. 1974)

Prenatal development of the gland. Concerning prenatal and perinatal development of the mammary gland, it is established that the whole mammary parenchyma arises from a single epithelial ectodermal bud. Recent work of Kordon and Smith (1998) shows that any epithelial portion of a normal mouse mammary gland can reproduce an entire functional gland when transplanted into an epithelium-free mammary fat pad. Moreover, they show that an entire functional mammary gland may comprise the progeny from a single cell (Kordon and Smith, 1998)

Formation of milk streak is first observed during the 4th week of embryonal life. This becomes the mammary ridge or milk line during the 5th week. Mammary ridge thickens, and forms milk hill stage at 6th week. By the 7th and 8th week parenchymal cells start to invade the underlying stroma, the mammary disk arises, progressing to a globular stage. Mammary parenchyma grows inward, and protrusion of the overlying skin regresses to form cone stage at the 9th week. Epithelial buds sprout from the invading parenchyma, which occurs between 10th and 12th weeks. With notching at epithelial-stromal border, buds become lobular between 12th and 13th weeks. Branching stage starts between 13th and 20th weeks, by further branching into 15-25 epithelial strips or solid cords. Between 20th and 32th weeks, canalization stage occurs by desquamation and lysis of the central epithelial cells to canalize solid cords. Lobuloalveolar development occurs between 32 and 40 weeks of gestation, in which end vesicles are composed of a monolayer of epithelium and contain colostrum (Dabelow *et al.* 1957).

Postnatal development of the gland. Postnatal period is very important for the completion of the development of a functional mammary gland. Postnatal development of the gland varies greatly from woman to woman, and makes it impossible to categorize mammary gland structure based on age. Mammary gland

development can be defined from the external appearance of the breast or by determination of mammary gland area, volume, degree of branching, or degree of structures whose appearance indicates the level of differentiation of the gland, such as lobule formation.(Dabelow *et al*. 1957)

The adolescent period begins with the first signs of sexual change at puberty and terminates with sexual maturity **.** Rudimentay mammae begin to grow both in glandular tissue and in the surrounding stroma with the approach of puberty. Small bundles of primary and secondary ducts grow and divide partly dichotomous basis. Ducts grow, divide and form clubshaped terminal end buds. These buds give origin to new branches, twigs, and alveolar buds. These buds are morphologically more developed than the terminal end bud, but more primitive than the ductules in mature organ.

Mammary gland development during pregnancy. Development of the gland in pregnancy can be divided into two main phases, early and late stages of pregnancy (Bassler *et al.* 1970; Salazar and Tobon *et al.* 1974)**.** In the early stage, distal elements of the ductal tree proliferates, and grow forming acini by neoformation of ductules. By the third month of pregnancy, these well-formed lobules exceeds the number of primitive buds (Salazar and Tobon *et al.* 1974). The epithelial cells in each acinus is greatly increase in number, and in size (Salazar and Tobon *et al.* 1974). At the mid-pregnancy, lobules become enlarged further and increased in number. In the second half of pregnancy branching continues in parallel with the formation of true secreting units. Proliferation of new acini reduces to minimum, while secretory material begins to accumulate into luminae of acini, and at the end of pregnancy mature secretory acinus is formed. It is important to say that during these events there is still growing of undifferentiated area in glands.

Gestational changes. After postpartum withdrawal placental lactogen, and sex steroids lactation starts. No major morphological changes of the mammary gland are observed during lactation. As long as milk is removed regularly from the mammary gland, the alveolar cells continue to secrete milk almost indefinitely.

After weaning, a reduction in breast volume and secretary ability of epithelial cells is observed. Mammary lobules undergo atrophy, and stroma shows a marked desmoplastic reaction and fat infiltration. Cell autolysis, collapse of acinar structures, narrowing of the tubules, appearance of round cell infiltration, and phagocytes in and about disintegrating lobules occurs. Finally periductal and perilobular connective

tissue regeneration with renewed budding and terminal tubule proliferation occurs. (Salazar and Tobon, 1974).

1.2.2 Hormonal Regulation of Mouse Mammary Gland Morphogenesis

Ductal growth is stimulated by various factors. One of them is estrogens. Estrogen receptors are concentrated in stromal cells around end buds not in rapidly dividing cap cells have role in stimulation of ductal growth. Another factor, EGF is a secondary mammotrophic signal acting on stromal cells for ductal growth. Stromal cells are also targets of a third hormone, growth hormone (GH). GH stimulates IGF-1 synthesis in synergy with low doses of estrogen, and stimulate ductal growth in hypophysectomized animals (Kleinberg *et al.* 1997).

Prolactin (PRL) and progesterone (PR) are essential for labuloalveolar development. By PRL receptor-null epithelium transplantation to wild type virgin animal fat pads, it was observed that ducts developed normally, but at pregnancy labuloalveolar development is inhibited. PRL receptor is essential for targeting epithelial cells in labuloalveolar development (Brisken et al. 1999). Progesterone (PR) is another hormone found to have effects on labuloalveolar development. Loss of PR action caused ablation of lobuloalveolar development, while ductal morphogenesis seemed to be normal (Brisken *et al.* 1998). PR receptors are found exclusively in a subset of stem-type cells in the lumen of the duct (Silberstein *et al.* 1996), and PR act by inter-epithelial, paracrine signaling (Brisken *et al.* 1998).

Not only growth stimulating factors but, also inhibitory factors play vital roles in mammary gland morphogenesis, by preventing infilling interductal space by lateral branching , and inhibiting of end bud extension at the fat pad boundaries, and TGFβ1 is one of these inhibitory factors (Silberstein *et al.* 1992). TGFβ family of growth factors inhibit proliferation and differentiation of target cells.

Experiments done by ectopic expression of TGFβ in the mammary ducts, and mammary ducts with mutant TGFβR, it is found that TGFβ regulates lateral branching by acting on TGFβ receptors on stroma cells, by inhibiting HGF expression in the periductal extarcellular matrix (ECM) (Joseph *et al.* 1999). It is found that site-specific inhibitory effect of TGFβ in lateral branching depends on TGFβ localization in mammary gland by ECM most probably via collagen-TGFβ binder decorin (Yamaguchi *et al.* 1990).

1.2.3 Identification of NIS in the Mammary Gland

It has been known since the classical article of Honour published in 1952 that the breast also concentrates iodide, secreting it into milk (Honour et al. 1952). Nursing infants synthesize essential thyroid hormones using iodide from breast milk (Carrasco et al. 1993). A tragic consequence of iodide transport in mammary gland and thyroid cells was observed after the Chernobyl nuclear power plant accident in 1986. In Ukraine and Belarus, this accident caused more than 800 children who drank milk from exposed dairy herds to develop thyroid cancers (Pacini *et al*., 1999).

Two separate research groups addressed the question whether transporters responsible of I^- transport in thyroid and in lactating mammary gland are identical, or two entirely different proteins. Immunoblots were performed on lactating mammary gland cell membrane fractions using antibodies (Ab) raised against thyroid NIS. These experiments have shown that Abs recognized a single band on immunoblots, albeit at a different molecular weight than the thyroid NIS. When the same experiment is repeated after treatment of membrane fractions from thyroid and lactating mammary gland with N-glycanase F enzyme (removing asparagine bound glycosyl residues), a single band at the same molecular weight is detected (Tazebay *et al.,* 2000). This was a first evidence showing that these two proteins could be identical. Conclusive evidence about the identity of lactating mammary gland detected protein came from cyanogen bromide (CnBr) treatment of membrane fractions before the immunoblots. CnBr restricts proteins from the methionine residues, revealing an identical fragmentation pattern in identical proteins.CnBrn was cutting the NIS of thyroid and mammary gland at N terminus which had same glycosylation patterns. This was the result obtained for the NIS ab detected proteins in thyroid and mammary gland after treatment of membrane fractions from these two organs (Tazebay *et al*., 2000). Therefore, as previously suggested by Spitzweg *et al*. (1998) in result of their sequencing of NIS cDNAs from thyroid and lactating mammary gland cells, it was definitively shown that the Na^+/I^- Symporter that functions in the thyroid gland is also present in the lactating mammary gland (Tazebay *et al*., 2000; Cho *et al*., 2000).

1.2.4. Regulation of NIS in the Mammary Gland

In healthy animals, mammary gland NIS (mgNIS) is expressed solely during lactation. Actually, the functional expression of mgNIS starts at mid-pregnancy (day 11 of the 19 days gestation period in mice), and reaches the peak at end-pregnancy (day 18). Interestingly, after birth mgNIS expression is suckling-dependent in a reversible manner: when pups are separated from the mother for 24 hours, mgNIS expression drops significantly, and when they are re-united, again it increases (Tazebay *et al*., 2000; Cho *et al*., 2000). Based on these observations, in an effort to identify hormones responsible of mgNIS regulation, virgin mice (either ovariectomised or surgically unmodified) were treated with combinations of lactogenic hormones and steroids, and it was shown that 17-β-estradiol, prolactin, and oxytocin up-regulates functional NIS expression, whereas progesterone has a down-regulatory effect (Tazebay *et al*., 2000; Cho *et al*., 2000). Moreover, an increase in mgNIS protein expression in cultured mid-pregnant mammary gland explants is seen after 24 h PRL treatment. This study has shown that PRL increase NIS gene expression both at the transcriptional and translational level (Rillema *et al.* 2000).

In a series of experiments using scintigraphic imaging accompanied with immunological techniques, mgNIS expression was also shown in mammary tumors of female transgenic mice carrying either activated *Ras* or *Neu* oncogenes under control of Murine Mammary Tumor Virus (MMTV) promoter. Significantly, these studies demonstrated functional mgNIS expression in both types of tumors (Tazebay *et al*., 2000). Subsequently, human breast specimens were examined by immunohistochemistry, and it was found that in contrast to no expression in 8 normal breast specimens from reductive mammoplasties, 20 out of 23 invasive carcinomas, and 5 out of 6 ductal carcinomas in situ did express mgNIS (Tazebay *et al*., 2000). This indicates that mgNIS is upregulated with a very high frequency during malignant transformation in human breast. This finding raised the question whether radioiodide could be effective for the diagnosis and as an adjuvant to surgical treatment of breast cancer, as it is the case in thyroid diseases.

The hormonal regulation of NIS in mammary gland is studied, but cis-, and trans-acting elements that are important in transcriptional regulation is unknown.

1.3. Regulation of Transcription in Eukaryotes

1.3.1. A General Overview

Most gene regulation occurs at the initiation of transcription. With a reductionist view, we can state that there are two types of genetic elements that help initiate or prevent this first step of gene expression: 1) *cis*-acting regulatory region in proximity of regulated genes; 2) *trans*-acting proteins (transcription factors) that interact with *cis*-acting regulatory regions. All regulated genes in eukaryotes contain two kinds of major *cis*-acting elements: The first one, *the promoter*, is always very close to the gene's protein coding region. It includes an initiation site, where transcription begins, and a TATA box, consisting of roughly seven nucleotides of the sequence $TATA^{A}/_{T}A^{T}/_{A}$. This region is usually located 30 nucleotides upstream of the initiation site. The second type of elements, *enhancers*, are regulatory sites that can be quite distant –up to tens of thousand of nucleotides away– from the promoter. Enhancers are often called as "upstream activator sites" or UASs. The binding of *trans*-acting proteins (transcription factors) to a gene's promoter or enhancer(s) controls the initiation of transcription. Different types of proteins bind to each of the *cis*-acting regulatory regions: Basal factors which assist recruitement of RNA Polymerase II (RNA Pol II) bind to the promoter, whereas activators and repressors bind to the enhancers (Kassavatis *et al.* 1990). When activator proteins bind to enhancer regions, they can interact directly or indirectly with basal factors at the promoter to cause an increase in transcriptional initiation (Mueller-Storm *et al*. 1989). Another group of proteins that are shown to interact with transcriptional activators are chromatin remodelling enzymes such as histone acetyl transferases (HATs) and deacetylases (HAD), as well as SWI/SNF family of ATPases (see below).

DNA in eukaryotes are highly organized, and packed as chromatin. Basic structure of this packed DNA is nucleosome, which is a 146 bp of DNA wrapped twice around a histone core (Arents *et al.* 1991). This core is composed of two copies of histone H2A, H2B, H3, and H4 proteins. This wrapped DNA is then bound to histone H1 protein. In some parts of the genome, this packing is more condense as compared to other parts. More condensed regions are named as heterechromatins, and less condensed regions are called euchromatins. Heterchromatins are mostly untranscribed regions, while euchromatins are transcribed. In different cell types,

localization of euchromatin, and heterochromatin can change according to the transcribed genes (Felsenfeld *et al.* 1992).

In mammals, coactivator proteins that interact with transcription factors –or nuclear receptors, as called for hormone activated transcription factors– may be classified into two families of proteins: One family identified as proteins of 160 kDa, consists of related proteins: the steroid receptor coactivator proteins SRC-1A and SRC-1E (Kamei *et al*., 1996; Onate *et al*., 1995) transcription intermediary factor TIF2 (Voegel *et al*., 1996) also called as GRIP-1 (Hong *et al*., 1996), and CREB binding protein (CBP) interacting protein p/CIP (Torchia *et al*., 1997). The second family comprises CBP and p300, which were originally shown to function as coactivators for CREB, the transcription factor that mediates responses to protein kinase A stimulation. Subsequently, however, CBP/p300 were shown to function as coactivators for many other transcription factors and may play a central role in many signalling pathways (see below; Janknecht and Hunter, 1996); Shikama *et al*., 1997

SRC-1 and p/CIP encode histone acetyl transferases (HATs) and they are capable of recruiting P/CAF, another histone acetyl transferase (Spencer *et al*., 1997; Yang *et al*., 1996). Therefore, they play a role in chromatin remodelling by acetylating histones. They may also play a role in the recruitment of the basal transcription machinery since CBP has been shown to interact with TFIIB (Parker *et al*., 1998)

1.3.2. Regulation of NIS Transcription in Thyroid Gland

Thyroid stimulating hormone (TSH) is the main hormonal regulator of thyroid function overall. This hormone is a glycoprotein of 30 kDa which is synthesized in the adenohypophysis by basophilic cells known as thyrotropes. Most actions of TSH take place through activation of adenylate cyclase via the GTP binding protein Gα (Vassart *et al*., 1995). The interaction of TSH with its receptor (TSHr) is necessary for the initiation of TSH related events in thyrocytes. Early observations made before the isolation of NIS cDNA suggested that TSH stimulation of thyroid I^- accumulation results from the cAMP mediated increased biosynthesis of NIS (Kaminsky *et al*., 1994). Later, using high affinity anti-NIS antibody, Levy *et al*. (1997) demonstrated that in rats NIS protein expression is upregulated by TSH *in vivo*. At around the same time, Kogai *et al*. (1997) has shown that TSH induces NIS gene mRNA expression as well. A detailed molecular mechanism leading to TSH regulation of NIS gene in thyrocytes was later proposed by Ohno *et al*. (1999) (see below).

