THE EFFECT OF n$Na_V1.5$ GENE EXPRESSION IN BREAST CANCER METASTASIS

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ABSTRACT

THE EFFECT OF $nNa_v1.5$ GENE EXPRESSION ON BREAST CANCER METASTASIS

Breast cancer is the most common cancer among women. Its metastasis is lethal that can not be detected at microscopic levels using current techniques. Thus, there is need for reliable early metastasis markers. Neonatal $Na_v1.5$ ($nNa_v1.5$) is a voltage-gated sodium channel (VGSC) and one of the potential early markers for breast cancer metastasis.

In this study, we determined that $nNa_v1.5$ expression was in parallel with breast cancer metastasis and estrogen receptor (ER) expression in a group of patients. To provide data for future drug development, we analyzed the expression pattern of $nNa_v1.5$ protein in normal human tissues. The protein was not expressed in skeletal and heart muscle, brain, small intestine, colon, stomach, esophagus, urinary bladder and prostate but expressed in breast at basal level. We also investigated the distribution of VGSCα in these non-excitable human tissues. Except urinary bladder, VGSCα protein was determined mostly in secretory cells in all of the tissues above that may indicate a role in secretion. Upon identification of VGSCα upregulation in tumor regions of different cancers including, colon, stomach, urinary bladder, kidney and lung it is possible that VGSCα expression could be a widespread mechanism in cancer metastasis.

Within the scope of this thesis, we also investigated the possible role of estrogen on $nNa_v1.5$ upregulation and activity in metastatic breast cancer. Estrogen had no effect on proliferation of cells but slightly increased motility through $nNa_v1.5$ in highly metastatic cells that express the protein. In weakly metastatic cells that do not posses $nNa_v1.5$, estrogen decreased motility slightly. The quantity of $nNa_v1.5$ protein was not affected by estrogen but functionally available form on the plasma membrane was increased only in the highly metastatic cells. These results may suggest that estrogen increases motility capacity of breast cancer cells by regulating $nNa_v1.5$ activity.

ÖZET

$nNa_v1.5$ GEN ANLATIMININ MEME KANSERİ METASTAZI ÜZERİNDEKİ ETKİSİ

Meme kanseri kadınlarda en sık görülen kanser tipidir. Metastazı ölümcüldür ve günümüzdeki tekniklerle mikroskopik düzeyde tanımlanamamaktadır. Bu nedenle, güvenilir erken metastaz belirteçlerine gerek duyulmaktadır. Neonatal $Na_v1.5$, ($nNa_v1.5$), voltaj-kapılı sodyum kanalı (VGSC) alttiplerinden biridir ve erken metastaz belirteci adayı olabilir.

Bu çalışmada, $nNa_v1.5$ anlatımı ile meme kanseri metastazı ve östrojen reseptörü (ER) arasındaki paralel ilişki bir grup hastada belirlendi. İleride ilaç geliştirilmesine yönelik veri sağlamak için normal insan dokularında $nNa_v1.5$'in dağılımı incelendi. İskelet ve kalp kası, beyin, ince bağırsak, kalın bağırsak, mide, özofagus, mesane ve prostat dokularında $nNa_v1.5$ belirlenmese de memede bazal seviyede anlatıldığı gösterildi. Ayrıca VGSCα proteininin elektrikle uyarılamayan dokulardaki dağılımı incelendi. Mesane dışında adı geçen tüm dokularda genellikle salgı yapan hücrelerde belirlenmesi proteinin salgılamada rolü olabileceğini gösterdi. VGSCα'nın kolon, mide, mesane, böbrek ve akciğer kanserlerinin tümörlü bölgelerindeki anlatımının artmış olması nedeniyle proteinin metastaz mekanizmasında yaygın rolü olabileceği düşünülmektedir.

Bu tez çerçevesinde, östrojenin metastatik meme kanserinde görülen $nNa_v1.5$ artışı ve aktivitesi üzerindeki olası etkisi incelendi. Östrojenin hücre hatlarında çoğalmayı etkilemediği ancak $nNa_v1.5$ proteinini anlatan yüksek metastatik kapasitesi olan hücrelerde hareketliliği bu protein üzerinden az miktarda arttırdığı gösterildi. $nNa_v1.5$ proteini içermeyen düşük metastatik hücrelerde ise az miktarda azalttığı görüldü. Yüksek derecede metastatik hücrelerde östrojenin $nNa_v1.5$ protein miktarını değiştirmediği ancak işlevsel proteinin hücre membranında yoğunlaşmasına neden olduğu belirlendi. Bu sonuçlar, östrojenin meme kanseri hareketliliğini $nNa_v1.5$ ile etkileşerek artırabileceğini gösterdi.

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AC	Adenylyl cyclase
AF	Activation function
Akt	Thymoma viral proto-oncogene-1
ATM	Ataxia telangiectasia
Bcl2	B-cell CLL/lymphoma 2
BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2
cAMP	Cyclic adenosine monophosphate
CAM	Cell adhesion molecule
CDK-1	Cyclic dependent kinase
c-erb-B2	Human epidermal growth factor 2
CHECK2	Cell cycle checkpoint kinase 2
CREB	Cyclic AMP response element binding protein
CRE	Cyclic AMP response element
DBD	DNA-binding domain
DCIS	Ductal carcinoma *in situ*
DRG	Dorsal root ganglion
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERE	Estrogen response elements
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
FHF	FGF homolog factor
GDNF	Glial derived neurotrphic factor
GIRK	G protein-activated inward rectifier potassium channel
GPCR	G-Protein coupled receptor (GPR)
HER2	Human epidermal growth factor 2
hCytb5R	Human cytochrome b5 reductase

HIN-1	High in normal-1
hMLH1	Human mutL homolog 1
hMSH2	Human mutS homolog 2
HUVEC	Human umbilical vein endothelial cells
HSVEC	Human saphenous vein cells
IGF-1	Insulin–like growth Factor-1
IGFR	Insulin growth factor-1 receptor
LBD	Ligand-binding domain
LCIS	Lobular carcinoma *in situ*
LNM	Lymph node metastasis
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MRC	Medical research council
Na^+, K^+-ATPase	Sodium/Potassium ATPase
NESOpAb	Neonatal sodium channel polyclonal antibody
NGF	Nerve growth factor
P21	Protein 21
P27 KIP1	Cyclin-dependent kinase inhibitor p27(Kip1)
p53	Protein 53
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PNS	Peripheral nervous system
PTEN	Phosphatase and tensin homolog
RARβ2	Retinoic Acid receptor Beta 2
REST	RE1-silencing transcription factor
RPTPβ	Receptor-like protein phosphatese β
SCLC	Small-cell lung carcinoma
Src	v-src sarcoma
SKG1	Serum and glucocorticoid inducible kinases
TGF-α	Transforming growth factor-α
TIMP3	Tissue inhibitor of metalloproteinase-3
TTX	Tetrodotoxin

uPA	Urokinase protein activator
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VGCC	Voltage-gated calcium channel
VGPC	Voltage-gated potassium channel
VGSC	Voltage-gated sodium channel

1. INTRODUCTION

1.1. Breast

The breast is a modified apocrine gland that is arranged in several lobes and embedded into the fibroadipose tissue of the chest wall. Breasts reach complete development in females and are fully differentiated during pregnancy and lactation. It is made up of four main parts; nipple, ducts, lobules and fibroadipose tissue (Figure 1.1).

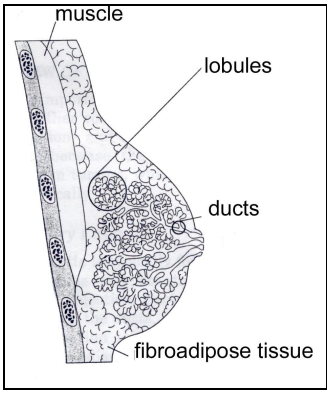


Figure 1.1. A schematic diagram of a normal female breast (Curran, 1994)

The nipple is composed of epidermis with sebaceous units (Montgomery glands) and excretory lactiferous ducts (Figure 1.2a). The ducts extend from the nipple into the fibroadipose tissue and terminate in breast lobules that consist of a central terminal duct and outer alveolar duct (Figure 1.2b). These structures are embedded in a loose connective tissue stroma. Lobules are the functional units of the breast. The lumen of both the ducts and the lobules are lined by a continuous single layer of cuboidal to low columnar epithelium surrounded by an array of myoepthelial cells (Figure 1.2c) (Fawcett, 1976).

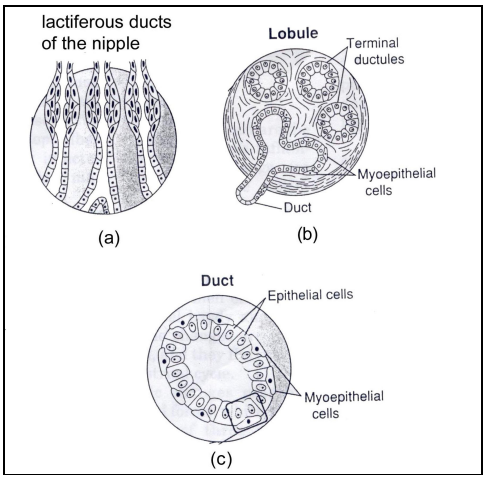


Figure 1.2. Detailed diagram of a normal breast (Curran, 1994)

There is not much difference between the breast tissue of males and females until puberty. This is the period when females achieve lengthening and further branching of ducts, development of lobules and proliferation of fibrous stroma and fat (Russo and Russo, 1987; Monaghan *et al*., 1990). During pregnancy terminal ducts undergo hyperplasia and form acini that regress with the end of pregnancy. After the third decade of life, proliferation rate of epithelial cells decrease but overall proportion of epithelium increases until menopause. In later stages, progressive epithelial atrophy, increased density of collagen and fat is observed (Hutson *et al*., 1985).

1.2. Diseases of the Breast

A variety of breast diseases exist that usually do not contribute to the development of cancer. One of them is inflammation that can be caused by staphylococci and anaerobic infections. It can be observed 2-3 weeks after being born or later in life (Scholefield *et al*., 1987; Rogers, 1990). Periductal mastitis is a chronic inflammatory lesion where the ducts are filled with grumous material and present attenuation of the epithelium. Fat necrosis is clinically difficult to diagnose since it interferes with invasive mammary carcinoma (Lee and Adair, 1920). It forms a painless hard mass sometimes causing skin or nipple retraction but it is self-limited and heals with scarring. Fibrocystic change is another non-neoplastic breast disease that affects approximately 10 per cent of women of reproductive age. The breasts feel beady and are tender on palpation. It is a complex of lesions that include fibrosis, epithelial proliferation and cyst formation (Foote and Stewart, 1945). The

presence of epithelial hyperplasia is the most important risk factor for tumor formation. The rate and type of proliferation in these cells is a major indicator for the subsequent development of mammary carcinoma (Page *et al*., 1978).

1.2.1. Neoplastic Lesions

1.2.1.1. Fibroadenoma. It is the most common benign lesion encountered in women up to 25 years of age (Ferguson and Powell, 1989). It is composed of proliferation of epithelium and connective tissue. The ductal epithelium is usually double layered. It can be in two forms: pericanalicular and intracanalicular. In case of the former, the epithelial and myoepithelial cells form round to elongated ducts surrounded by loose fibroblastic stroma. Intracanalicular fibroadenomas contain elongated ducts that are also lined by cuboidal epithelial and myoepithelial cells. The ducts look distorted and are compressed by stroma. Fibroadenoma poses only a small risk for the development of breast cancer (Carter *et al*., 1988).

1.2.1.2. Phyllodes Tumor. Like fibroadenoma it is a combination of proliferation in epithelium and stroma. It is usually observed at late ages. The epithelia may display atypical hyperplasia or *in situ* carcinoma. The overgrowth of the stroma may extend into the epithelium. Studies have shown that 20 per cent is histologically malignant and more than 50 per cent metastasize (Grimes, 1992).

1.2.1.3. Adenoma. It is not as frequent as fibroadenoma. It is characterized as tubular or lactating adenomas. They produce distinct masses within the breast and are composed of densely packed terminal ducts with little surrounding stroma. The nipple-adenoma is quite different which involves proliferation of ductal and stromal cells.

1.2.1.4. Papilloma. It is a benign largely intraductal lesion that causes nipple discharge and rarely a palpable mass. Epithelial hyperplasia without cytological atypia is often present. Papillomas can be solitary or multiple. Solitary ones usually occur in subaorelar ducts but multiple forms are usually at the periphery involving terminal duct lobular units. Papillomas slightly increase the risk of cancer development especially in patients with multiple form (Murad *et al*., 1981).

1.3. Breast Cancer

1.3.1. Epidemiology of Breast Cancer

Carcinoma of the breast is the most common malignancy in women (Parkin *et al*., 1990). One in eight women is at risk of developing breast cancer in Europe and USA. In Turkey, the disease accounts for 24,1 per cent of women cancers. However, the rate of breast cancer in Japan is 20 per cent that of USA (www.emedicine.com). This lower incidence in Japan indicates the presence of important genetic, cultural and environmental factors in the development of the disease. The risk increases for Japanese when they move to Europe or USA, proving the involvement of non-genomic factors like hormones or lifestyle (Muir, 1992). Several breast cancer risk factors have been known for years and are still being investigated. The risk and relative factors are summarized in Table 1.1 (Hankinson *et al*., 2004).

Table 1.1. Confirmed risk factors and protective factors of breast cancer

Risk factor	**Effect on breast cancer development**
Family history in first-degree relatives	Moderate to high increase
Breast benign disease	Moderate to high increase
Mammographically dense breast	Moderate to high increase
Age at first birth (>30 versus <20)	Moderate to high increase
Late menopause (>54 versus <45)	Moderate to high increase
High endogenous estrogen levels	Moderate to high increase
Ionizing radiation exposure during childhood	Moderate to high increase
Advanced age	Moderate to high increase
Height	Slight to moderate increase
Postmenopausal hormone therapy	Slight to moderate increase
Early menarche (<12 versus >14)	Slight to moderate increase
Postmenopausal high body mass index	Slight to moderate increase
High-fat diet	Slight to moderate increase
Alcohol use (~ one or more drinks/day)	Slight to moderate increase
Parity	Slight to moderate decrease
Lactation (long duration)	Slight to moderate decrease
Premenopausal high body mass index	Slight to moderate decrease

According to substantial data, use of oral contraceptives, *in utero* exposures, high prolactin levels and high premenopausal insulin-like growth factor I (IGF-I) levels are factors that may pose a risk for breast cancer development. Conversely, physical activity may have a role in the prevention of the disease by delaying the onset of menarche and controlling weight. The relation between height and breast cancer risk can be due to the influence of IGF-I. The timing of exposure to radiation is also important in the development of breast cancer. Radiation exposure that occurs at early life can pose a higher risk than those in later life (Miller *et al*., 1989; Land *et al*., 1994). Early life events may determine the number of susceptible breast stem cells that are at risk and whether mutations occur in these cells. The effect of birth weight and height on breast cancer development strongly suggests an influence of early events, even those occurring *in utero*. Low levels of folate together with deprivation of its cofactors (vitamin B6, B12) and particularly in conjunction with high alcohol intake can lead to abnormal DNA synthesis and repair, as well as aberrant DNA methylation (Mason and Levesque 1996), causing breast cancer.

Pregnancy, particularly at an early age, decreases the risk of breast cancer. This may be due to shortening the time of breast being susceptible to mutations that starts from menarche and lasts until first pregnancy (Russo and Russo, 1997). Prolonged exposure to estrogen increases the risk of developing the disease. The factors that increase estrogen exposure involve early menarche and late menopause due to increase in the number of ovulatory cycles. The risk of developing breast cancer decreases by 20 per cent for each year that menarche is delayed (Henderson *et al*., 2003). Since lactation also delays ovulation it is protective against breast cancer.

Women who drink one alcoholic beverage daily have 10-30 per cent higher risk of developing the disease than non-drinkers (Smith-Warner *et al*., 1998; Longnecker M, 1994). Several hypotheses have been developed to explain this effect. First one proposes that alcohol increases the circulating hormone levels (Schatzkin and Longnecker 1994). The other hypothesis suggests that alcohol may increase the production of IGF that functions as a potent mitogen (Yu, 1998).

Human diet contains a variety of carcinogens that may act through generation of free radicals. For example well-done meat consumption (Zheng *et al*., 1998) and high fat diet correlates with increased breast cancer risk (Howe *et al*., 1991). However, particular types of polyunsaturated fatty acids like omega-3 may decrease the risk (Bartsch *et al*., 1999). High fiber diets may protect against breast cancer, which may be due to the role of fibers in reduction of intestinal reabsorption of estrogens excreted via the billary system (Hunter and Willett, 1994).

1.3.2. Pathology of Breast Cancer

Breast carcinomas derive from the epithelial cells lining the ducts or lobules, therefore being classified as ductal or lobular carcinomas. Both of these can remain localized or can be invasive and form secondary tumors.

1.3.2.1. Non-invasive Breast Cancer.

Ductal carcinoma in situ (DCIS). It accounts for 5 per cent of all breast carcinomas. DCIS is the result of proliferation of the ductal luminal cells that fill the lumen but do not enter the basement membrane and the surrounding stroma (Figure 1.3). Deposition of calcium, called microcalcification, is a typical feature of the disease. All intraductal carcinomas have the tendency to invade unless treated. Comedo ductal carcinoma is a subtype of DCIS where central area of the duct shows necrosis. Tumor cells may extend to lobules, called cancerization. Non-comedo DCIS is less aggressive than comedo DCIS with lower recurrence rate after excision (Silverstein *et al*., 1992) and with favorable biological features like presence of hormone receptors (Poller *et al*., 1993) and low proliferation rate (Meyer, 1986). Solid non-comedo DCIS is another subtype with solid proliferation without forming acini or micropapillae. The cribriform pattern is composed of cells forming evenly spaced, uniform microacini. The cells of micropapillary non-comedo DCIS pose small papillary projections.

Lobular carcinoma in situ (LCIS). It accounts for 5-10 per cent of breast cancer cases. It consists of uniform small cells with round nuclei and distinct cell membranes and moderate amount of relatively clear cytoplasm (Figure 1.4). Occasionally, it is difficult to

distinguish between DCIS and LCIS, since both may extend into breast lobules and extralobular ducts. In some cases histology pattern may present an intermediate form.

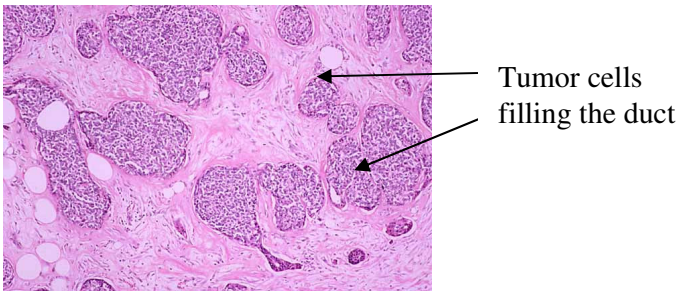


Figure 1.3. An example of DCIS section stained with hematoxylene (purple) and eosin (pink) where tumor cells fill the duct (http://genome-www.stanford.edu/breast_cancer/molecularportraits/histology.shtm -BC14)

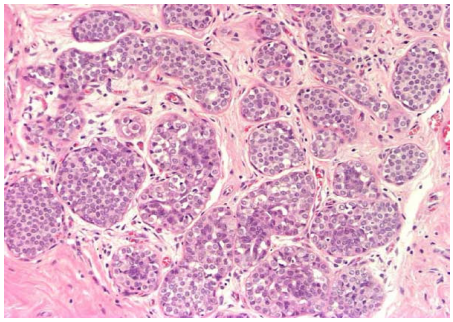


Figure 1.4. An LCIS section stained with hematoxylene and eosin (http://conganat.sld.cu/6CVHAP/conferencias/figure-3g-Masarelli.jpg)

1.3.2.2. Invasive Breast Cancer. Invasive carcinomas constitute 90 per cent of breast cancers. Although categorizing these cancers is quite difficult due to heterogeneity, it is important in providing information about prognosis, pattern of metastatic spread and behaviour.

Infiltrating ductal carcinoma (Invasive DCIS): It accounts for 60 per cent of all breast cancers and 70 per cent of invasive ones. It is the most aggressive type within other invasive carcinomas of the breast. It is firm on palpation during macroscopic examination and has a microscopic gritty texture. Infiltrating DCIS consists of glands or solid nests enclosed within strands of connective tissue but do not have a typical histology (Figure 1.5). The cytologic features are also heterogenous where the size of the cells may vary

from small to large and shape can be round, pleomorphic or intermediate. The amount of cytoplasm is highly variable as well. The nuclear features that consist of the size, shape, chromatin and nucleoli, may show mild to severe atypical forms. To categorize invasive DCIS, different grading systems have been established (eg. Bloom-Richardson grading system) (Bloom and Richardson, 1957). Bloom-Richardson grading system depends on major histologic features like the percentage of tubule formation, the degree of nuclear pleomorphism and the number of mitosis in a field.

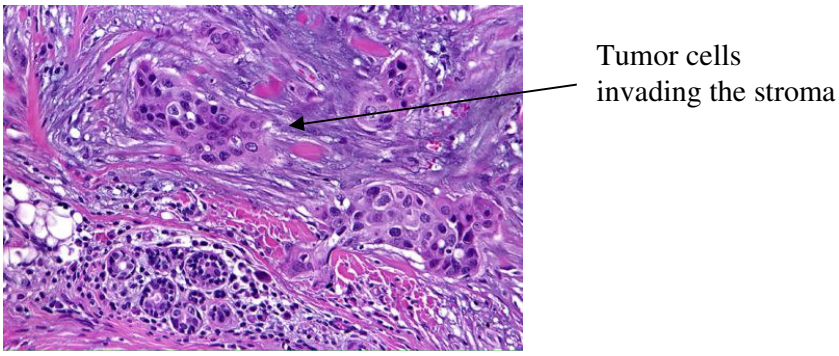


Figure 1.5. An example of invasive DCIS where tumor cells invade the surrounding stroma (http://www.breastpath.com/photos/s99=2520A420xinv.htm)

Infiltrating lobular carcinoma (Invasive LCIS): It accounts for 15 per cent of invasive breast cancer cases (Simpson and Page, 1992; Page, 1991; Anderson *et al*., 1991). It may form a palpable mass that can be detected but presence of a diffuse lesion does not allow its detection either by palpation or mammography. Histologic appearance of classic invasive LCIS includes small, round, poorly cohesive cells with low-grade nuclear features (Figure 1.6). The cells are dissociated from each other, form single files (Indian file) or targetoid patterns around uninvolved ducts. Several variants have been identified as the solid, alveolar, mixed and pleomorphic subtypes. The cytologic features of all types except the pleomorphic subtype are the same in the classical invasive LCIS and are distinguished by their growth patterns. The pleomorphic LCIS shows a diffuse growth pattern and is distinguished by its high-grade nuclear feature. It may be confused with invasive DCIS.

Medullary carcinoma: It is a relatively uncommon type of breast carcinoma (5 per cent) that occurs in younger people. It is usually palpable. The microscopic features of the typical medullary carcinoma are solid-syncytial groups of cells with pushing edge high

grade nuclei, loose stroma and a lymphocytic infiltration. The atypical subtype does not have a well defined pushing edge and presents less lymphoid infiltration.

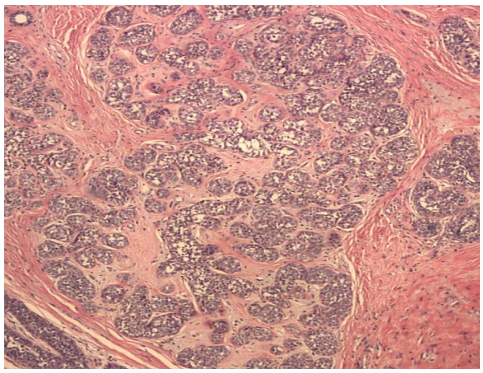


Figure 1.6. An example of infiltrative LCIS where tumor cells invade the stroma (http://interpath1.uio.no/norcyt/arm2002/wk/c2/hist.htm)

Tubular carcinoma: It accounts for 5 per cent of all breast cancers and involves formation of tubules. The prognosis of the typical subtype is stated to be the best of all the invasive breast carcinomas. It may present a palpable mass that can be detected by mammography. Microscopically they are composed of small glands or tubules with varying shapes. The nuclei do not show high grade features.

Invasive cribriform carcinoma: It is biologically similar to tubular carcinoma (Venable *et al*., 1990) since it is composed of masses of small regular cells as in the previous lesion. The invasive islands present a cribriform appearance. Nuclei do not show high grade degrees of atypia.

Mucinous carcinoma: It is also known as mucoid, gelanitous or colloid carcinoma and accounts for 2-3 per cent of all breast cancers. It has typical and variant subtypes in which the former presents a better prognosis. The variant subtype is more common in old women (Rosen *et al*., 1985). It is characterized with soft palpable mass. Microscopically it is composed of small islands of malignant cells suspended in extracellular mucin. Cytological and nuclear features are of a low-grade to intermediate-grade tumor.

Rare types of breast cancer: Collectively they account for less than 1 per cent of all breast cancer cases. Adenocystic carcinoma, carcinosarcomas, metaplastic carcinomas

(Wargotz and Norris, 1990), invasive papillary carcinoma (Fisher *et al*., 1980), basal cell carcinomas, signet-ring cell carcinoma (Merino and LiVolsi, 1981), and the so-called lipid-rich carcinomas have been observed in few patients. Since they are rare, clinical correlations could not be performed (Damjanov, 1996).

1.3.3. Molecular Biology of Breast Cancer

Breast cancer is mainly caused by accumulation of many genetic changes and/or mutations in different genes. Mutations in the breast stem cells and acquired somatic mutations due to ionizing radiation, chemical carcinogens or oxidative damage may be responsible for the disease.

Hereditary form of the disease represents 5-10 per cent of all cases. BRCA1 and BRCA2 are the major breast cancer susceptibility genes (Marcus *et al*., 1996; Miki *et al*., 1994; Stratton and Wooster, 1995). Both of these genes maintain genomic stability through homologous recombination and repair of double-strand breaks. They also have a role in transcription and cell cycle control (Venkitaraman, 2002) acting as tumor suppressor genes. BRCA1 was found in complex with RNA polymerase II through RNA helicase A (Scully *et al*., 1997) and regulate transcription. BRCA1 was shown to stimulate transcriptional activity of p53 and androgen receptor (Park *et al*., 2000; Yeh *et al*., 2000), repress the activity of estrogen receptor-α (ERα) (Fan *et al*., 1999, 2001; Ma *et al*., 2005) and c-myc (Wang *et al*., 1998). BRCA1 also stimulated the expression of several growth inhibitory genes (Somasundaram *et al*., 1997; Jin *et al*., 2000; Williamson *et al*., 2002). Various studies have shown transactivation of several cyclin-dependent kinase inhibitors via BRCA1 (p21 and p27KIP1) that block the S-phase entry (Somasundaram *et al*., 1997; Williamson et al 2002). However, less evidence is found for BRCA2 as a transcriptional regulator. Mutations in BRCA1 make up 40 per cent and those of BRCA2 make up 25 per cent of hereditary cases (Berry *et al*., 1997; Couch *et al*., 1997). The mutations at either end of the *BRCA1* gene are associated with more aggressive tumors. Mutations at the 5' end are observed in breast and ovarian cancers, and ones closer to the 3' end are observed only in breast cancer. Epigenetic inactivation of BRCA1 due to promoter hypermethylation has been observed in some of the breast cancer cases (Dobrovic and Simpfendorfer, 1997).

However, BRCA1 mutations have incomplete penetrance where 16-55 per cent of BRCA1/BRCA2 mutation carriers do not develop breast cancer (Antoniou *et al*., 2003; Ford *et al*., 1998).

Other than BRCA genes, germline mutations of p53, ATM, CHECK2, and PTEN were found to be associated with breast cancer (Hill *et al*., 1997, Bell *et al*., 1999, Cantor *et al*., 2001). Mutations of a tumor supressor gene, p53, were observed in less than 75 per cent of all breast cancer cases (Norberg *et al*., 1998; Geisler *et al*., 2001) and 20-40 per cent of sporadic ones (Beroud and Soussi, 1998; Soussi *et al*., 2000). p53 has been known to be involved in cell cycle regulation, DNA damage repair, apoptosis and inhibition of angiogenesis. Therefore loss of functional protein was suggested to eliminate the growth arrest in response to DNA damage and allow the replication of mutated DNA. Altered expression of p53-regulated genes could also be observed.

ATM protein acts via detection and repair of double strand breaks caused by ionizing radiation. Individuals homozygous for ATM mutations suffer from a degenerative disease, Ataxia Telangiectasia, that causes susceptibility to cancer (Thompson and Easton, 2004). CHECK2 is a G2 check point kinase that repairs DNA breaks. Deletion mutations of CHECK2 gene were identified in 5 per cent of familial breast cancer cases with no BRCA1/2 mutations (CHECK2 breast cancer case-control symposium, 2004). PTEN is a lipid phosphatase that was identified as a candidate tumor suppressor gene. It is suggested to inhibit PKB/Akt that is required for cell growth and survival (Downward J, 1998) and block integrin-mediated cell migration thus preventing metastasis (Tamura *et al*., 1998). This finding has suggested the formation of metastasis in the case of loss of PTEN function.

The role of HER2 in breast cancer was identified 20 years ago. HER2 (c-erb-B2) is one of the epidermal growth factor (EGF) receptors. EGF receptors are known to interact with many ligands (eg EGF, TGF-α, hergulin) that induce hetero- or homodimerization of the receptors. This was followed by autophosphorylation and activation of the intrinsic catalytic domain. EGF stimulates proliferation, angiogenesis, metastasis and prevention of apoptosis through EGFR binding (Sunpaweravong and Sunpaweravong, 2005). The HER2 gene was known to function as a proto-oncogene and was overexpressed in 25-30 per cent

of all breast cancer cases (Slamon *et al*., 1987). The overexpression was associated with faster disease progression and metastasis, more aggressive clinical course, decreased survival time and lower response to chemotherapy (Horton, 2002; Slamon *et al*., 1987; Tetu and Brisson, 1994). A monoclonal antibody against HER2, Trastuzumab, has been developed that inhibits proliferation, cell growth and induce breast cancer cell apoptosis (Kita *et al*., 1996; Kunisue *et al*., 2000). These findings have enabled the use of Trastuzumab for therapy in patients that overexpress HER2.

