CHAPTER 1

Introduction

Breast Cancer

Cancer is the leading cause of death in humans and every year more than 1 million people die in the world due to cancer. Cancer is the complex tumour tissues characterized with uncontrolled cell growth. To spread neoplastic agenda, the mutant cancer cells in tumours have acquired and developed special properties, which are different from normal cell types. Malignant cells are self-sufficient in growth signals with limitless replication potential that develop into tumours. During the course of progression, the tumours acquire sustained angiogenesis with tissue invasion and metastasis, evading apoptosis. The tumours are insensitive to antigrowth signals and control mechanisms [1]. Important types of cancer in humans, their Percent of occurrence and mortality rates are listed in table 1.1. Although percent of occurrence of breast cancer is 15 % more than lung cancer, the second leading cause of cancer death is mammary carcinoma in women and adenocarcinoma in men.

Breast cancer is originating from breast tissue, most commonly from the inner lining of epithelial cells of milk ducts (ductal carcinomas) or the lobules (lobular carcinomas) [2]. Breast cancer occurs in mammals including humans. The frequency of breast cancer disease occurrence is more common among women than men.

The benefit versus harms of breast cancer screening is controversial. The characteristics of the cancer determine the treatment, which may include surgery, medications (hormonal therapy and chemotherapy), radiation and/or immunotherapy [3]. Surgery provides the single largest benefit, and to increase the likelihood of cure, several chemotherapy regimens are

commonly given in addition. Radiation is used after breast-conserving surgery and substantially improves local relapse rates and in many circumstances also overall survival.

Table 1.1Percent of occurrence and mortality rates of different types of human cancer

Males	% Occurrence	% Mortality	Females	% Occurrence	% Mortality
Prostate	33	10	**Breast**	32	15
Lung	13	31	**Lung**	12	27
Colorectal	10	10	**Colorectal**	11	10
Pancreatic	-	5	**Ovarian**	-	6
Leukemia	-	4	**Pancreatic**	-	6

The symptom of breast cancer is development of a lump that gives a feeling different from the rest of the breast tissue or can be detected early by a mammogram or lumps found in lymph nodes located in the arm pits. Other symptoms include breast inflammation sometimes characterized with itching, pain, swelling, redness and nipple inversions, including Paget's disease with eczema. About 20 % of lumps turn out to be non-cancerous and benign; however appearance of lump must be taken seriously by the patients.

The primary risk factors for breast cancer are female sex, older age [3], lack of childbearing or lack of breastfeeding [4], higher levels of certain hormones i.e. hormonal therapy [5], high fat diet [6], alcohol intake and obesity [7]. Certain life style also contributes to increase in the risk of cancers such as smoking [8], a lack of physical activity [9] and exposure to radiation [10] and environmental pollutants [11] including pesticides [12].

In less than 10 % of cases, the genetics is a primary cause for the risk of developing breast cancer that is the mutations in BRCA1 and BRCA2 genes [13]. Both these genes are tumour

suppressor genes play a role in transcriptional regulation. Women who inherit loss-of-function mutations in either of these genes have risk of developing cancer by the age of 70 [14]. The carriers of these mutations are also have elevated risk of developing cancer of the ovary and pancreas [15] Surprisingly, somatic disease causing mutations of BRCA1 or BRCA2 proteins are extremely rare in sporadic breast cancers [14].

Diagnosis of the breast cancer is normally done by simple screening method that is the physical examination of the breasts and mammography for a lump or lesions. If the physical examination is inconclusive, on the doctor's advice, the patient has to undergo microscopic examination of a sample or biopsy of the affected area by fine needle aspiration and cytology (FNAC). Under special circumstances additional tests such as imaging by ultrasound or MRI and other tests may be required to confirm the breast cancer with accuracy [16].

Classification and Prognostic factors

Pathologists/oncologists, classify the breast cancer by several grading systems that help in the prognosis of the disease and subsequent treatment. By histological appearance, the cancers are classified as ductal or lobular carcinoma. Carcinoma *in situ* confined to a particular tissue compartment such as mammary duct with precancerous cells while, *invasive carcinoma* does not confine itself to the initial tissue compartment characterized with the invasion of the surrounding tissue [17].