A through caracterization of the upstream enhancer of the rat thyroid NIS gene revealed that the regulatory region contains a basal promoter between –564 and –2 bp and an enhancer between –2,264 and –2,495 bp. This enhancer mediates thyroid specific gene expression by its interaction with two transcription factors; namely TTF-1 (Thyroid Transcription Factor-1) and Pax8. TTF-1 is a homeodomain region-containing transcription factor present in the developing thyroid, lung, forebrain and pituitary. Pax8 is an activator which is a member of the murine family of paired domain containing genes, and it is present in thyroid, kidney and developing midbrain. The interaction between Pax8 and the enhancer region of NIS gene is under control of a novel cAMP-dependent pathway (Ohno *et al*., 1999). Together with two Pax8 binding sites, this enhancer region contains two TTF-1 binding sites and a degenerate CRE (5'-TGACGCA-3') sequence that is essential for the transcriptional activity. Both Pax8 and the unidentified CRE-like binding factor act synergistically to obtain full TSH/cAMP-dependent transcription. Interestingly, this enhancer is also able to mediate cAMP dependent transcription by a novel PKA-independent mechanism (Ohno *et al*., 1999).

1.3.3. Regulation of Transcription by Estrogens, Retinoic Acids, and Prolactin

1.3.3.1. Gene Regulation by Estrogens

Estrogens are steroid hormones, which are mainly produced in ovaries, and testes. There are three main forms of estrogens, 17-β-estradiol, estriol, and estrone. 17-β-estradiol is the most potent one among them. Estrogens diffuse into cells through membranes and bind to their intranuclear binding proteins (estrogen receptors) and regulate the expression of target genes by activating these receptors (Gronemeyer and Laudet *et al.* 1995). It is important to note that may also activate MAPK pathway via membrane bound G protein-coupled receptors (Filardo *et al.* 2000)

Estrogens have many effects on many tissues like mammary gland, uterus, vagina, ovary, testis, epididymis, prostate, bone, cardiovascular system, and central nervous system for growth, differentiation and function of these tissues (Clark *et al.* 1992).

ERα and ERβ are the two types of estrogen receptors (ER) which are nuclear receptor superfamily members. ERα was first cloned in 1986 by Green *et al.,* and after 10 years a second ER receptor, ERβ was cloned by Kuiper *et al*. (1996) and Mosselman *et al.* (1996). ERs can be subdivided into several functional domains

(Beato *et al.* 1995). A/B-region is highly variable in sequence and length, and usually contains a transactivation domain, which activates target genes by interacting with components of the core transcriptional machinery. C-region is DNA binding domain, and contains two zinc fingers which are important for specific DNA binding, and receptor dimerization. D-region is hinge domain, which gives flexibility to the receptors. E-region is ligand binding domain, and has role in ligand binding, receptor dimerization, nuclear localization, and interactions with transcriptional co-activators, and co-repressors. F-region is C-terminal extension domain and it contributes to the transactivation capacity of the receptor.

N C

A/B	C	D	E	F
Transactivation	DNA binding	Hinge domain	ligand binding	transactivation

Rat ERα and ERβ have %55 amino acid identity in their ligand binding domains, and %95 amino acid identity in their DNA binding domains. Two receptors' ligand binding affinities, and specifities change from ligand to ligand (Barkhem *et al.* 1998). While two receptors have similar affinities to many ligands, 17-β-estradiol, 17-α-ethynyl are ERα selective agonist, and 16-β, and 17-β-epiestriol are ERβ selective agonists. Additionally, two receptors responsed differentially to some antagonists. For example, tamoxifen, 4-OH-tamoxifen, raloxifene, and ICI 164,384 have an ERα-selective partial agonist/antagonist function, but they are pure antagonists for ERβ (Barkhem *et al.* 1998). While they have some differences in binding and activation potencies to different ligands, both receptors have similar activating potencies at estrogen responsive element (ERE) sites .

Beside low homology in amino acid sequence, and ligand binding affinities of these two estrogen types, their tissue distribution is also different. In rat, while ERα is mostly expressed in mammary gland, uterus, testis, pituitary, overy, kidney, epididymis, and adrenal , ERβ has a higher expression in mammary gland, prostate, ovary, lung, bladder, brain, and epididymis (Kuiper *et al.*1996b).

1.3.3.1.1 Transcriptional Activation Pathways of Estrogen Receptor

It has been reported by Pettersson *et al.* in 1997 that ERα, and ERβ can form heterodimers in solution, or while they are bound to DNA. Heterodimer formation is greatly enhanced by the presence of a ligand.

ERs use two mechanisms to activate target genes upon ligand binding. The first, and well known one is via binding to estrogen response elements (ERE) on promoters of target genes by its AF-2 domain, and the second one is via interacting with AP-1 sites or jun/fos proteins on AP-1 sites by its AF-1 domain.

An example to AF-1 domain activation of ERα, is tamoxifen partial agonism. Tamoxifen is one of the antagonists of ERα, and used as a chemo preventive agent for high breast cancer risk patients, and as a therapeutically agent in chemotherapy of breast cancer patients. But it was observed that tamoxifen therapy could cause endometrium, and uterus cancer (Kedar *et al.* 1994). Tamoxifen aganonism is defined first by Berry *et al.* in 1990, showing that tamoxifen binds to ER and allows ER to bind DNA. Then, AF-1 domain of ER and the target promoters get close to each other. In spite of weak transcriptional activity of AF-1, it could activate target genes in three situations, cell type specificity, strong promoter specific activity, and MAPKK phosphorylation of AF-1 domain (Ali *et al.* 1993). Also ERβ possess a site in its amino terminus that is regulated by MAP kinases (Tremblay *et al.* 1997).

Some genes that ER/AP1 pathway regulate are Insulin-like growth factor I, matrix metalloproteases, which have roles in cancer formation, and metastasis. ER action at AP1 responsive reporter genes correlate with tamoxifen, and estrogen effects on growth, since tamoxifen, and estrogen activate target genes by different mechanisms in cell type-specific manner via ER.

1.3.3.2. Gene Regulation by Retinoic Acids

Retinoids are both naturally occuring or synthetic vitamin A metabolites and analogs. Retinoids are essential for embryonic development, vision, reproduction, bone formation, metabolism, hematopoiesis, differentiation, proliferation and apoptosis (Gudas *et al.,* 1994; DeLuca *et al.,* 1991; Lotan *et al.,* 1995; Nagy *et al*., 1998).

Retinoids bind to their receptors, and activate them to promote cell growth, differentiation, and apoptosis. Retinoid receptors belong to nuclear hormone receptor superfamily. These recptors have ligand binding domain (LBD) which is approximately 225 a.a., and DNA binding domain (DBD) which is approximately 66 a.a. (Giguere *et al.* 1994). There are two types of retinoic acid receptors. These are RAR, and RXR receptors which are are activated by different ligands, while RARs are activated by both 9-cis, and all-trans retinoic acid (ATRA), RXR is activated by

only 9-cis RA. RARs, and RXRs are encoded by different genes and each type contains 3 subtypes α, β, and γ.

Retinoic acid receptors activate target genes upon ligand binding, in dimerized forms. They can either form homodimers, or heterodimers with each other. RXRs can also for dimmers with other nuclear receptors like thyroid hormone receptor. In the absence of ligand, retinoic acid receptors are bounded to co-repressor proteins like N-CoR or SMRT, mSin3, and histone deacetylases and they stay in an inactive state (Alland *et al.* 1997; Heinzel *et. al*., 1997; Nagy *et al.,* 1997). Upon ligand binding, their conformation changes, and they bind to co-activator proteins like CBP/p300, and ACTR in order to mediate gene transcription (Freedman *et al.* 1999).

1.3.3.3 Gene Regulation by Prolactin

Prolactin (PRL) is an anterior pituitary hormone. It binds and activates prolactin receptor (PRLR) which belongs to a cytokine class-1 receptor superfamily (Taga *et al.* 1992). Rat PRLR has 3 main isoforms. These isoforms are short, intermediate, and long isoforms which are coded by alternatively spliced forms of PRLR transcripts(Goffin *et al.* 1996). In humans, a soluble PRLR was also identified, named as PRL binding protein (PRLbp) which does not have transmembrane, and cytoplasmic domain (Fuh *et al.* 1995).

Membrane-bound PRLRs contain three main domains, extracellular domain (ECD), transmembrane domain, and cytoplasmic domain (CD). ECD is composed of cytokine receptor homology region (CRH) which can be subdivided into D1, and D2 domains, each showing analogies with the fibronectin type III module (Bazan *et al.* 1990). PRL binding to its receptors is primarily driven by these conserved fibronectin type modules. Transmembrane domain contains 24 a.a. in rat PRLRs, and its functional activity is unknown yet. Intracellular domain of PRLR differs in isoforms of PRLR. In all isoforms of rat PRLRs, box1 region, which is membrane proximal region, is found. Box1 includes a 8 a.a. region which is enriched in prolines and hydrophobic residues, that are important for Janus kinase 2 (Jak2) association and activation (Goupille *et al.* 1997). Jak2 subsequently phosphorylates PRLR at tyrosine residues. After phosphorylation, PRLR interacts with SH2 domains of STAT (Signal Transduction and Activation of Transcription); molecules which mediate cytokine and growth factor induced signals; and mediates STAT

phosphorylation, and thus activation. Phospho-STATs dimerize, enter to nucleus, and activates target genes which includes GAS (γ-IFN-activated sequence) sequences in their upstream regulatory regions.

Beside Jak2 pathway, PRLR also activates MAPK pathway, Src kinases, IRS-1, SHP-2, PLC γ, PKC and intracellular Ca^{+2} (Bole-Feysot *et al*. 1998).

1.4. Aim of Study

Recent identification of NIS protein in the lactating mammary gland opened the path to many interesting studies. Some of the hormones that regulate NIS in mammary gland (mgNIS) were identified in previous studies (Cho *et al*., 2000; Tazebay *et al*., 2000; Rillema *et al*., 2000). One question that still remains without an answer is the molecular determinants of mgNIS regulation. Which transcription factors regulate NIS expression in the mammary gland? Which *cis*-acting enhancer elements located in NIS upstream regulatory region interact with regulatory proteins? How does the chromatin structure in NIS regulatory region changes upon activation of this gene?

Some of the previously published studies indicated that estrogens may play a role in mgNIS regulation (Tazebay *et al*., 2000; Cho *et al*., 2000; Kogai *et al*., 2001). Yet, clear evidences showing the role of estrogen receptors in regulation of mgNIS are missing. Similarly, PRL was shown to be a hormone that upregulates mgNIS protein expression and iodide transport activity in various experimental settings, both *in vivo* and *in vitro* (Tazebay *et al*., 2000; Rillema *et al*., 2000). In this Master's thesis project, we aimed to inquire 1) the contribution of estrogens to mgNIS regulation; 2) a possible role of human estrogen receptors α and β, as well as the prolactin receptor (PRLR) in mgNIS regulation in human mammary tumor cell lines *in vitro*. Insofar as NIS is functionally expressed to a sufficient degree in cancerous cells, whether of thyroid, breast, or any other origin, radioiodide emerges as a potential diagnostic and therapeutic tool. A considerable amount of work has already been carried out concerning transcriptional regulation of NIS in thyroid gland (Magliano *et al*., 2000). Yet, molecular determinants of mammary gland NIS transcription are entirely unknown. Therefore, besides its scientific impact, an extensive study of *cis*- and *trans*-acting factors regulating the NIS gene in mammary gland might prove extremely valuable and informative for the efforts of establishing novel diagnostic and/or therapeutic protocols against the breast cancer.

2.MATERIALS AND METHODS

2.1. Bacterium Strain

The bacterial strain used in this study is DH5α which is a kind gift from Dr. Uğur Yavuzer from Department of Molecular Biology in Bilkent University.

2.2. Growth And Maintenance Of Bacteria

Bacterial strains were stored as glycerol stock at –70 ºC. Glycerol stock was prepared by mixing 500 µl overnight grown cultures with 500 µl sterile glycerol, and keptat–70 ºC. Bacteria were recovered by growing some amount of stock in LB with an appropriate antibiotic at 37 ºC for 18 hours by shaking at approximately 190 rpm.

To grow bacteria on a solid medium, bacteria were spread onto LB agar, which contains appropriate amount of antibiotic.

2.3. Mammalian Cells

We used MCF-7, and MDA-MB-231 breast cancer cell lines, CHO-K1 Chinese hamster ovary cell line, and Hek-293 embryonic cell line. MCF-7, and CHO-K1 was taken from SAP Institute, MDA-MB-231 cell line was a kindly gift from Dr. Işık Yuluğ, and Hek-293 cells were a kindly gift from Dr. Mehmet Öztürk from Department of Molecular Biology and Genetics at Bilkent University

2.4. Oligonucleotides

All oligonucleotides were designed by using primer.exe program (Copyright 1990,91 Scientific & Educational Software), and they were synthesized in Department of Molecular Biology at Bilkent University. All oligonucleotide sequences, and product lengths are given at table1.