Epigenetic changes in breast cancer initiation and progression has been studied for the last decade. Hypermethylation and global hypomethylation of certain genes have been correlated with breast cancer. Hypermethylation of a tumor suppressor gene p16 was proposed to increase the cell growth regulatory signals (Herman *et al*., 1995; Silva *et al*., 2003) and associated with lymph node metastasis (Hu *et al*., 2003). Methylation of DNA damage response genes eg. BRCA1 (Niwa *et al*., 2000) and mismatch repair genes hMLH1 and hMSH2 (Murata *et al*., 2002) have been reported in breast cancer cases. Disruption of repair genes may increase the accumulation of sporadic mutations that is favorable for cancer cells. Silencing of estrogen receptor, progesterone receptor and retinoic acid beta 2 (RARβ2) through methylation have been detected *in vitro* and *in vivo* (Piva *et al*., 1990, Lapidus *et al*., 1996, Sirchia *et al*., 2000; Widschwendter *et al*., 2000). Methylation of E-cadherin gene (cell adhesion molecule) and TIMP-3 gene (inhibitor of proteases) (Graff *et al*., 2000; Bachman *et al*., 1999) may promote metastasis in breast cancer. Cyclin D2 and a putative cytokine, High in Normal-1 (*HIN-1*) genes were shown to be methylated in invasive breast cancer cases (Fackler *et al*., 2003; Krop *et al*., 2001). The 14-3-3σ gene that has a role in signal transduction, cell cycle regulation, apoptosis and malignant transformation, was found to be methylated in more than 50 per cent of primary invasive breast carcinomas (Evron *et al*., 2001). Global hypomethylation was correlated with genomic instability in breast cancer as well (Vilain *et al*., 1999). This was suggested to be due to activation of tumor promoting genes or pro-metastatic genes. Although no proto-oncogenes has been shown to be demethylated, certain metastasis genes like HEPARANASE, that degrades the heparan sulphate proteoglycans, and uPA that is a serine protease were hypomethylated in invasive breast cancer cells (Shteper *et al*., 2003; Guo *et al*., 2002).

1.3.4. Breast Cancer Metastasis

Metastasis is the most life-threatening aspect of cancer. Benign and malignant forms of breast cancer are differentiated by metastasis. It involves a complex process including (i) detachment of cells from a primary tumor, (ii) degradation of basal membrane via secretion of proteolytic enzymes, (iii) entry to blood or lymphatic circulation system (intravasation), (iv) adhesion to a secondary tissue and angiogenesis (Figure 1.7).

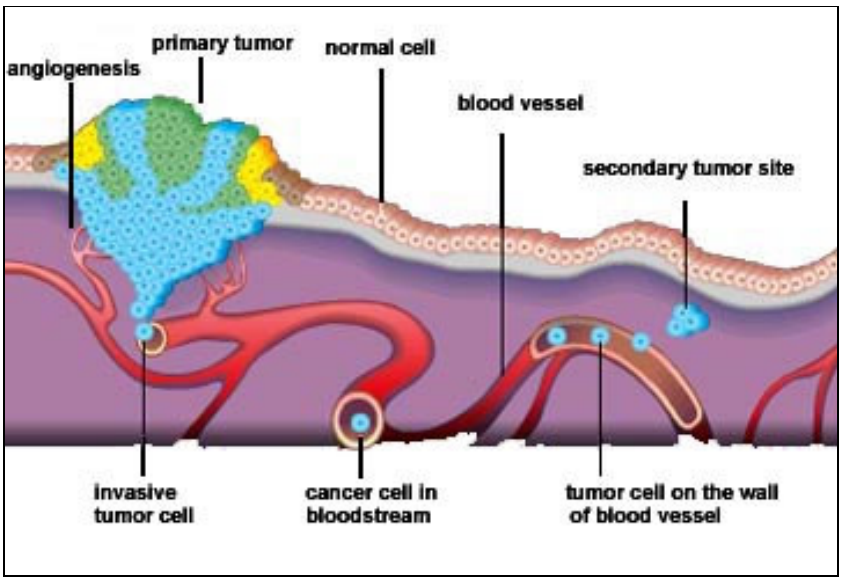


Figure 1.7. Schematic diagram of major steps of metastasis (http://www.sciencemuseum.org.uk/exhibitions/lifecycle/77.asp)

1.3.4.1. Detachment of Cells from a Primary Tumor. For a tumor cell to metastasize, first, it has to lose attachment to the primary tumor. Immunoglobulin-like adhesion molecules (CAMs) and cadherins are two families of CAMs that attach the cells to each other. Cadherins are transmembrane glycoproteins that interact with the cytoskeleton via catenins. E-cadherin, identified as a tumor suppressor gene, is lost in some of the carcinomas enabling cancer cells to lose coherence and gain invasive characteristics (Behrens *et al*., 1989). Studies on adhesive capacity of metastatic and non-metastatic cancer cell lines have shown that metastatic ones have much less adhesion capacity than non-metastatic ones (Mycielska *et al*., 2004). Integrins are molecules that mediate cell-extracellular matrix (ECM) interactions and intracellular signal transduction. Altered

expression pattern of integrins can confer motility to tumor cells and invasion through the basement membrane.

1.3.4.2. Degradation of Basal Membrane via Secretion of Proteolytic Enzymes. The basement membrane and its underlying stroma contain the extracellular matrix and make up the major connective tissue units separating organ compartments. Migrating tumor cells need to penetrate the epithelial basement membrane and enter the stroma during metastasis. The basement membrane contains type IV collagen, glycoproteins (e.g. laminin), fibronectin, proteoglycans, and embedded growth factors. The organization and distribution of basement membrane is subject to change during the transition to an invasive carcinoma. Tumor cells have to secrete proteolytic enzymes to degrade the extracellular matrix barriers. Almost all migrating tumor cells overexpress one or more of these enzymes (Nagase and Woessmer 1999). Degradation of the basement membrane depends not only on the amount of proteolytic enzymes present but also on the balance of active proteases and their inhibitors.

1.3.4.3. Intravasation. Migrating tumor cells need to penetrate the circulation to reach a secondary tumor site. This is achieved by attachment to the stromal face of the vessel, proteolysis of the basement membrane, movement between the endothelial cells and survival in the vascular system. Only a few cells manage surviving and get arrested in the capillary beds of target organs and invade, forming secondary tumors. A protease receptor (urokinase receptor) is found to play role in intravasation process (Hollas *et al*., 1991). Extravasation is required for the escape of the tumor cells from the vessel and is the same but reverse process of intravasation. E-selectin, a transmembrane adhesion molecule, is expressed on endothelial cells and is important for the attachment of cancer cells to the endothelium (Pecorino, 2005).

1.3.4.4. Adhesion to a Secondary Tissue. To successfully establish a metastatic colony, the circulating tumor cells should escape the immunologic surveillance, arrest at a distant site and extravasate. Loss or alteration of cell adhesion molecules, integrins and cadherins can facilitate cell motility thus invasion through the organ parenchyma (Chan *et al*., 1991). Proteolytic degradation of extracellular components of the basement membrane and the connective tissue is required for this step.

1.3.4.5. Angiogenesis. Once tumor cells attach to their secondary tissue they need to proliferate and survive in this new environment. Nutrients and oxygen needed for cells' proliferation are supplied via angiogenesis. Tumor vascularization is, therefore, one of the rate-limiting steps for tumor metastasis and growth (Folkman *et al.*, 1974). It has been hypothesized that without angiogenesis, balance between cell proliferation and apoptosis is steady thus the volume of primary tumors is kept constant (Holmgren *et al.*, 1995). A tumor mass larger than 0.125 mm^2 cannot survive only with nutrient diffusion therefore initiates angiogenesis (Folkman *et al.*, 1974). Angiogenesis requires destabilization of the mature vessel, proliferation and migration of endothelial cells and maturation. The angiogenic switch is dependent on the balance of angiogenic inducers and inhibitors. Growth factors like fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and their receptors VEGFR, angiopoetins, and Tie receptors, ephrins and ephrin receptors are examples of angiogenic inducers (Pecorino, 2005). Angiostatin (that blocks annexin) and endostatin (that blocks MAPK pathway and MMPs in endothelial cells) are well known inhibitors of angiogenesis.

1.3.4.6. Metastasis Suppressor Genes: These genes encode proteins that specifically suppress metastasis without affecting the primary tumor growth. Non-metastatic 23 (Nm23) is the first such gene identified (Steeg *et al.*, 1988). To this date, 11 metastasis suppressor genes have been identified in different cancers (Dong *et al.*, 1995; Seraj *et al.*, 2000; Lee *et al.*, 1996; Gildea *et al.*, 2002; Goldberg *et al.*, 2003; Xia *et al.*, 2001; Fu *et al.*, 2003; Guan *et al.*, 2000). The common feature of these genes is reduced expression in highly metastatic cancer cells when compared to non-metastatic ones (Steeg, 2003). Metastatic suppressor genes affect various cellular functions including invasion, growth factor receptor signaling, MAP kinase pathway, cell-cell communication and transcriptional regulation (Bogenrieder and Herlyn, 2003).

1.3.4.7. Mechanisms of Metastasis: Several hypotheses have been proposed to explain the metastatic potential in cancer. The most prevailing one is established by Stephen Paget (1889) that states the 'seed and soil' phenomena. This hypothesis proposes the metastasis of specific cancers to certain organs. It is based on the affinity of metastasizing tumor cells (seeds) to certain organs (soil). Paget proposed metastatic heterogeneity, where only selected cells succeed in invasion, survive in circulation and attach to a secondary tissue, thus achieve metastasis. For a targeted metastasis, these cells require specific cell surface

receptors and growth factors expressed by the organ (Fidler, 2003). Chemokines are found to be one of these receptors that recruit metastatic breast cancer cells to lungs and lymph nodes. The cancer cells that metastasize express the chemokine receptors, and the target sites express the molecules that bind to these receptors (Muller *et al.*, 2006). Cancers of the esophagus, head and neck and others that express cytokines were determined to form secondary tumors at specific sites (Kaifi *et al*., 2005; Muller *et al*., 2006; Ishikawa *et al*., 2006; Saur *et al*., 2005). Growth factors like PDGF can also influence the success and site of metastasis (Uehara *et al*., 2003). Many studies have supported Paget's hypothesis for different cancers (Holleran *et al*., 2002; Gauthier *et al*., 2004). Results of genetic analysis brought the idea that a metastatic tumor cell requires accumulation of mutations whereas a non-metastatic cell does not. Analysis of the genetic changes in tumors of different stages showed that the number of alterations in late stage tumors was more than those in early stage ones (Yokota, 2000). The animal experiments showed that metastasis is rare and only 0,001 per cent of tumor cells contribute to form a secondary lesion (Weiss, 1990; Egan *et al*., 1987). In several studies metastasis was formed by injecting breast cancer cells to mice. The results showed differences in gene expression profiles of metastasis formed in different organs (Kang *et al*., 2003; Minn *et al*., 2005). The authors concluded on the presence of organ-specific gene expression profiles. The major drawback of these studies is the difficulty in identifying whether the differences result from the secondary events that occurred during metastasis or are required for the dissemination of the primary tumor.

However, clinical evidence showed the presence of a classic mutation accumulation profile only in a small percentage of cancers. Furthermore, a positive correlation between increasing number of mutations and histologic aggresiveness could not be established for breast, prostate and lung carcinoma (Schedin and Elias, 2004). These observations led to establishment of a novel hypothesis for tumor progression and metastasis. It proposes that metastasis does not require accumulation of mutations and it is not a late event but there are tumor cells that are born to be metastatic. Expression profiles of primary tumors suggested that mutations causing metastasis are acquired relatively early in tumor progression (Veer *et al*., 2002). Supporting results came from DNA microarray studies that showed similarities in gene expression patterns of both metastatic cancer cells and primary tumor cells (Bernards and Weinberg, 2002; Weigelt *et al*., 2003; Budhu *et al*., 2005). This hypothesis may explain the reason of metastasis observed in small-sized primary tumors.

Another study that analyzed the gene expression patterns of primary and secondary tumors, has proposed the establishment of the route of metastasis early in primary tumor development (Braun *et al*., 2001). Since metastatic potential could be present from the start of tumor development researchers state that oncogenes and tumor suppressor genes that initiate cancer could also trigger metastasis. This statement was confirmed by the findings of mouse models in tumorigenesis studies that showed the initiation of metastasis in response to mutations in *ras* and *myc* oncogenes. According to this hypothesis there are no metastasis-causing genes but already identified cancer genes play a role in metastasis (Bernards and Weinberg, 2002).

A more recently proposed hypothesis indicates the importance of inheritance in the metastasis process (Park *et al*., 2005). Signal-induced proliferation-associated gene 1 (*Sipa1*) is found to be involved in strain specific differences in metastasis efficiency, an evidence for a predisposition model. In this study, altered expression of *Sipa1* allelic variants was shown to modulate metastatic efficiency by changing the cellular adhesion and proliferation. This finding indicates the involvement of polymorphisms in the metastatic potential. In another study 17-gene expression profile was determined in mice that could predict metastasis in primary breast tumors. The same profile was also identified in normal mammary tissues of the same mice suggesting that metastasis-associated gene expression pattern was pre-existing in normal tissue. The authors thus suggested that metastasis could be constitutional or a heritable trait (Qiu *et al*., 2004) and does not require accumulation of mutations.

Another alternative hypothesis proposed the origin of metastasis to be the response of the body against a systemically acting carcinogen but not a primary tumor (Freireich *et al*., 2005). The authors suggested that the difference between the primary and secondary tumors was not the result of clonal evolution and that they were different primary tumors arised from a systemic carcinogen. Observation of tumors with an unknown primary source supports this hypothesis. This alternative hypothesis has been established from a biologic point of view and may not fulfill the exact mechanism of metastasis at molecular level.

1.3.5. Breast Cancer and Estrogen

The role of estrogen in the promotion and development of breast cancer was first suggested by Beatson (1896). The biological effects of estrogen are mediated through an interaction with the estrogen receptor (ER). ER is a ligand-activated transcription regulator that acts mainly on genes that promote breast cancer cell proliferation and survival eg. insulin-like growth factor 1 receptor (IGFR), cyclin D1 and antiapoptotic Bcl2 (Klinge, 2001; Lee *et al*., 2001; Sanchez *et al*., 2002; Schiff *et al*., 2004).

Estrogen receptor contains a DNA-binding domain (DBD), two transcriptional activation function (AF) domains and a hinge domain. AF1, that is located on the amino terminal of the protein, is constitutively active and ligand independent whereas AF2 is ligand-dependent and resides in the carboxyl terminal (Figure 1.8). Phosphorylation of AF1 can regulate its activity and gain the ligand independent property to the ER (Ali and Coombes, 2002). The hinge domain contains the nuclear localization signal (Picard *et al*., 1990).

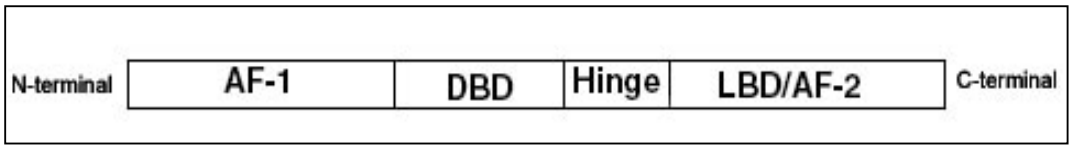


Figure 1.8. ERα structure and organization. AF; Activation Function domain, DBD; DNA-binding domain, LBD; Ligand-binding domain (modified from Platet *et al*., 2004)

ER was first cloned subsequently by two groups (Green *et al*., 1986; Greene *et al*., 1986). It is currently named as ERα since another ER (ERβ) was discovered in 1996 (Mosselman *et al*., 1996). Although ERα and ERβ are highly homologous in DNA- and ligand-binding domains (96 per cent) and have similar responsiveness to estrogen, there are many structural and functional differences between ERα and ERβ. These differences can be summarized as; diverse chromosomal locations of the coding genes (Enmark *et al*., 1997), presence of many different nonconserved domains (eg. activation function 1 (AF1), hinge region), (Mosselman *et al*., 1996), differential tissue expression and different roles in the development of mammary duct and breast cancer (Korach, 1994; Krege *et al*., 1998). ERα and ERβ have been shown to form homo/heterodimers complicating their individual

and/or combined function within the cell. Although there is evidence for ERα expression at the initial steps of breast cancer, the exact roles of ERα and ERβ in initiation and progression of the disease are still unclear. It has been suggested that the ERα:ERβ ratio but not the individual levels of ERα and ERβ determines the progression of cancer (Leygue *et al*., 1998).

1.3.5.1. Estrogen Receptor-alpha (ERα). The ERα gene is located on chromosome 6q25.1 and 140 kb of size. The coding region contains eight exons and eight introns shown as shaded area in Figure 1.9 (Gosden *et al*., 1986; Menasce *et al*., 1993). In addition to this, eight upstream untranslated exons are present (A-E, T1 and T2). The alternative exons enable tissue specific expression, for example exons T1 and T2 are expressed in testis and epididymis respectively (Brand *et al*., 2002).

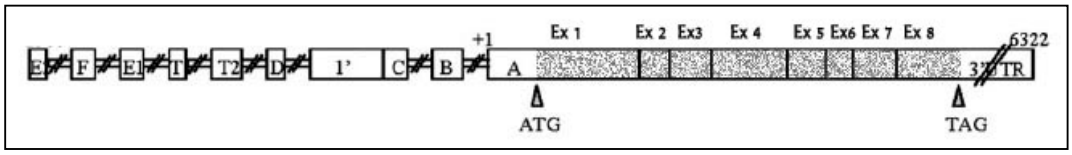


Figure 1.9. The structure of ERα gene (modified from Hernyk and Fuqua, 2004)

Various splice variants of ERα mRNA also exist that are expressed in different normal and cancerous tissues (Hernyk and Fuqua, 2004). It is suggested that expression of these variants is required for normal physiological processes (Petursdottir *et al*., 2001).

ER signaling is required for the development and maturation of the mammary gland by stimulating DNA synthesis and promoting bud formation (Anderson *et al*., 1982; Dickson *et al*., 1986; Huseby *et al*., 1984; Mueller *et al*., 2002). Studies have shown that only 7-10 per cent of the normal breast epithelial cells express ERα (Ricketts *et al*., 1991) Overexpression of ERα was determined in breast tumors through the distal promoter B (Figure 1.19) (Hayashi *et al*., 2003). Another cis-acting element downstream of the transcription start site was found to enhance ERα transcription in breast cancer cells (Tanimoto *et al*., 1999). Loss of ERα gene expression that usually causes hormone insensitivity in breast cancer patients is suggested to be through methylation of the promoter region. Loss of transcriptional activators can also explain the decrease in ERα gene expression (Hayashi *et al*., 2003).

ERα and ERβ are co-expressed in breast, brain, cardiovascular system, urogenital tract and bone (Enmark *et al*., 1997; Kuiper *et al*., 1997; Gustafsson, 1999; Taylor and Al-Azzawi, 2000) whereas ERα is predominant in the liver and uterus (Hayashi *et al*., 2003).

1.3.5.2. Estrogen Receptor-beta (ERβ). Unlike ERα, ERβ is expressed in 80-85 per cent of the normal breast epithelia (Roger *et al*., 2001). However, the mode of action of ERβ in breast cancer is not well known. Some contrasting functions have been suggested for ERβ e.g. as a protector of carcinogenesis (Leygue *et al*., 1998, Jarvinen *et al*., 2000) or as an inducer of malignancy (Hu *et al*., 1998). In terms of prognosis, ERβ expression seems to have both beneficial (Leygue *et al*., 1998, Jarvinen *et al*., 2000; Mann *et al*., 2001) and harmful effects (Speirs *et al*., 1999; Speirs and Kerin., 2000; Shaaban *et al*., 2003). These inconsistent results may be caused by difficulties in PCR-based analysis (use of tumor samples that also contain normal cells like stroma or amplification of different splicing products), patient selection criteria and the low number of cases analyzed. Usually studies analyzing the ERβ protein level have shown that ERβ was accompanied with decrease in proliferation and invasion (Lazennec *et al*., 2001), absence of relapse (Omoto *et al*., 2001) and likely response to hormone therapy (Mann *et al*., 2001, Jarvinen *et al*., 2000). But the results of few studies in small groups of patients suggested association of ERβ expression with high proliferation and high tumor grade (Miyoshi *et al*., 2001; Jensen *et al*., 2001).

ERβ gene is localized on chromosome 14q and has several splice variants. The wild type ERβ (ERβ1) encodes a full-length 530-amino acid receptor protein, whereas ERβ2 (ERβcx) uses an alternative exon 8 and encodes 495 amino acid long protein. This alteration leads to a protein with a different C-terminus and relatively poor binding to estrogen (Ogawa *et al*., 1998) (Figure 1.10).

Both ERβ1 and ERβ2 are found to inhibit ERα function causing growth inhibition in ERα-positive breast cancer cells (Hayashi *et al*., 2003). A putative ERβ5 differs from ERβ1 at amino acid position 469 by alternative splicing (Moore *et al*., 1998). This results in truncation of C-terminus and loss of AF-2 domain and difference in ligand binding (Peng *et al*., 2003).

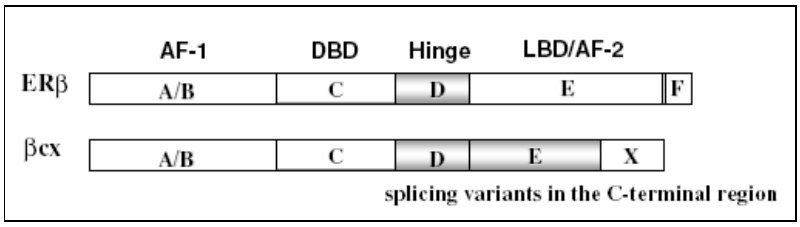


Figure 1.10. Structure of wild type ERβ and ERβ2 (ERβcx) (Hayashi *et al*., 2003)

In addition to the splice variants at the C-terminus of ERβ mRNAs, divergence at the 5' untranslated regions due to alternative splicing at the upstream of exon 1 was also determined (Hirata *et al*., 2001). Presence of these upstream exons, named 0K and 0N, indicated the transcription of ERβ gene through at least two different promoters (promoter 0K and promoter 0N) (Figure 1.11). Expression of various splice variants were shown in different cell lines and tumors (Leygue *et al*., 1999; Iwao *et al*., 2000a, b; Omoto *et al*., 2002; Tong *et al*., 2002) and suggested to affect the breast cancer phenotype and response to endocri ne therapy (Davies *et al*., 2004).

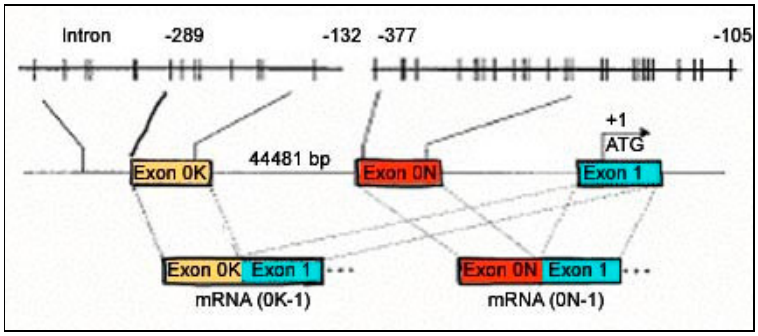


Figure 1.11. Schematic illustration of alternative promoter usage at 5' untranslated region of ERβ promoters (Hirata *et al*., 2001)

1.3.5.3. Genomic Action of Estrogen. The transcriptional activity of ER is mediated through AF1 and/or AF2 domains. Since AF1 is not active in ERβ, its activity is achieved by AF2. Transcriptional activation via ER requires the recruitment of general transcriptional factors, coactivators, corepressors, cointegrators, histone acetyltransferases and histone deacetylases (McKenna *et al*., 1999; Klinge, 2000; Tremblay and Giguere, 2002). Ligand binding to AF1 and/or AF2 domains induces a conformational change in the receptor and causes receptor dimerization (Schiff and Fuqua, 2002). This enables binding of coregulatory proteins (Schiau *et al*., 1998) that alter ER transcriptional activity on

specific DNA elements (Estrogen Response Elements, ERE) present on the promoter regions of target genes. If the coregulatory protein is an estrogen agonist, ER transcriptional activity is enhanced. If it is an estrogen antagonist (e.g. Tamoxifen), ER transcription is repressed (McKenna *et al*., 1999; Schiau *et al*., 1998). ER can also regulate gene expression through protein-protein interactions with transcription factors e.g. c-Jun, NFκB, and Sp1 (Kushner *et al*., 2000). These complexes play an important role in recruitment of transcriptional machinery, the modulation of chromatin structure and in the regulation of ER target gene expression (McKenna *et al*., 1999). The genomic action of estrogen is slow since the diffusion of estrogen to the nucleus to exert its effect takes time. However, rapid effect of estrogen has also been determined that is described in the following section.

1.3.5.4. Non-Genomic Action of Estrogen. A non-genomic action of estrogen has been proposed due to its rapid effect (in a few seconds or minutes), insensitivity to inhibitors of mRNA and protein synthesis, stimulation of secondary messengers including cAMP (Nakhla *et al*., 1990), inositol phosphate (Le Mallay *et al*., 1997) and calcium (Morley *et al*., 1992; Audy *et al*., 1996). It is independent of the classic gene transcription and is probably initiated outside the nucleus. Some of the studies suggested that, this activity was mediated through traditional ERs or by a closely related splice variant located on the plasma membrane (Li *et al*., 2003, Figtree *et al*., 2003). However, a role for an unrelated membrane ER has been proposed whose action was blocked by inhibitors of G-protein signaling (Le Mallay *et al*., 1997; Filardo, 2002). The non-genomic action of estrogen through G-proteins has been shown in a variety of cell types (Gu *et al*., 1999; Nadal *et al*., 2000; Qiu *et al*., 2003). Activation of Erk via estrogen in breast cancer cells that do not express ERα/β, but a type of G-protein (GPR30), and the requirement of GPR30 for estrogen action proposed a possible role of this G-protein in these cells (Filardo *et al*., 2000). This finding was confirmed by further studies where estrogen-mediated signaling was sensitive to G-protein inhibitors in GPR30 transfected cells (Revenkar *et al*., 2005; Thomas *et al*., 2005). It has been shown that GPCRs mediate transphosphorylation of EGFR, via metalloproteinase-dependent (MMP) cleavage of heparan-bound EGF (Figure 1.12) (Prenzel *et al*., 1999). Through this pathway estrogen is proposed to promote EGF-like effects. Previous studies have also shown the relationship between EGFR and estrogen (DiAugustine *et al*., 1988; Mukku and Stancel 1985; Nelson *et al*., 1991) but the

mechanism was not known. In another study, stimulation of ER-EGFR interaction with estrogen has been shown in breast cancer cells (Yang *et al*., 2004). Lately, the effect of estrogen on the motility and morphology of breast cancer cells were reversed with the use of EGFR inhibitor showing the relation between estrogen and EGFR (Azios and Dharmawardhane, 2005).

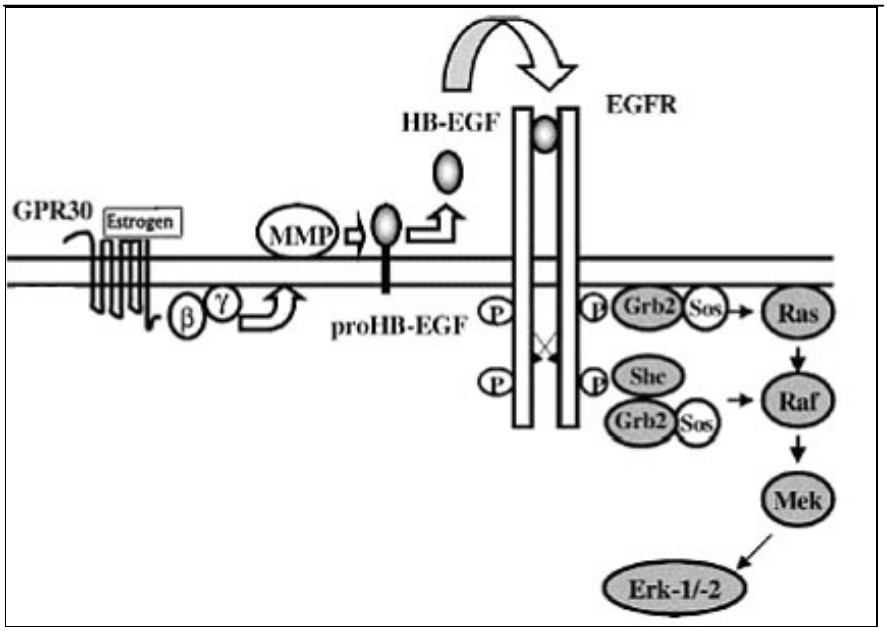


Figure 1.12. The schematic diagram of estrogen action through GPCR and transactivation of EGFR (Filardo, 2002)

1.4. Estrogen and Ion Channels

It is well known that steroids like estrogen, progesterone, androgens and corticosterone have a role in developmental organization of the nervous system. For example, steroids organize the brain circuits during embryogenesis and perinatal development (Matsumoto, 1991; Roselli and Klosterman, 1998). In addition to these, determination of ERα/β in dorsal root ganglion (DRG) indicated the implication of estrogen in sensory and autonomic functions (Papka *et al*., 1997; Taleghany *et al*., 1999; Patrone *et al*., 1999). Further *in vitro* and *in vivo* analysis showed the effect of estrogen on dendritic spines, synapses within the hippocampus and in hippocampal, cortical and basal forebrain neurons (Yankova *et al*., 2001; Brinton *et al*., 2000; Brinton *et al*., 1997). The

mechanism of estrogen action in these cells was proposed to be through genomic and non-genomic pathways (Brinton *et al*., 1997). Detailed investigation showed that estrogen induced Ca^{+2} influx was followed by activation of Src/MAPK pathway and CREB (Zhao *et al*., 2005). With this study the effect of estrogen on neuroprotection and electrophysiological plasticity was enlightened. However, other studies showed that estrogen inhibited the Ca^{+2} current in a subpopulation of DRG neurons (Lee *et al*., 2002) and smooth muscle cells (Kitazawa *et al*., 1997; Nakajima *et al*., 1999). Inhibitory effect of estrogen on K^+ currents in neurons enhancing the excitability of the cells was also shown (Fatehi *et al*., 2005; Kelly *et al*., 2002; Carrer *et al*., 2003). Estrogen is also found to activate the Ca^{+2} channels in colon (Doolan *et al*., 2000) but inhibit in myocytes (Tanabe *et al*., 1999; Nakajima *et al*., 1999). It is suggested that estrogen achieves vasodilation in coronary smooth muscle cells by inhibiting the Ca^{+2} channels and activating the K^+ channels in these cells (Ruehlman *et al*., 1998).

In some studies, tamoxifen was shown to activate K^+ channels through its β-subunit in smooth muscle cells (Dick *et al*., 2001; Dick and Sanders, 2001; Dick, 2002) but inhibit in the arterial smooth muscle of the cardiovascular system (He *et al*., 2003; Thomas *et al*., 2003). Lately, this inhibitory effect of tamoxifen was suggested to be due to the level of channel activity and that β-subunit was not necessary. Since the channel inhibition was reversible β-subunit was proposed to stabilize the binding of tamoxifen (Perez, 2005). Another type of antiestrogen (clomiphene) was shown to inhibit ion currents generated by voltage-gated sodium and potassium channels and L-type calcium channels in heart myocytes (Borg *et al*., 2002).

The relation between estrogen and ion channels in cancer has also been analyzed. Studies showed that tamoxifen could be useful in prevention of brain tumors and their metastasis (Smitherman and Sontheimer, 2001). The mechanism of this action was suggested to be mediated through the astrocytes that control the brain microenvironment and express both TTX-sensitive and TTX-resistant sodium channels. Although these channels do not produce action potentials they can have a role in cell cycle progression. Therefore, blockage of these channels by tamoxifen could disrupt cell division thereby prevent tumor progression.

The detection of K^+ channels in breast cancer cells (Quadid-Ahidouch *et al*., 2004) initiated the investigation of its mechanism of action. Further studies have shown that estrogen increased cell proliferation via activation of K^+ channels through both genomic and non-genomic pathways in breast cancer cells (Coiret *et al*., 2005). Estrogen was suggested to exert its effect on K^+ channels either by binding to the modulatory β-subunit (Valverde *et al*., 1999; Dick and Sanders, 2001) or by binding directly to the functional α-subunit of the channel (Korovkina *et al*., 2004). In addition to these, estrogen upregulated the mRNA expression level of both α- and β-subunits of K^+ channels (Jamali *et al*., 2003; Benkusky *et al*., 2002).

The effect of estrogen on ion channels through the genomic pathway involves phosphorylation of cyclic AMP Response Element (CRE) via Protein Kinase A (PKA) and transcriptional regulation of certain genes that in turn acts on ion channels (Figure 1.13). The non-genomic pathway involves the G-protein linked receptors that upon estrogen binding, activate PKA and Protein Kinase C (PKC) via adenylyl cyclase (AC) and hydrolysis of PIP_2, respectively. PKC phosphorylates AC that in turn activates PKA. This would inhibit the activities of various K^+ channels (eg SK, GIRK) via direct phosphorylation (Figure 1.13). In addition to this, binding of estrogen to GPCR directly activates the K^+ channel, GIRK.

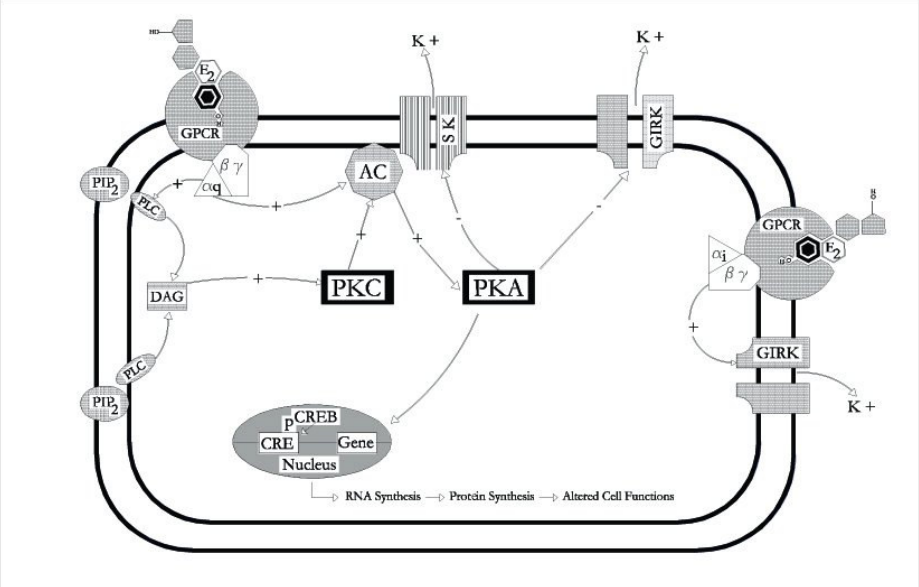


Figure 1.13. The schematic diagram showing both the genomic and non-genomic estrogen action and its effect on ion channels (modified from Kelly *et al*., 2002)

1.5. Ion Channels and Cancer

Ion channels are well known to be involved in excitation, muscle contraction, volume regulation and hormone secretion. Ion channels in tumor formation and progression have been studied in different cancers for more than a decade. Various types of voltage-gated potassium channels (VGPC) have been correlated with cancers of colon, breast, prostate, lung, stomach, urinary bladder, pancreas, lymphoma and melanoma (Abdul and Hoossein, 2002a; Lastraioli *et al*., 2004; Kim *et al*., 2004; Pardo *et al*., 1999; Quadid-Ahidouch *et al*., 2001, 2004a, b; Abdul *et al*.,2003; Mu *et al*., 2003; Abdul and Hoosein, 2002b; Fraser *et al*., 2003; Rane, 2000; Takanami *et al*., 2004; Shao *et al*., 2005; Jäger *et al*., 2004; Smith *et al*., 2002; Meyer *et al*., 1999; Schwab *et al*., 1999). Detailed analysis has shown the upregulation of some VGPCs in cancer and a role in proliferation of tumor cells. The activity of one of the K^+ channel subtypes was shown to change during cell cycle progression where an increase at M/G1 phase but a decrease at S/G2 phase was observed (Day *et al*., 2001). This channel was found to contain binding sites for MAPK, CDK-1 (Camacho *et al*., 2000) and other cell cycle regulatory proteins that may explain the above oscillation (Piros *et al*., 1999). On the other hand, changes in microtubules and intracellular Na^+ concentration affected the activity of K^+ channel throughout the cell cycle (Day *et al*., 2001). Regulation of K^+ channels via growth factors and kinases during cell division was also determined (Roderick *et al*., 2003). For examle, serum and glucocorticoid kinase (SGK1) that is activated through PI-3 kinase was found to inhibit the ubiquitin ligase Nedd4-2 thereby preventing endocytosis of ion channels. This finding may explain the reason of ion channel activation via mitogenic signals (Lang *et al*., 2003). Kinases like p21ras, Raf, and ras activate (Huang and Rane 1994) and Src kinase directly phosphorylate the K^+ channels (Sobko *et al*., 1998). Such post-translational modifications on K^+ channels may have an effect on cancer development and progression.

Upregulation of voltage-gated calcium channels (VGCC) was found to correlate with cancers of colon cancer, lung, prostate, melanoma (Wang *et al*., 2000; Tsavaler *et al*., 2001; Wissenbach *et al*., 2001; Peng *et al*., 2001). However, a role in cancer development is not suggested yet.

Voltage-gated sodium channel (VGSC) is another group of ion channels that has been correlated with breast cancer, prostate cancer and small cell lung carcinoma (Fraser *et al*., 2005; Roger *et al*., 2003; Diss *et al*., 2001; Abdul and Hoosein, 2002b; Onganer and Djamgoz, 2005). Functional analyses have indicated a role for VGSCs in directional motility (Djamgoz *et al*., 2001; Fraser *et al*., 2003); secretory membrane activity (Krasowska *et al*., 2004; Mycielska *et al*., 2003); adhesion (Palmer *et al*., 2006) and invasion (Fraser *et al*., 2004, 2005; Grimes *et al*., 1995; Laniado *et al*., 1997; Roger *et al*., 2003; Smith *et al*., 1998). Since these cellular behaviours are involved in metastasis, VGSC was suggested to have a role in the dissemination of cancer.

Sodium/potassium ATPase (Na^+, K^+-ATPase) that is a ubiquitous plasma membrane ion pump maintaining the normal Na^+, K^+ gradient in most of the eukaryotic cells was also shown to be involved in cancer (Skou and Esmann, 1992). Na^+, K^+-ATPase enables normal resting potentials and various cellular activities. The ionic homeostasis was found critical for cell growth, differentiation and cell survival. Na^+, K^+-ATPase was also shown to modulate cell migration and (Vogel *et al*., 1993; Woo *et al*., 2000), cell to cell interaction (Shoshani *et al*., 2005; Barwe *et al*., 2005; Contreras *et al*., 1995). Several studies determined altered expression and activity of Na^+, K^+-ATPase in cancers of the bladder, gastric, prostate, urothelial, renal clear carcinoma and breast. The disturbed ionic homeostasis of cancer cells was suggested to be due to changes in Na^+, K^+-ATPase activity. This statement was based on the findings where expression of Na^+, K^+-ATPase subunits were increased in gastric and bladder cancer (Lee *et al*., 2002). However, Na^+, K^+-ATPase activity and β-subunit expression was decreased in invasive renal clear carcinoma cells (Rajasekaran *et al*., 2003). Several studies showed that estrogen increased activity of Na^+, K^+- ATPase and regulated expression of subunits. Inhibitors of Na^+, K^+-ATPase were shown to prevent proliferation and due to their structural similarities act as ER antagonists specially for membrane bound ERs. These properties of Na^+, K^+- ATPase and its inhibitors support the idea that Na^+, K^+- ATPase in combination with ERs could be potential targets for the development of anti-breast cancer drugs (Chen *et al*., 2006).

1.6. Voltage-Gated Sodium Channels

VGSCs mediate Na^+ ion influx into the cells to generate action potentials in many excitable cells. VGSC was first isolated from electric eel electroplax and functional expression analysis was done in *Xenopus* oocytes (Goldin *et al*., 1986). VGSCs are composed of a functional catalytic α-subunit and regulatory β-subunits. At least 10 different human isoforms of α-subunit (Na_v 1.1-Na_v1.9 and Na_x) (260 kDa) and four different β-subunits (β1-β4) was identified (33- 36 kDa) (Figure 1.14). When expressed alone, α-subunit was sufficient for a functional channel in *Xenopus* oocytes (Goldin *et al*., 1986). The β-subunits act to modulate electrophysiological properties of the channel; accelerating inactivation, voltage dependence and gating (Isom, 2001). As well as influencing the kinetics of VGSC, β-subunits facilitates trafficking and anchoring of VGSCα on the plasma membrane thus increasing the availability of functional VGSCs. Since the β-subunits have immunoglobulin-like motifs similar to those found in many cell adhesion molecules, they have been proposed to have a role in cell-cell attachment (Isom, 2002). This was confirmed in a study where β1 and β2 were found to recruit ankrin protein to the site of cell-cell contact and interact with extracellular matrix proteins (Xiao *et al*., 1999; Srinivasan *et al*., 1998).

VGSCα is a glycoprotein made up of four homologous transmembrane domains (D1-D4) each of which contains six segments (S1-S6) (Figure 1.14). The four domains fold together to create a central pore whose structural constituents determine the selectivity and conductance properties of the channel. The voltage sensor is located in the S4 segment that contains a repeated motif made up of positively charged amino acid residues (Lys or Arg) (violet segments in Figure 1.14). These amino acids are stabilized by ionic interactions with negatively charged residues in adjacent transmembrane segments. Depolarization causes release of S4 segment outwards leading to a conformational change that opens the pore. A loop is present between helices S5-S6 (orange segments in Figure 1.14) that forms the ion selective pore. Glutamate and aspartate residues found in analogous positions in all four domains are thought to form the negatively charged outer and inner rings that serve as a receptor site for the selectivity filter. The carboxyl side chains of these amino acids interact with Na^+ passing through the channel thus determine its selectivity.

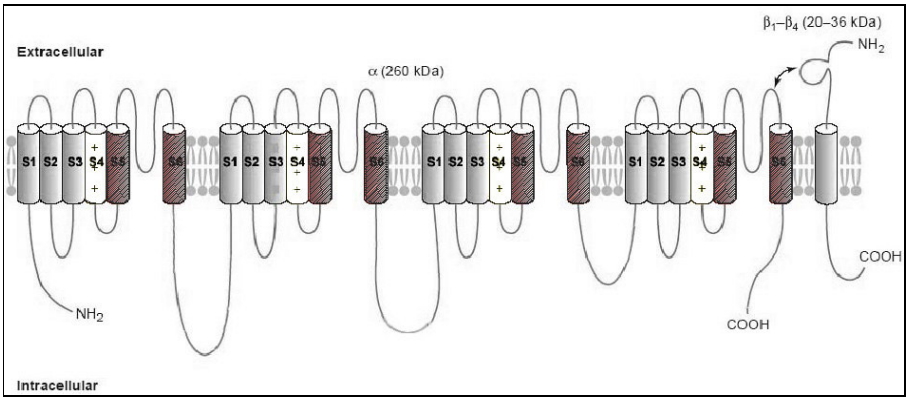


Figure 1.14. Structure of voltage-gated sodium channel α-subunit (modified from Chahine *et al*., 2005)

The inactivation gate is on the intracellular loop between D3-D4. When the cell membrane is depolarized, the VGSC is inactivated for a few milliseconds after its opening. The three hydrophobic amino acid residues, Ile-Phe-Met, (IFM motif) interacts with the pore blocking it just like a 'hinged-lid' (Figure 1.15).

VGSCα genes are made up of at least 20 exons that are localized on different chromosomes (Goldin, 2001). Na^+ channels are mainly characterized by their pharmacological properties. Genes found on the same chromosome usually show similarities in sequence, biophysical and pharmachological characteristics. Tetrodotoxin (TTX) blocks neural (eg. $Na_v1.1$-1.3) and skeletal muscle ($Na_v1.4$) isoforms at nanomole range whereas the cardiac ($Na_v1.5$) isoform requires micromolar concentrations to be blocked (Table 1.2). The glutamate at position 387 in D1 determines the TTX-sensitivity (Cummins *et al*., 1999; Akopian *et al*., 1996; Satin *et al*., 1992). However in the cardiac isoform a change of tyrosine or phenylalanine to cysteine at position 385 in D1 causes 200-fold decrease in TTX affinity when compared to brain and skeletal muscle isoforms (Heinemann *et al*., 1992).

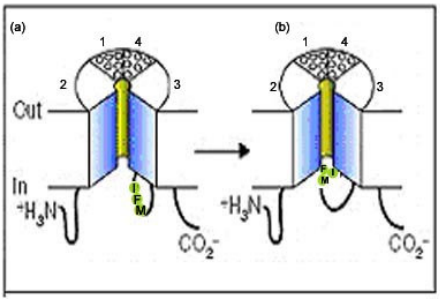


Figure 1.15. Inactivation gate of the VGSC (Yu and Catterall, 2003)

Table 1.2. The chromosomal and tissue distribution and TTX sensitivity of VGSCα

VGSC subtype	**Chromosome**	**Tissue localization**	**TTX sensitivity**
$Na_v1.1$	2q24	CNS, PNS, heart	TTX-S
$Na_v1.2$	2q23-24	CNS	TTX-S
$Na_v1.3$	2q24	CNS, heart	TTX-S
$Na_v1.4$	17q23-25	Skeletal muscle	TTX-S
$Na_v1.5$	3p21	Heart, CNS	TTX-R
$Na_v1.6$	12q13	CNS, PNS, heart, glia, nodes of Ranvier	TTX-S
$Na_v1.7$	2q24	PNS, Schwann cells	TTX-S
$Na_v1.8$	3p22-24	PNS	TTX-R
$Na_v1.9$	3p21-24	PNS	TTX-R
Na_x	2q21-23	Heart, uterus, lung, PNS smooth muscle, glia	TTX-R

1.6.1. Tissue Distribution of VGSCs

VGSCs are known to be expressed in excitable cells like nerve, heart and skeletal muscle. The studies have shown the presence of VGSC mRNA and proteins in these cells. Electrophysiological studies determined the functional VGSC protein on plasma membrane of heart muscle cells and located the protein immunohistochemically, in clusters, where they ensure uniform conduction of electric depolarization along and within the myocytes (Cohen, 1996). In the central nervous system (CNS) and the peripheral nervous system (PNS) different cells contain different VGSC subtypes. In brain, VGSC mRNA and protein were detected in the axons of granule cells ($Na_v1.2$), Purkinje cells

($Na_v1.2$), pyramidal cell layers of the cortex and neuronal cell bodies ($Na_v1.1$) (Whitaker *et al*., 2001; Chung *et al*., 2000). In the PNS, the VGSCs were mainly clustered at the nodes of Ranvier and internodal zones of the axons (Shrager, 1989). They were also detected in DRG neurons, Schwann cells and neuroendocrine cells (Sangameswaran *et al*., 1997; Belcher *et al*., 1995; Klugbauer *et al*., 1995).

Non-excitable cells were also investigated for the presence of VGSCs. Electrophysiological studies determined VGSC in glia (Chiu *et al*., 1984), osteoblasts (Black and Waxman, 1996), fibroblasts (Bakhramov *et* al., 1995) and endothelial cells (Walsh *et al*., 1998; Gosling *et al*., 1998). In another *in vitro* study, human umbilical endothelial cells (HUVEC) were found to express $Na_v1.4$ and $Na_v1.6$ that inhibited shear stress-mediated ERK1/2 activation. This finding proposed a role for VGSCs in signal transduction (Traub *et al*., 1999). According to an RNA based study, $Na_v1.9$ was expressed in non-neural cells like spermatogonia and spermatocytes of testis, granular cells surrounding the oocytes, syncytiotrphoblasts of placenta and goblet cells of small intestine (Ogata *et al*., 2000). Soon after this, an isoform of cardiac VGSC, $Na_v1.5$, was identified in the jejunal circular smooth muscle. The identified $Na_v1.5$ was found to have eight amino acid differences from the known $Na_v1.5$ at the linker between Domain 2 and 3 (Ou *et al*., 2002). Lymphocytes that move to the site of infection were known to contain VGSCs over a decade (Gaspar *et al*., 1992). Detailed investigation in a model system identified the expression of $Na_v1.5$ that gained the invasive capacity to the cells (Fraser *et al*., 2004).

1.6.2. VGSC and Cancer Metastasis

The presence of VGSCs in some cancer cells like small-cell lung carcinoma (SCLC) were known for more than a decade (Champigny *et al*., 1991; Marx *et al*., 1991; Pancrazio *et al*., 1989). However, a correlation between cancer cell metastasis and VGSC expression was shown by Grimes (1995) in prostate cancer cells. Later molecular analysis identified $Na_v1.7$ as the cause of these currents in metastatic cells (Diss *et al*., 2001). Further *in vivo* analysis has shown that the VGSCα protein was localized to epithelial cell membranes in different grades of prostate cancer tissues (Abdul and Hoosein, 2002b; Diss *et al*., 2005). Functional studies determined that VGSCα related cellular activities were required for metastasis in prostate cancer (Fraser *et al*., 1999; Fraser *et al*., 1998; Djamgoz *et al*., 2001;

Mycielska *et al*., 2000; Mycielska and Djamgoz, 2004; Grimes *et al*., 1995; Laniado *et al*., 1997.

Studies on breast cancer cells with different metastatic abilities determined the presence of a functional VGSC protein only in strongly metastatic breast cancer cells (Roger *et* al., 2003; Fraser *et al*., 2005). Investigation of the effect of VGSC on various cellular behaviour showed that VGSC was not involved in proliferation but in invasion of metastatic breast cancer cells (Roger *et al*., 2003). VGSC was also shown to be involved in directional motility and endocytosis that are basic steps of metastasis (Fraser *et al*., 2005). In this study, authors determined upregulation of the cardiac sodium channel, $Na_v1.5$, in its neonatal spliced form ($nNa_v1.5$) in metastatic breast cancer cells and tissues. A positive correlation was identified between $nNa_v1.5$ expression and metastasis in a small group of patients (Fraser *et al*., 2005). In a further study, site of alternative splicing in $nNa_v1.5$ was found to be on the D1:S3 extracellular loop resulting in 31 nucleotide difference (seven amino acids) when compared to the adult isoform (Figure 1.16). Such a difference between amino acids enabled development of a specific antibody against $nNa_v1.5$ (NESOpAb). Since the epitope was on the extracellular loop, *in vitro* application of the antibody specifically targeted and blocked the Na^+ current in $nNa_v1.5$ transfected cells. The sensitivity and selectivity of the NESOpAb was determined to be better than VGSC blockers, e.g. Tetrodotoxin (TTX) (Chioni *et al*., 2005).

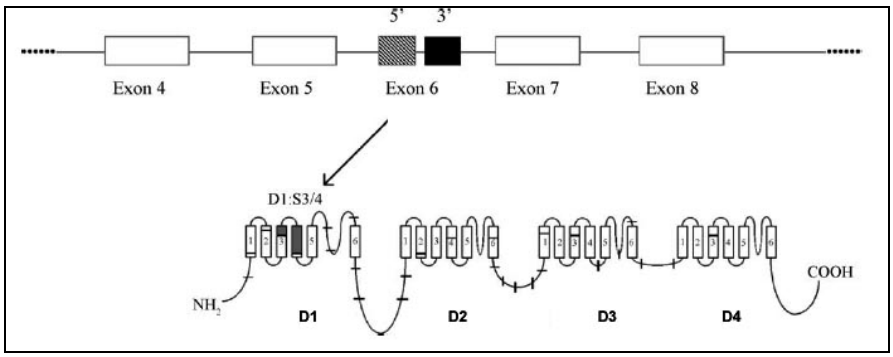


Figure 1.16. The location of alternative splicing in $Na_v1.5$ gene and protein (modified from Chioni *et al*., 2005)

Recently the presence of VGSCs in a highly aggressive cancer, small-cell lung carcinoma (SCLC) has been analyzed (Onganer and Djamgoz, 2005). *In vitro* analysis on

SCLC cells showed the role of VGSC in endocytosis as in the case of prostate and breast cancer (Fraser *et al*., 2005; Mycielska *et al*., 2003). Initial observation of this study state the expression of $Na_v1.3$, $Na_v1.5$ and $Na_v1.6$ genes in the cell lines tested.

Analysis of neuroblastoma tumor cells showed that these cells contain different VGSCs (Urbano *et al*., 1997). Characterization studies identified the expression of two different splice variants of $Na_v1.5$ whose effect on the pathophysiology of the disease is not known yet (Ou *et al*., 2005).

1.6.3. Regulation of VGSCs

VGSC regulation can be at transcriptional and post-translational stages. Such modifications can lead to differences in functional properties, level of transcription/translation and localization of the channel. Several growth factors like nerve growth factor (NGF), fibroblast growth factor (FGF) and its homologous factor 1B (FHF1B/FGF12), glial derived neurotrophic factor (GDNF), epidermal growth factor (EGF) and hormones (e.g. androgen and dexamethasone) affect the VGSC expression and/or activity depending on the cell type and subtype of the channel (Avila *et al*.,. 2003; Cummins *et al*., 2000; Tabb *et al*., 1994; Waxman *et al*., 2000; Zakon 1998; Zur *et al*., 1995: Liu *et al*., 2003). Both NGF and GDNF were found to upregulate $Na_v1.8$ and $Na_v1.9$ but downregulate $Na_v1.3$ in spinal sensory neurons (Black *et al*., 1997; Fjell *et al*., 1999). FHF1B was shown to bind to $Na_v1.5$ and modulate the inactivation state of the channel (Liu *et al*., 2003).

The analysis of VGSC regulation at transcriptional level has determined a binding site for transcription repressor REST on the RE-1 element on the promoter of $Na_v1.2$ (Chong *et al*., 1995, Kraner *et al*., 1992; Mori *et al*., 1992). Upon binding, VGSC expression was restricted to neuronal cells. Alternative splicing is another way of increasing the functional diversity of VGSCα. Up to now five different splice sites have been identified in different VGSCα genes. These occur at D1:S3, interdomain 1-2 (ID1-2), ID2-3, D3 and D4:S3 sites (Diss *et al*., 2004). The spliced products may have major differences in the highly conserved regions of the protein. These may associate with other VGSCα variants or prevent the synthesis/activity of a full-length protein or produce a non-

functional protein. Trans-splicing may cause increase in the number of exons or changes in the exon order. Such events may gain novel functions to VGSCs (Diss *et al*., 2004).

Post-translational modifications of VGSCs involve phosphorylation, glycosylation, and palmitoylation of the α-subunit. VGSCα can be phosphorylated by PKA, PKC, calcium calmodulin kinase II (CAM kinase II) and tyrosine kinase (Figure 1.17). PKA phosphorylates the VGSCα on four serine residues at positions Ser 573, 610, 623 and 687 on the Loop 1-2 in Figure 1.17 (Murphy *et al*., 1993). PKC phosphorylation takes place on serine residue at the inactivation gate (Ser 1506) and ser 576 at L1-2 (Numann *et al*., 1991; West *et al*., 1991; Cantrell *et al*., 2002) (Figure 1.17).

The effect of phosphorylation depends on the cell-type and subtype of VGSC. For example PKA phosphorylation of $Na_v1.2$ and $Na_v1.1$ decreased the amplitude of the ion current causing a reduction in the activity of the channel (Figure 1.17) (West *et* al., 1992; Smith and Goldin, 1996) whereas it increased the ion conductance of $Na_v1.5$ (Marban *et al*., 1998) and activation rate of $Na_v1.8$ (Fitzgerald *et al*., 1999). PKC phosphorylation of $Na_v1.2$ decreases the ion currents and activity of the channel as in PKA (Schreibmayer *et al*., 1991; Dascal and Lodan, 1991) (Figure 1.18). Detailed analysis on single channels have shown that the decrease in ion current is either due to the reduction of the number of available channels or to the reduction in open form of the channel (Numann *et al*., 1991). On the other hand, PKC increases the Na^+ current of $Na_v1.8$ (Gold *et al*., 1998).

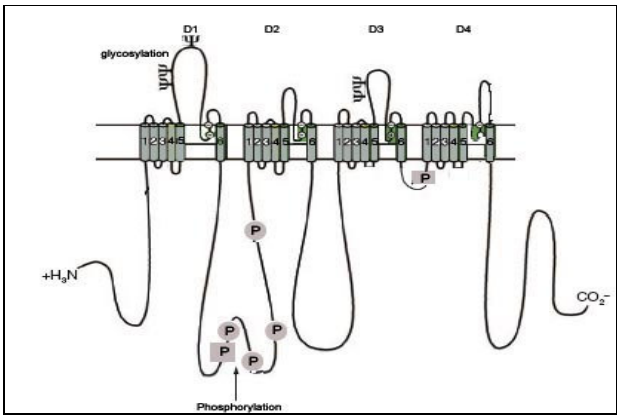
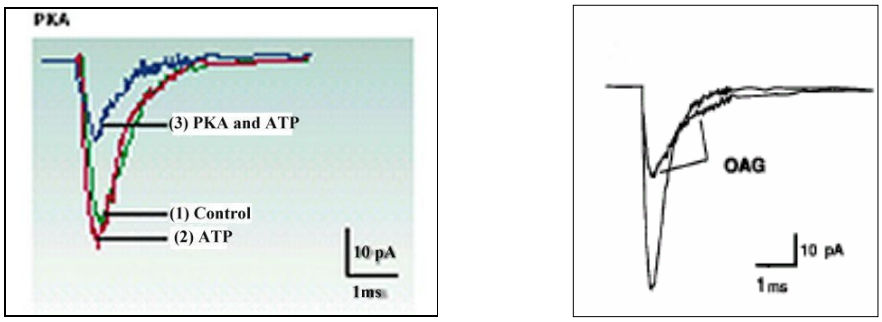


Figure 1.17. Phosphorylation and glycosylation pattern of VGSCα via different kinases; circles and squares are phosphorylation sites of PKA, and PKC respectively. Ψ; glycosylation site (modified from Yu and Catterall, 2003)



(A) (B)

Figure 1.18. The effect of PKA (A) and PKC (B) phosphorylation on $Na_v1.2$ (Cantrell and Catterall, 2001)

Phosphorylation of VGSC is suggested to affect the localization of the channel on the plasma membrane that is essential for its function. The processing of a functional VGSC was found to be through transition from the ER to Golgi. It is well known that PKA activates Na^+ currents in different cells. Zhou (2000) and Vijayaragavan (2004) have suggested that this activation was due to the transport and incorporation of additional VGSC from the Golgi to the cell membrane since an inhibitor of intracellular trafficking blocked the ion influx.

Protein kinases, like serum and glucocorticoid inducible kinases SGK1 and SGK3, increased the Na^+ current produced by $Na_v1.5$ and regulate the channel function (Boehmer *et al*., 2003). CAM kinase II also increased the amplitude of VGSC current (Carlier *et al*., 2000). Other CAM kinases are known to modulate the channel inactivation and various electrophysiologic properties of VGSC in a subtype specific manner (Deschenes *et al*., 2002).

The phosphorylation of VGSC is reversed by protein phosphatases. Receptor-like protein phosphatese β (RPTPβ), Ca+2/calmodulin-dependent protein phosphatese 2B, calcineurin, protein phosphatese1-2 have been shown to dephosphorylate VGSCs and increase the Na^+ currents (Ratcliffe *et al*., 2000).