Primer Pairs		Primer Sequence
hERα (256 bp)	F R	5’-ATTCGGATCCT-CAAGGAGACTCGCTACTGTGC-3’ 3’-ATCTCTCGAG-CATTCTCCCTCCTCTTCGGTC-3’
hNIS (602 bp)	F R	5’CTCATCCTGAACCAAGTGAC-3’ 5’-GTGCTGAGGGTGCCACTGTA-3’
hGAPDH (142 bp)	F R	5’-GGCTGAGAACGGGAAGCTTGTCAT-3’ 5’-CAGCCTTCTCCATGGTGGTGAAGA-3’
hPS2 (389bp)	F R	5’-CCATGGAGAACAAGGTGATCTGC-3’ 3’-GTCAATCTGTGTTGTGAGCCGAG-3’
rPRLR (1990 bp)	F R	5’-GATCCCGGAATTCAGTGCACAGCCTCTGGTATG GC-3’ 3’-AAGGAAAAAAGCGGCCGCGTGAAAGGAGTGCA TGAAGC-3’

Table 1. The oligonucleotide sequences used in cloning and PCR experiments.

2.5. Standard Solutions and Buffers

LB Medium:

10 g bacto-tryptone

5 g yeast extract

10 g NaCl

50X TAE Buffer:

2 M Tris Base

57.1 ml Glacial Acetic Acid

50 mM EDTA

10 X Phosphate-buffered Saline (PBS):

80 g NaCl

2 g KCl

11.5 g Na2HPO4.7H2O

2 g KH2PO4o

2X SDS PAGE Loading Buffer:

1 ml 0.5 M Tris-HCl pH 6.8

0.8 ml glyserol

1.6 ml 10% SDS

0.4 ml 2-Beta-MercaptoEthanol

0.4 ml 0.05% Bromophenol Blue

3.8 ml ddH2O

Transfer Buffer:

25 mM Tris

192 mM Glysine

10% Methanol

1X TE:

10 mM Tris, pH 8.0

1 mM EDTA, pH 8.0

Bradford Solution:

Stock solution (15 ml)	Working Solution (100 ml)
5 ml 95% Ethanol	85.15 ml ddH2O
10 ml 85% Phosphoric Acid	2.85 ml 95% Ethanol

17.5 mg Coomassie Brilliant Blue 3 ml Phosphoric Acid

3 ml Bradford stock

5X Tris-Glycine Electrophoresis Buffer:

22.5 g Tris

108 g Glycine

7.5 g SDS

Adjust volume to 1 L

TBS:

100 mM Tris (pH 7.5)

500 mM NaCl

2.6. Recombinant DNA Techniques:

2.6.1. Polymerase Chain Reaction

Polymerase chain reaction (PCR), is a rapid procedure for in vitro enzymatic amplification of specific sequence of DNA (Mullis and Faloona et al. 1987), was performed to amplify the partial coding sequences of hER-α, hPS2, hGAPDH, and hNIS genes, and complete mRNA coding region of rPRLR gene. PCR reactions were all performed in 0.2 ml Thermowell^{TM} tubes (Corning Costar Corp.) using the GeneAmp PCR system 9600 (Perkin Elmer).

All PCR reactions were performed in final volume of 25 µl, containing 1-2 ng of template genomic DNA, or 1 µl cDNA , 1X PCR buffer (MBI Fermentas), 1.5 mM $MgCl_2$ (MBI Fermentas), 0.2 mM of each dNTP (MBI Fermentas), 10 pmol of each primer except hGAPDH primers which were used 5 pmol, and 1 unit Taq DNA polymerase (MBI Fermentas).

The reaction was preheated to 94 ºC for 5 minutes, and then subjected to 30 cyles of denaturation (30 seconds at 95 ºC for all except rPRLR, it was 40 seconds for rPRLR), annealing (40 seconds at 60 ºC for hNIS, hGAPDH, hPS2primers, 40 seconds at 50 ºC for ER-α, and 30 seconds at 58 ºC .), and elongation (at 70 ºC for 40 seconds for hNIS, hGAPDH, hPS2, hER-α, and 50 seconds for rPRLR primers). At the end of 30 cycles a final extension at 72 ºC for 10 minutes was also applied.

Agarose gel electrophoresis and Ethidium Bromide (EtBr) staining assessed 20 µl of the PCR products.

2.6.2.Semi-quantitative PCR

Semi-quantitative PCR is a suitable method for comparing the relative amount of the used templates between different samples. Normally, the PCR reaction does not have a linear graph for the product amount versus cycle number. In algebraic terms, it is the graph of the combination of two functions. This is due to the saturation of the PCR. In order to have a dependable comparison, the cycle number, in which the PCR reaction is not saturated, should be determined for each gene. This was done by performing the PCR reaction for different cycles for each gene, and drawing a graph from the intensity of the DNA bands assessed by agarose gel electrophoresis. Optimized cycle numbers for pS2 was 23, for GAPDH was 19, and for NIS was 27.

2.6.3. Purification of DNA Fragments by Agarose Gel Electrophoresis

DNA purification from agarose gel is performed by Qiagen Gel Purification Kit according to manufacturer's manual after running the PCR products on agarose gel, and cutting the desired band from agarose gel.

2.6.4. Restriction Enzyme Digestion of DNA

1-5 µg DNA was digested with appropriate amount of buffer and units of enzyme in a 20 ml final volume according to the manufacturer's recommendations at 16 ºC for overnight.

2.6.5. DNA Ligation

DNA fragments were ligated with plasmid vectors according to the protocol described in Molecular Cloning (Maniatis et al. 1982). Prior to ligation vector and insert concentrations are checked by agarose gel electrophoresis. For directional cloning, vector:insert ratio was kept close to 1 in the ligation reactions. Ligations were performed in 15 µl reaction volumes containing approximately 0.1-0.2 µg of plasmid DNA, and the corresponding amount of insert in the presence of 4 Weiss units of T4 DNA ligase, 1mM ATP, and standard ligation buffer, supplied by the manufacturer. The reaction was performed at 16 ºC for overnight.

2.6.6. Plasmids

Plasmids that are used in this study are, ERα-pSG5puro,ERE-bglob-Luc . were kindly provided by Dr. Ediz Demirpence, and pcDNA3.1c was kindly provided by Dr. Uğur Yavuzer.

2.6.7. Recombinant Expression Constructs

rPRLR-pcDNA-3.myc-his

Erα-pcDNA3.1c

2.7. Preparation Of Competent Cells And Transformation Of *E.coli*

2.7.1. Simple and Efficient Method (SEM)

A single colony of DH5α was inoculated into 15 ml of LB, and grown overnight. The starter culture was diluted to an $O.D._{600}$ of 0.2-0.3 in 250 ml of SOB medium (2% Bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCL, 10 mM $MgCl_2$, 10 mM $MgSO_4$, sterilized through autoclaving), and grown to an OD600 of 0.6 at 18 ºC with shaking at 200 rpm. The culture was chilled on ice for 10 minutes, centrifuged at 2500 g for 10 minutes at 4 ºC. The pellet was then resuspended in 80 ml of ice-cold TB (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCL, pH 6.7, sterilized by filtration through 0.45 um filter, stored at 4ºC), and incubated on ice for 10 minutes. The mixture was precipitated as above, resuspended gently in 20 ml of TB. DMSO was added to a final concentration of 7%, mixed gently and incubated on ice for 10 minutes. 500 µl aliquots of this mixture were then immediately frozen in liquid nitrogen, and stored at –70 ºC up to 3 months without loss of transformation efficiency (Inoue et al. 1990)

2.7.2. Transformation of *E.coli*

With super-competent cells (look to 7.1), 0.1 µg plasmid DNA is sufficient for high transformation efficiency. 0.1-0.5 µg of plasmid DNA was mixed with 200 µl supercompetent cells, and incubated on ice for half an hour in a 15 ml round bottom tube (Greiner Labortechnik). Then the cells were heat shocked at 42 ºC for 30 seconds, chilled on ice for 2 minutes, and then grown at 37 ºC for 1 hour at 200 rpm, after adding 0.8 ml of SOC (SOB with 20 mM glucose) onto them . After 1 hour, 50 µl of culture was spread onto LB agar plates containing appropriate antibiotics. (Inoue et al. 1990)

2.8. Plasmid DNA Isolation

2.8.1. Small Scale Plasmid DNA Isolation

Single colonies were picked up from LB plate, and inoculated in LB with appropriate antibiotic in an 15 ml falcon tube. Culture was grown overnight at 37 ºC with shaking at 160 rpm. 1.5 ml culture was taken into an eppendorf tube andcells were precipitated. Supernatant was discarded, and the pellet was dissolved in 150 µl Solution I (50 mM Glucose; 25 mM Tris.Cl pH8.0; and 10 mM EDTA pH8.0) by vortex. Freshly prepared 200 µl Solution II (0.2 N NaOH; 1% SDS) was added immediately and mixed by inversion for 5 times. 150 µl Solution III (60 ml 5M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml H2O=100ml) was added immediately and mixed by inversion 5 times. The mixture was centrifuged for 5 minutes at 16,249 xg, and the supernatant was transferred to a clean eppendorf tube. 400 µl phenol:chlorophorm (24:1) mixture was added and vortexed for 1 minutes and centrifuged for 5min at 16,249 xg. The upper layer was transferred to a new tube and 800 µl ice cold absolute ethanol was added and mixed by inversion. Mixture was centrifuged at 18,845 xg for 20 minutes at 4ºC. The EtOH was removed and the pellet was washed with 70% EtOH and dried at RT. The pellet was dissolved in appropriate amount of water.

2.8.2. Medium Scale Plasmid DNA Isolation

Positive bacteria colony which was screened after miniprep was grown in 100 ml LB with appropriate amount of antibiotic overnight. To perform midiprep, the Nucleobond AX-100 kit (Macherey-Nagel) was used. The culture was spinned down at 6000 rpm for 3 minutes in 50 ml falcons. Supernatant was discarded and cell pellet was dissolved with 4 ml S1 buffer. 4 ml S2 was added and mixed by inversion for 5 times. Immediately 4 ml S3 buffer was added and mixed by inversion, and filtered by filter paper immediately. After equilibration of the cartridges with 2.5 N2 buffer, filtered suspension was loaded onto cartridge. After elution, cartridge was washed with 5 ml N3 buffer 2 times. The plasmid DNA was eluted with 5 ml N5 buffer in a clean polypropylene centrifuge tube. 3.6 ml isopropanol was added and mixed. Centrifugation was performed by centrifuge for 30 min at 20,000 g. The supernatant was discarded, and the plasmid DNA pellet was washed with 70% (ethanol) EtOH

for 3 times. The pellet was dried and dissolved in an appropriate amount of sterile ddH_2O.

2.8.3. Spectrophotometric Quantification of DNA

5 µl of each sample was diluted in 1:200 ratio with sterile ddH_2O. With spectrophotometer (Beckman), O.D. measurements were done at 260 and 280 nm. O.D. 260/ O.D.280 ratios were calculated inorder to understand if there was any protein contamination. The expected ratio was between 1.6-2.0.

2.9. Cell Culture Techniques

2.9.1. Thawing a frozen Cell Line

Frozen cell line stock was transferred from liquid nitrogen tank to 4 ºC, and taken immediately to 37ºC water bath. After it was completely dissolved, it was diluted in growing medium, and centrifuged for 4 minutes at 400 rpm. After centrifugation the supernatant was aspirated, and the cell pellet was gently resuspended with growing medium, and plated on desired plate, or flask. The culture was incubated at 37ºC, in a humidified chamber with 5% CO_2.

2.9.2. Sub-Culturing of Monolayer Cells

After cells reached to 80-90 % confluency, they were subcultured into new plates, or flasks. Medium of cells was aspirated, and then cells were washed with 1X PBS two times before putting 1 ml trypsin-EDTA to 10 cm plates. Cells were incubated with trypsin-EDTA at 37 ºC for 3 minutes, and after all were detached, they were mixed with 5 ml growing medium, and split into fresh plates or flasks at desired ratio, and were grown at 37 ºC humidified chamber with 5% CO_2.

2.9.3. Cryopreservation

Cells were detached from the plates by trypsin as told above, and after this step, cells were collected in a falcon tube, and centrifuged at 400 rpm for 4 minutes. After that the supernatant was aspirated , and the cell pellet was dissolved in frozen medium in a 5 millions of cells/1 ml frozen medium ratio. Then this mixture was put into cryotubes, and were frozen overnight at –80 ºC from where transferred into liquid nitrogen (Doyle and Griffiths et al. 1997).

2.9.4. Transient Transfections

Two transfection methods were used.

2.9.4.1. Electroporation

Cells were splitted 18 hours prior to transfection. The confluence of cells were 90% at the time of transfection. Cells were trypsinized, collected and counted. Appropriate amount of cells 15 $X10^6$/cuvette centrifuged at 400 g for 5 minutes. Medium was aspirated, and cells were washed with 10 ml Ca^{+2}, Mg^{+2} free 1X PBS, and centrifuged at 400 g for 5 minutes. Washing was repeated once more. Cell pellet was dissolved in appropriate amount of Ca^{+2}, Mg^{+2} free 1X PBS (800 ml/cuvette). 800 ml cell suspension was put into an ice cold electrophorese cuvette. 40 μg plasmid DNA was added onto the cells, mixed, and incubated on ice for 5 min. The cuvette was placed into the electrophoresis machine, and electric pulse was given for 18-22 seconds, at 220 mV, and 950 μF setted apparatus. Cuvettes were incubated on ice for 5 min, and transfected cells were plated onto two 15 cm plates.

2.9.4.2. $CaCl_2$ Transfection

Cells were splitted 18 hours prior to transfection, and the confluency was between 50-70% at the time of transfection. 20 μg plasmid DNA was mixed with sterile ddH2O to a final volume of 450 μl in a 15 ml falcon tube. During vortexing the falcon, 50 μl 2.5 M $CaCl_2$ was added drop by drop. The mixture was incubated for 30 min at RT. 500 μl of BES pH 6.95 (for some cell lines pH optimization was required) was added during vortexing the mixture in falcon. The final solution was incubated at RT for 40 min, and 1 ml from this solution was added onto cells with 9 ml medium drop by drop. 12-18 hours after transfection cells were washed with 1X PBS for 3 times and fresh medium was added.

2.9.5. Stable Transfection

72 hours after transfection of mammalian cell lines with 10% confluency, antibiotic selection was started. After 2-3 weeks of selection colonies were formed, and with sterile pipette tips they were picked up and plated onto 96 well plates. Then they were plated onto 24 well plates, 12 well plates, 6 well plates, and 10 cm plates

respectively. They were frozen immediately after protein isolation from each colony. By western blotting each colony was screened for positive expression of desired exogenous gene.

2.9.6. Cell Treatments

2.9.6.1. Culturing Cells in Media

Minimal Media: MM, DMEM without phenol red containing 3% charcoal treated FBS, and 1% P/S

Normal growth media: NGM,DMEM with phenol red containing 10% FBS, and 1% P/S

Cells were grown in minimal medium, which is DMEM without phenol red containing 3% charcoal treated FBS(fetal bovine serum), and 1% P/S (penicillin/ streptomycin), for 1-2 weeks to get rid of the steroids and phenol red in media, and in cells.

2.9.6.2. Treatment of Cells with Various Substances

Cells were treated with various substances. All the substances were powder, and dissolved in 95% (ethanol) EtOH as a 10^{-2} M stock solutions. The stocks were diluted into final concentrations, by diluting them in medium.

2.10. Gel Electrophoresis

2.10.1. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To separate proteins with different masses in denaturing conditions by electrophoresis SDS-page is used according to the following method described in Current Protocols (Ausucel 1987). All SDS-pages in this study was prepared by using Thermo EC mini vertical GEL system. The apparatus was prepared according to the instructions described by manufacture. 10% resolving gel was used, and both resolving gel and stacking gel is prepared by 30% acrylamide stock solution. 10 ml 10% resolving gel is prepared by mixing 5 ml 30% acrylamide (29,2% acrylamide, 0.8% bisacrylamide), 2.5 ml 1.5 M Tris pH 8.8, 50 µl 20% SDS, 100 µl 10% ammonium persulfate, and 4 µl Temed, and poured into the gap between glass plates, and immediately iso-propanol was poured onto resolving gel to give a sharp edge. After polymerization 5ml stacking gel was prepared by mixing 0.83 ml 30% acrylamide (29,2% acrylamide, 0.8% bisacrylamide), 0.63 ml 1M Tris pH 6.8, 50 µl 10% SDS, 50 µl 10% ammonium persulfate, and 5 µl Temed, and poured onto

washed resolving gel with ddH2O. And immediately avoiding not forming bubbles the comb was placed. After polymerization comb was taken out, and the wells were washed with running buffer to get rid off unpolymerized gel.
Protein samples with appropriate amount (30-40 μg), are mixed with 5X SDS loading buffer and incubated in boiling water for 5 min, or at 70 ºC for 10 min. These samples were incubated on ice for 5 min and loaded onto gel. Gel was run at 80 V until samples run to resolving gel, after the voltage was increased to 100 V. After running proteins were transferred onto an appropriate membrane.

2.11. Biochemical Techniques

2.11.1. RNA Isolation From Mammalian Tissue Culture Cell Lines

RNA isolation from cells was performed by acid guanidinium thiocynate-phenol-chloroform extraction method . 0.1 g tissue was homogenized with 1 ml solution D (4 M guanidinium thiocyanate; 25 mM sodium citrate pH 7; 0.5% sarcosyl; 0.1 M 2-mercaptoethanol)(cells in 10 cm plate was lysed with 1 ml solution D). 0.1 ml 2M sodium acetate pH 4 was added, mixed by inversion, then 1 ml phenol was added and vortexed. 0,2 ml chloroform-isoamyl alcohol (49:1) was added, vortexed and incubated on ice for 15 minutes. The mixture was centrifuged at 10,000 g for 20 min at 4 ºC. Aqueous layer was transferred into new tube, and mixed with 1 ml isopropanol and incubated at –20 ºC for 1 hour. The mixture was centrifuged at 10,000 g for 20 min at 4 ºC, and supernatant was removed. The RNA pellet was washed with 70% (ethanol) EtOH for 3 times, and dried at RT. The pellet was dissolved in DEPC treated ddH2O

2.11.1.1. RNA quantification

5 μl of each sample was diluted in 1:200 ratio with sterile ddH2O, and with spectrophotometer (Beckman) O.D. measurements were done at 260 and 280 nm.

Concentration of RNA was calculated with this formula:

[RNA]=O.D.260Xdilution factor(200)X40

O.D. 260/ O.D.280 ratios were calculated in order to understand if there was any protein contamination. The expected ratio was between 1.6-2.0.

2.11.1.2. RT-PCR:

The cDNA samples were synthesized from the total RNA samples with the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas) by using the manufacturer's protocol.

5 µg of RNA was mixed with 1 µl random hexamer primer, and mixed with appropriate amount of deinozed water in a final volume of 12 µl. The mixture was incubated at 70 ºC for 5 minutes, and chilled on ice. After quick spin, 4 µl of 5X reaction buffer, 1 µl of RNase inhibitor, 2 µl of 10 mM dNTP mix was added respectively and incubated at 25 ºC for 5 min, and 1 µl of RevertAid M-MuLV reverse transcriptase was added and incubated at 42 ºC for 1 hour. Incubating the mixture at 70 ºC for 10 minutes, the reaction was stopped.

2.11.2. Protein Isolation from Whole Cell Extracts

2.11.2.1 Eukaryotic Cell Lysis

After aspirating medium, cells were washed with cold 1X PBS two times. 1 ml cold 1X PBS was put onto cells, and on ice all cells were scraped, and cell suspension was collected into 1.5 ml eppendorf tube. Cells were precipitated at 4 ºC. PBS was aspirated and cells were frozen in liquid nitrogen and stored at –80 ºC. 4X w/m lysis buffer was put onto cells, the cell pellet was dissolved by gentle mixing, and waited on ice for 30 minutes. Mixture is mixed gently by 10 minute periods. Mixture was centrifuged at 116,249 xg for 20 minutes 4 ºC. Supernatant was aliquoted as total protein sample.

2.11.2.2. Bradford Assay

Protein concentration was determined by Bradford assay. Bovine serum albumin (BSA) was used as protein standard and the standard curve was calculated by taking OD595 of different BSA concentrations from 2,5 µg/ml to 20 µg/ml mixed with Bradford working solution. 5 µl of protein samples were mixed with Bradford working solutions and OD595 was measured and concentration of these protein samples was calculated by standard BSA curve.

2.11.3. Immunological Detection of Immobilized Proteins

2.11.3.1. Transfer of Proteins onto Membranes

Semi-dry transfer of proteins from SDS-PAGE gel to PVDF membrane was performed by semi-dry transfer machine. Whatman papers and membrane was prepared by cutting them on the same size as the resolving part of gel. Membrane was waited in methanol for 30 sec, then washed with transfer buffer and incubated with whatman papers in transfer buffer for 5 min. 3 whatman papers were placed on the transfer apparatus, onto them the nitrocellulose membrane was placed, and then the gel was placed and 3 whatman papers were placed on the gel. Bubbles should not be formed, to avoid this a cylinder was used. After closing the apparatus the transfer was obtained at 12 V for 30 min. If 2 gels were used , then 50 min should be applied.

2.11.3.2. Detection of Immobilized Proteins

After transfer of proteins to nitrocellulose membrane, membrane was incubated in blocking solution (TBS-T, 3% dried milk) for 1 hour at room temperature or overnight at 4 ºC. Then membrane was washed with TBS-T for 5 minutes at RT three times. After wash, membrane was incubated with 1st antibody, which was diluted in blocking solution (1:1000 dilution for c-myc, 1:500 dilution for ER-α, and 1:100 dilution for ER-β) for 1 hour at RT, or overnight at 4 ºC. Membrane was washed with TBS-T for 5 minutes at RT three times. And the membrane was incubated with 2nd antibody which is diluted in blocking solution (3% dried milk in TBS-T) at RT for 1 hour . The membrane was washed with TBS-T for 5 minutes three times. Using Amersham LIFE SCIENCE ECL Western blotting detection reagents according to the manufacturer's instructions carried out the immunodetection with HRP conjugated secondary antibody. The bands were visualized by exposing the membrane on photograph films.

2.11.4. Luciferase Assay

$5X10^5$ cells were plated on 6 well plates 18 h prior to transfection. Transfection was performed by calcium phosphate method (Look to 9.4.2.). 20 µg plasmid DNA was transfected to the cells in each well. 24 h after transfection, cells were treated with desired substances (17-est in our case) for 36 hours. Luciferase assay was performed according to manufacture's instructions.

2.11.5. Southern Blot

2.11.5.1.Genomic DNA digestion and membrane blotting

Genomic DNAs (8 μg) were digested with 4 units of BglII overnight at 37°C in a final volume of 50 μl. Then the digests were run on a 1% agarose gel in TAE buffer. Running is performed at 50V for 7 hours. The gel is then put into the denaturation solution 1.5 M NaCl, 0.5 M NaOH for 30 min. Then it is placed into the renaturation solution, which was 1.5 M NaCl, 0.5 M Tris-HCl pH: 7.2, EDTA 10 mM. The gel is left in this solution for 30 min, with constant shaking at 100 rpm on a bench-top shaker. The gel was carefully put on the whatman paper and the area covering the gel is surrounded by saran wrap to avoid leaks from this region due to capillary forces. A nylon membrane (Hybond-N, Amersham) of precisely the size of the gel was put on top of the gel and covered with two pieces of 3MM whatman paper and enough tissue papers on top. The stack was pressed with a book of around 500g on a glass plate. After disassembly of the stack, the positions of the wells on the membrane were marked with a pencil. Upper left edge of the membrane was cut off to allow easy orientation. The membrane is the removed, and air dried for 10 minutes. Then the DNA is fixed on membrane by U.V. using a Stratalinker (Stratagene, CA) apparatus.

2.11.5.2. Labelling the DNA probe

A DNA fragment corresponding to the EcoRI-BglII fragment (1286 bp) of human ERα gene is cut and purified from a 0.8% agarose gel. 50 ng of linearized DNA is taken in 20 μl ddH_2O and denatured by incubation in boiling waterbath. The labelling was performed with a Random Prime DNA labelling kit. The reaction carried out by adding 2μl dNTP mix solution (0.5 mM each nucleotide) 2 μl random hexanucleotide solution, 6 μl radioactively labelled nucleotide (α^{32}P-dCTP; specific act. 10 mCi/ml), 10 μl ddH_2O and 1μl Klenow enzyme (2U/μl). The reaction was carried out for 30 min at 37°C. To remove unincorporated nucleotides from the DNA probe, labelled samples was purified using G-50 Sephadex quickSpin™ columns, using the manufacturers instructions. Specific activity of 1μl (out of around 40μl) of probe is measured with a radioactivity counter, and the probe is used in hybridization reaction.

Labelling is done twice. Specific activities were as follows: probe 1: $4,6x10^5$ cpm; probe 2: $5,5x10^5$ cpm. Probe 2 was used in experiment shown in this thesis.

2.11.5.3. Hybridization with a labelled probe

The membrane is prehybridized in a hybond cylinder and oven for 3 hours at 65°C in a buffer containing 0.5 M Na_2HPO_4 pH: 7.0, 1 mM EDTA, 7% SDS, 1% BSA. Then the radioactively labelled probe is denatured in boiling waterbath for 5 min and it is added in prehybridization solution. Hybridization is performed for 14 hours. Then, the membrane is washed once at RT with 2x SSC, 0.2 SDS for 15 min, and twice at 65°C in 0.2x SSC, 0.1 SDS. Afterwards, the membrane is wrapped in saran wrap and exposed to an autoradiography film for overnight.

2.11.6 Semiquantitative PCR optimization

In order to perform semi-quantitative PCR reactions, we first to optimize the cycle numbers for pS2, hNIS, hGAPDH. Seen below.

3.RESULTS

3. RESULTS

3.1. Introduction

Hormonal regulation of mammary gland NIS (mgNIS) was previously studied and the roles of estrogen (17-β-estradiol), oxytocin, prolactin, and progesterone on regulation of mgNIS expression or activity was established (Tazebay *et al*., 2000, Cho *et al*., 2000, and Rillema *et al*., 2000). It is important to state that among the hormones tested in these studies, 17-β-estradiol (17-Est) is the only one that activates mgNIS protein expression in mammary when administered alone to overiectomized virgin mice (Tazebay *et al*., 2000). The effect of oxytocin on mgNIS was surprising: this hormone does not activate mgNIS expression in ovariectomized mice, but it does so in animals that are not surgically modified, i.e. animals with intact ovaries (Tazebay *et al*., 2000, Cho *et al*., 2000). Moreover, a decrease in radioiodide uptake in lactating mammary glands after treatment with an oxytocin inhibitor, and an increase in hNIS mRNA after oxytocin treatment of 3-D histo-cultures of human breast tumors were also demonstrated (Cho *et al*. 2000). Prolactin is another hormone that is shown to activate mgNIS expression when administered together with oxytocin, and estrogen in overiectomized virgin mice (Tazebay *et al*. 2000). Also in cultured mouse mammary tissues, prolactin was shown to stimulate mgNIS expression (Rillema *et al*. 2000).

Above summarized studies have identified several hormones that may contribute to mgNIS regulation in animal models. Next logical step in analysis of mgNIS gene regulation is going to be the identification of *cis*- and *trans*-acting elements responsible of the regulation of this gene in the mammary gland. In this study, we aimed to focus on several candidate *trans*-acting factors, such as estrogen receptors, and candidate signalling pathways such as the prolactin receptor/Stat5 signalling pathway, and to assess their possible contribution to this regulation in *in vitro* conditions.