Glycosylation of VGSCα has been determined on specific sites of the pore-lining regions of D1 and D3 (Figure 1.17) (Bennett, 2002; Marban *et al*., 1998). The rate of glycosylation in each VGSCα subtype is quite different eg. heavy glycosylation was

observed in $Na_v1.1$-Na_v 1.4 (15-30 per cent) but not in $Na_v1.5$ and $Na_v1.9$ (5 per cent) (Marban *et al*., 1998; Tyrrell *et al*., 2001). The presence of carbohydrate groups affects the structure, localization/cell surface expression and electrophysiological properties of the channels (Bennett *et al*., 1997; Zhang *et al*., 2003). Studies have shown that glycosylation could be developmentally regulated (Tyrrell *et al*., 2001) and alteration in the glycosylation pattern could impair VGSC function in certain diseases like cardiac arrhythmia and neuropathic pain (Ufret-Vincenty *et al*., 2001; Zhang *et al*., 2003).

Palmitoylation of VGSCs (addition of fatty acid palmitate) has been first shown by Schmidt and Catterall (1987). Palmitoylation occurs in the Golgi and was suggested to mediate VGSC transport to the plasma membrane. It is a dynamic process that is reversible thus may be an important tool for regulating diverse functions of the channel (Huang and El-Husseini, 2005).

Apart from modifications on the VGSC, interaction with proteins and VGSCβ regulate the function and availability of the channel. The VGSC-interacting proteins are cytoplasmic elements, linker proteins (eg. actin), enzymes, ion channels (eg. voltage-dependent anion channel), membrane associated proteins and motor proteins (dynein, intermediate and light chain) (Malik-Hall *et al*., 2003). The VGSC-protein interactions may be subtype specific and regulate the VGSC expression and/or cellular response. For example, compact myelin was found to regulate the localization of $Na_v1.2$ and $Na_v1.6$ in retinal ganglion cells and nodes of Ranvier, respectively (Boiko *et al*., 2001). An FGF family member FHF2B interacts with $Na_v1.6$ and colocalize at nodes of Ranvier (Wittmack *et al*., 2004). Also dystrophin and syntrophin are suggested to interact with $Na_v1.4$ and $Na_v1.5$ that may regulate their localization to neuromuscular junction (Caldwell, 2000). Binding of dystrophin to VGSCα may link the channel to actin cytoskeleton and the extracellular matrix. Interaction with cytoskeletal elements induces VGSC open probability and persistent activity (Maltsev and Undrovinas, 1997; Undrovinas *et al*., 1995). Ubiquitin-protein ligases Nedd4 and Nedd4-2 were shown to decrease the Na^+ current mediated by $Na_v1.5$. Later Nedd4 and Nedd4-2 were found to bind the neuronal VGSCs and through ubiquitination and endocytosis, inhibit the channel activity (Fotia *et al*., 2004). The authors suggest that since seven of the nine VGSCs

contain binding motif for Nedd4 and Nedd4-2 their turnover could be through ubiquitination via these proteins.

The data available in the literature indicates the presence of problems in the diagnosis and treatment of breast cancer. The currently used markers can not determine microscopic metastasis. Therefore, a significant proportion of patients receive adjuvant therapies that decrease their quality of life. In addition to these, resistance to hormone therapy is developed at later stages of the disease showing the requirement of reliable markers. Several studies implicated VGSCα in cancer metastasis and a preliminary study proposed the use of $nNa_v1.5$ as an early marker. However, little knowledge is available regarding the molecular mechanisms controlling the expression of the channel and the signaling pathway through which $nNa_v1.5$ regulates the cellular behaviours. It is well known that growth factors and hormones (e.g. estrogen) are important for breast cancer progression and regulation of VGSC activity in excitable cells. The involvement of $nNa_v1.5$ expression in cancer metastasis needs to be confirmed in a large number of cases and its specific expression in breast cancer metastasis should be tested to accomplish the requirements of a prognostic marker. In addition to this, the underlying mechanism of $nNa_v1.5$ regulating the metastatic cell behaviour needs to be identified.

2. AIM

This study aims to provide further evidence for the possible association of $nNa_v1.5$ expression with breast cancer metastasis and to determine the possible role of estrogen on $nNa_v1.5$ expression and function.

Within the scope of this thesis, we aimed to analyze possible simultaneous presence of $nNa_v1.5$ and breast cancer metastasis using RT-PCR and immunohistochemistry on human tissues. Since estrogen is involved in breast cancer development and progression through its receptors (ER) and regulates VGSCα function in several tissues, we aimed to analyze the possible relation between $nNa_v1.5$ expression and ER in breast cancer tissues.

Due to the differences between adult and neonatal splice-forms of $Na_v1.5$, drugs could be developed that specifically target $nNa_v1.5$ and treat breast cancer with minimal side effects. In this respect, it is of great importance to determine the distribution of VGSCα (and specifically $nNa_v1.5$) proteins in normal human tissues using immunohistochemistry. Since VGSCα expression has been related with prostate cancer and small-cell lung carcinoma (SCLC), we also aimed to test whether VGSCα expression was a widespread mechanism in cancer metastasis**.**

To unravel the mechanism of VGSCα upregulation in metastatic breast tissue, possible effect of estrogen on $nNa_v1.5$ expression and function was investigated. For this purpose, cell lines with different metastatic capacities were exposed to estrogen and/or VGSC blocker, tetrodotoxin, and the effect on cell proliferation and motility and $nNa_v1.5$ protein expression and location were analysed.

3. MATERIALS

3.1. Human Tissues

The frozen breast biopsies for $nNa_v1.5$ gene expression analysis were obtained from Hammersmith Hospital, London, UK.

Frozen and paraffin embedded breast cancer, normal tissues (small intestine, colon, stomach, urinary bladder, prostate, esophagus) and various cancers (lung, kidney, prostate, stomach, colon, urinary bladder) used in VGSCα and $nNa_v1.5$ protein analysis were obtained from Marmara University Hospital, Istanbul, Turkey. Both frozen and paraffin embedded skeletal muscle sections were provided by Istanbul University, Istanbul Medical School Neurology Department. Informed consent was obtained for the human tissues studied. Ethical permission was obtained from Boğaziçi University and Marmara University, Faculty of Medicine. Brain and heart sections were provided by Forensic Medicine Faculty, Cerrahpaşa from autopsies with ethical permission.

3.2. Human Breast Cancer Cell Lines

Human breast cancer cell lines, MDA-MB-231 and MCF-7, were kindly provided by Prof. Mustafa Djamgoz, Imperial College Science Technology and Medicine, London, UK. MDA MB-231-ERα transfected cells were provided by Prof. Craig Jordan, Robert H. Lurie Comprehensive Cancer Center, Chicago, USA.

3.3. Fine Chemicals

3.3.1. Primers

Two different sets of primers were used in this study (Table 3.1). The first set of primers, hH1 and hH2, was used to amplify both adult and neonatal forms of the $Na_v1.5$

gene. The primer set HCYTBL and CB5B recognizes a housekeeping gene, human cytochrome b5 reductase (hCytb5R), and is used to test the efficiency of cDNA synthesis.

Table 3.1. Primer sequences used in this study

Primer Name	Primer Sequence
hH1 (F):	5'-CAT CCT CAC CAA CTG CGT GT-3'
hH2 (R):	5'-CAT TGA GGT AAA GGT CCA GG-3'
HCYTBL (F):	5'-TAT ACA CCC ATC TCC AGC GA-3'
CB5B (R):	5'-CAT CTC CTC ATT CAC GAA GC -3'

3.3.2. Antibodies

Voltage-gated sodium channel specific antibody, Pan-VGSC (Upstate) was used on breast cancer, other cancers and normal tissues. The antibody recognizes a highly conserved region at the intracellular loop D3-D4 on the channel. The antibody peptide sequence is given in Table 3.2.

Antibody against adult form of Na_v 1.5, developed by William Catterall, was used on breast cancer tissues and heart as a positive control (Table 3.2). The peptide corresponds to residues 1122-1137 on the intracellular loop of D2-D3 of $Na_v1.5$. The antibody was kindly provided by Prof. Mustafa Djamgoz

A poly-clonal antibody specific to the neonatal spliced form of $Na_v1.5$ was developed in Imperial College MRC Clinical Sciences Centre (Table 3.2). The antibody was a kind gift of Prof. Mustafa Djamgoz.

Table 3.2. Antibody Peptide Sequences

Antibody Name	Peptide Sequence
Anti-Na^+ Channel (PAN)	TEEQKKYYNAMKKLGSKKC
Anti-Adult $Na_v1.5$	KTEPQAPGCGETPEDS
Anti-n$Na_v1.5$ (NESOpAb)	VSENIKLGNLSALRC

3.3.3. Commercially Available Kits

The kits used throughout the thesis are given in Table 3.3.

Table 3.3. Kits used

Technique used	Kit
RT-PCR	Strataprep Miniprep RNA extraction kit MMLV Superscriptase II cDNA synthesis kit, Invitrogen
Immunohistochemistry	Avidin-biotin blocking kit,Vector Horseradish peroxidase (HRP) linked streptavidin-biotin complex (ABC), Dako Diaminobenzidine (DAB), Vector

3.4. Buffers and Solutions

The ingredients of the buffers used in this study are given in Table 3.4 through Table 3.8. All the chemicals are supplied from Sigma or Merck unless stated.

Table 3.4. Buffers and solutions for immunohistochemistry

Fixative for frozen sections	50 % Acetone 50 % Methanol pre-cooled to -20°C
10X PBS	3,43 g NaCl 14,4 g Na_2HPO_4 8,97 g NaH_2PO_4
0,1 % saponin/PBS	0,1 g saponin (Applichem) 100 ml 1X PBS
0,2 % Triton X/PBS	200 µl Triton X 100 ml 1X PBS
3 % H_2O_2/PBS	1 ml 30% H_2O_2 99 ml 1X PBS

Table 3.4. Buffers and solutions for immunohistochemistry (continued)

Citric Acid Buffer	0,01 M Citric Acid (pH: 6,0)
Washing solution	0,1 g BSA (Applichem) 100ml 1X PBS

Table 3.5. Buffers and solutions for immunocytochemistry

Poly-L-Lysine	0,01 mg/ml poly-L-Lysine in sterile distilled water
Washing solution	0,1 % BSA/PBS

Table 3.6. Primary and secondary antibody dilutions for immunohisto/cytochemistry

Pan-VGSCα	1:100 in serum/1X PBS
NESOpAb	1:52 in serum/1X PBS in immunohistochemistry 1:200 in serum/1X PBS in immunocytochemistry
Swine anti-rabbit biotin	1: in immunohistochemistry (Dako)
Goat anti-rabbit FITC	1:300 in serum/1X PBS in immunocytochemistry (Dako)

Table 3.7. Buffers and solutions for cell culture

Complete Medium for MDA-MB-231 and MCF-7 ce	DMEM without phenol red, with sodium pyruva 100 mg/L glucose, pyridoxine (Invitrogen) 10 % FBS (Invitrogen) 4mM L-Glutamine (Biochrom) 0,01 % pennicilin/streptomycin (Biochrom)
Complete Medium for MDA-MB-231-ERα	DMEM 5 % FBS 4mM L-Glutamine 0,01 % pennicilin/streptomycin 500 µg/ml Geneticin (G418) (Invitrogen)
Medium mixture for serum starving MDA-MB-231 and MCF-7 cells	DMEM 0 % FBS 4 mM L-Glutamin 0,01% pennicilin/streptomycin

Table 3.7. Buffers and solutions for cell culture (continued)

Medium mixture for serum starving MDA-MB-231-ERα	DMEM 5% DC-FBS 4 mM L-Glutamin 0,01 % pennicilin/streptomycin 500 µg/ml Geneticin 6 ng/ml insulin (Roche)
Trypsin solution	0,25 g Trypsin (Invitrogen) 100 ml 1X PBS
Freezing medium	20% FBS 10% DMSO 70% DMEM mixture

Table 3.8. Buffers and solutions for BrdU proliferation assay

Fixative	100 % Methanol (precooled at-20°C)
Trypsin solution	0,05 % Trypsin in 1X PBS
Blocking solution	0,5 % BSA 0,1 % Tween 20 In 1X PBS
Anti-BrdU-FITC	1:500 in blocking solution (Roche)

3.5. Equipment

Autoclave	Midas 55, Prior Clave, UK
Balances	DTBH 210, Sartorius, GERMANY
Electronic Balance	VA 124, Gec Avery, UK
CCD camera	CCD Camera, JAI Corporation, JAPAN
CO_2 incubator	WTB Binder, GERMANY
Centrifuges	ProFuge 10K, Stratagene, USA Mini Centrifuge 17307-05, Cole Parmer, USA Genofuge 16M, Techne, UK Centurion K40R, UK

	Centrifuge B5, B. Braun Biotech International, GERMANY
Deep-freezers	-20°C, 2021D Arçelik, TURKEY
	-20°C, Bosch, GERMANY
	-86°C ULT Freezer, ThermoForma, USA
Documentation System	Gel Doc XR System, Bio-Doc, ITALY
Electrophoresis System	Horizon, Life Technologies, USA
Filters	DAPI Chroma 11000, GERMANY
	FITC Chroma 41001, GERMANY
	Texas Red Chroma 41004, GERMANY
Hand tally counter	Milky Way Counter, TAIWAN
Hemocytometer	Improved Neubauer, Weber Scientific International Ltd, UK
Laminal flow cabinet	Labcaire BH18, UK
Magnetic Stirrers	M221 Elektro-mag, TURKEY
	Clifton Hotplate Magnetic Stirrer, HS31, UK
Microscopes	B3000, Prior, UK
	CM110 Inverted Microscope, Prior, UK
	Phase contrast Inverted microscope, Olympus,
	Zeiss Axioscope, GERMANY
Microwave oven	M1733N, Samsung, MALAYSIA
pH meter	WTW, GERMANY
Pipettor	Pipetus-akku, Hirscmann Labogerate, GERMANY
Refrigerators	2082C, Arçelik, TURKEY
	4030T, Arçelik, TURKEY
Shakers	VIB Orbital Shaker, InterMed, DENMARK
	Nüve SL350, TURKEY
Software	Isis digital FISH imaging system, Metasystems, GERMANY
	MATLAB 6.5, technical computing
	Image Pro Plus 5.1v, Digital Microscopy imaging analysis system
Spectrophotometer	Agilent Technologies, G1103A, Germany

Thermocyclers	MyCycler, Bio-Rad, ITALY
Vortex	Vortexmixer VM20, Chiltern Scientific, UK
Water bath	TE-10A, Techne, UK
Water purification	WA-TECH ultra pure water purification system, GERMANY

4. METHODS

4.1. Total RNA Extraction from Breast Cancer Tissues

Total RNA was extracted from frozen breast cancer tissues using Strataprep RNA extraction kit (USA). The tissues (0,2 g) were chopped into pieces with the aid of a scalpel and homogenized using a homogenizor in guanidium thiocyanate containing lysis buffer and β-mercaptoethanol. After centrifugation at 14000 rpm (maximum speed) for 1 minute, homogenization was repeated. The supernatant was transferred to a clean eppendorf and mixed with a pipette for several times. After vortexing for 5 seconds centrifugation was performed at maximum speed for 1 minute. The supernatant was transferred to pre-filtration columns and centrifuged at maximum speed for 5 minutes. Equal volume of 70 per cent ethanol was added on top of the filtrate and the mixture was transferred to an RNA-binding column. Centrifugation was performed at 14000 rpm for 1 minute. After washing the columns with low-salt buffer, the colons were treated with DNAse I at 37°C for 15 minutes. The columns were washed once with high-salt buffer and twice with low-salt buffer that was finally transferred to a clean eppendorf. The bound RNA was eluted by incubating the columns with 30 µl elution buffer at room temperature for 2 minutes followed by centrifugation at 14000 rpm for 1 minute. This step was repeated twice for efficient elution. The quantity of RNA was measured by spectrophotometric analysis at OD_{260}. OD_{260}/OD_{280} ratio was measured to reveal a possible protein contamination. The quality of the extracted RNA was analyzed by loading 0,5 µg RNA onto 0,8 per cent agarose gel.

4.2. cDNA Synthesis of RNA Extracted from Breast Cancer Tissues

Single stranded cDNA synthesis was performed using 1 µg total RNA. The RNA sample was mixed with random hexamer (2,5 µg/µl) and the volume was completed to 11 µl with sterile distilled water. The mixture was incubated in 70°C heat block for 10 minutes to eliminate secondary structure of RNA. After cooling the sample on ice, 5X First Strand Buffer, 10 mM dNTP, 0,1 M DTT and RNAse inhibitor (20 U) were added and

incubated at 25°C incubator for 5 minutes. Upon addition of 200 U MMLV Superscript II reverse transcriptase incubation at 25°C for 10 minutes was performed. The cDNA was synthesized in 42°C water bath for 105 minutes and the reaction was terminated in 70°C heat block for 10 minutes. To eliminate RNA contamination cDNA samples were incubated with RNAse H at 37°C followed by 10 minute-incubation at 70°C.

4.3. RT-PCR

The synthesized cDNA was used in the amplification of the human cytochrome b5R (hCytb5R) and $Na_v1.5$ genes. The housekeeping gene hCytb5R was used as a control to measure the RNA level of the tissue and the efficiency of cDNA synthesis. The RT-PCR reaction was prepared using 1 µl cDNA, 0,5 µM from each primer, 1 µM dNTP and 1 U Taq polimerase at a final volume of 20 µl. The RT-PCR reaction for hCytb5R was initiated with a hotstart for five minutes at 94°C that was followed by 30 cycles at 94°C, 60°C, and 72°C 1 minute each. The reaction was extended for 10 minutes at 72°C. The reaction for $Na_v1.5$ was the same as the control except the cycle number (45) and the annealing temperature (58°C).

The RT-PCR products were analyzed on 1 percent agarose gel. Tissues that do not show any amplification for hCytb5R were not used for further analysis. Consistency between samples was achieved by duplicating the cDNA synthesis reactions.

4.4. Immunohistochemistry

4.4.1. Frozen Tissues

Human tissues obtained from biopsies (skeletal muscle, normal and cancerous breast) were immediately frozen in liquid nitrogen. Sections were cut on slides (~8 µm) and stored at -20°C until use. To protect the proteins, tissues were fixed in 1:1 Acetone:Methanol that was previously cooled to -20°C for 15 minutes. The fixative was removed by washing the sections in 1X PBS three times for five minutes. The cell

membrane was permeabilized by incubation with 0,1 per cent saponin for 15 minutes and washed in 1X PBS three times for 5 minutes. Endogenous peroxidase activity of the tissue was suppressed by washing with 0,3 per cent H_2O_2 for 15 minutes. Washing with 1X PBS was repeated and background caused by the secondary antibody was suppressed by incubation with 5 per cent serum for one hour. To block the endogenous biotin activity of the tissue, avidin-biotin blocking kit (Vector) was applied. After a short 1X PBS wash, primary antibody incubation (pan-VGSC, Upstate) was performed in a moist chamber for one hour. Unbound antibody was removed by washing the slides with 1X PBS three times for 10 minutes. Sections were incubated with the secondary antibody for one hour. Excessive antibody was removed with 1X PBS as in the previous step. Then Horseradish peroxidase (HRP) linked streptavidin-biotin complex (ABC) (Dako, Denmark) was applied for 30 minutes. The sections were washed in 1X PBS three times for 10 minutes. The chromogen Diaminobenzidine (DAB) was added on top of sections and formation of colour reaction was followed under the light microscope. To protect the stained sections, slides were covered with mounting medium and detailed analysis was performed using the light microscope.

4.4.2. Paraffin Embedded Tissues

Immunohistochemistry was applied on normal and tumor human tissues. All the tissues were fixed in 4 per cent paraformaldehyde, de-hydrated in alcohol series, embedded in paraffin and sectioned on poly-L-lysin coated slides (3-5 μm). Sections were deparaffinized in toluene and re-hydrated in 100 per cent, 90 per cent and 70 per cent alcohol series. The endogenous peroxidase activity of the tissue was blocked by washing in 3 per cent H_2O_2/1X PBS for 20 minutes. Duration of the washing was extended to one hour in case of high background. The cross-links formed during fixation were broken by heat-mediated antigen retrieval using 0.01 M citric acid buffer (pH 6) for three or 10 minutes in a microwave depending on the tissue type. The sections were allowed to cool down at room temperature for 20 minutes. Permeabilization of the membrane was achieved by washing the sections in 0.2 per cent Triton X/PBS for 10 minutes when needed. Sections were washed in 1X PBS three times for 5 minutes. The background staining caused by the secondary antibody was blocked for one hour by 10 per cent serum. Endogenous biotin activity of the tissue was blocked by avidin biotin complex (Vector). Sections were

incubated with the primary antibody diluted in 10 per cent serum/Triton X/PBS in a moist chamber for one hour or overnight (depending on the tissue). Pan-VGSC antibody was used in all normal and tumor tissues whereas NESOpAb was used in normal and breast cancer tissues. The negative control was incubated only with 10 per cent serum/Triton X/PBS. Then sections were washed in 0,1 per cent BSA/PBS for three times 10 minutes and incubated with biotinylated secondary antibody for one hour (1:125 diluted in 10 serum/1X PBS). The washing step was repeated and followed by horseradish peroxidase ABC incubation for one hour. Sections were washed in washing solution, 0,1 per cent BSA/PBS (3x10 minutes). Colour development caused by conversion of diaminobenzidin (DAB) to a coloured reagent via HRP enzyme was followed under a light microscope. The colour reaction was protected using a mounting medium. Image analysis was performed using Image Proplus 7 imaging system.

4.5. Maintenance of Breast Cancer Cells

MDA-MB-231 and MCF-7 cell lines were grown in DMEM medium (without phenol red) containing 10 per cent Fetal bovine serum (FBS), 4 mM L-Glutamine and 0.1 per cent penicillin-streptomycin. MDA-MB-231-ERα cells were grown in the same complete medium as above except the use of 5 per cent FBS and 500 μg/ml Geneticin (Gibco-BRL). All the cells were plated in 100 mm petri dishes at the 37°C in a humid atmosphere with 5 per cent CO_2.

Cells were passaged when they reached 80 per cent confluency. After removing the medium, cells were washed with sterile 1X PBS. Trypsinization was performed by treating the cells with 1,5 ml 0,25 per cent Trypsin/1X PBS for 3 minutes at 37°C incubator. Since extended trypsinization can be toxic to cells, activity of trypsin was blocked by adding 2 ml complete medium. Cells were centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and the cell pellet was resuspended in fresh complete medium and split into new petri dishes.

In vitro assays were performed by incubating the cells with 10 µM TTX and/or estrogen (10 and 100 nM). The control samples were devoid of TTX and estrogen application. All the treatments were performed for 72 hours.

4.5.1. Cell Counting

Cells were counted using a haemacytometer for seeding at a certain concentration in each assay. The haemacytometer and coverslip were cleaned with alcohol to avoid contamination. Cell pellet was resuspended in 4 ml of complete medium and 100 µl of this suspension was delivered into the haemacytometer. Using 10 X objective number of cells in one big square were counted. At least four different areas were counted and average was multiplied by 10^4 to give the number of cells in 1ml.

4.5.2. Cell Storage

The cells were frozen when they reached 80-90 per cent confluency. To achieve this, cells were trypsinized as described in section 4.6.1. The cells were resuspended in a 1 ml mixture of 70 per cent complete medium, 20 per cent FBS and 10 per cent DMSO and transferred into cryovials. The cells were frozen in multiple steps where cryovials were kept at -20°C freezer for 1 hour then transferred to -80°C deepfreezer. For long term storage frozen cells were placed in liquid nitrogen after keeping them at -80°C overnight. When needed the cells were defrosted quickly in 37°C water bath and transferred into 100 mm dishes containing 7 ml complete medium. The medium of the attached cells were changed the next day to remove the DMSO in the freezing mixture.

4.5.3. Trypan Blue Exclusion Assay

Trypan blue is a negatively charged dye that diffuses into the cells when their membrane is no longer intact e.g. in case of cell death. Cells excluding the dye are alive and those stained into blue are dead. Trypan blue exclusion assay was performed on cells that were exposed to 10 µM BrdU, with estrogen (10 nM, 100 nM) and/or 10 µM TTX and controls for 72 hours. Their medium was removed and cells were washed in 1X PBS twice. Trypan blue (0,4 per cent) and complete medium was added in 1:1 ratio and incubated for

7 minutes. The number of stained and unstained cells were counted at least from four different areas using 10 X objective of inverted microscope. The viability of the cells were determined by calculating the ratio of live cells to total number of cells.

4.6. Cell Proliferation Assay

BrdU incorporation assay was used to analyze the proliferation rate of MCF-7, MDA-MB-231 and MDA-MB-231-ERα cell lines in response to TTX and/or estrogen treatments. Cells were seeded at a concentration of $2x10^4$ cells/dish on poly-lysine covered 12mm coverslips placed in 35 mm dishes and grown for 24 hours. After overnight serum starvation (0 per cent serum), cells were treated with 10 µM BrdU for 1 hour. The complete medium was removed and the cells were treated for 72 hours with a medium change and 10 µM BrdU addition every 24 hours. The cells were fixed in methanol (cooled at -20°C) for 30 minutes and washed with 1X PBS three times for 5 minutes. Enzymatic digestion was performed in 0,05 per cent Trypsin for 5 minutes in 37°C CO_2 incubator. The nuclear membrane of the cells were permeabilized with 0,5 per cent Triton X and DNA was denatured in 2 M HCl for 1 hour in 37°C CO_2 incubator. Each treatment was followed by 1X PBS wash three times for 5 minutes. The cells were blocked with the blocking solution for 10 minutes at room temperature. FITC labelled anti-BrdU antibody incubation was performed for 1 hour in 37°C CO_2 incubator. The unbound antibody was removed by 1X PBS wash three times for 5 minutes. DAPI counterstaining (1:10,000) was applied for 10 minutes and the results were analyzed under fluorescence microscope. Each assay was performed as three individual experiments where approximately 400 cells were counted for each treatment. Statistical evaluation was performed using Student's t-test.

4.7. Wound Heal Motility Assay

Breast cancer cells were seeded on 35 mm dishes at a concentration of $15x10^4$ cells/dish and grown for 24 hours. Serum starvation (0 per cent serum) was performed overnight and the next day three wounds were created using a 100 µl pipette tip. To discard the removed cells plates were washed twice with medium deprived of additives. The cells were treated with estrogen and/or TTX for 72 hours and non-treated ones were

used as controls. All treatments were performed in triplicates. The distance of the gaps created by the wound was measured under inverted microscope using an ocular with a micrometer. Measurements of the same sites were recorded at 24, 48 and 72 hours after treatments. Motility index was calculated using the formula given below,

$$MI= 1-(W_t/W_0)$$

where MI is motility index, W_t is width of the wound t hours after treatment and W_0 is initial wound width. Statistical evaluation was performed using Student's t-test (Fraser *et al*., 2003).

4.8. Immunocytochemistry

Immunocytochemistry was applied on in 35mm dishes grown on duplicates of 12mm coverslips. The coverslips were cleaned with 70 per cent alcohol and coated with poly-L-lysine by incubation for 15 minutes at room temperature. After washing the coverslips several times with sterile distilled water, the dishes were allowed to dry in 37°C incubator for approximately 30 minutes. Cells were seeded at a concentration of $15x10^3$ cells/well and grown for 48 hours. Serum starvation was performed overnight and cells were treated with estrogen and/or TTX for 72 hours changing the complete medium every 24 hours. Cells were fixed in methanol for 5 minutes. After washing the cells with 1X PBS three times for 5 minutes, permeabilization was performed using 0,1 per cent saponin for 5 minutes. The washing step was repeated and followed by 5 per cent serum treatment for 1 hour in a moist chamber to block background staining. NESOpAb primary antibody incubation was performed for 1 hour at room temperature. The unbound antibodies were removed by washing the cells with washing solution three times for 5 minutes. The FITC labeled secondary antibody was incubated for 1 hour that was followed by a washing step. The counterstaining was achieved by treating the cells with DAPI (1:40,000) for 5 minutes. The results were pictured using Zeiss Axioskop fluorescence microscopy and ISIS digital imaging system.

4.8.1. Quantification of Immunocytochemistry Staining

The quantification of staining obtained during immunocytochemistry was performed using MATLAB 6.5 computing software modified by Boğaziçi University, Institute of Biomedical Engineering. FITC-coupled NESOpAb stained pictures were opened using MATLAB 6.5. By selecting 'green' option only green coloured pixels were made visible. Background pixels were removed by limiting the pixel size and adjusting the threshold. Since the software defines each separate pixel as an object, when a cell was partially stained a square tool was used to define the borders of the cell. All the green pixels per cell were counted in the picture by the software. The data were automatically transferred to a Text file where pixels in each individual cell, total number of objects and total number of pixels in the picture were given. For each type of treatment pixels of at least 100 cells were counted. Student's T-test was performed to determine the significance of changes in the pixel numbers upon estrogen and/or TTX treatment.

5. RESULTS

In this study, we investigated VGSCα and $nNa_v1.5$ expression in metastatic breast cancer cases. In this context, we compared $nNa_v1.5$ expression with status of lymph node metastasis (LNM) and estrogen receptor (ER) and analyzed whether its expression is unique to metastatic breast using normal human tissues. To define VGSCα expression in non-excitable tissues and to test its contribution to metastasis, we analyzed its distribution in normal and tumor tissues.

The effect of estrogen on breast cancer metastasis was investigated in strongly metastatic and weakly metastatic human cell lines that express estrogen receptor by performing *in vitro* motility and proliferation assays. The modulation of $nNa_v1.5$ expression and localization upon exposure of cells to estrogen was examined by immunocytochemistry.

5.1. Analysis of $nNa_v1.5$ Gene Expression in Breast Cancer and Normal Breast Tissues

In this part of the study, frozen biopsy samples of 60 breast cancer patients and two normal individuals were analyzed. The double-blind study involved determination of $nNa_v1.5$ gene expression in each patient and comparison with lymph node metastasis and ER status.

5.1.1. Total RNA Extraction from Breast Cancer and Normal Breast Tissues

Total RNA was extracted from frozen breast cancer biopsies and normal breast samples using Stratagene RNA extraction kit. The RNA quality was tested by loading 0,5 μg RNA on 0,8 per cent agarose gel. Presence of sharp 28S and 18S RNA bands indicated good quality RNA (Figure 5.1).

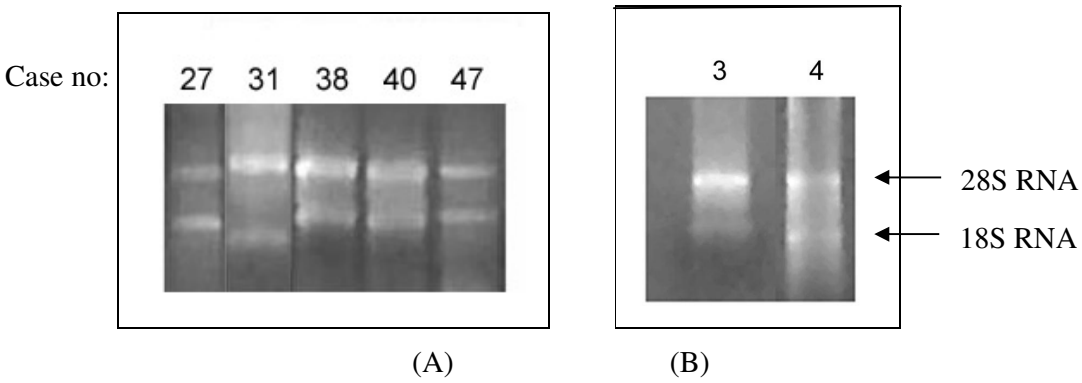


Figure 5.1. Agarose gel electrophoresis of total RNA from breast cancer (A) and normal breast tissues (B)

5.1.2. RT-PCR of $nNa_v1.5$ in Breast Cancer and Normal Breast Tissues

cDNA samples of 60 breast tumor and two normal tissues were prepared in duplicates. The RT-PCR products were visualized by loading 5 µl of the sample together with 10X loading dye on a 1,2 per cent agarose gel. The RT-PCR of the control transcript, hCytb5R, produced a 491 bp fragment in samples with sufficient amount of RNA and $nNa_v1.5$ was amplified, producing a 500 bp product in cancerous and normal breast tissues (Figure 5.2A and B, respectively). Two breast cancer samples that produced faint or no amplification of hCytb5R gene were not used in the analysis of $nNa_v1.5$ gene expression. Figure 5.2A shows the result of $nNa_v1.5$ amplification in four of the five breast cancer cases. However, $nNa_v1.5$ gene expression was also detected in both of the normal breast tissues (Figure 5.2B) that is not quantified and might indicate expression of $nNa_v1.5$ gene in various cells within the breast tissue e.g. fibroblasts, endothelial cells and epithelial cells.

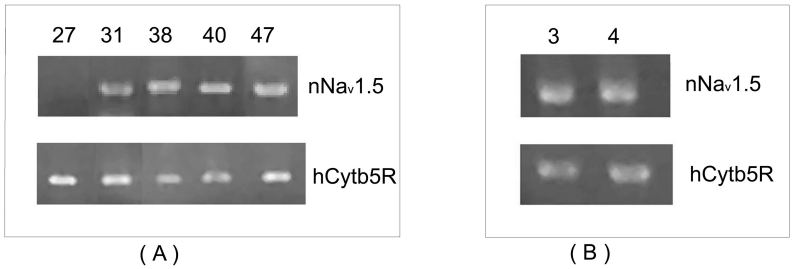


Figure 5.2. $nNa_v1.5$ and hCytb5R expression in breast cancer (A) and normal breast (B) tissues

5.1.3. $nNa_v1.5$ Gene Expression and Lymph Node Metastasis

Lymph node metastasis (LNM) data was available for 45 of the 60 breast cancer patients analyzed for $nNa_v1.5$ gene expression. The gene was found to be expressed in 23 patients that had LNM at the time of diagnosis (e.g. samples 31 and 40 in Figure 5.2). Nineteen patients were $nNa_v1.5$ positive but did not show LNM at initial diagnosis for (e.g. samples 38 and 47 in Figure 5.2). LNM was not encountered in any of the $nNa_v1.5$ (-) cases (e.g. sample 27 in Figure 5.2) (Table 5.1). Fourteen $nNa_v1.5$ (+) and one $nNa_v1.5$ (-) cases could not be included in the study since LNM data was not available. However, only in 26 of the 45 cases (23 cases that were $nNa_v1.5$(+)/LNM(+) and three cases that were $nNa_v1.5$(-)/LNM(-)) $nNa_v1.5$ expression was in parallel with LNM status (χ^2 =2,8, 0,1>p>0,05).

Table 5.1. Analysis of $nNa_v1.5$ gene expression and LNM status

n= 45	**LNM (+)**	**LNM (-)**
$nNa_v1.5$ (+)	23 (51 %)	19 (42 %)
$nNa_v1.5$ (-)	0	3 (7 %)

n: Total number of patients analyzed

5.1.3. Expression of $nNa_v1.5$ Gene and Estrogen Receptor Protein in Breast Cancer

Estrogen, acting through its receptors (ER), regulates VGSC in several tissues (Borg *et al*., 2002; Smitherman and Sontheimer, 2001). On the other hand, ER is one of the most commonly used prognostic markers in breast cancer. Therefore, $nNa_v1.5$ gene expression and ER status was investigated in 36 cases that were used in RT-PCR analysis with available ER data. Of these, 23 patients expressed both $nNa_v1.5$ gene and ER protein. In 10 patients, $nNa_v1.5$ gene was expressed but not the ER protein. $nNa_v1.5$ gene was not expressed in two ER (+) and one ER (-) patients (Table 5.2). However, only in 24 of the 36 cases (23 cases that were $nNa_v1.5$(+)/ER(+) and one case that was $nNa_v1.5$(-)/ER(-)) $nNa_v1.5$ expression was in parallel with ER status (χ^2=1,04, p<0,05, df=1). Among the patients for which ER status was not known 23 were $nNa_v1.5$ (+) and one $nNa_v1.5$ (-).

Table 5.2. Comparison of $nNa_v1.5$ Gene Expression with ER status

n= 36	**ER (+)**	**ER (-)**
$nNa_v1.5$ (+)	23 (64 %)	10 (28 %)
$nNa_v1.5$ (-)	2 (5 %)	1 (3 %)

n: Total number of patients analyzed

5.2. VGSCα and $nNa_v1.5$ Protein Expression in Normal Human Tissues

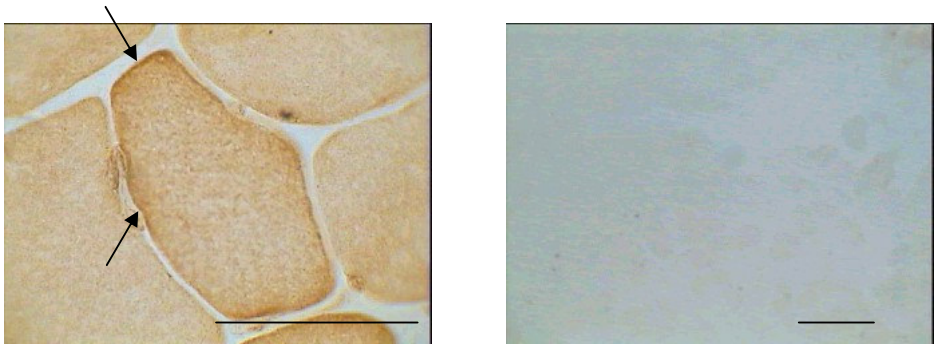
Immunohistochemistry (IHC) was applied both on frozen and paraffin-embedded normal and tumor tissues. On frozen sections, an antibody that recognizes VGSCα protein (pan-VGSCα, Upstate) and on paraffin embedded tissues pan-VGSCα or antibody raised against $nNa_v1.5$ protein (NESOpAb) (Chioni *et al*., 2005) were used.

The presence of VGSCα and $nNa_v1.5$ proteins were investigated on paraffin embedded normal small intestine, colon, stomach, esophagus, prostate, urinary bladder and breast tissues. Skeletal, heart muscle and brain were used as positive controls for VGSCα. A range of two to eight samples were studied for each type of normal tissue. In frozen IHC skeletal muscle (positive control) and normal breast were used for VGSCα analysis.

5.2.1. IHC for VGSCα at Frozen Normal Human Tissues

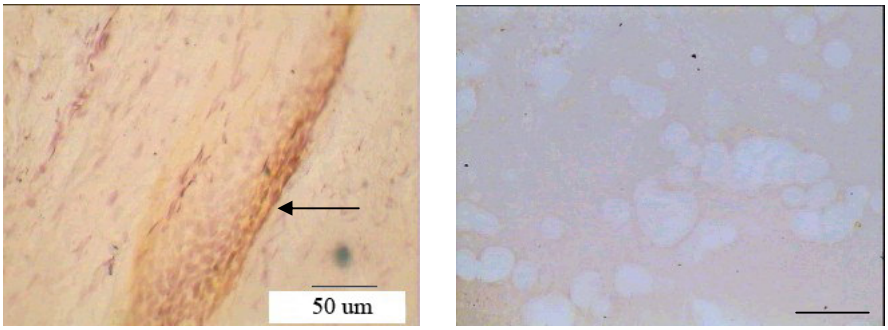
In frozen skeletal muscle tissue, VGSCα protein was localized on the membrane of myocytes (single skeletal muscle cells) in the form of clusters that is consistent with previous findings (Ribaux *et al*., 2001) (Figure 5.3A). Control sections processed without primary antibody produced no staining (Figure 5.3B).

VGSCα protein expression was investigated in seven frozen normal breast tissues. Due to difficulties in sectioning frozen tissues high background staining was observed in most of the samples. However, VGSCα immunoreactivity was primarily detected in the epithelial cells in two cases (Figure 5.4).



(A) (B)

Figure 5.3. Frozen skeletal muscle tissue incubated with (A) and without pan-VGSC antibody (B). Clustering of VGSCα is indicated by arrows (Scale bar: 500 µm)



(A) (B)

Figure 5.4. VGSCα protein expression in frozen normal breast tissue (A). The specific staining is absent when pan-VGSCα antibody is omitted (B). Scale bar represents 50 µm

5.2.2. IHC for VGSCα on Paraffin Embedded Normal Human Tissues

In paraffin embedded sections the results of frozen IHC were confirmed in all of the excitable tissues where VGSCα expression was determined on membranes of skeletal muscle and heart myocytes in clusters (Figure 5.5A, and B). The VGSCα protein was also present in neuronal cell bodies in the brain (Figure 5.5C) as reported previously (Whitaker *et al*., 2001). In non-excitable tissues VGSCα protein was determined in some of the cases analyzed (summarized in Table 5.3.). In VGSCα (+) small intestine tissues, the mucus secreting goblet cells (Figure 5.5D) and secretory cells of Brunner glands were shown to posses the protein (Figure 5.5E). Goblet cells in different parts of the colon (transverse, descending, sigmoid, rectum, cecum) were also found to express VGSCα protein (Figure

5.5F) consistent with the previous findings that suggests the role of VGSCα in secretion (Mycielska *et al*., 2003). In VGSCα (+) cases the epithelial cell lining of stomach (Figure 5.5G) and parietal cells that secrete HCl (Figure 5.5H) were immunoreactive for the protein. The stratified squamous epithelia in esophagus were found to express VGSCα protein. The protein was clustered on the cell membrane of the epithelial cells (Figure 5.5-I). VGSCα was also present in prostate, specifically on the apical membrane of epithelial cells facing the lumen where the secretion product is released (Figure 5.5J). Breast epithelial cells (Figure 5.5K) and endothelial cells (Figure 5.5L) were found to express VGSCα protein as well but the protein was absent in urinary bladder. (Figure 5.5M). In some cases (e.g. Figure 5.5.H and M background staining was observed due to extensive fixation of the tissues.

Table 5.3. IHC results for VGSCα expression in normal human tissues

Tissue	**N**	**VGSCα (+)**	**VGSCα (-)**	**Stained cell type**
Small intestine	8	6	2	Goblet cells, Brunner Glands
Colon	8	7	1	Goblet cells
Stomach	7	2	5	Parietal cells, epithelial cells
Esophagus	2	2	0	Stratified Squamous Epithelia
Prostate	5	3	2	Epithelial cells
Breast	7	5	2	Epithelial cells
Urinary bladder	2	0	2	None

n: The number of cases analyzed

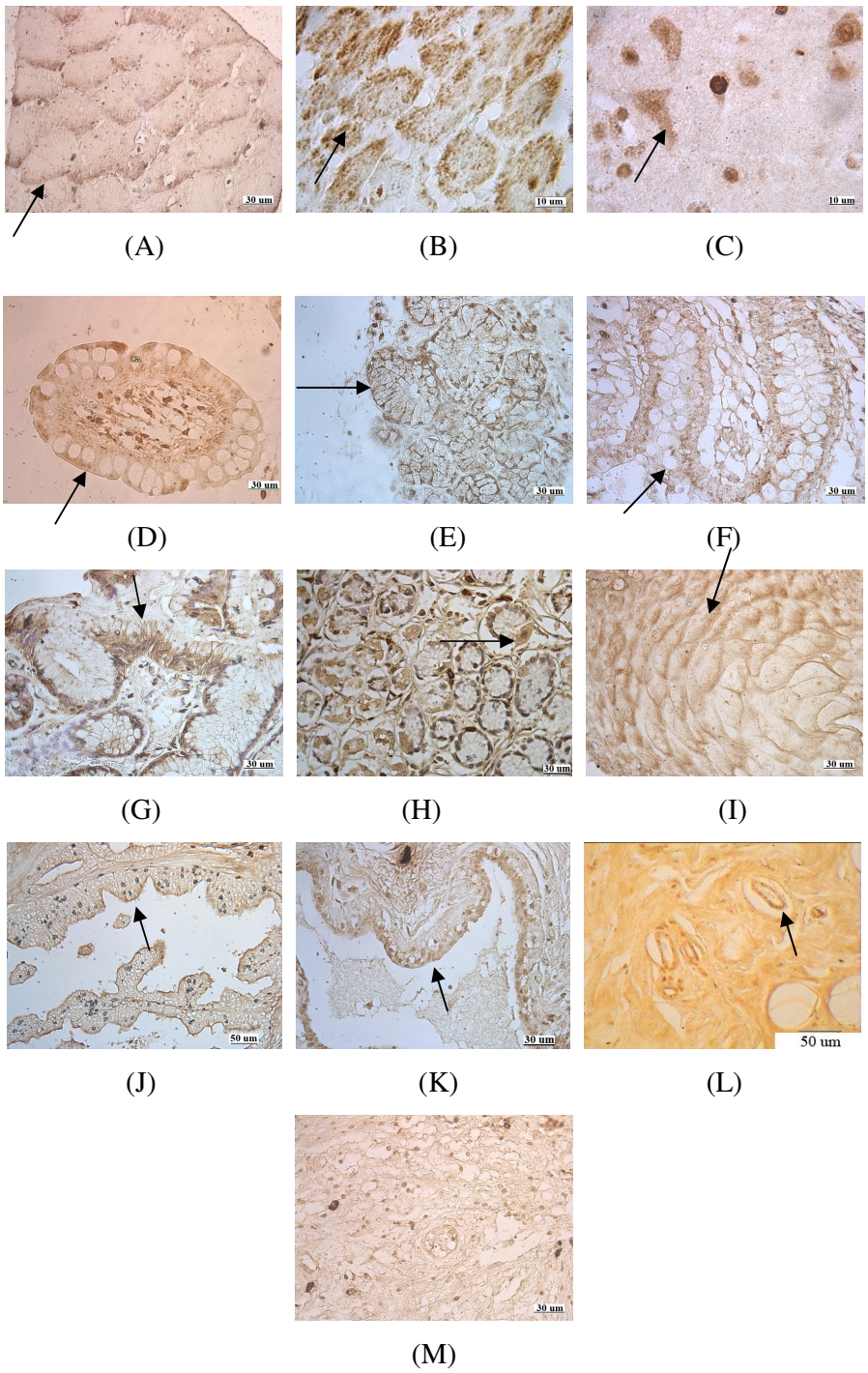


Figure 5.5. Distribution of VGSCα protein in normal human tissues. (A): skeletal muscle, (B): heart muscle, (C): brain, (D): goblet cells of small intestine, (E): Brunner glands in small intestine, (F): colon, (G): epithelial cell lining of stomach, (H): parietal cells in stomach, (I): esophagus, (J): prostate, K: epithelial cells of breast, L: endothelial cells of breast, M: urinary bladder. Arrows indicate VGSCα specific staining.

5.2.3. IHC for nNa$_v$1.5 on Paraffin Embedded Normal Human Tissues

The expression of nNa$_v$1.5 protein was investigated in all of the normal paraffin embedded human tissues tested for VGSCα presence. The nNa$_v$1.5 was absent in skeletal (Figure 5.6A) and heart muscles (Figure 5.6B), brain (Figure 5.6C), small intestine (Figure 5.6D), colon (Figure 5.6E), stomach (Figure 5.6F), esophagus (Figure 5.6G), prostate (Figure 5.6H) and urinary bladder (Figure 5.6-I). However, nNa$_v$1.5 protein expression was determined in five of the seven breast tissues (71 per cent) only in some epithelial cells of few ducts (Figure 5.6J).

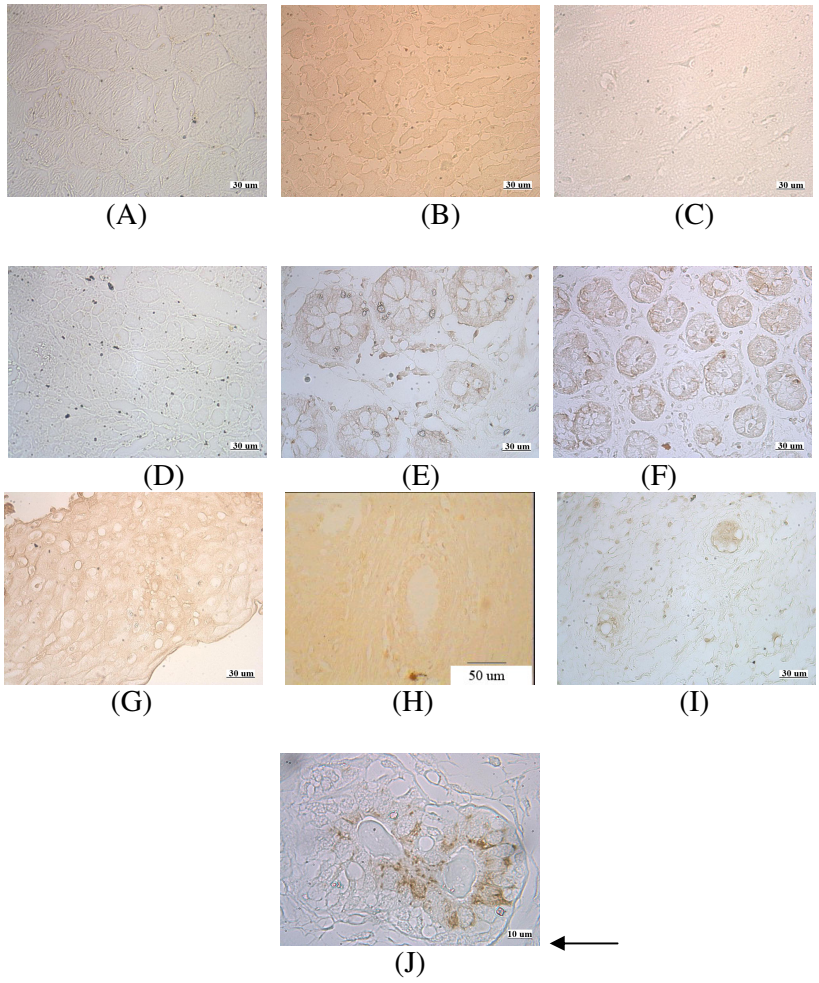


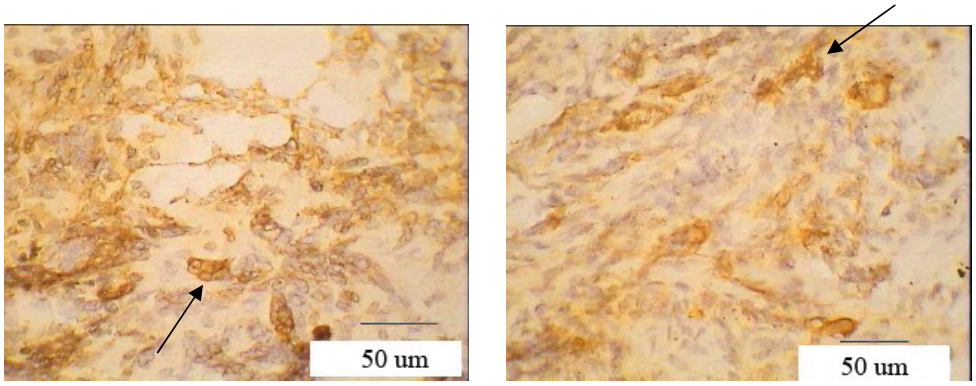
Figure 5.6. Expression of nNav1.5 in paraffin embedded normal human tissues. (A): skeletal muscle, (B): heart muscle, (C): brain, (D): small intestine, (E): colon, (F): stomach, (G): esophagus, (H): prostate (I): urinary bladder, (J): breast. Arrow indicate nNa$_v$1.5 specific staining

5.3. Investigation of VGSCα and $nNa_v1.5$ Protein Expression in Breast Cancer

To analyze the expression of VGSCα protein on frozen and paraffin embedded breast cancer tissues, pan-VGSCα antibody was used. Since this antibody can not discriminate the VGSCα subtypes and $nNa_v1.5$ is the predominant VGSCα expressed in breast cancer, a specific antibody was used to determine the $nNa_v1.5$ expression in paraffin embedded tissues.

5.3.1. VGSCα Protein Expression on Frozen and Paraffin Embedded Breast Cancer Tissues

A total of 10 frozen and 21 paraffin embedded breast cancer cases were investigated for the presence of VGSCα protein using pan-VGSC antibody. The protein expression was determined on the membrane and/or cytoplasm of epithelial cells in eight frozen (80 per cent) and 12 paraffin embedded tissues (57 per cent). Hematoxylene counter staining was performed to identify the specific localization of the VGSCα protein in the sections (Figure 5.7).



(A) (B)

Figure 5.7. Expression of VGSCα protein in frozen breast cancer tissues. A and B shows VGSCα protein on the epithelial cell membrane of the tumor cells in two different cases. Hematoxylene counterstaining was used to localize the nuclei in both sections

Breast tumor sections, in which arteries were observable showed immunoreactivity in endothelial cells (Figure 5.8) that is consistent with previous findings (Gordienko and Tsukhara, 1994; Gosling *et al*., 1998; Traub *et al*., 1999).

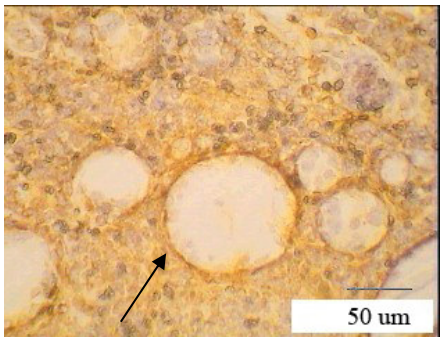


Figure 5.8. Expression of VGSCα protein in endothelial cells of arteries in breast cancer. Hematoxylene counterstaining is performed

5.3.2. VGSCα Protein Expression and LNM

Amongst the 10 frozen cases, six of the VGSCα (+) patients had LNM at the time of diagnosis. VGSCα protein expression was not determined in one patient who did not have LNM. However, one of the VGSCα (+) patients did not present LNM at initial diagnosis. LNM status was not investigated in one VGSCα (+) and one VGSCα (-) patient (Table 5.4).

Table 5.4. Analysis of VGSCα protein expression and LNM status in frozen breast cancer tissues

n=8	**LNM (+)**	**LNM (-)**
VGSC (+)	6	1
VGSC (-)	0	1

n: Number of cases analyzed

Investigation of VGSCα protein expression in 21 paraffin embedded breast cancer tissues revealed parallel results with the frozen samples where cell membrane and/or cytoplasm of epithelial cells were stained (Figure 5.9). The protein expression was determined in 12 but not in 10 cases. In this double blind study, since LNM data for most

of these cases was not present, VGSCα expression could not be compared with lymph node metastasis.

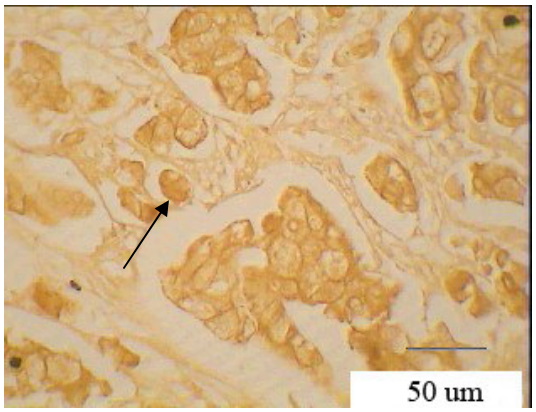


Figure 5.9. VGSCα protein expression on epithelial cells in paraffin embedded breast cancer tissues

In cases where observable, other than epithelial cells, endothelial cells of blood vessels were also found to express VGSCα protein (Figure 5.10) as in frozen breast cancer tissues.

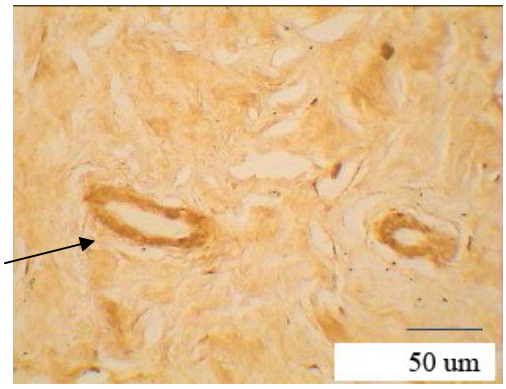
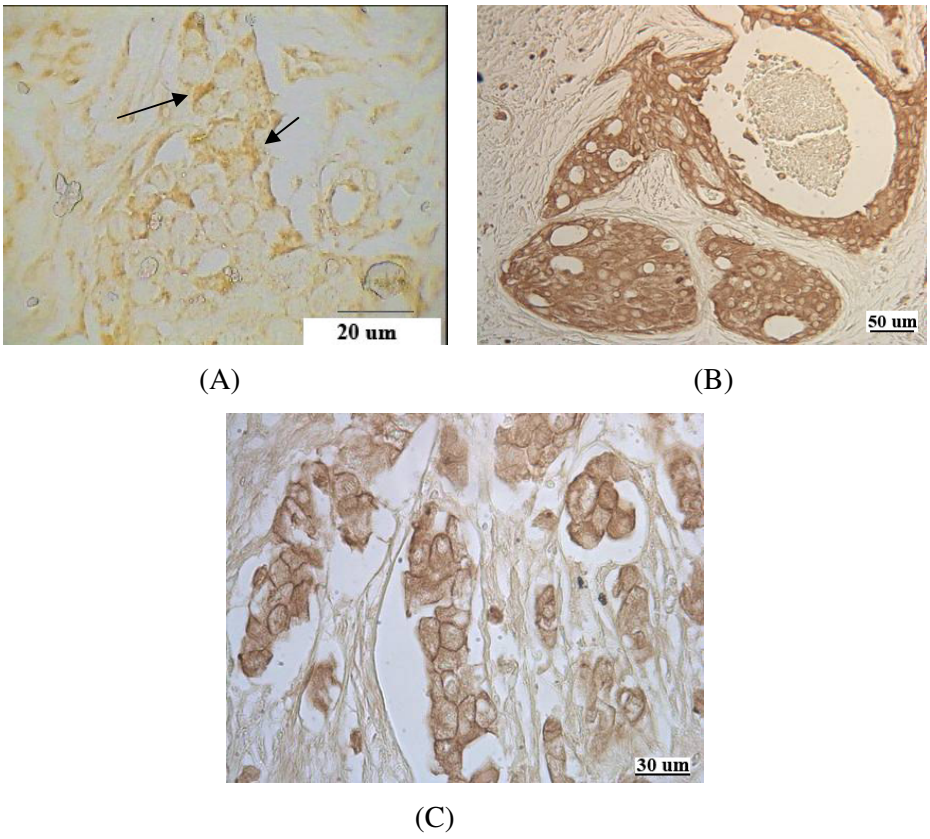


Figure 5.10. VGSCα protein expression in endothelial cells of a breast cancer tissue

5.3.3. Investigation of nNa$_v$1.5 Protein Expression in Breast Cancer

The expression and cellular location of nNa$_v$1.5 protein was analyzed using immunohistochemistry on 51 paraffin embedded breast cancer tissues obtained from Marmara University, Department of Pathology. In some cases the protein was present only on the membrane, and/or cytoplasm of the tissues (17 cases each). Figure 5.11A shows an

example of a breast cancer case where $nNa_v1.5$ was determined only on the tumor cell membrane. Cytoplasmic localization (Figure 5.11B) and membrane staining together with fainter cytoplasmic staining (Figure 5.11C) was shown in different cases.



(A) (B)

(C)

Figure 5.11. Breast tumor cells expressing nNav1.5 protein only on the membrane (A), on cytoplasm only (B) and membrane and cytoplasm (C)

In cases where observable endothelial cells of the arteries were also stained indicating the expression of $nNa_v1.5$ protein in these cells (Figure 5.12). This result defines the possible subtype of VGSCα expressed in endothelial cells that was determined using pan-VGSCα antibody (Figure 5.10).