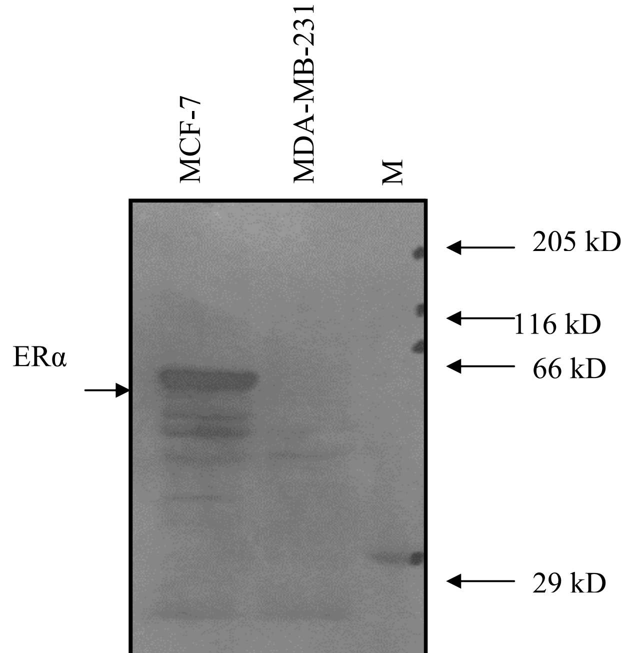


Fig. 1. ERα status of MCF-7, and MDA-MB-231 cell lines. Total protein was isolated from MCF-7 and MDA-MB-231 cell lines and a Western blot analysis was performed using monoclonal anti-human ERα Ab. ERα protein is detected in MCF-7 cell line (indicated with arrowhead), whereas it is not detected in MDA-MB-231 cell lines. M, is a size marker; sizes indicated with arrowheads.

3.2. The effect of retinoic acid on NIS gene expression in MCF-7 human mammary cancer cell line.

Despite a very broad and time consuming search by several groups, only one mammary gland cell line was, so far, identified to express mgNIS mRNA and protein in an inducible manner. This is the estrogen receptor positive [ER(+)] MCF-7 human mammary gland cancer cell line (Kogai *et al.*, 2001). In this cell line, mgNIS was shown to be inducible by retinoic acid (RA), a ligand that was known to induce the iodide transport activity in dedifferentiated thyroid tumor metastatic tissues in humans (Schmutzler and Kohrle, 2000; Kogai *et al.*, 2001). In the study of Kogai *et al.* (2001), mgNIS mRNA levels were highest after treatment with 10^{-6} M RA for 24 hours. The absence of a similar increase in mgNIS mRNA levels in ER(-) MDA-MB-231 cell line after a similar treatment led them propose that the ERs may have a

role in this regulation of the iodide transporter gene under these conditions (Kogai *et al*., 2001). In an effort to study a possible role of ERs in mgNIS regulation, we decided to use the same experimental system, and inquire about the role of these transcription factors (ERs) in mgNIS regulation.

We have started our experiments by checking the ERα status of mammary cell lines used in the study of Kogai *et al*. (2001). This has confirmed the ERα(-) phenotype of the MDA-MB-231 cell line, and the ERα(+) phenotype of the MCF-7 cell line (Fig. 1). Then we have repeated the RA treatment of MCF-7 cell lines, simply to check the reported effect of RA on these cell lines in our laboratory conditions. By RT-PCR, we observed the expression of mgNIS mRNA in MCF-7 cells after 24h, and 48h RA treatments as Kogai *et al*.(2001)(Fig. 2). There were no amplification without RA treatment, similar to what was observed in the study performed by Kogai *et al*. (2001).(Fig. 2.)

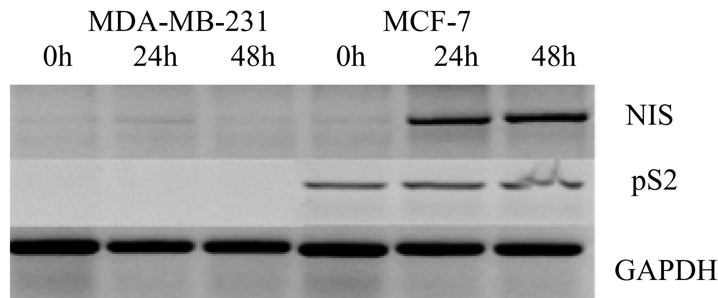
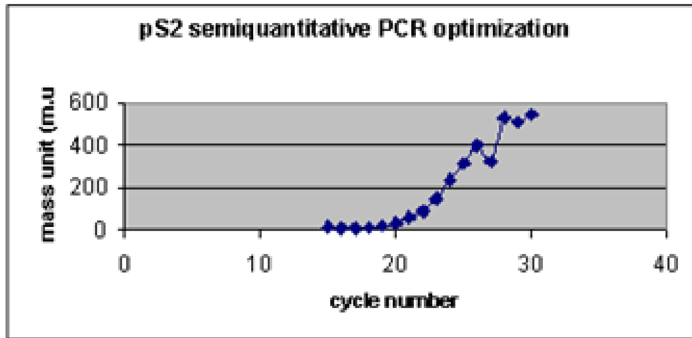


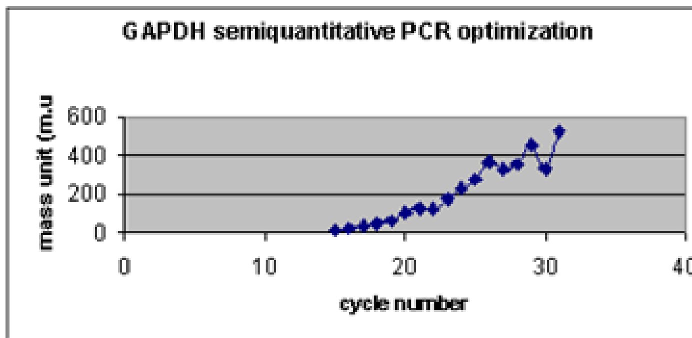
Fig. 2. Response of the mgNIS gene to RA treatment. MDA-MB-231, and MCF-7 cells are treated with 10^{-6} M RA in DMEM growth medium (DMEM (including phenol red) + 10% FBS + 1% P/S) for indicated time periods. RT-PCR is made from total RNA by using hNIS, hGAPDH, and hPS2 primers (see Materials and Methods). House keeping gene GAPDH is used as a control of RT-PCR amplification. PS2 is an estrogen responsive gene of unknown function (Jakowlew et al. 1984), and it is used as a control of ER activity.

3.3. RA treatment of MCF-7 cells grown in minimal media.

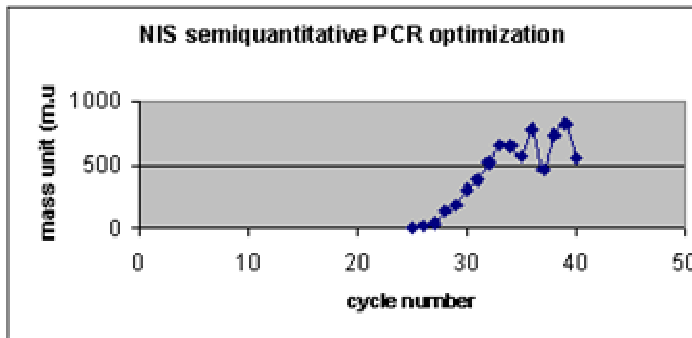
In order to perform semi-quantitative PCR reactions to detect mRNA level differencies, we first optimized the cycle numbers for pS2, hNIS, hGAPDH. Seen below.



Cycle 23 is selected



Cycle 19 is selected



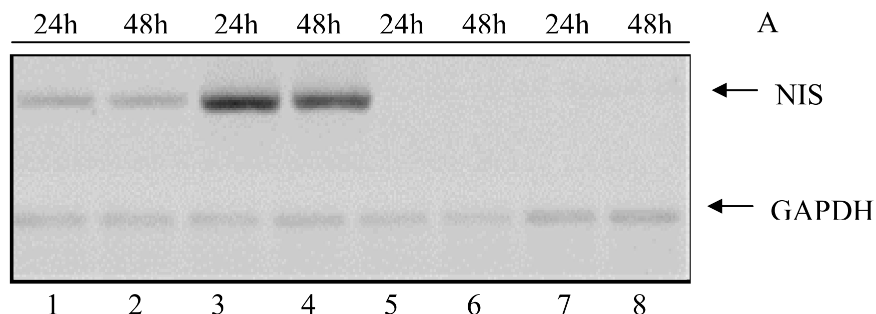
Cycle 27 is selected

Graph. 1. Semi-quantitative optimization of GAPDH, PS2, and NIS primers. For GAPDH, cycle 19, for NIS cycle 27, for pS2 cycle 23 is chosed.

Phenol red that is present in most cell culture medium (and that gives the red color) was shown to have low but significant estrogenic activity. Besides, FBS that is

used to supplement the media may also contain various steroids and other hormones that might affect the response of cultured cells to RA. We have asked the question whether RA by itself is sufficient for mgNIS upregulation, or other additional ligands are essential for, or contribute to, the effect of RA in this regulation. In order to clarify this point, we have grown cells in DMEM medium without phenol red. Charcoal treatment is a well-known method to remove lipidic substances and ligands, such as steroids, from the growth media. Therefore, in addition to using phenol red-free medium, we have treated the FBS that was added into the media with charcoal. We are referring to this supposedly relatively low steroid, or steroid-free media as the "minimal media" (MM) in this text (also see Materials and Methods). After culturing MCF-7 cells in this medium for 1-2 weeks, these cells are treated with 10^{-6} M RA for 24, and 48 hours and we compared mgNIS mRNA levels in MCF-7 cells which are grown in DMEM with phenol red supplemented with 10% FBS (normal growth medium (NGM)) after a similar hormonal treatment. By performing semi-quantitative PCR it is found that, hNIS mRNA level is 2,5 times decreased in minimal media grown RA treated cells as compared to normal (phenol red and untreated 10% FBS containing) media grown RA treated cells (Fig. 3). Besides, there were no significant difference in mgNIS mRNA levels between 24h and 48h RA treatments (Fig. 3).

RA	+	+	+	+	-	-	-	-
NGM	-	-	+	+	-	-	+	+
MM	+	+	-	-	+	+	-	-



B

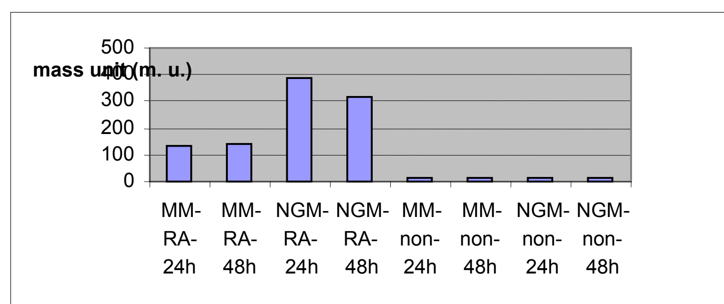


Fig. 3. Comparison of RA treatment of MCF-7 cells grown in minimal media vs. normal media. MCF-7 cells are treated with 10^{-6} M tRA for 24h and 48h. MCF-7 cells which are grown in MM (B) gave 2,5 times decrease in hNIS mRNA level when compared with cells grown in NGM. There is no significant difference between 24h, and 48h treatments. A) Lane 1,2,5,6 are MCF-7 cells grown on MM. Lane 3,4,7,8 are MCF-7 cells grown on NGM. GAPDH is used as control for equalizing cDNA amounts.(mass intensity: intensity/pixel)

This experiment has clearly shown that in the presence of RA, additional factor(s) that are absent in minimal media contribute to mgNIS regulation in MCF-7 cells. Identification of this (or these) factor(s) may lead to important clues about the molecular mechanisms operating for mgNIS regulation under these conditions.

3.4. 17-Est treatment of MCF-7 cells

Bearing in mind that 17-β-estradiol (17-Est) upregulates mgNIS expression when it is administered to overiectomized mice (Tazebay *et al*. 2000; Cho *et al*., 2000), and that the MDA-MB-231 cell line which is ERα(-) does not express mgNIS upon RA treatment, we asked the question whether 17-Est can be one of these additional factors. Thus, we desired to see whether this ligand can induce mgNIS expression in MCF-7 cell lines. Therefore, we treated MCF-7 cells with 10^{-10} M 17-Est for the time periods of 0h, 12h, 24h, and 48h. Then, we assessed the mgNIS gene activity by RT-PCR. In this experiment the MDA-MB-231 cell lines were used as negative control because of their ERα(-) status. As it is seen in Fig. 4, we were not able to detect mgNIS mRNA in MCF-7 cells, while PS2 gene which is under control of ERs was upregulated in MCF-7 cells as compared to MDA-MB-231.

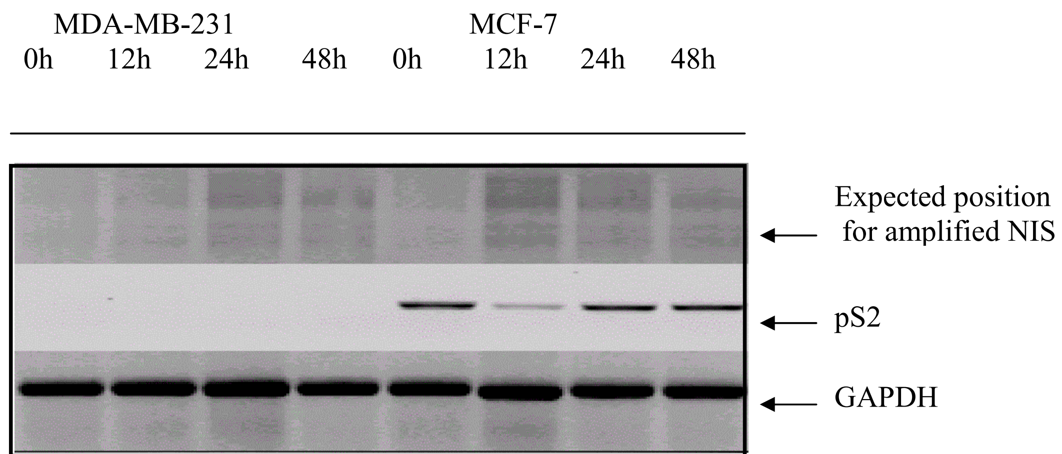


Fig. 4. Effect of an estrogen on mgNIS gene expression in MCF-7 cells. MCF-7, and MDA-MB-231 cells are treated with 10^{-10} M 17-Est in normal growth medium(DMEM wh ph. Red. + 10% FBS + 1% P/S) for 0h, 12h, 24h, and 48h. RT-PCR is made from total RNA by hNIS, hGAPDH, and hPS2 primers. NIS was undetectable in all cDNA samples. GAPDH is used as control to check the integrity of cDNAs. PS2 is an estrogen responsive gene of unknown function (Jakowlew et al. 1984), and it is used as a control of ER activity.