Amongst the 51 breast cancer cases analyzed, $nNa_v1.5$ protein was present on epithelial cells with variable intensity of staining in 42 patients (82 per cent). A conclusion about the expression of $nNa_v1.5$ protein could not be made for two cases due to technical problems and LNM data was not available for 18 cases. Nineteen of $nNa_v1.5$ (+) patients (58 per cent) had LNM at the time of diagnosis. $nNa_v1.5$ protein was also expressed in 12

of LNM (-) patients (36 per cent) mostly at a relatively lower level compared to that of LNM (+) cases. In neither of the two $nNa_v1.5$ negative cases LNM was positive (6 per cent) (Table 5.5). Expression of the protein on the membrane alone and together with cytoplasm was observed in 14 of the LNM (+) cases (n=19). Whereas, six cases that express the protein at the cytoplasm were usually LNM (-) (n=10). Simultaneous presence or absence of $nNa_v1.5$ protein and LNM was observed in 64 per cent of the cases (χ^2=2,88, 0,1>p>0,05, df=1).

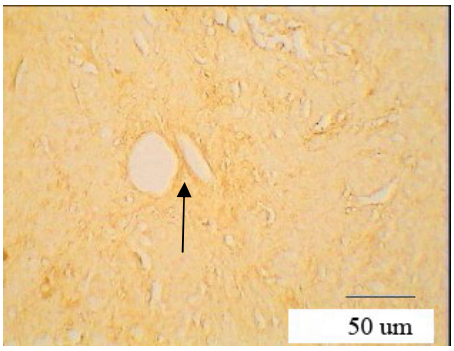


Figure 5.12. $nNa_v1.5$ protein expression in endothelial cells of arteries in breast cancer

Table 5.5. Analysis of $nNa_v1.5$ and LNM status of breast cancer cases

n=33	**LNM (+)**	**LNM (-)**
$nNa_v1.5$ (+)	19 (58 %)	12 (36 %)
$nNa_v1.5$ (-)	0	2 (6 %)

n: Total number of cases studied

Analysis of ER status showed that 27 ER (+) patients (71 per cent) and 8 ER (-) patients (21 per cent) expressed $nNa_v1.5$. Simultaneous expression of $nNa_v1.5$ and ER was observed in 71 per cent of the cases analyzed (χ^2=1,5, 0,1>p>0,05, df=1). In three $nNa_v1.5$ (-) patients (8 per cent) ER was present. However, ER data was not available for 13 patients (Table 5.6).

Since overexpression of HER2 protein is associated with aggressive type of breast cancer $nNa_v1.5$ expression was also compared with HER2 expression. The pathology reports of the patients showed that 16 cases (43 per cent) expressed both of the proteins but 18 (49 per cent) expressed only $nNa_v1.5$ protein. Two patients (5 per cent) were $nNa_v1.5$ (-)/HER2 (+) and one (3 per cent) was $nNa_v1.5$ (-) /HER2 (-). HER2 status of 27 per cent

was not available, therefore not included in the study (Table 5.7). $nNa_v1.5$ expression could not be associated with that of HER2 proteins (χ^2=0,07, 0,1>p>0,05, df=1).

Table 5.6. Analysis of $nNa_v1.5$ and ER status of breast cancer cases

n=38	**ER (+)**	**ER (-)**
$nNa_v1.5$ (+)	27 (71 %)	8 (21 %)
$nNa_v1.5$ (-)	3 (8 %)	-

n: Total number of cases studied

Table 5.7. Analysis of $nNa_v1.5$ and HER2 status of breast cancer cases

n=37	**HER2 (+)**	**HER2 (-)**
$nNa_v1.5$ (+)	16 (43 %)	18 (49 %)
$nNa_v1.5$ (-)	2 (5 %)	1 (3 %)

n: Total number of cases studied

The summary of LNM, hormone receptor status and histological grades of the cases analyzed for $nNa_v1.5$ protein expression are given in Table 5.8.

Table 5.8. Summary of breast cancer cases studied

No	**Age**	**$nNa_v1.5$**	**LNM**	**ER**	**PR**	**cErbB2**	**Diagnosis**	**Hist Grade**
1	NR	-						
2	NR	+						
3	NR	+						
4	NR	-						
5	NR	+						
6	NR	-						
7	40	+	N2 (2)	10-20%	70%	ND	Infiltr Ductal Ca	2
8	35	+	N2 (4)	20%	>%15	+3	Infiltr Ductal Ca	3
9	67	+	ND	ND	ND	ND	Infiltr Ductal Ca	2
10	48	+	N0	-	-	-	Infilt papiller Ca	2
11	74	+	N2 (7)	60%	70%	-	Infiltr Ductal Ca	2
12	78	+	ND	90%	70%	-	Infiltr musinoz ca	2
13	50	-	ND	ND	ND	ND	Infiltr Ductal Ca	2
14	38	+	N2 (5)	60%	40%	+1	75% Infiltr Ductal Ca + 25% lobular Ca	2
15	60	-	ND	ND	ND	ND	Infiltr Ductal Ca	2
16	50	+	N2 (14)	90%	10%	+1	Infiltr Ductal Ca	2
17	54	+	N0	90%	-	-	Infiltr Ductal Ca	2
18	49	+	N0	90%	10%	+1	Infiltr Ductal Ca	2
19	47	+	N0	Low	-	ND	Microinv DuCa, multifocal	ND
20	63	NC	ND	ND	ND	ND	Tubular Ca	1
21	49	+	N0	40%	60%	-	Infiltr Ductal Ca	2
22	36	+	N2 (7)	-	70%	-	Infilt papiller Ca	2
23	NR	-						
24	65	-	ND	70%	30%	+1	Infiltr Ductal Ca	2
25	42	+	N0	40%	30%	+2 only 1area	Infiltr Ductal Ca	2
26	77	-	N0	100%	70%	+1	Infiltr Ductal Ca	2

Table 5.8. Summary of breast cancer cases studied (continued)

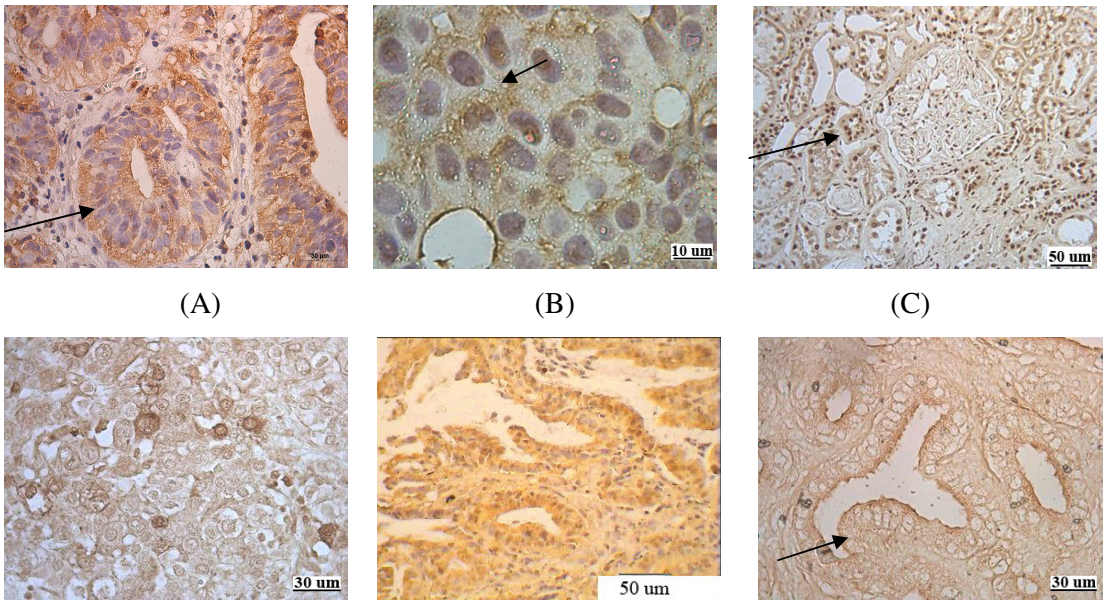
27	59	+	N0	-	-	+1	Infiltr Ductal Ca	3
28	?	+	ND	90%	70%	-	Musinoz Ca	2
29	36	+	N1 (1)	90%	-	+3	Infiltr Ductal Ca+ musinoz Ca	2
30	40	-	N0	70%	70%	-	Infiltr Ductal Ca	3
31	80	NC	ND	70%	60%	-	Musinoz Ca	2
32	49	+	N0	-	-	+3	Infiltr apocrine Ca	2
33	55	+	N2 (4)	-	-	-	Infiltr Ductal Ca	3
34	52	+	N1 (1)	90%	70 %	-	Infiltr Ductal Ca	ND
35	70	+	N1 (1)	70%	30%	+1	Infiltr Ductal+micropapiller signet ring Ca	2
36	61	+	ND	70%	40%	-	Infiltr Ductal Ca	2
37	60	+	N1(2)	-	-	+1	Infiltr Ductal Ca, multifocal	2
38	47	+	N1 (2)	-	-	+1	Infiltr Ductal Ca	3
39	44	+	N2 (8)	90%	-	+1	Multicentric infiltr Ductal Ca	3
40	59	+	ND	90%	90%	-	Infiltr Ductal Ca	2
41	77	+?	ND	90%	90%	ND	Infiltr Ductal Ca	2
42	82	+	N0	60%	60%	-	Infiltr Ductal Ca	3
43	54	+	N1 (1)	70%	30%	2+	Infiltr Ductal Ca	3
44	63	+	N0	%30	-	+1	Infiltr Ductal Ca+tubulolobular	2
45	60	+	N0	90%	70%	-	Infiltr Ductal+solid micropapiller differentiation	2
46	42	+	N0	40%	30%	+1	Infiltr Ductal Ca	2
47	44	+?	ND	-	-	-	Infiltr Ductal Ca	ND
48	59	+	ND	-	10%	-	Infiltr Ductal Ca	3
49	42	+?	ND	-	30%	-	Infiltr Ductal Ca	ND
50	65	+	N1(1)	70%	-	-	Micropapiller Ca, multicentric	3
51	80	+	N0	70%	20%	-	Infiltr Ductal Ca	2
52	50	+	N2(7)	-	-	-	Infiltr Ductal Ca (multifocal)	2
53	64	+	N1(3)	80%	45%	+1	Infiltr Ductal Ca	1

PR: progesterone receptor; HER2: epidermal growth factor receptor; NR: Not reported; ND: Not done; N2: LNM present with grade 2; N0: no LNM; NC: No conclusion; +?: suspected positive; Hist Grade; histological grade

5.4. Investigation of VGSCα Protein Expression in Other Cancers

The expression of VGSCα protein was analyzed in cancers of the stomach, urinary bladder, kidney and lung. Prostate cancer was used as a positive control. For each type of cancer, 3-4 cases were analyzed. Cases where VGSCα was expressed only at the non-tumoral (normal) regions of the section were not included in analysis. In stomach cancer, VGSCα protein was determined on the membrane and cytoplasm of tumor cells (Figure 5.13A). In high grade urothelial carcinoma the protein was localized on the membranes of tumor cells (Figure 5.13B). The tubular epithelium of a kidney cancer case was shown to express VGSCα protein (Figure 5.13C). In case of lung cancer, VGSCα immunoreactivity was observed in the cytoplasm of individual tumor cells dispersed within the tumor area (Figure 5.13D). The colon adenocarcinoma possessed the VGSCα both at the normal and

tumoral area (Figure 5.13E). Prostate cancer was immunoreactive to pan-VGSCα antibody on the apical membrane of epithelial cells facing the lumen (Figure 5.13E), that is consistent with previous findings (Diss *et al*., 2005). Amongst the cases studied, metastasis was clinically evident in one of the lung cancer patients. Histopathological findings of VGSCα (+) cases in stomach, urothelial carcinoma, kidney and prostate cancers indicated poor prognosis, suggesting future metastasis in these patients. The results of VGSCα analysis in the stomach, urinary bladder kidney, lung, and prostate cancer are summarized in Table 5.9.



(A) (B) (C)

(D) (E) (F)

Figure 5.13. Investigation of VGSCα protein in different cancers. Stomach cancer (A), urinary bladder cancer (B), kidney cancer (C) lung cancer (D) and prostate cancer (E) and colon cancer (F). Arrows indicate VGSCα specific staining. Hematoxylene counterstaining is performed in (A), (B) and (F)

Table 5.9. Summary of the VGSCα investigation in different cancers

Tissue	**n**	**VGSCα (+)**	**VGSCα (-)**
Stomach cancer	4	1	3
Urinary bladder cancer	4	1	3
Kidney cancer	3	2	1

Table 5.9. Summary of the VGSCα investigation in different cancers (continued)

Lung cancer	4	3	1
Prostate cancer	3	2	1
Colon cancer	4	2	2

n: Total number of cases analyzed

5.5. Investigation of a Possible Role of Estrogen on VGSC Function During Metastasis of Breast Cancer Cells

Three different breast cancer cell lines MDA-MB-231, MDA-MB-231 transfected with ERα (MDA-MB-231-ERα) and MCF-7 were used to analyze the possible effect of estrogen on VGSC function during metastasis. For this purpose, proliferation and motility of breast cancer cells were investigated using BrdU incorporation assay and wound heal motility assay, respectively. The assays were performed by exposing the cells to estrogen and/or TTX (VGSC blocker) for 72 hours.

5.5.1. Determination of Toxicity of BrdU, Estrogen and/or TTX on Breast Cancer Cells

The possible toxic effect of BrdU, estrogen and TTX was determined by Trypan blue exclusion assay after treating the cells with estrogen (10 nM or 100 nM) and/or 10 μM TTX for 72 hours. During BrdU incorporation assay, breast cancer cells were treated with estrogen and/or TTX, in combination with 10 μM BrdU. Our results showed the absence of toxic effect of estrogen and/or TTX when compared to non-treated controls (Figure 5.14). Treating the cells with BrdU did not affect the viability and/or proliferation rate of the cells either (Figure 5.15).

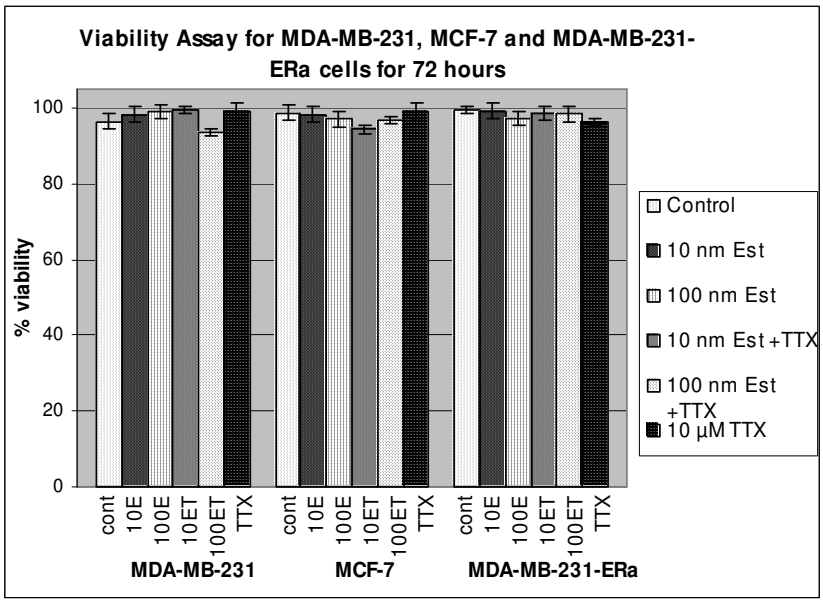


Figure 5.14. No toxic effect of estrogen and/or TTX was observed on MDA-MB-231, MCF-7 and MDA-MB-231-ERα at 72 hours. Cont: control, 10E: 10 nM estrogen, 100E: 100 nM estrogen, 10ET: 10 nM Estrogen + TTX, 100ET: 100 nM estrogen

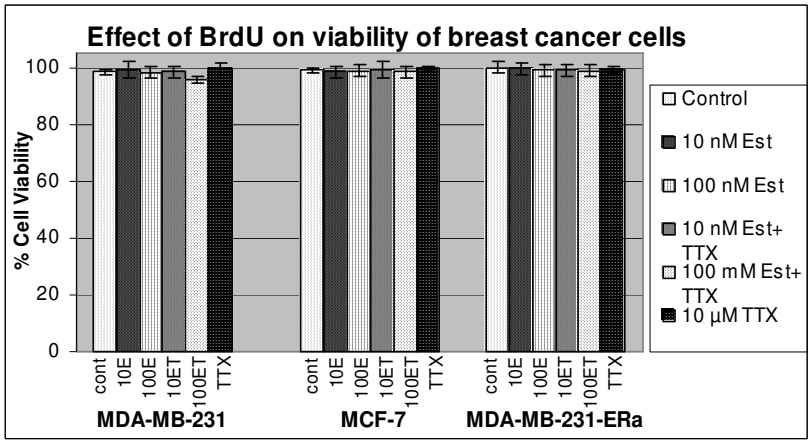
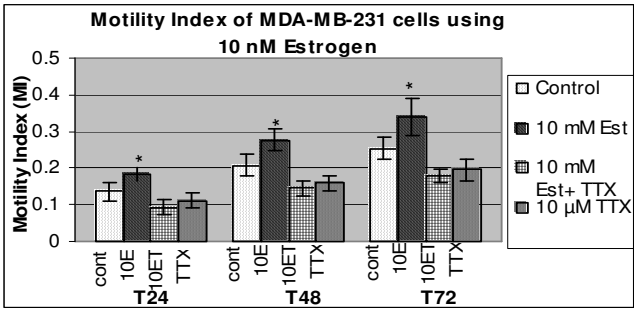


Figure 5.15. No Toxic effect of BrdU was observed on breast cancer cells

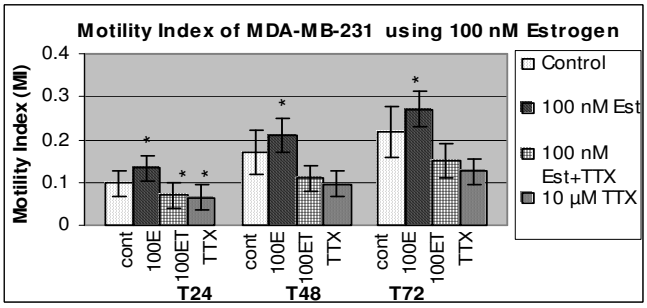
5.5.2 Analysis of the Effect of Estrogen and/or TTX on Motility of Breast Cancer Cells

The wound heal assay was performed using metastatic MDA-MB-231 cells that express functional VGSC but not ER, weakly metastatic MCF-7 cells that do not express functional VGSC but ER and MDA-MB-231-ERα. Minimally three individual

experiments were performed where cells were treated with estrogen and/or 10 µM TTX. Dosage sensitivity to estrogen was tested using 10 nM and 100 nM estrogen. Each treatment was performed in triplicates and non-treated cells were used as controls. The changes in motility upon treatments were based on visual measurements that may cause difficulties in determining the actual motility rate of the cells. The results may indicate that treatment with both 10 nM and 100 nM estrogen increased the motility of MDA-MB-231 cells ($p<0,05$) (Figure 5.16A and B, respectively). Treatment with estrogen together with TTX reversed the effect of estrogen, causing a decrease in the motility of the cells ($p<0,001$). Incubation with 10 µM TTX alone, also decreased the motility ($p<0,001$). The results were consistent within each other throughout 72 hours both in 10 nM and 100 nM estrogen treatment groups (Figure 5.17A and B). The motility increased within time in each type of application. Cells treated with estrogen showed higher motility in time than those treated with TTX with/without estrogen. The phase contrast pictures of MDA-MB-231 cells are shown in Figure 5.18 where more cells are observed in estrogen treated sample (B) than in control (A). Whereas less number of cells was observed in samples exposed to TTX with/without estrogen (C and D, respectively) within the wound area.

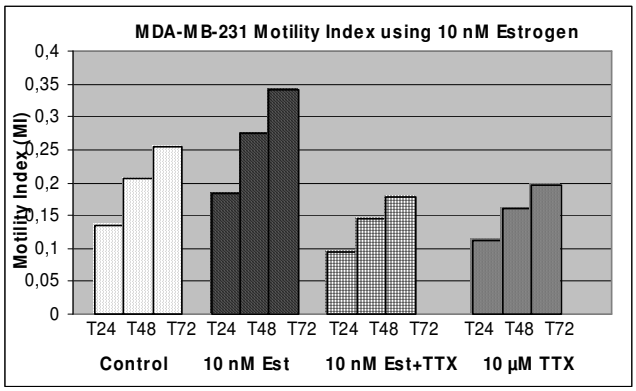


(A)

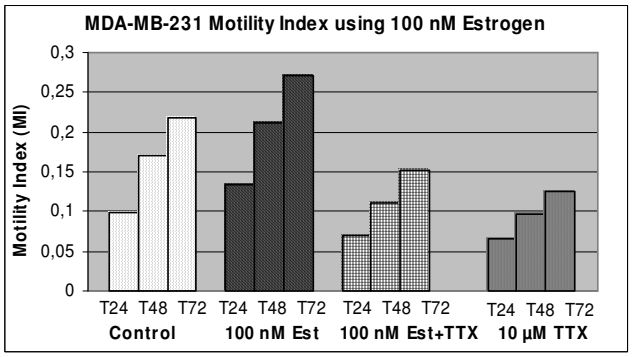


(B)

Figure 5.16. Effect of 10 nM (A) and 100 nM (B) estrogen and/or TTX on motility of MDA-MB-231 breast cancer cells (*: $p<0,05$; the rest $p<0,001$)

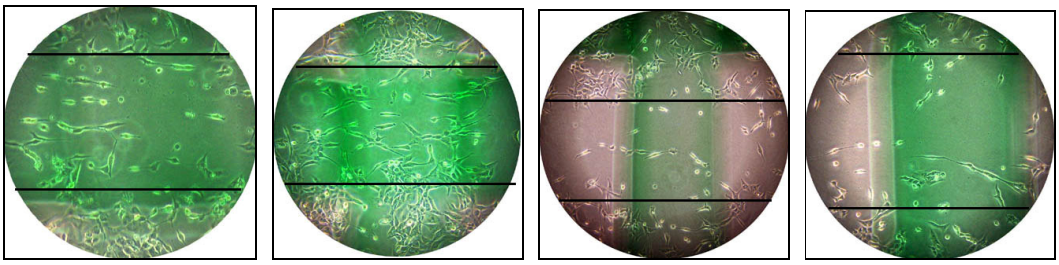


(A)



(B)

Figure 5.17. Change of motility of MDA-MB-231 cells upon 10 nM (A) and 100 nM (B) estrogen with/without TTX incubation during 72 hours

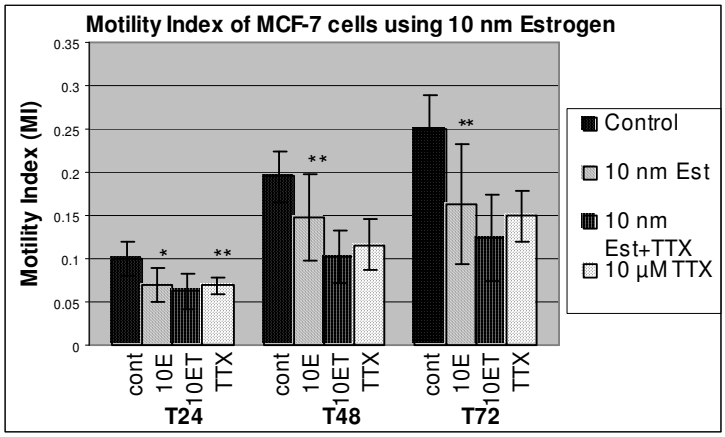


(A) (B) (C) (D)

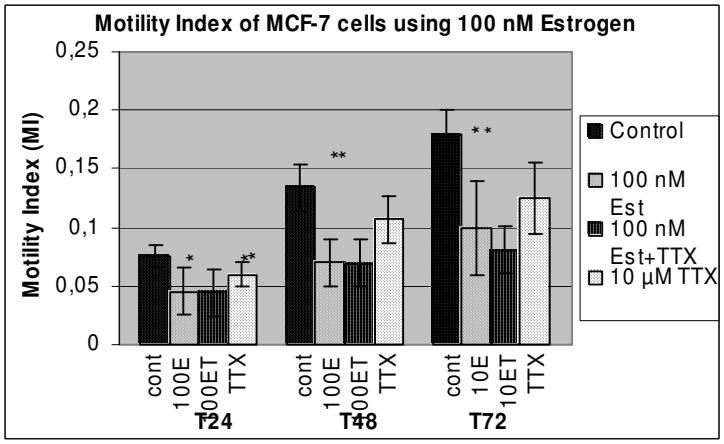
Figure 5.18. Phase-contrast pictures of MDA-MB-231 untreated cells (A), treated with estrogen (B), estrogen and TTX (C) and TTX (D) (100X magnification). Initial wound widths are indicated by black lines. Green color is from the permanent marker on the dish

Analysis of the motility of MCF-7 cells after incubation with estrogen and/or TTX has shown that estrogen may decrease motility of cells at both 10 nM and 100 nM

concentrations (Figure 5.19A and B, respectively). This effect is not significant for 10 nM concentration at T24. Treatment with 100 nM estrogen inhibited motility more than that observed with 10 nM estrogen. TTX alone and in combination with estrogen also decreased motility. The effects of estrogen and/or TTX were consistent where motility increased within 72 hours (Figure 5.20). The microscopic observation of cells upon treatment is shown in Figure 5.21 where fewer cells are observed within the wound area in samples exposed to estrogen alone estrogen and/or TTX.



(A)

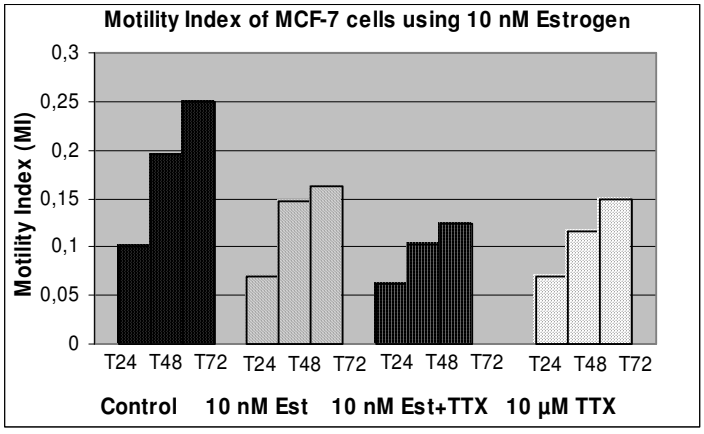


(B)

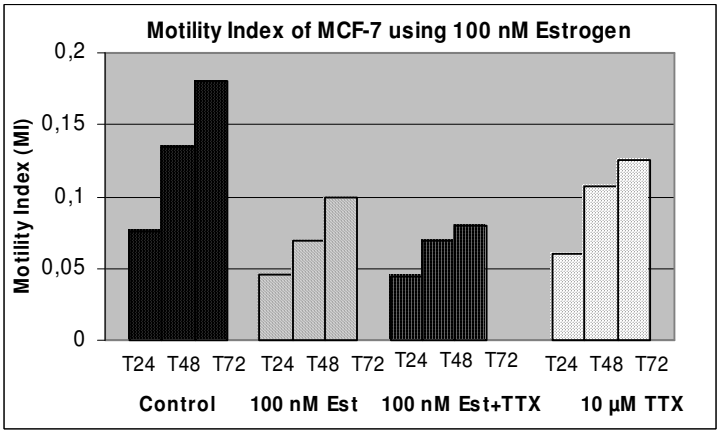
Figure 5.19. Effect of incubation with 10 nM (A) and 100 nM (B) estrogen with/without TTX on motility of MCF-7 cells (*: p=0,07; **: p<0,05; the rest p<0,005)

Motility index was also analyzed for MDA-MB-231-ERα. The effect of estrogen on MDA-MB-231-ERα was different at short term and long term treatments. After 24 hour of

estrogen incubation (10 nM and 100 nM) motility decreased (Figure 5.22. However, an opposite effect of estrogen was observed at 48 and 72-hour incubation where motility was increased. The analysis at 48 and 72 hours indicated the threshold concentration of estrogen as 10 nM since higher concentrations of estrogen (100 nM) did not enhance the result. Incubation with TTX and estrogen decreased the motility at all times ($p<0,005$) reversing the effect of estrogen at 48 and 72 hours. TTX treatment inhibited motility at all times ($p<0,005$). Motility index of MDA-MB-231-ERα was consistent within each treatment where gradual increase in motility was observed during 72 hours (Figure 5.23). The microscopic observation of MDA-MB-231-ERα upon treatment is shown in Figure 5.24.

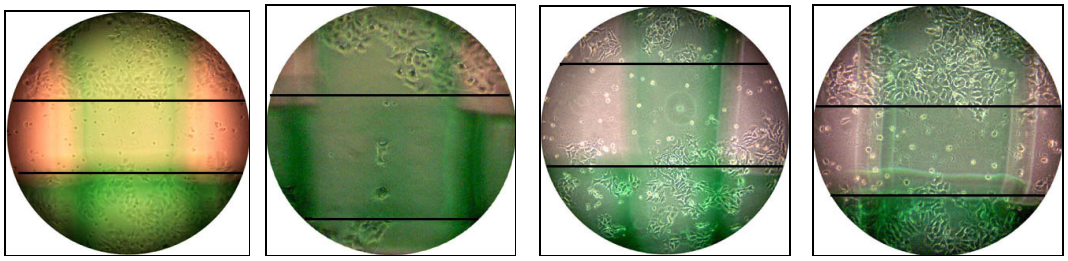


(A)



(B)

Figure 5.20. Change of motility of MCF-7 cells upon 10 nM (A) and 100 nM (B) estrogen with/without TTX incubation during 72 hours



(A) (B) (C) (D)

Figure 5.21. MCF-7 cells that are untreated (A) (40X magnification), treated with estrogen (B), estrogen and TTX (C) and TTX alone (D) (100X magnification). Black lines indicate initial wound width

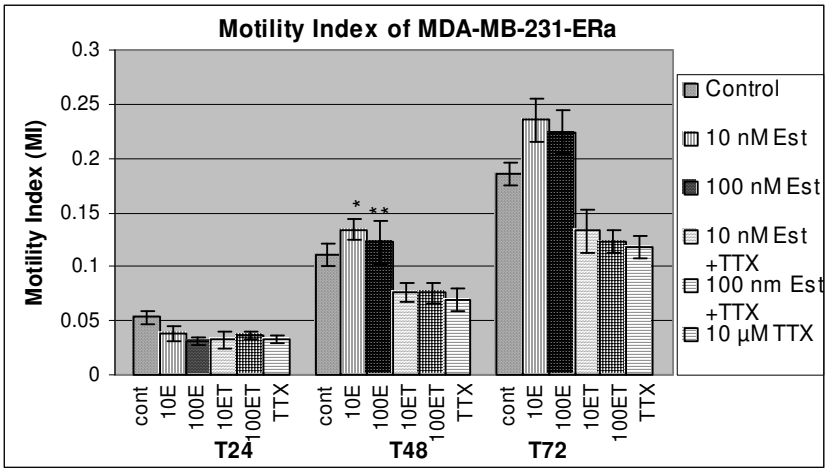


Figure 5.22. Effects of estrogen with/without TTX on motility of MDA-MB-231-ERα cells. *: p<0,05; **: p=0,08; rest p<0,005

The motility of MDA-MB-231, MCF-7 and MDA-MB-231-ERα was compared within each other at both 10 nM and 100 nM estrogen treatment groups. The highly metastatic MDA-MB-231 cells are the most motile ones amongst the other cell lines. The motility of MDA-MB-231 cells was highest at all times and in each type of treatment (Figure 5.25(A) and (B)). However, transfection of these cells with ERα has decreased the motility capacity dramatically, causing the cells to be less motile even than the weakly metastatic MCF-7 cells. Treating the breast cancer cells with estrogen caused different effects on each cell line. These effects may depend on the metastatic capacity and the ER content of the cells, where estrogen increased the motility of highly metastatic MDA-MB-231 cells but decreased in weakly metastatic MCF-7 cells. In case of MDA-MB-231-ERα,

short term effect of estrogen was a decrease but long term effect was an increase in the motility of cells. However, treatment with estrogen and TTX has reversed the effect of former in MDA-MB-231 with/without ERα but not in MCF-7 cells.

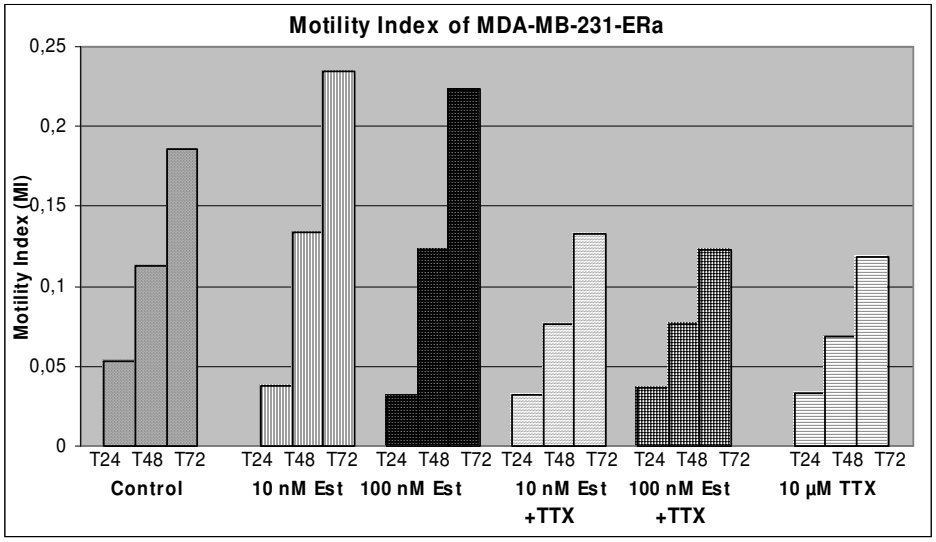
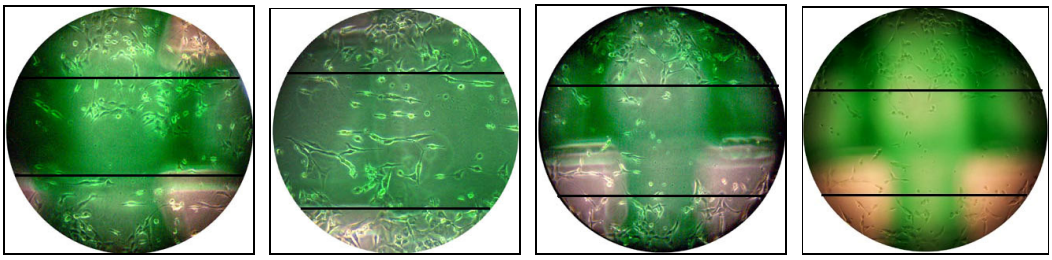
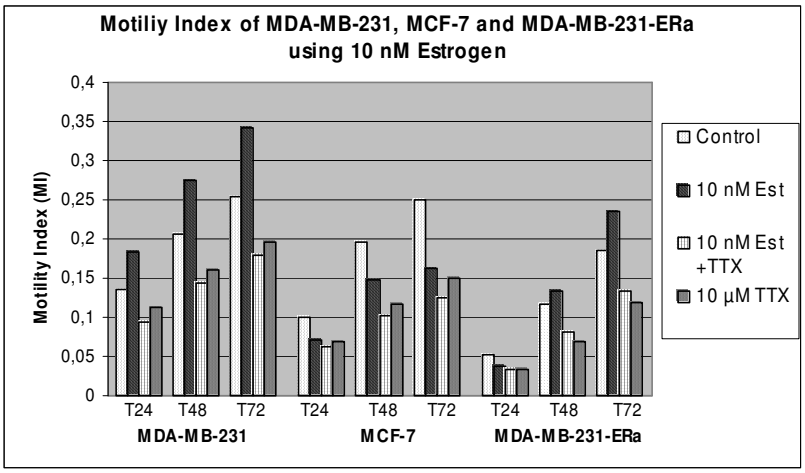


Figure 5.23. Change of motility of MDA-MB-231-ERα cells upon estrogen with/without TTX incubation during 72 hours

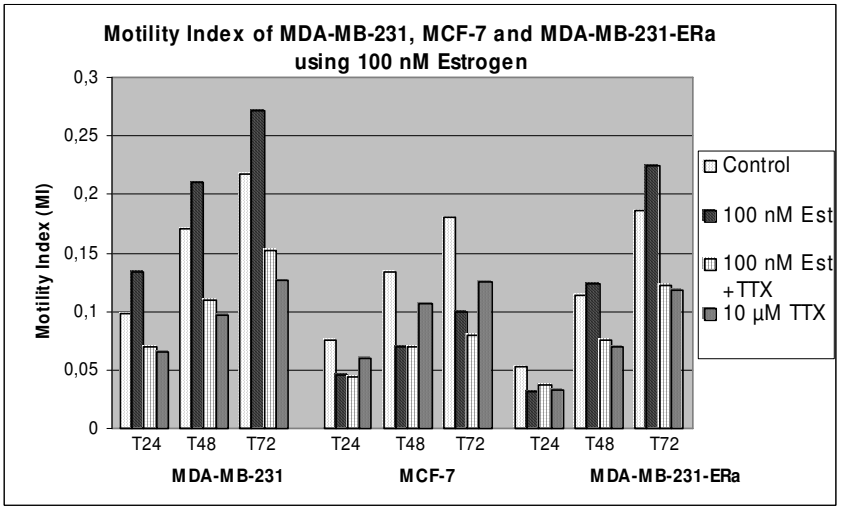


(A) (B) (C) (D)

Figure 5.24. Pictures of MDA-MB-231-ERα untreated cells (A) and treated with estrogen (B), estrogen and TTX, (C) and TTX alone (D) (A and D, 4X objective, B and C 10X objective). Black lines represent initial wound width



(A)



(B)

Figure 5.25. Effects of estrogen with/without TTX on motility of MDA-MB-231, MCF-7 and MDA-MB-231-ERα cells using 10 nM estrogen (A) and 100 nM estrogen (B)

5.5.3. The Analysis of the Effect of Estrogen and/or TTX on Proliferation of Breast Cancer Cells

The proliferation rate of MDA-MB-231, MCF-7 and MDA-MB-231-ERα cell lines after 72-hour incubation with 10 mM or 100 mM estrogen and/or 10 µM TTX was determined using the BrdU incorporation assay. Three individual experiments, in duplicates, were performed for each cell line. The results showed that neither estrogen nor TTX significantly modulates proliferation of the cells after 72 hour incubation (Figure

5.26) (p= 0,4-0,9). The absence of the effect of TTX on proliferation of MDA-MB-231 and MCF-7 cells was previously reported (Fraser *et al*., 2005) in agreement with our data.

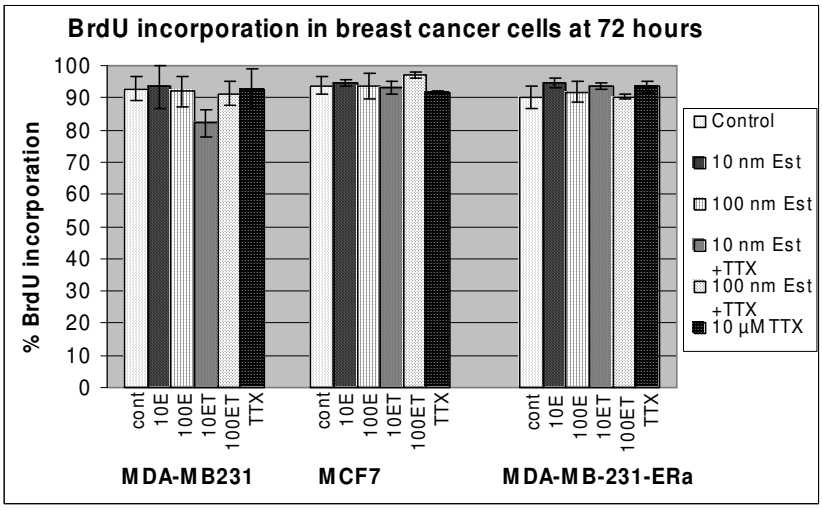


Figure 5.26. Rate of proliferation of breast cancer cells in response to estrogen and/or TTX at 72 hours

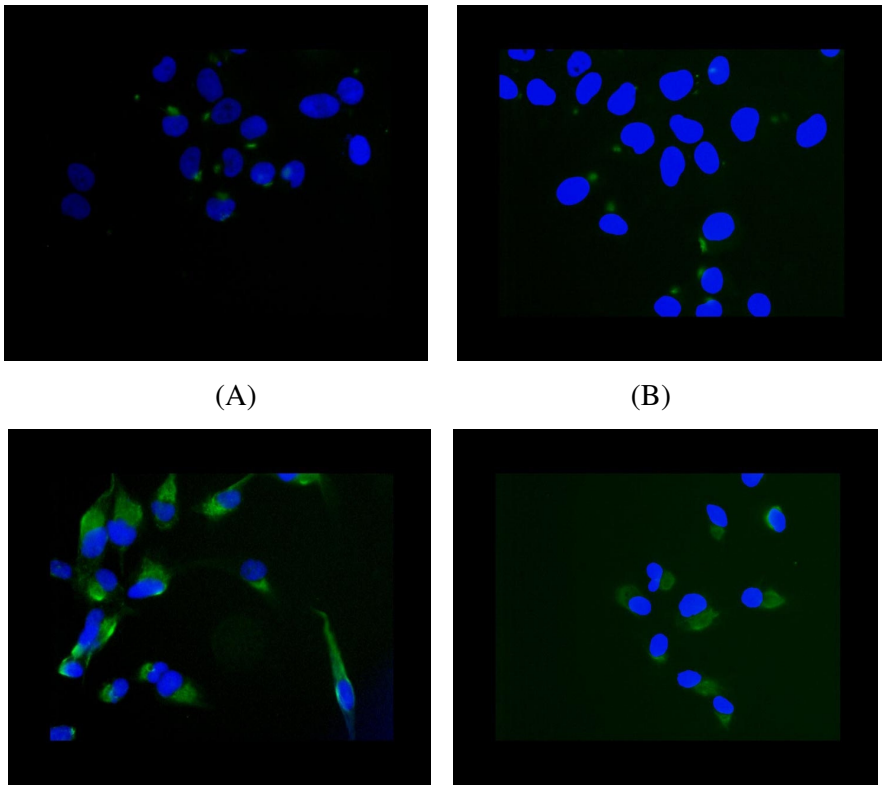
5.6. Investigation of Possible Effect of Estrogen on nNa$_v$1.5 Protein Expression and Localization in Breast Cancer Cells

The possible effect of estrogen on nNa$_v$1.5 protein expression and location was analyzed on MDA-MB-231, MCF-7 and MDA-MB-231-ERα cell lines using immunocytochemistry. The staining intensity was quantified using Matlab 6.5 software. The cells were treated with 10 nM or 100 nM estrogen and/or 10 µM TTX for 72 hours, fixed and used for immunocytochemistry.

5.6.1. Effect of Estrogen on nNa$_v$1.5 Protein Expression

The expression of nNa$_v$1.5 protein upon treatment with estrogen and TTX was analyzed using Matlab 6.5 software. For each type of treatment the pixels of approximately 100 cells were counted. In MDA-MB-231 cells estrogen caused no change in nNa$_v$1.5 protein level ($p<0,5$). Treating the cells with TTX alone or in combination with estrogen decreased the protein amount but the changes were not significant ($p<0,5$). The nNa$_v$1.5

expression in MCF-7 cells was quite low when compared to MDA-MB-231 cells, as expected (Figure 5.27).Treating the cells with estrogen and/or TTX had only minor changes in the protein level (p=0,2-0,7). In case of MDA-MB-231-ERα cells estrogen increased $nNa_v1.5$ expression as in non-transfected ones (p=0,5). Upon treatment using TTX with/without estrogen, no change in the protein amount was observed (p=0,4-0,7). The results are summarized in the bar chart shown in Figure 5.28.



(A) (B)

(C) (D)

Figure 5.27. The expression of nNav1.5 protein in MCF-7 (A-B) and MDA-MB-231 (C-D) cells. Blue colour of DAPI indicates the nuclei and green FITC is specific for $nNa_v1.5$

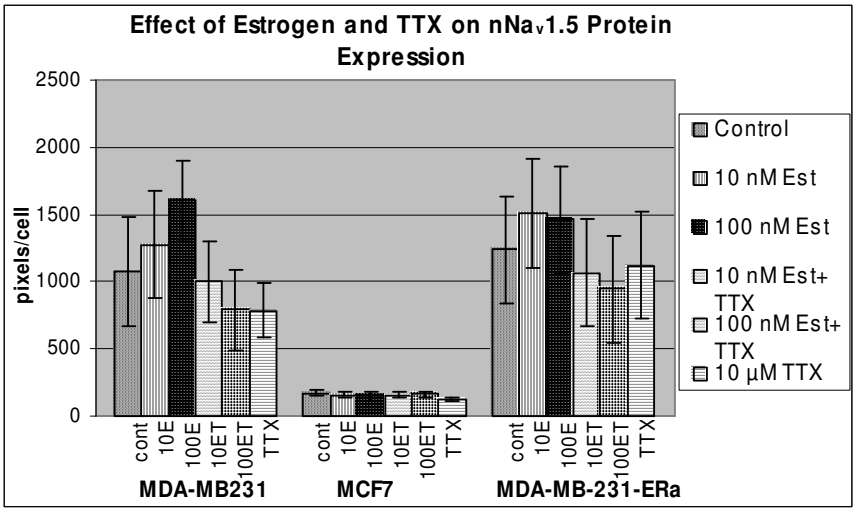


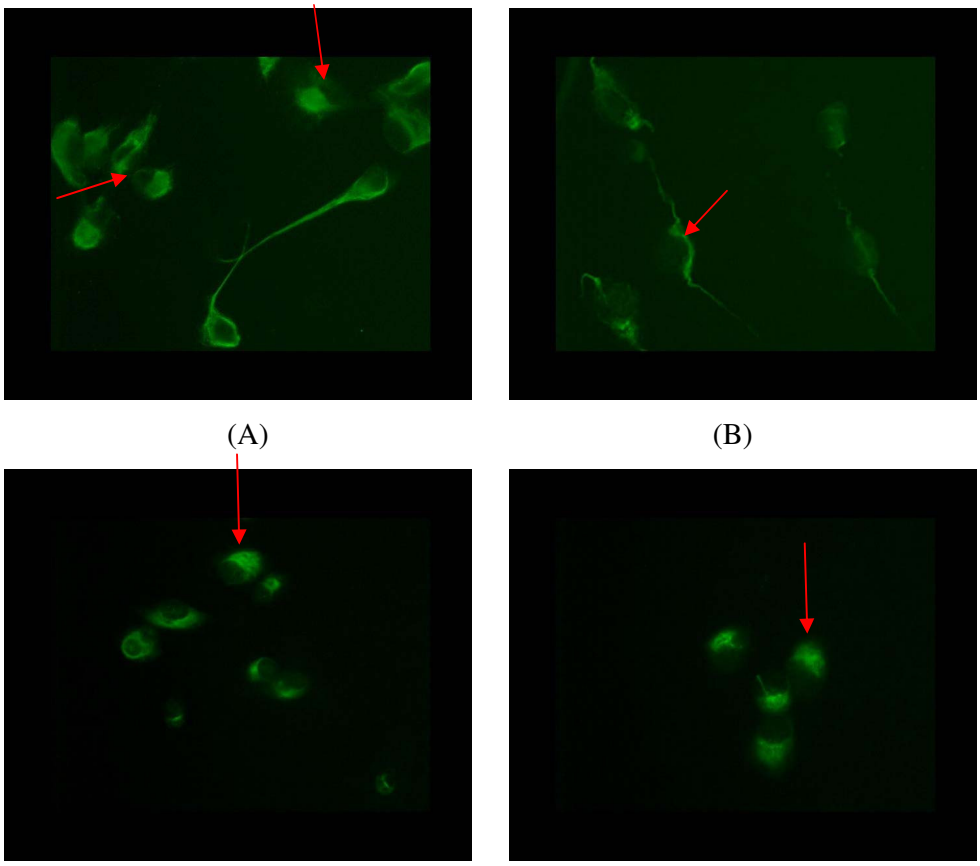
Figure 5.28. Changes in $nNa_v1.5$ protein expression with respect to estrogen and TTX

5.6.2. Effect of Estrogen on $nNa_v1.5$ Protein Localization

The change in the localization of $nNa_v1.5$ protein upon treating the cells with estrogen and TTX was analyzed in ~100 cells for each group. $nNa_v1.5$ protein was observed either in the cytoplasm, or plasma membrane or both. In untreated MDA-MB-231 cells, the protein was mostly expressed in the cytoplasm (59 per cent), some on the membrane (12 per cent) and some on the membrane and cytoplasm (29 per cent) (data not shown). Upon treating the cells with estrogen, $nNa_v1.5$ was transported to the membrane (36 per cent) significantly ($p<0,05$). When the cells were incubated with estrogen and TTX, cytoplasmic localization was again favored (55 per cent) reversing the effect of estrogen ($p<0,05$). TTX alone has also caused transport of the protein to the cytoplasm ($p<0,05$) (Figure 5.29). The results are summarized in bar charts in Figure 5.30.

MDA-MB-231-ERα cells were also tested for the regulation of $nNa_v1.5$ location upon estrogen and TTX treatment. Estrogen caused transport of the protein from the cytoplasm to the membrane. The significance of this effect was increased with increasing concentrations of estrogen ($p<0,05$ with 100 nM estrogen). The effect of estrogen was reversed with simultaneous incubation of cells with estrogen and TTX where protein was transported back to the cytoplasm ($p<0,05$). TTX alone has also caused increased

cytoplasmic localization of nNa$_v$1.5 protein ($p<0,05$) (Figure 5.33). The results are summarized in bar charts in Figure 5.34.



(A) (B)

(C) (D)

Figure 5.29. Localization of nNa$_v$1.5 protein in MDA-MB-231 control (A), and cells treated with estrogen (B), estrogen and TTX (C), TTX only (D). Arrows indicate cytoplasmic staining in A, C and D and membrane staining in B

MCF-7 cells shown to express very low amounts of nNa$_v$1.5 protein were also analyzed for the effect of estrogen and TTX on localization. The results showed that neither of the treatments affected the localization of nNa$_v$1.5 protein significantly ($p=0,4-0,9$) (Figure 5.31).

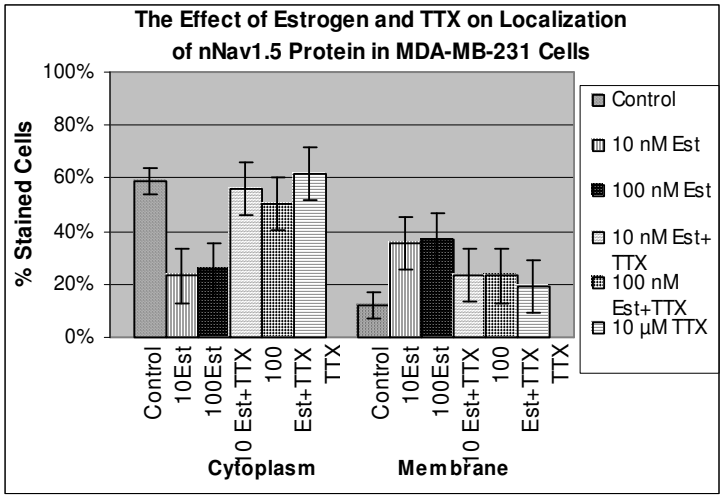
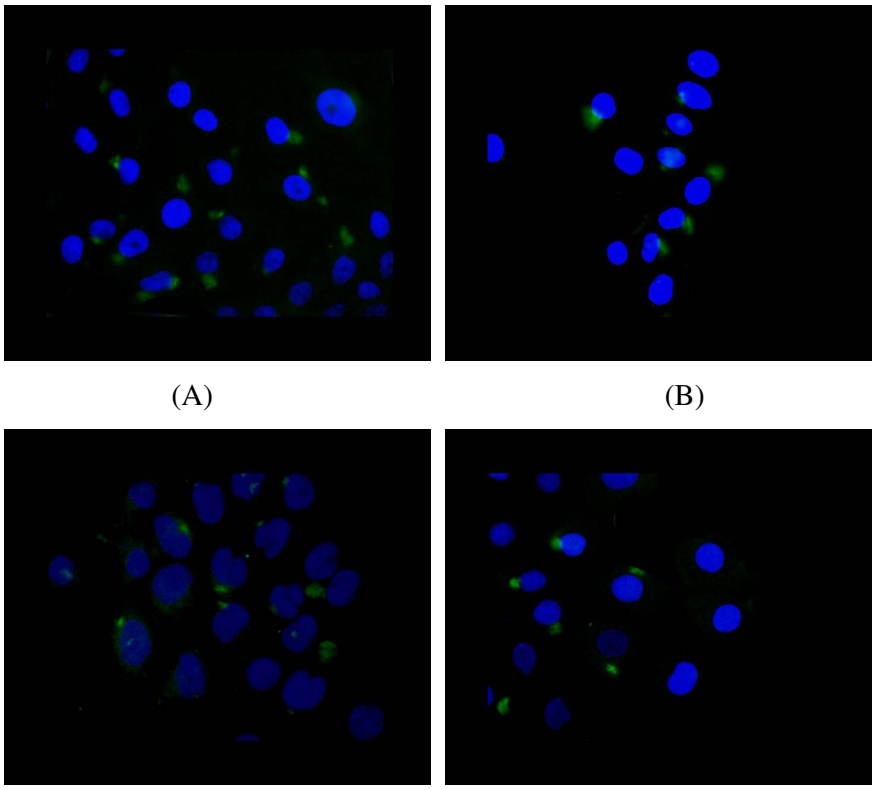


Figure 5.30. Localization of nNa$_v$1.5 protein in MDA-MB-231 cells



(A) (B)

(C) (D)

Figure 5.31. Localization of nNa$_v$1.5 protein in MCF-7control (A), and cells treated with estrogen (B), estrogen and TTX (C), TTX only (D)

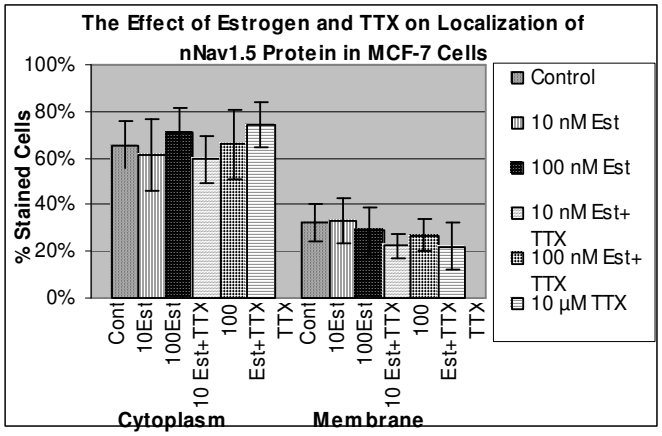
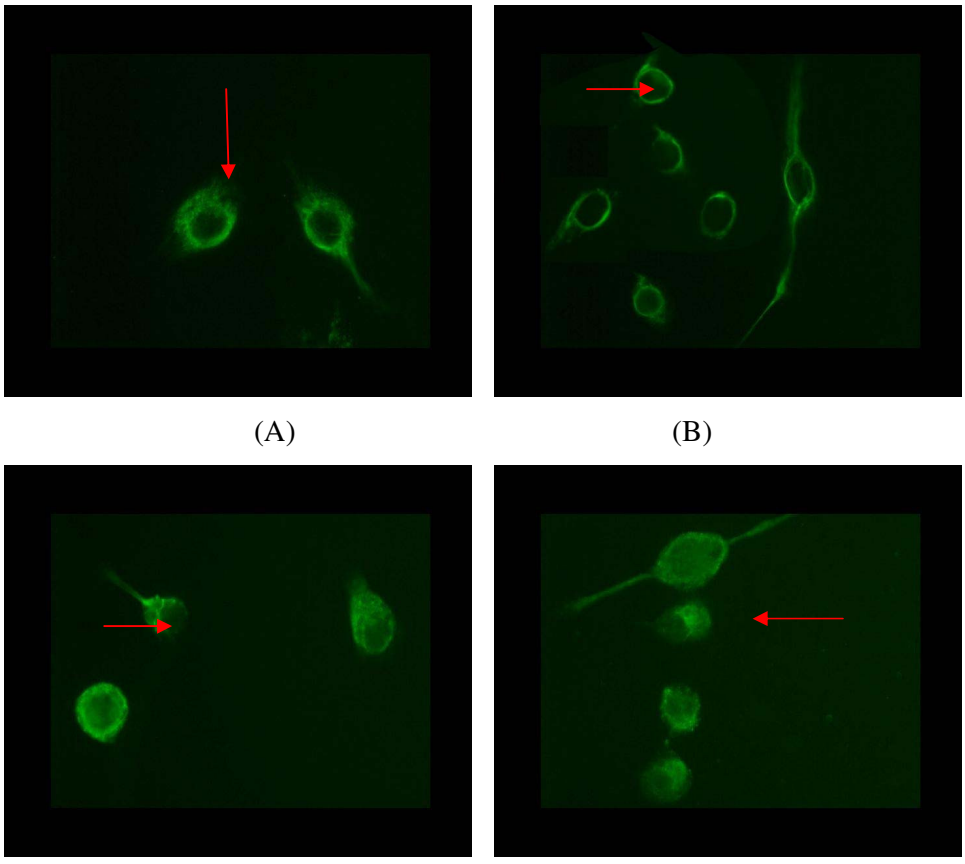


Figure 5.32. Localization of $nNa_v1.5$ protein in MCF-7 cells



(A) (B)

(C) (D)

Figure 5.33. Localization of nNav1.5 protein in MDA-MB-231-ERα control (A), and cells treated with estrogen (B), estrogen and TTX (C), TTX only (D). The arrows indicate staining in cytoplasm and membrane (A), membrane (B) and cytoplasm in (C and D)

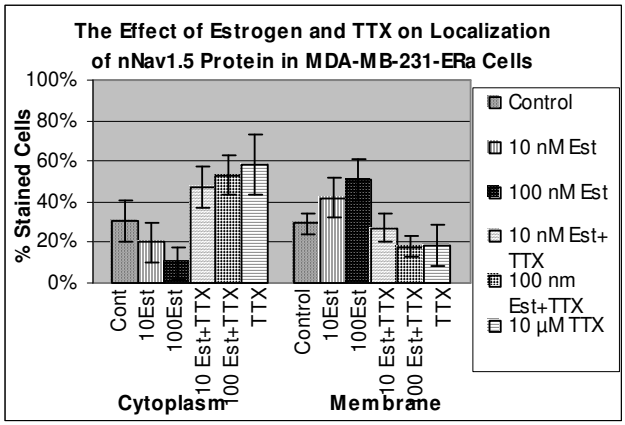


Figure 5.34. Localization of $nNa_v1.5$ protein in MDA-MB-231-ERα cells

6. DISCUSSION

This study aimed to further our understanding in the implication of $nNa_v1.5$ expression in breast cancer metastasis and to investigate the mechanism of $nNa_v1.5$ upregulation in the metastatic disease. The results of RT-PCR and immunohistochemistry showed that $nNa_v1.5$ expression was in parallel with breast cancer metastasis and ER expression only in a group of patients. Specific expression of $nNa_v1.5$ protein was observed in the metastatic disease where the protein was determined in normal breast at basal level amongst the normal tissues.

Analysis of the expression of VGSCα protein in non-excitable human tissues implicated the presence of the protein mostly in secretory cells of samples investigated. To elucidate whether VGSCα expression was a widespread mechanism in cancer metastasis we analyzed its expression in different cancer types. The preliminary results in a small number of patients may indicate possible contribution of VGSCα expression and cancers of stomach, colon, urinary bladder, kidney, and lung.