3.5. Dose dependent estrogen treatment of MCF-7 cells grown in steroid free minimal medium.

We were not able to activate NIS expression by treating MCF-7 cells with 10^{-10} M 17-Est for 48 hours (Fig. 4). Then, we treated MCF-7 cells with increasing 17-Est concentrations. In these experiments we have again used the special minimal media composition as described above and in Materials and Methods. It is also important to keep the cells in this estrogen-free special media in order to prevent any interference of intracellular and media-borne estrogens with the administered doses of the same hormone. Accordingly, we have cultured MCF-7 cells for one week in this special media prior to treatment with various concentrations of 17-Est from 10^{-10} to 10^{-7} M. In the same experiment, we also wanted to treat separate groups of MCF-7 cells with same concentrations of 17-Est together with tamoxifen which is an antagonist of both ERα and β (Elgort *et al*., 1996; Zou *et al*., 1999; and references within). These last conditions were added to the experiment thinking that, in case an increase is seen in mgNIS expression in response to higher concentrations of 17-Est, then a negation of this increase in presence of tamoxifen would give an experimental evidence concerning the role of ERs in this upregulation. Accordingly, in a preliminary experiment groups, MCF-7 cells are treated for 24h with these ligands at following concentrations: 10^{-10} M 17-Est + 10^{-8} M tamoxifen; 10^{-9} M 17-Est + 10^{-7} M tamoxifen; 10^{-8} M 17-Est + 10^{-6} M tamoxifen, 10^{-7} M 17-Est + 10^{-5} M tamoxifen. Unfortunately, last 2 groups of cells were dead in a very short time like half an hour. This may be due to the high concentrations of tamoxifen, which might be toxic. As seen as a result of an RT-PCR analyses shown in Fig. 5, very surprisingly, the combination of 17-Est with tamoxifen resulted in induction of mgNIS while there were no induction in 17-Est treated samples.

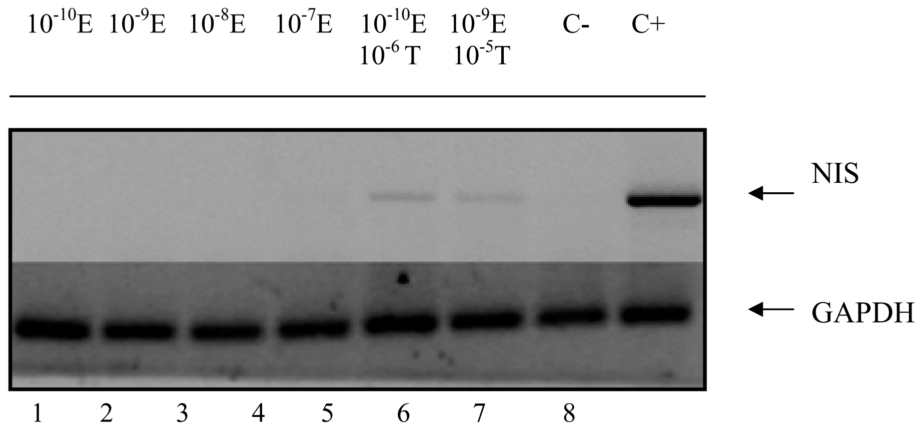


Fig. 5. Effect of increasing concentrations of estrogen on mgNIS gene expression in MCF-7 cells. MCF-7 cells which are grown in MM (see text) are treated with 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M 17-Est (indicated as E in the Fig.), and combinations of 10^{-10} M 17-Est plus 10^{-6} M Tamoxifen (T in the Fig), 10^{-9} M 17-Est plus 10^{-5} M T for 24 h. C- is non treated, and C+ is RA treated MCF-7 cells. RT-PCR is made from total RNA by hNIS, and hGAPDH primers. GAPDH is used as control to check the integrity of cDNAs. Combination of 17-Est and tamoxifen resulted in an activation of NIS gene in mRNA level.

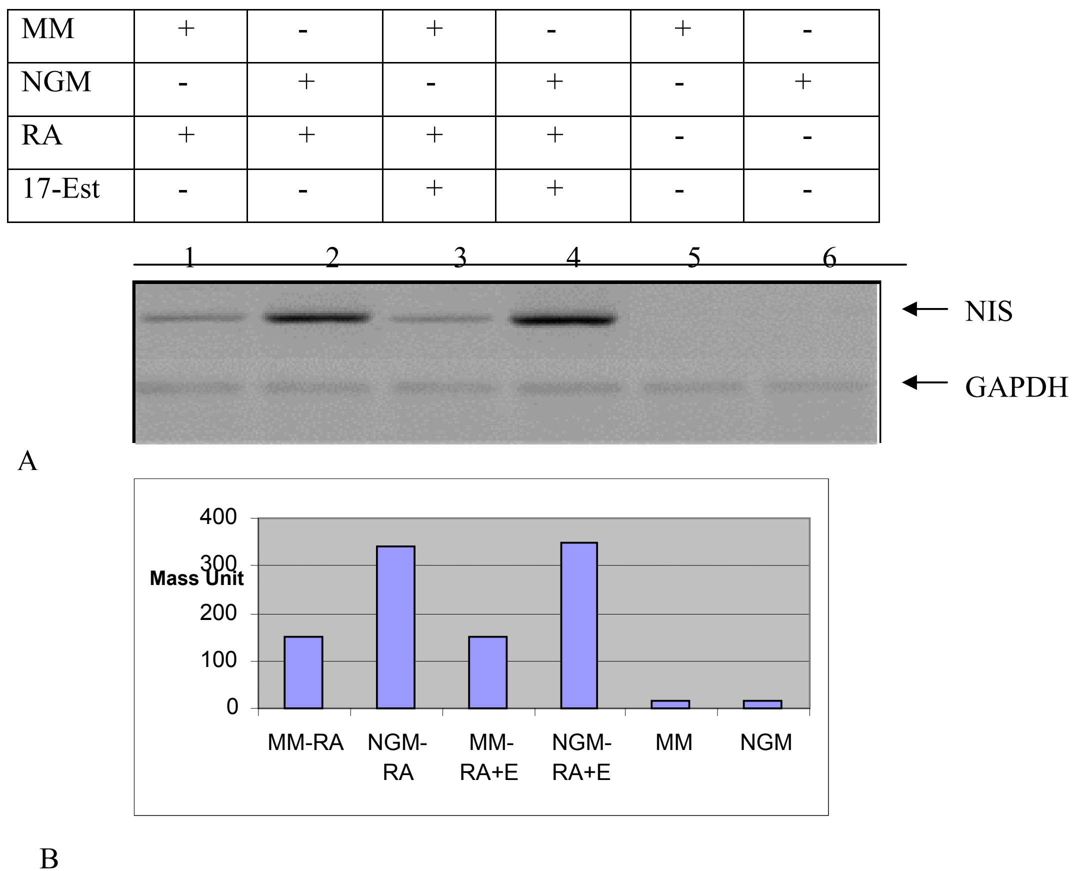
Activation of human RARα gene promoter by ERβ in presence of tamoxifen was previously discovered (Zou *et al.*, 1999). Surprisingly enough, they have shown that tamoxifen is a ligand that acts as the agonist of ERβ in this promoter (hRARa

promoter) context. Even more surprisingly, again in this promoter context 17-Est acts as the *antagonist* for ERβ (Zou *et al.*, 1999). Our result on NIS gene expression in presence of tamoxifen as presented in Fig. 5 fits well to this scheme (see the Discussion part).

3.6. Estrogen and RA treatment of MCF-7 cells:

Estrogen is known to be upregulate the RA receptor (RAR) protein levels in human cells in culture (Zou *et al.*, 1999). Then, RAR upregulates the genes that are under its control. If such a regulatory cascade applies to mgNIS regulation also, this would explain why mgNIS expression is not observed in ERα(-) MDA-MB-231 cells upon RA treatment, whereas it is seen in ERα(+) MCF-7 cells under the same conditions. Therefore, we could speculate that estrogen might have an indirect effect

on mgNIS transcription by upregulating the RAR protein level, leading to indirect activation of mgNIS via ERα. To study this possibility, we have grown MCF-7 cells in DMEM without phenol red with 3% charcoal treated FBS (MM), or normal growth medium (NGM) for 1-2 weeks. Then these cells were treated with either 10^{-6} M RA, or 10^{-6} M RA plus 10^{-10} M 17-Est for 24 hours. Between two groups of cells Fig6, lane 1-3, and lane 2-4 there were no difference in mgNIS mRNA level (which were grown in same medium), so between 17-Est and RA vs only RA, no difference is observed in mgNIS mRNA accumulation in MCF-7 cells.



MM	+	-	+	-	+	-
NGM	-	+	-	+	-	+
RA	+	+	+	+	-	-
17-Est	-	-	+	+	-	-

Fig. 6. Treatment of MCF-7 cells that were grown in different media with RA, 17-Est, and 17-Est+RA. MCF-7 cells are treated with 10^{-6} M RA, and 10^{-6} M +10^{-10} M 17-est for 24h. MCF-7 cells which are grown in MM (see text) gave 2,5 times decrease (B) in hNIS mRNA level when compared with cells grown in NGM upon treatment with either RA, or RA with estrogen. There is no significant difference between 17-Est+RA or RA treatments. A) Lane 1,3,5 are grown in MM, Lane 2,4,6 are grown in NGM.(mass unit : intensity/pixel)

Thus we can state that under these experimental conditions, not only 17-Est does not induce mgNIS expression in tested mammary cell lines, but also it does not have a significant additive up regulatory effect when given together with RA.

To understand the decrease reason of mgNIS expression in MM grown cells compared to NGM grown cells, we designed another experiment. We used the same concentration of normal and charcoal treated FBS at this experiment. We grown MCF-7 cells in DMEM with phenol red, and DMEM without phenol red with either 3% FBS, or 3% charcoal treated FBS. These cells were treated with either 10^{-6} M RA, or 10^{-7} M 17-Est, or 10^{-6} M RA after treatment with 10^{-10} M 17-Est for 24 hours.

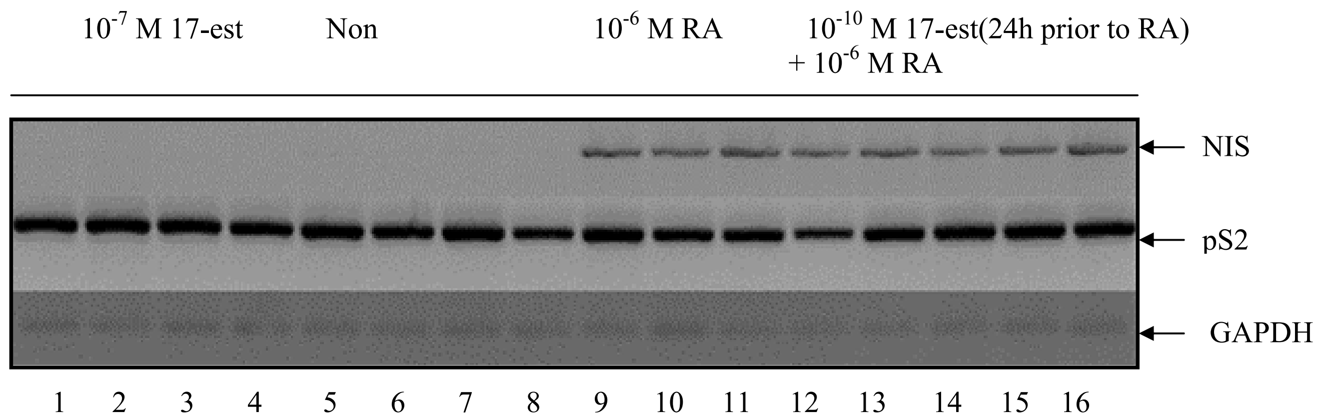


Fig 7. RA, 17-est,and 17-est plus RA, treatments of MCF-7 cells grown on different medium compositions. MCF-7 cells were grown in four different growth mediums DMEM w/o phenol red, DMEM with phenol red either with 3% FBS or 3% char-coal treated FBS. Lane 1,5,9,13,are grown in DMEM with phenol red plus 3% FBS, Lane 2,6,10,14 are grown in DMEM with phenol red plus 3% treated FBS, lane 3,7,11,15 are grown in DMEM without phenol red plus 3% FBS, lane 4,8,12,16 are grown in DMEM without phenol red plus 3% treated FBS. All group of MCF-7 cells which are treated with 10^{-7} M 17-est did not induced NIS transcription. All group of cells which were treated with either RA, or estrogen plus RA did not gave a difference in NIS mRNA level. pS2, ER responsive gene, levels in each group of cells, and in each treatment did not significantly changed. The reason can be that pS2 mRNA level can be huge in these cells, and semi-quantitative PCR may not sufficient enough to detect changes in mRNA levels.

If we look at 17-Est treatment again there were no induction of hNIS expression in none of the samples(Fig.7 lanes 1,2,3,4). In RA treated cells after 17-Est treatment (Fig.7. Lanes 13, 14, 15, 16), we tried to analyze 17-Est effect on NIS induction via RA, if 17-est is introduced before RA treatment, but we could not

detect any induction in none of the samples. In both two sets of treatments (RA and EST+RA) of 4 MCF-7 cell groups, there were no significant change in hNIS mRNA level.