In this study, we also investigated the possible role of estrogen in upregulating $nNa_v1.5$ expression and modulating its function in metastatic breast cancer cells. We showed that estrogen had no effect on proliferation in all types of cell lines tested. However, it caused a slight increase in motility through $nNa_v1.5$ in highly metastatic cells that express the protein but had an opposite effect on weakly metastatic ones that do not express it. We also found that estrogen did not change the $nNa_v1.5$ protein level but increased the functionally available form on the plasma membrane in metastatic cells. These results suggest that estrogen may increase motility of breast cancer cells by acting through $nNa_v1.5$ and enabling its translocation to the plasma membrane where it is functional.

6.1. Breast Cancer Metastasis and $nNa_v1.5$ Expression

Breast cancer is the most common cancer among females and its metastasis is observed in 40 per cent of the cases, causing death (Weigelt *et al*., 2005). In Turkey, breast cancer accounts for 24,1 per cent of the female cancer cases (http://www.saglik.gov.tr/ extras/istatistikler/ apk2001/092.htm). Currently used prognostic markers for breast cancer include, tumor size, histological grade, axillary lymph node metastasis, estrogen and progesterone receptors, and HER2 expression (Daidone *et al*., 2004). However, these indirect markers are not sufficient for detection of microscopic metastasis that is present at initial diagnosis in a significant proportion of patients (20-40 per cent). Thus, clinicians require direct gene markers for early diagnosis of metastasis. None of the ongoing studies like microarray analysis and detection of metastatic tumor cells in blood circulation (Bernards and Weinberg, 2002; Ring *et al*., 2004) accomplished the requirements of a prognostic marker. These studies either could not be reproduced by independent researchers (Veer *et al*., 2005) or were not prospective.

VGSC may be one of the potential early prognostic markers for breast cancer metastasis and was implicated in the basic metastatic steps of metastatic prostate (MatLy-Lu and PC3), breast cancer cells (MDA-MB-231) and small-cell lung carcinoma (Diss *et al*., 2001; Fraser *et al*., 2005; Onganer and Djamgoz, 2005). Preliminary results based upon 20 breast cancer patients have previously shown $nNa_v1.5$ gene expression in metastatic human tissues and prior to metastasis (Fraser *et al*., 2005). Within the framework of this thesis, a double-blind study was performed in 45 patients to further our understanding in association of $nNa_v1.5$ gene expression and metastasis. The results of RT-PCR and immunohistochemistry showed that expression of $nNa_v1.5$ was in parallel with LNM only for 26/45 and 21/33 patients, respectively. Based on previous findings (Fraser *et* al., 2005), some patients that did not have clinically evident metastasis but expressed $nNa_v1.5$ (19/45 and 12/33, in RT-PCR and immunohistochemistry, respectively) may have the likelihood to develop metastasis in the future. However, the prognosis of these patients should be followed to confirm this suggestion. In our *in vivo* study, $nNa_v1.5$ expression was determined in 93 and 94 per cent of the cases using RT-PCR and IHC, respectively. This finding is in consistence with presence of invasive tumors in breast cancer (90 per cent of all cases) that are likely to metastasize. On the other hand, we can not disregard the finding

that $nNa_v1.5$ mRNA and protein was detected in normal breast tissues. Further imaging analysis by our collaborators showed that the $nNa_v1.5$ protein in normal breast was 1000 times less than that in cancer (Fraser S. P., personal communication). Based on our observation, the intensity of $nNa_v1.5$ staining was very low and rare in epithelial cells of the normal tissue. We have shown by immunohistochemical analysis that expression was of epithelial origin excluding the possibility that it could have been expressed in fibroblasts and/or endothelial cells of the normal breast. The identity of the VGSCα subtype expressed both in normal and cancerous breast was determined to be $nNa_v1.5$ in paraffin embedded sections of identical tissues. However, since the staining intensity of $nNa_v1.5$ was less than that of VGSCα, we suggest simultaneous expression of other VGSCα subtypes.

Since availability of new breast cancer cases and normal tissues was restricted, immunohistochemical analysis could be performed only in a limited number of frozen tissue samples. In addition to this, immediate freezing procedure performed prior to sectioning led to fracturing of the sections, causing background staining and difficulties in obtaining specific immunoreactivity.

We have also investigated the relation between the location of $nNa_v1.5$ protein and LNM status in breast cancer cases. Since the protein is functional on the membrane detection of its expression on the membrane with/without cytoplasm in 14 of the 19 LNM (+) cases may indicate a parallel link with $nNa_v1.5$ expression and the LNM positivity. Absence of LNM in $nNa_v1.5$ positive cases, where $nNa_v1.5$ can be on the membrane or in the cytoplasm, can be expected since the protein is a strong candidate early marker for breast cancer metastasis; however these patients should be carefully followed clinically for providing further evidence that Nav is associated with metastasis.

6.2. Analysis of $nNa_v1.5$ and ER Expression

A major problem in breast cancer treatment is the management of the hormone therapy. Tamoxifen is a widely used anti-estrogen drug in treating breast cancer patients who express ER but with less benefit in patients with metastatic disease than in patients

with primary tumors. However, a significant group of patients develop resistance to Tamoxifen where ER expression is either lost or protein becomes non-functional due to mutations (Ali and Coombes, 2000; 2002). Furthermore, estrogen is known to regulate VGSC expression in several tissues (Borg *et al*., 2002; Smitherman and Sontheimer, 2001) through its receptors. We, therefore, investigated whether expression of $nNa_v1.5$ is in parallel with ER status, a widely used prognostic marker in breast cancer. The results showed that $nNa_v1.5$ gene and protein expression was in parallel with ER expression only in 23/36 patients and 27/38 patients, respectively.

Simultaneous expression of a possible marker for aggressive phenotype ($nNa_v1.5$) together with ER that is usually associated with good prognosis in about 60-70 per cent of our patients may be surprising, but the role of ER in breast cancer development is not well established. Although estrogens are potent mitogens for normal breast cells and extended exposure is a risk factor for development of cancer, some studies showed that presence of ER may decrease metastatic potential (Platet *et al*., 2004, Marsden and Backs, 1996). These findings suggest a protective role for ERα/β in tumor progression. Parallel results were obtained in *in vitro* studies where proliferation and invasion of breast cancer cell were decreased upon transient transfection of ER (Platet *et al*., 2000; Lazennec *et al*., 2001). Other *in vitro* studies also showed that majority of ERα/β expressing cells do not have increased proliferation rate (Zeps *et al*., 1999; Saji *et al*., 2000). However, contrasting results were also found where ERα/β expression caused high grade tumor formation and metastasis (Speirs *et al*., 1999a and b). In the light of the above findings and the implication of estrogen in control of VGSC activity our data suggests poor prognosis for patients that simultaneously express ER and $nNa_v1.5$. On the other hand, we cannot exclude the possibility that ER can have a protective role for the patients that are positive for $nNa_v1.5$. In these patients different variants of ERα and ERβ can be expressed and their ratio in tissues may vary that may affect the overall clinical prognosis.

6.3. Analysis of $nNa_v1.5$ and HER2 Expression

HER2 protein is well known to be associated with aggressive phenotype in breast cancer and is used as a prognostic marker for breast cancer metastasis. Since we propose

that $nNa_v1.5$ expression could be a marker for poor prognosis, parallel expression of the two proteins was expected. However, 16 cases expressed both of the proteins but 18 cases expressed only $nNa_v1.5$ protein (n=37). Investigation of $nNa_v1.5$, HER2 and LNM in patients with available data, showed the absence of HER2 in six LNM (+) cases that were positive for $nNa_v1.5$ (n=37) (Table 5.9). Thus, we suggest that $nNa_v1.5$ could be expressed earlier than HER2 in the metastatic cascade. Disturbance of the homeostasis in the cells by $nNa_v1.5$ activity, increases the Na^+ concentration that may affect gene expression, primarily that of HER2.

In our *in vivo* study, the age of the patients analyzed ranged from 24-88. Out of 44 patients that were over 50 years of age, 28 expressed both $nNa_v1.5$ and ER but had no evident metastasis. This is consistent with previous findings that state the expression of ER in elderly women with breast cancer (Sweeney *et al*., 2004). Among the 22 patients that were above the age of 50, 12 had metastasis and expressed both $nNa_v1.5$ and ER. This finding may show that the disease progresses with age. However, seven patients (n=12) who developed metastasis and express $nNa_v1.5$ and HER2 are relatively young (30-50). This is consistent with the literature that states aggressive breast cancer in young women (Sweeney *et al*., 2004).

6.4. Possible Involvement of $nNa_v1.5$ in Angiogenesis

Angiogenesis is one of the critical and rate-limiting steps of metastasis that provides the tumor its essential needs to grow and disseminate. Previous studies have shown the expression of $Na_v1.5$ in human saphenous vein cells (HSVECs) (Gosling *et al*., 1998) and emerging knowledge is increasing in the field. Recent *in vitro* studies have shown the expression of functional VGSCα in human umbilical vein endothelial cells (HUVEC) cells that functions in angiogenesis (Djamgoz, unpublished data). We have also determined VGSCα expression in endothelial cells of both normal and cancerous breast tissues, consistent with these findings. Our data together with that of Fraser *et al*., (2005) indicate the possible contribution of $nNa_v1.5$ in various steps of metastasis including motility, invasion and secretion. Determination of $nNa_v1.5$ protein expression in endothelial cells of breast cancer but not of normal breast tissues may indicate possible involvement of the

protein in angiogenesis. The staining intensity of $nNa_v1.5$ was lower than that of VGSCα in identical cases proposing the expression of other VGSCα subtypes apart from $nNa_v1.5$. VGSCs are known to be regulated by growth factors e.g NGF and FGF that are known to be involved in angiogenesis (Toledo-Aral *et al*., 1995; Liu *et al*., 2003; Mosesson, 2005). The major angiogenesis growth factor vascular endothelial growth factor (VEGF) and/or NGF and FGF may regulate expression and function of VGSC in endothelial cells of cancer cells. However, further analysis should be performed to confirm this statement.

6.5. Expression of VGSCα and $nNa_v1.5$ Proteins in Normal Human Tissues

The expression of VGSCα protein in excitable tissues like muscle and brain is well known. Several studies have identified presence of VGSCα in glia (Chiu *et al*., 1984), osteoblasts (Black and Waxman, 1996), fibroblasts (Bakhramov *et* al., 1995), endothelial cells (Walsh *et al*., 1998; Gosling *et al*., 1998) spermatogonia, spermatocytes, granular cells, syncytiotrophoblasts, goblet cells (Ogata *et al*., 2000), jejunal circular smooth muscle (Ou *et al*., 2002) and lymphocytes (Fraser *et al*., 2004). However, most of these studies used electrophysiology or RNA based techniques and did not show the protein expression. In this study, we analyzed the expression of VGSCα protein in small intestine, colon, stomach, esophagus, prostate, breast and urinary bladder where muscle and brain were used as positive controls. Our results showed that VGSCα was present in all of the tissues above, except urinary bladder that posed background staining due to extensive fixation of the tissue. The cells expressing the protein mostly had secretory functions, confirming the results of Mycielska *et al*., (2003) that proposed VGSCα activity during secretion. Expression of VGSCα in endothelial cells of breast tissue is also in concordance with previous findings (Walsh *et al*., 1998; Gosling *et al*., 1998; Traub *et al*., 1999) where VGSCα was suggested to function in shear-stress mediated Erk1/2 inhibition in endothelial cells.

Analysis of $nNa_v1.5$ distribution in normal human tissues is of great importance since it would give us information about the specificity of its expression in metastatic breast cancer. In this respect, $nNa_v1.5$ protein expression was investigated in muscle, brain, small

intestine, colon, stomach, esophagus, prostate and urinary bladder. The results showed the absence of the protein in all of the tissues tested except normal breast where $nNa_v1.5$ protein was expressed 1000x less than that of metastatic breast.

6.6. Possible Involvement of VGSCα Protein Expression in Different Types of Cancers

There is increasing evidence for the involvement of ion channels in cancer development and progression. Amongst these, VGSCα was shown to contribute metastasis of prostate, breast cancer and SCLC (Diss *et al*., 2001; Abdul and Hoosein, 2002; Fraser *et al*., 2005; Roger *et al*., 2003; Onganer and Djamgoz, 2005). To test whether VGSCα expression was a widespread mechanism in metastasis, cancers of stomach, urinary bladder, kidney, and lung were investigated in a limited number of patients. One of the VGSCα (+) lung cancer cases had metastasis at the time of diagnosis. On the other hand, the pathology reports of cases with VGSCα (+) expression in stomach, urothelial and kidney cancer indicated the presence of histopathologically poorly differentiated tumors (Grade 3) that are likely to metastasize. In case of prostate cancer, one of the VGSCα (+) cases had wide perineural and capsule invasion with Glison score 7. These histopathological findings indicate the possibility of future metastasis in this patient. However, pathology reports were not available for any of the colon cancer patients that were immunoreactive to pan-VGSC antibody. Since VGSCα was also expressed in normal stomach and colon (but less than that in cancer), only if the VGSCα expression was at the tumoral area of the cancer tissues, the sample was accepted to be VGSCα (+). In addition to this, absence of VGSCα protein in kidney and minimal expression in lung of adult normal mice was shown in previous studies (Chioni *et al*., 2005). Our results may indicate upregulation of VGSCα in the cancer types studied. Analysis of the pathology reports of the cases studied showed absence of metastasis in VGSCα (-) cases as well as presence of high grade tumors in VGSCα (+) cases. This finding may indicate the possibility of VGSCα protein expression being in parallel with metastasis. However, a detailed imaging analysis should be performed and higher number of cases should be analyzed to confirm this suggestion.

6.7. Role of Estrogen on $nNa_v1.5$ Function During Metastasis of Breast Cancer Cells

Our findings indicate the upregulation of $nNa_v1.5$ in metastatic breast cancer. Since estrogen is well known to act on breast cancer development and regulate VGSC activity we analyzed the possible effect of estrogen on VGSC function in motility and proliferation that are basic steps of metastasis.

The motility was investigated using wound heal assay on three breast cancer cell lines: strongly metastatic MDA-MB-231 cells that express functional VGSC but not ER, MDA-MB-231-ERα and weakly metastatic MCF-7 cells that do not express functional VGSC but ER. The results showed that estrogen increased the motility of MDA-MB-231 cells slightly ($p<0,05$). However, simultaneous treatment of the cells with estrogen and TTX reversed the effect of estrogen and decreased the motility significantly ($p<0,001$). Depending on these findings we suggest that estrogen may increase the motility of these cells that lack ER through $nNa_v1.5$. On the other hand, presence of recently identified membrane bound receptors that bind estrogen (e.g. GPCR) can explain its effect in an ER (-) cell line (Filardo, 2002; Revankar *et al*., 2005; Thomas *et al*., 2005). GPR30, a GPCR family member, has been shown to bind estrogen in MDA-MB-231 cells and its effect was abolished with the use of GPR30 siRNA and a GPCR blocker, pertussis toxin (Thomas *et al*., 2005; Le Mallay *et al*., 1997; Filardo, 2002). In another study use of an EGFR blocker was shown to abolish estrogen induced motility and morphology of MDA-MB-231 cells. Thus, it has been suggested that binding of estrogen to GPCR causes release of EGF that binds and activates EGFR (Azios and and Dharmawardhane, 2005). In the light of these findings, we suggest that estrogen may exert its effect on motility of breast cancer cells through both genomic and non-genomic pathway via GPCR and EGFR (Figure 6.1). Since TTX reversed its effect, we suggest that estrogen could be upstream of VGSCα in the signal transduction pathway. These results can be confirmed with further analysis involving the use of inhibitors against GPCR, EGFR, and VGSCα.

The VGSCα blocker TTX, alone decreased the motility of MDA-MB-231 cells ($p<0,001$) as expected and consistent with previous findings (Fraser *et al*., 2005).

Availability of ER (-) MDA-MB-231 cells transfected with ERα has enabled us to see the effect of ERα on motility and its relation with VGSCα. When motility index was compared for non-treated controls of MDA-MB-231 transfected/non-transfected with ERα a ligand independent decrease was observed. However, the significance of motility index change decreased in time (T24: $p<0,01$; T48: $p<0,05$; T72: $p<0,5$) (Figure 5.26A). Treating the transfected cells with estrogen initially (T24) decreased the motility significantly ($p<0,005$). We propose that the short term effect of estrogen could be due to the protective role of ERα in these cells that is also observed in clinics. Long term estrogen incubation, however, abolished this effect and motility was increased at T48 and T72 ($p<0,005$) as in the case of MDA-MB-231 cells. This long term effect of estrogen could be in parallel with our *in vivo* data that indicated poor prognosis in the patients that express both $nNa_v1.5$ and ER and may be consistent with clinical findings where ER (+) patients who receive long term anti-estrogen therapy become unresponsive after some time. The insensitivity to hormones was suggested to be due to the loss of ER expression that could be caused by methylation of the ER promoter, mutations in the ER gene or loss of transcriptional activators (Hayashi *et al*., 2003).

Treating the MDA-MB-231-ERα cells with TTX alone decreased the motility at all times, as expected. Simultaneous treatment of estrogen and TTX also decreased the motility significantly ($p<0,005$). This result indicates the possibility that estrogen may act through $nNa_v1.5$ in increasing motility of the cells.

MCF-7 cells that express both ERα and ERβ are weakly metastatic. Treatment of these cells with estrogen has decreased the motility slightly at all times. We propose that estrogen may act through nuclear ERs, via genomic pathway and may have a protective effect in a weakly metastatic cell. These results are in consistence with previous studies that has showed decrease in proliferation and motility after treatment with estrogen in cells that express ER (Platet *et al*., 2004; Lazennec *et al*., 2001). The negative effect of estrogen on motility of these cells suggests the presence of a different gene expression profile that confers resistance to $nNa_v1.5$ upregulation. The basal level of VGSCα was blocked with TTX alone and in combination with estrogen, thus decreasing the motility significantly.

The results of wound heal motility assay was based upon visual measurements using inverted microscope. Therefore errors caused by the experimenter and culture conditions may affect the results. Use of Transwell chambers to determine the motility capacity of cells could give more accurate results by avoiding visual measurements.

BrdU incorporation assay results showed that estrogen had no effect on proliferation rate of none of the breast cancer cells used in this study, either alone or via acting through $nNa_v1.5$. *In vitro* studies supporting our findings show that in majority of ERα/β expressing cells proliferation rate was not increased (Zeps *et al*., 1999; Saji *et al*., 2000). Since the rate proliferation was stable in our cells, the motility changes that were observed in wound heal motility assay was probably not due to increase in the number of cells.

6.8. Role of Estrogen on $nNa_v1.5$ Expression and Localization in Breast Cancer Cells

Since estrogen may modulate $nNa_v1.5$ function during motility of breast cancer cells, we tested whether estrogen achieves this through regulating expression and/or localization of the protein. Using immunocytochemistry and Matlab 6.5 software, we analyzed the changes in the $nNa_v1.5$ expression level and localization upon treatment with estrogen and/or TTX. The results showed that neither estrogen nor TTX affected the quantity of $nNa_v1.5$ protein significantly in the cell lines tested. However, there was a significant difference between $nNa_v1.5$ expression level in MDA-MB-231 and MCF-7 cells. This result was expected since MCF-7 cells are weakly metastatic and express $nNa_v1.5$ only minimally.

Although the total protein level was not modified, localization of $nNa_v1.5$ was determined to vary upon estrogen treatment in MDA-MB-231 and MDA-MB-231-ERα but not in MCF-7 cells. Incubation of highly metastatic cells with estrogen increased the availability of $nNa_v1.5$ on the plasma membrane, that may indicate the transport of the protein from the cytoplasm to its functional location ($p<0,05$). TTX alone caused clustering of the protein mostly in cytoplasm significantly ($p<0,05$). This result is consistent with previous findings in prostate cancer where TTX was shown not to affect

the overall VGSCα protein expression but cause translocation to the cytoplasm (Brackenbury and Djamgoz, 2006). Our results may indicate that application of both estrogen and TTX at the same time localized the protein mostly in the cytoplasm ($p<0,05$). These results indicate that estrogen may modulate the level of functionally available form of $nNa_v1.5$ protein in breast cancer cells. However, more sensitive methods like confocal microscopy should be used to confirm these results.

6.9. The Mechanism of Estrogen Action on $nNa_v1.5$ Protein

The mechanism of estrogen modulating $nNa_v1.5$ protein in metastatic breast cancer may be both through genomic or non-genomic pathway that is summarized in Figure 6.1. The genomic pathway can be both GPCR and ER-mediated. Upon estrogen binding, GPCR activates PKA that can phosphorylate pCREB. pCREB binds CRE altering transcription of genes that may affect functional availability of $nNa_v1.5$. GPCR activation via estrogen activates PI3K by stimulating the binding of its regulatory subunit (p85α). This in turn stimulates Akt that increases VGSC activity and mRNA (Djamgoz, 2004). In case of ER mediated pathway estrogen directly diffuses into the cell and binds its nuclear receptor causing transcriptional regulation of ERE harboring genes.

In the non-genomic pathway, binding of estrogen to its membrane receptor (GPCR) can activate PLC either by estrogen bound to GPCR or EGFR. This causes Adenylyl cyclase (AC) activation through PKC. AC then phosphorylates PKA. Phosphorylation of VGSC via PKA is suggested to affect the localization of the channel on the plasma membrane and increase its activity in prostate cancer cells (Brackenbury and Djamgoz, 2006). In return VGSC activates PKA, forming a positive feedback mechanism. In an alternative non-genomic pathway, Grb2 mediated binding of PKA to EGFR causes activation of VGSC.

In vitro analysis of breast cancer cells may indicate the role of estrogen in modulating $nNa_v1.5$ function and its availability. In highly metastatic cancer cells estrogen increased the motility of the cells slightly probably by increasing their functional form. This finding may be in parallel with our *in vivo* study where some patients simultaneously expressed

nNa$_v$1.5 and ER. We propose that estrogen may achieve this role via PKA or PI3K through genomic and non-genomic pathways. Use of PKA and PI3K inhibitors can test this hypothesis. Also the presence of non-genomic pathway in MDA-MB-231 cells can be tested by using blockers of GPCR and EGFR.

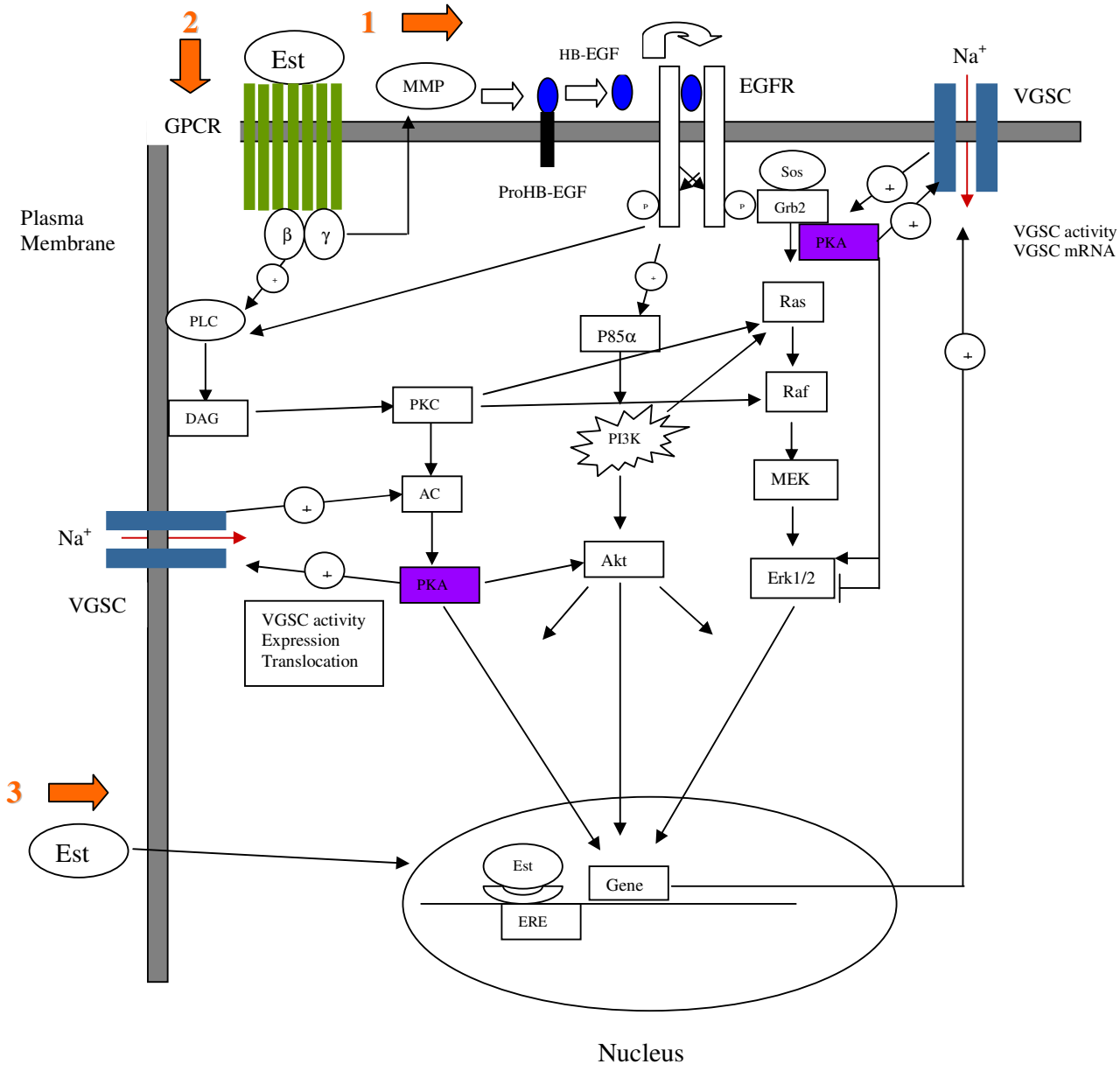


Figure 6.1. Signal transduction pathway proposed to involve estrogen regulating VGSC

CONCLUSION

Our results have provided more support for a role of $nNa_v1.5$ in breast cancer metastasis. A high number of cases that present $nNa_v1.5$(+)/LNM(-) should be followed clinically with caution to further support our data. However $nNa_v1.5$ is a strong candidate as an early prognostic marker for breast cancer. Drug development is promising since the amino acids that differ in adult and neonatal forms of the protein are located on the extracellular loop. Use of drugs specific to $nNa_v1.5$ is suggested to cause minimum side effects because expression of this protein is specific to metastatic breast cancer and is expressed minimally only in normal breast. We suggest that $nNa_v1.5$ may be a direct, reliable and early marker saving patients from metastasis thus increasing their quality of life. What is more, use of a specific drug could decrease the need for expensive chemotherapy. However, more research needs to be done to verify use of it as a prognostic marker, its role in breast cancer metastasis and enlighten its mechanism of action in the cells.

APPENDIX A: EXAMPLES OF PROTEIN QUANTIFICATION IN BREAST CANCER CELLS USING MATLAB 6.5 SOFTWARE

A.1. $nNa_v1.5$ Quantification in MDA-MB-231 Cells

MDA-MB-231-FILE NAME	No	Total	Total-Page	1	2	3	4	5	6	7	8
	Cells	**Cell-pix**	**Pix**	**Pix**	**Pix**	**Pix**	**Pix**	**Pix**	**Pix**	**Pix**	**Pix**
rezicc2.407.a.JPG	4	1824	273504	357	263	452	752				
rezicc2.409.a.JPG	2	3517	275872	2216	1301						
rezicc2.408.a.JPG	7	3210	275872	1020	376	262	278	368	801	105	
rezicc2.411.a.JPG	3	1484	279774	508	524	452					
rezicc2.412.a.JPG	8	2019	277648	538	97	599	111	112	281	147	134
rezicc2.413.a.JPG	1	1663	274008	1663							
rezicc2.414.a.JPG	3	1165	275872	172	48	945					
rezicc2.415.a.JPG	2	734	275872	404	330						
rezicc2.416.a.JPG	2	1464	275872	462	1002						
rezicc2.417.a.JPG	3	1651	275872	484	836	331					
Total no of cells	**35**		**Total no of Pixels**		**18731**			**Average**		**535.2**	

A.2. $nNa_v1.5$ Quantification in MCF-7 Cells

MCF-7-FILE NAME	**No**	**Total**	**Total-Page**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
	Cells	**Cell-pix**	**Pixel**	**Pix**	**Pix**	**Pix**	**Pix**	**Pix**	**Pix**	**Pix**	**Pix**	**Pix**	**Pix**	**Pix**
rezicc2.592.a.JPG	7	682	274688	104	98	83	89	96	117	95				
rezicc2.593.a.JPG	11	737	266304	30	86	35	184	94	114	79	16	34	46	19
rezicc2.594.a.JPG	6	563	268584	142	90	40	66	76	149					
rezicc2.595.a.JPG	3	1310	270351	488	367	455								
rezicc2.596.a.JPG	7	965	271728	52	173	43	40	489	101	67				
Total no of cells	**34**		**Total no of Pixels**		**4257**			**Average**		**125.2**				

A.3. $nNa_v1.5$ Quantification in MDA-MB-231-ERα Cells

MDA-MB-ERa-FILE NAME	No	Total	Total-Page	1	2	3	4	5	6	7	8
	Cells	Cell-pix	Pixel	Pix	Pix	Pix	Pix	Pix	Pix	Pix	Pix
rezicc2.482.a.JPG	1	668	197944	668							
rezicc2.483.a.JPG	1	1206	271656	1206							
rezicc2.484.a.JPG	1	886	273504	886							
rezicc2.485.a.JPG	1	770	275872	770							
rezicc2.486.a.JPG	1	329	273504	329							
rezicc2.487.a.JPG	2	482	271656	339	152						
rezicc2.488.a.JPG	1	1636	271656	1636							
rezicc2.489.a.JPG	1	913	273633	913							
rezicc2.491.a.JPG	8	5214	273042	390	1759	512	663	328	872	297	383
rezicc2.492.a.JPG	2	2201	275872	626	1575						
Total no of cells	**19**		**Total no of Pixels**		**14304**			**Avarage**		**752.8**	

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