If we look at RA, and 17-est + RA treatments of cells grown in 4 different medium, it was easily seen that there was no difference in mgNIS mRNA expression between them. Thus serum concentration was important in mgNIS upregulation upon RA induction, and serum concentration increase gives additional effect on mgNIS upregulation obtained by RA. (Look Fig 3, and 7)

3.7. ERα transfection to MDA-MB-231:

In parallel to studies on the effect of various media compositions and ligands on mgNIS expression in MCF-7 cells in culture, we have asked the question whether ERs could contribute to, or necessary for, RA induction of mgNIS. MDA-MB-231 is a human breast cancer cell line, which is ERα negative (Fig. 1). This cell line can be considered RAR positive albeit the amount of RARα is lower compared to the MCF-7 line (Fig.8). mgNIS expression in this cell line (MDA-MB-231) is not responsive to RA (Kogai et al., 2001; and Fig. 2)

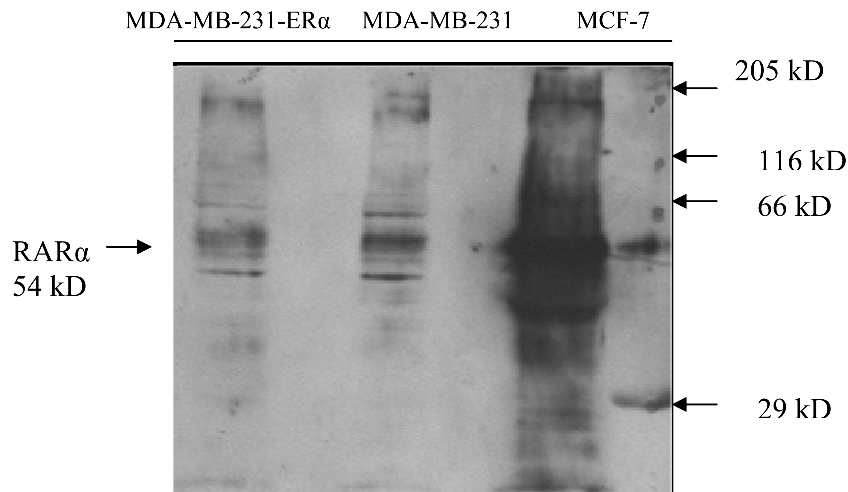


Fig. 8. RARα status of MDA-MB-231 and MCF-7 cell lines. Total protein was isolated from MCF-7 and MDA-MB-231 cell lines and a Western blot analysis was performed using a polyclonal anti-human RARα Ab. RARα protein is detected in both cell lines tested, albeit at different concentrations (indicated with arrowhead).

We hypothesized that if ERs have a direct or indirect role in RA regulation of mgNIS in MCF-7 cells in culture, then by transfecting the MDA-MB-231 (RA non-responsive) cell line with ERα we might activate RA responsiveness of mgNIS transcription in this line. To conduct this analysis, we stably transfected the MDA-MB-231 cell line first with the pcDNA3.1c vector plasmid containing the human ERα gene (see Materials and Methods). This plasmid was a kind gift of Dr. Ediz Demirpençe from The Hacettepe University Faculty of Medicine. ERα was subcloned to pcDNA3.1c by Hani Al Otabi. The vector was containing a geniticine (G-418) resistance selection marker gene. 20 clones were isolated after 3 weeks of antibiotic selection, and these clones were analyzed for ERα expression. First, we have screened these clones by a PCR based method for ERα genomic integration (see Materials and Methods). Primers used in this analysis are designed in such a way that only the integrated ERα cDNA and not the intron containing genomic copy is obtained. 7 clones are found to contain the ERα gene integrated in their genome (Fig. 9)

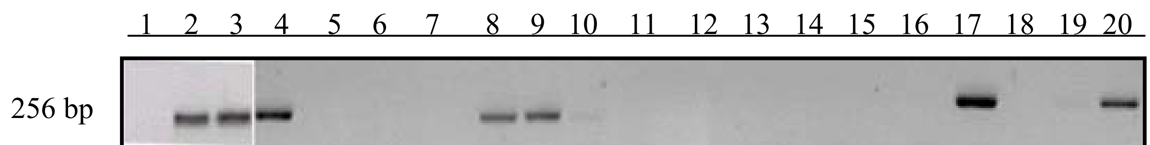


Fig. 9. Integration of ERα gene containing vector plasmids into the genome of MDA-MB-231 clones. MDA-MB-231 clones are screened for ERα gene integration to their genomes by a PCR based method. Clones with ERα gene integration gave an amplification of 226 bp DNA after the PCR. Clones 2, 3, 4, 8, 9, 17, 20 turned out to be positive in this analysis. The fragment corresponds to a portion of the ERα cDNA.

Expression of ERα mRNA in these 7 clones that were detected positive for Erα-pcDNA3.1c integration, are analyzed by RT-PCR analyses. Although the expression levels of ERα mRNA varied between different clones, they were all positive for transcriptional expression of genome integrated ERα gene (Fig. 10). This is also checked by Western blots (see below, Fig. 11).

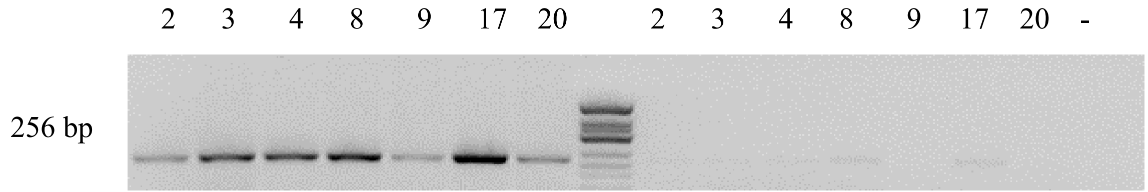


Fig. 10. Expression of the ERα gene in stably transfected MDA-MB-231 clones. Clones 2, 3, 4, 8, 9, 17, and 20 gave a significant amplification with primers corresponding to a partial sequence of ERα at expected size of 226 bp at the left side. – is negative control of PCR. The right site was a PCR that is done with RNA templates, that they were in a reaction of cDNA synthesis but reverts transcriptase is absent.

A Western blot analysis conducted on samples from these seven clones has shown that clone 17 is the only clone that does express the ERα protein (Fig. 11). Therefore we have conducted further studies using clone 17. From this point on, in this text, we are going to refer to this clone (17) as MDA-III.

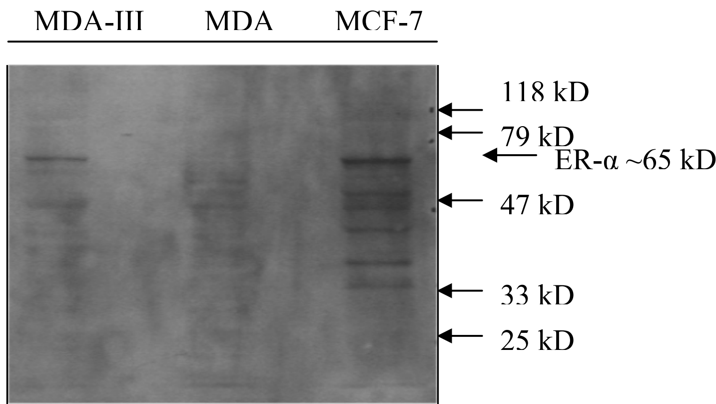


Fig. 11. Screening of MDA-MB-231 stable clones for ERα protein expression. Total protein was isolated from MCF-7, MDA-III and MDA-MB-231 cell lines and a Western blot analysis was performed using monoclonal anti-human ERα Ab. ERα protein is detected in MCF-7 and MDA-III cell lines (indicated with arrowhead), whereas it is not detected in MDA-MB-231 cell lines.

Subsequently, we have carried out a Southern blot analysis on MDA-III, simply to confirm the integration of ERα gene in the genome of this clone, and to identify the region in genome where the gene is integrated. This study also gave us an idea about the copy number of the ERα plasmid in MDA-III clone. Looking at this result we

can state that only one copy of the externally introduced gene was actually integrated into the genome (Fig. 12). For Southern analysis, the BglII restriction enzyme is used. This enzyme cuts only once in the ERα gene, and it does not have any site in the vector plasmid pcDNA3.1c (see Material and Methods). Thus in a Southern analysis after cutting the genomic DNA by BglII we could observe the integration of the transfected plasmid (Fig. 12)

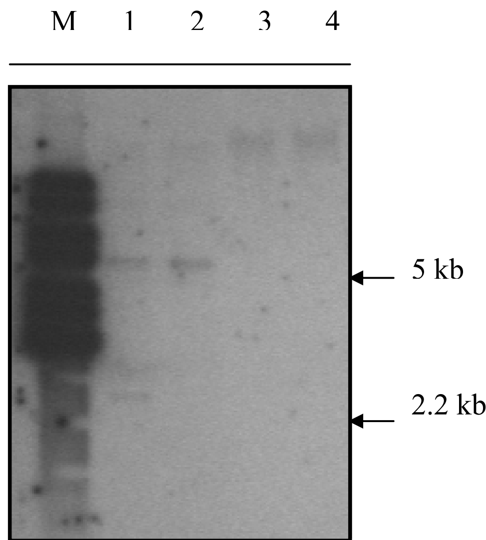


Fig. 12. Integration of externally introduced ERα gene into the genome of the clone MDA-III. Lane1, and lane2 are BglII cut genomic DNA of MDA-MB-231-III, and MDA-MB-231 respectively. Lane 3, and lane 4 are uncut genomic DNA of MDA-MB-231-III, and MDA-MB-231 respectively. Integrated ERα gene is detected around 2.2 kb (see Materials and Methods).

After observing the ERα expression in MDA-III cells at the protein level (Fig. 11), we aimed to check the ERα activity in this clone. For this purpose, MDA-III cells were transiently transfected with a vector plasmid containing the reporter gene, luciferase, under control of an estradiol responsive element (ERE). If the ERα protein detected in the clone III is biologically active, then it would interact with the ERE region in this plasmid and activate the reporter luciferase expression in response to 17-est. As seen in the graph in Fig. 13, MDA-III transfectants of ERE-reporter plasmid gave a 2 fold activation when compared to cells transfected with pGL3, a plasmid expressing the luciferase gene constitutively, thus we used as a luciferase

detection control. While it gives nearly same luciferase count when as pGL3 basic plasmid when they did not treated with 17-est, fig. 13. These results show us that ERα protein in MDA-MB-231-III cells are most probably functional. This experiment is performed only once, so it should be repeated to be sure that MDA-MB-231-III cells contain functional ERα protein.

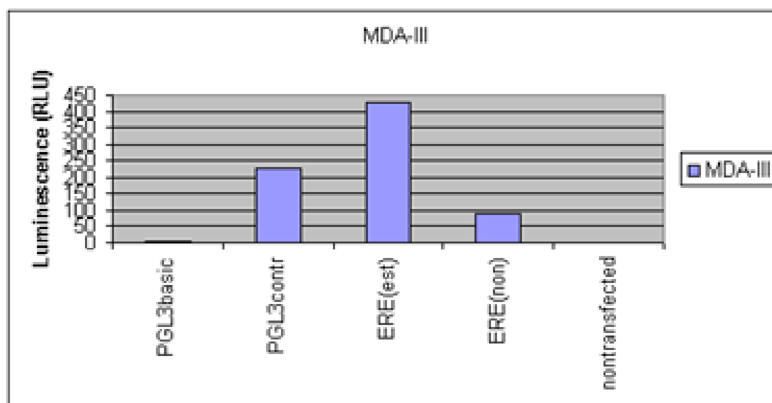


Fig. 13. Luciferase activity assay of ERE response element transfected to MDA-III stable clone. MDA-III cells are transfected with various reporter plasmids containing luciferase gene under control of different promoters. pGL3basic contains only the ORF of the luciferase gene. pGL3control contains the luciferase under control of a constitutive SV40 promoter, thus this is the positive control in this experiment. In ERE the reporter gene is under control of an estrogen responsive element cloned with a basal β-globulin promoter. ERE(est); ERE transfected MDA-III cells are treated with 10^{-10} M 17-Est for 24 hours and luciferase activity is compared with untreated cells, ERE(non).Luciferase activities was normalized with GFP transfection efficiencies of each transfection.

3.8. Absence of mgNIS upregulation in MDA-III cells in response to RA or 17-Est.

MDA-III cells and the MCF-7 cell line were treated with 10^{-6} M RA, or 10^{-6} M RA plus 10^{-10} M 17-est for 24 hours. RT-PCR analysis is made with treated samples, and as seen in Fig. 14, there were neither NIS expression nor PS2 expression in MDA-III, while GAPDH was expressed in all samples. This result may indicates that the presence of ERα in

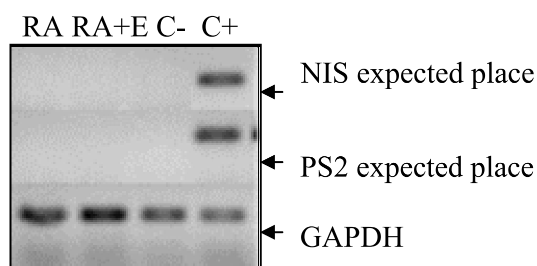


Fig. 14. MDA-III treatment with RA, and RA+Est. MDA-III is treated with either 10^{-6} M RA or 10^{-10}M 17-Est + 10^{-6} M RA. In presence of neither of two ligands mgNIS is expressed in MDA-III. GAPDH is used as control to check the integrity of cDNAs. PS2 is an estrogen responsive gene of unknown function (Jakowlew et al. 1984), and it is used as a control of ER activity.C+ is MCF-7 treated with RA. C- is non treated MDA-III

MDA-III cell line may not sufficient for mgNIS expression in presence of RA or 17-Est.

3.9. Sub-cloning of long form rat prolactin receptor

Besides our analysis on the role of ERα on mgNIS expression, we aimed to study a possible role of prolactin and rat prolactin receptor (rPRLR) in mgNIS regulation in mammary cells in culture. To this end, we have sub-cloned the long form of rat PRLR into pcDNA3.1 plasmid in frame with his, and myc tag (see below). cDNA was amplified from rat ovary, and RT-PCR was performed by using rPRLR primers (see Materials and Methods) using these cDNA as template (Fig. 15).

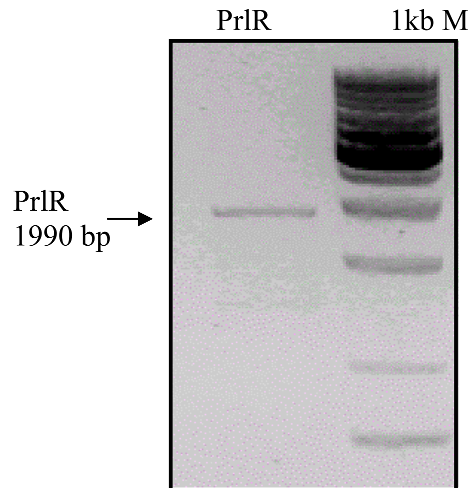


Fig. 15. Amplification of full length rPRLR coding sequence. Long form of prlR was amplified at expected size of 1.99 kb.

After cloning the amplified product into pcDNA3.1c mammalian expression vector, the recombinant plasmid was checked by restriction enzyme digestion.

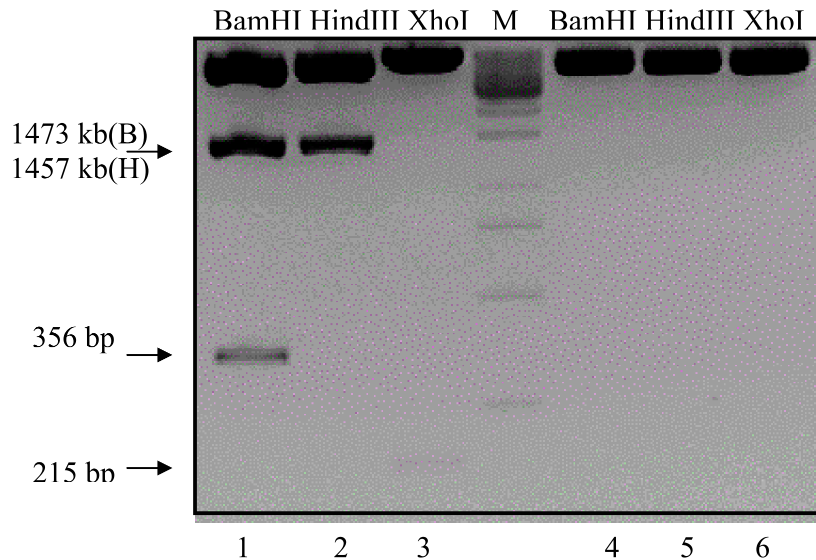


Fig. 16. Restriction enzyme digest analysis of recombinant vector.
Lane1,2,3 are prlR-pcDNA3.1c digests, lane 4,5,6 are pcDNA3.1c digests. Expected fragments are seen in figure.

This expression plasmid including rPRLR gene, was transfected to Hek-293 cells, and transfected cells were analysed by western blot for PRLR presence with myc antibody.

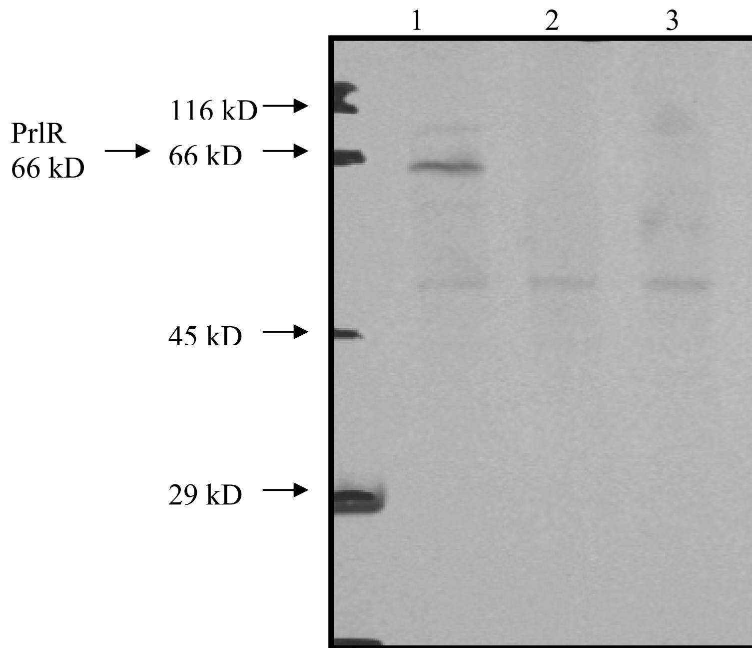


Fig. 17. Western blot analysis of transiently transfected Hek-293 cells with pcDNA3.1c-rPRLR. Lane 1 is Hek-293 transfected with PRLR-pcDNA3.1c, lane2 is Hek-293 cells transfected with pcDNA3.1c, lane3 is Hek-293 cells. PRLR protein is detected with α-myc antibody in PRLR-pcDNA3.1c transfected cells only.

In previous studies in overiectomized mice, PRL had an effect on mgNIS regulation, when it was administered with estrogen, and oxytocin (Tazebay et al. 2000). Additionally Rillemma et al. showed that prolactin increased mgNIS in mRNA, and protein level in 30D histocultured mid-pregnant mammary gland explants. Prolactin stimulatory effect on NIS expression was also established in Nancy Carrasco's laboratory, that when PRLR was introduced into CHO-K1 cells, and treated with prolactin and insulin, these cells expressed NIS protein. In parallel to these studies and results, we decided to construct stable CHO-K1 constructs with PRLR to study prolactin effects on exogenous rNIS promoter deletions constructed by Hani al Otabi. In order to achieve this goal we first cloned long form of rat prolactin receptor into pcDNA3.1c with his and myc tag (fig. 16, and fig. 17), and checked its expression in Hek-293 cells, fig. 17. Then we stably transfected rPRLR into CHO-K1 cells, and isolated 90 colonies. Screening procedure for rPRLR expression is not done yet.

4.Discussion

Identification of NIS protein in the lactating mammary gland was a start point to many interesting studies. One aspect of the mgNIS that is completely unknown is the molecular determinants of the transcriptional regulation of this gene. Several hormones that regulate NIS in mammary gland (mgNIS) were identified in previous studies (Cho *et al*., 2000; Tazebay *et al*., 2000; Rillema *et al*., 2000). However, transcription factors that regulate NIS expression in the mammary gland, and *cis*-acting enhancer elements located in NIS upstream regulatory region that are essential for regulation in mammary gland are to be studied.

Some of the previously published studies indicated that estrogens may play a role in mgNIS regulation (Tazebay *et al*., 2000; Cho *et al*., 2000; Kogai *et al*., 2001). Yet, clear evidences showing the role of estrogen receptors in regulation of mgNIS were missing. Similarly, prolactin was shown to be a hormone that upregulates mgNIS protein expression and iodide transport activity, in various experimental settings both *in vivo* and *in vitro* (Tazebay *et al*., 2000; Rillema *et al*., 2000). In this thesis, we have inquired 1) the contribution of estrogens to mgNIS regulation in human mammary gland cells in culture; 2) a possible role of human estrogen receptors α and β in mgNIS regulation in human mammary gland cell lines *in vitro*.

We have used two human breast carcinoma cell lines: the MCF-7, and the MDA-MB-231. MCF-7 cells are both ERα(+) and ERβ(+). Whereas, MDA-MB-231 cells are both ERα(-) and ERβ(-). MDA-MB-231 is commonly used in studies related with estrogen action in mammary cells (Kogai *et al*., 1999; Gross and Yee, 2002). We were, in a way, obliged to work with the MCF-7 cell line because this is the *unique* breast cell line in which mgNIS expression was detected. Importantly, RA was shown to be absolutely necessary for this induction (Kogai *et al*., 2001).

In experiments using minimal media we have inquired whether ligands or substances other than RA may affect mgNIS regulation in *in vitro* conditions (Figs. 3 and 7). In these experiments we have kept the RA concentration in media constant, but we changed the FBS concentration and we removed phenol red (see Materials and Methods). We have seen no difference in mgNIS expression between the medium containing phenol red vs the phenol red-free medium (Fig. 7). Also, we have seen no difference under conditions when treated vs non-treated serum are used in

media (Fig. 7). However, decreasing the FBS concentration from 10% to 3% affected mgNIS expression 2,5 fold in presence of constant amounts of RA (Figs. 3 and 7). This means that in addition to RA, serum-borne factors directly or indirectly contribute to mgNIS regulation without affecting GAPDH levels in MCF-7 cells.

Because the effect of 17-Est on mgNIS regulation was previously shown (Tazebay *et al*., 2000; Cho *et al*., 2000) in vivo, we inquired whether the unidentified serum component contributing to RA regulation may be estrogens or not. We first treated both MCF-7 cells, and RA non-responsive MDA-MB-231 cells, with 17-Est in physiological conditions (10^{-10} M). We did the treatment in different time periods between 0h, and 48h. We could not detect mgNIS mRNA by RT-PCR analyses in any time period in none of the cells. Estrogen in physiological conditions was not able to activate endogenous mgNIS promoter in these cell lines, as it was in overiectomized mice mammary glands *in vivo* (Tazebay *et al*., 2000). Either the transcription factor repertoire of MCF-7, and MDA-MB-231 cell lines are different from overiectomized mice mammary gland cells, or there are ligands that are essential for mgNIS gene transcription in vivo, and that they are absent in media we grow our cells.

Under *in vivo* conditions, concentration of estrogen may increase up to 10^{-8} M according to the physiological condition of the female body (Yoshinaga *et al*., 1969) Thus, we decided to gradually increase the concentration of 17-Est in cell culture media, and make an estradiol dose dependent analysis of mgNIS expression in MCF-7 cell lines. We used different concentrations of estrogen from 10^{-10} to 10^{-7}. In addition, in separate samples we treated cells with the same amounts of estrogen together with tamoxifen (from 10^{-6} to 10^{-5}). By RT-PCR we could not detect a significant amount of mgNIS gene expression when cells are treated with 10^{-7} M of 17-Est. But very interestingly, we detected a significant weak induction of mgNIS in samples treated with estrogen *together with tamoxifen*. This fits very well to the observations of Zou *et al*. (1999) on the activation of human RARα gene promoter by ERβ in presence of tamoxifen as its ligand. These experiments have shown that in HepG2 cells transiently transfected with ERβ gene, RARα promoter gets activated in response to tamoxifen. For this activation, ERβ does not bind to promoter DNA sequences of RARα gene, but cis-acting SP1 binding sites are necessary for the activation. Thus the effect of ERβ in this upregulation seems to be indirect.

Surprisingly, estrogen has an antagonistic effect on this activation of ERβ (and then RARα gene) by tamoxifen (Zou et al., 1999). This may also explain our results presented in Fig**.** 5. Besides, it may give hints about a possible link between ER action and RA effect in mgNIS regulation. In our case, by RT-PCR we have seen an amplification product corresponding to mgNIS after treatment of MCF-7 cells with 10^{-6} M tamoxifen together with 10^{-10} M 17-Est. We could propose two possible mechanisms based on Zou *et al* (1999)'s data in order to explain this observation: 1) ERβ may upregulate mgNIS gene directly or indirectly in response to a tamoxifen dependent activation; 2) as shown by these authors, ERβ may activate RARα expression in presence of tamoxifen, which in turn might activate mgNIS promoter by binding to RARα responsive elements (Schmutzler *et al*., 2002).

RA induction of mgNIS was seen by Kogai et al. (2001) in human MCF-7 cells, which is a cell line that is both ERα and ERβ (+). This induction is absent in the ERs (-) MDA-MB-231 cells. Thus, one of the hypotheses was on the role of ERs in RA regulation of mgNIS in human mammary cells in culture. In order to test this hypothesis, we decided to introduce ERα gene in MDA-MB-231 cells both by transient and stable transfections. Plasmid DNA transfections to MDA-MB-231 cells were quite cumbersome, with typical transfection efficiencies at about 5%. This has obstructed our experiments with transiently transfected cells, and thus we could not get significant data from these experiments. However, stable transfection experiments worked better and we obtained a transfected colony expressing human ERα protein. We named this clone as MDA-III (Fig. 11). In order to assess the activity of the externally introduced ERα, we have performed RT-PCR analysis to visualize the activation of an estrogen responsive endogenous gene, PS2. However, we could not see any significant PS2 or mgNIS upregulation upon 17-Est or RA treatment of MDA-III cells. One possible explanation is that the concentration of ERα in MDA-III cells is below the necessary threshold for the activation of PS2 by 17-Est. This might be due to lower expression levels of the introduced ERα gene in MDA-III cells as compared to its endogenous expression in MCF-7. In a preliminary experiment, we have transfected MDA-III with various plasmids in order to check the activity of the externally introduced ERα (Fig. 13). In cells transfected with plasmid ERE containing the luciferase reporter gene under control of an estrogen responsive promoter, we have detected significantly increased luciferase activity in

cells treated with 17-Est as compared to non-treated cells (Fig. 13). Although the experiment is to be repeated, it shows that the MDA-III cells contain 17-Est responsive ERα. Yet, the presence of this activator in MDA-III cells is not sufficient for mgNIS activation in response to RA or 17-Est treatment of cells.

Our future perspective is to put more emphasis on ERβ's role in mgNIS regulation in MCF-7 cells. It was known that ERβ activates human RARα gene promoter in presence of tamoxifen as the agonist (Zou *et al*., 1999). A similar mechanism may apply to mgNIS regulation in MCF-7 cells (Fig. 5), and ERβ may indirectly control the transcription of this gene. If this hypothesis turns out to be true, and mgNIS is upregulated by tamoxifen, then this result may have important medical applications: mgNIS was shown to be upregulated in ER(+) breast tumors in women (Tazebay et al., 2000). Thus, if our hypothesis is correct, then tamoxifen treatment of these tumors may lead to an even higher upregulation of mgNIS. Bearing in mind that radioactive iodide is used in thyroid cancer diagnosis and treatment (Daniels and Haber, 2000), this upregulation of mgNIS in response to tamoxifen may have interesting applications in fight against breast cancer.

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