The breast cancer grade is assessed by comparison of the breast cancer cells to normal breast cells. The closer to normal the cancer cells are, the slower their growth and the better the prognosis. If cells are not well differentiated, they will appear immature, will divide more rapidly, and will tend to spread. Histological grading compares the microscopic appearance of the breast cancer cells to the cells of the normal breast tissue. Normal cells in an organ are highly differentiated, that they take up specific shape and would normally line up in an

orderly fashion. In contrast, the cancer cells lose the shape, nuclei becomes less uniform, appears highly disorganized with uncontrolled cell division. Pathologists classify the cells as low grade 1 (well differentiated), intermediate grade 2 (moderately differentiated) and high grade 3 (poorly differentiated) as the cells progressively lose the features seen in normal breast cells.

The stage of the breast cancer is the most important component of traditional classification methods and has greater effect on prognosis of the disease. Breast cancer staging (TNM system) is based on the size of the tumour (T), whether or not the tumour has spread to the lymph nodes (N) in the armpits, and whether the tumour has metastasized (M) that is tumour has spread to a more distant part of the body. The main stages are Stage 0 (pre-cancerous condition) ductal carcinoma in situ or lobular carcinoma *in situ*, stages 1–3 cancer condition within the breast or regional lymph nodes and stage 4 (metastatic advanced cancer) [18]. The higher the stage at diagnosis, poorer the prognosis. The stage is raised by the invasiveness of disease to lymph nodes, chest wall, skin or beyond, and the aggressiveness of the cancer cells. The stage is lowered by the presence of cancer-free zones and close to normal cell behaviors (grading). Size is not a factor in staging unless the cancer is invasive. For example, ductal carcinoma *in situ* (DCIS) involving the entire breast will still be stage zero and consequently an excellent prognosis with a 10 yr disease free survival of about 98 % [19].

Breast cancer is also classified based on the presence of estrogen receptor (ER), progesterone receptor (PR), and HER2 receptors as positive or negative. ER+ cancer cells depend on estrogen for their growth, so they can be treated with the tamoxifen, the anti-estrogen drugs that block estrogen effects and generally have a better prognosis. HER2+ breast cancer had a worse prognosis [20]. Patients whose cancer cells are positive for HER2 have more aggressive disease and may be treated with the 'targeted therapy'. $HER2^{+}$ cancer respond to drugs such as the monoclonal antibody Trastuzumab in combination with conventional

chemotherapy and this has improved the prognosis significantly [21]. Breast cancer cells with none of these receptors are called as triple-negative and have worse prognosis.

Prognostic factors are reflected in the classification scheme for breast cancer including stage, (i.e., tumour size, location, whether disease has spread to lymph nodes and other parts of the body), grade, recurrence of the disease, and the age and health of the patient. The Nottingham Prognostic Index is a commonly used prognostic tool.

Younger women tend to have a poorer prognosis than post menopausal women due to several factors. Their breasts are active with their cycles, they may be nursing infants, and may be unaware of changes in their breasts. Therefore, younger women are usually at a more advanced stage when diagnosed. There may also be biological factors contributing to a higher risk of disease recurrence for younger women with breast cancer [22].

Disease management and treatment

Women can reduce their risk of breast cancer by maintaining a healthy weight, drinking less alcohol, being physically active with moderate exercise and breastfeeding their children [23]. Consumption of omega-3 polyunsaturated fatty acids also appears to reduce the risk of developing breast cancer [24]. Removal of both breasts with suspicious lump or lesion (prophylactic bilateral mastectomy) sometimes considered in the woman with BRCA1 and BRCA2 gene mutations [25].

Anti estrogen tamoxifen treatment reduces the risk of breast cancer but increase the risk of thromboembolism and endometrial cancer [26]. The management of breast cancer depends on various factors, including the stage of the cancer. Probably, the aggressive treatments are required with the poorer prognosis, but higher risk of recurrence of the cancer. Breast cancer is usually treated with surgery, which may be followed by chemotherapy or radiation therapy, or both. A multidisciplinary approach is preferable [27]. Hormone receptor ER+ cancers are

treated with hormone-blocking therapy by anti-estrogen tamoxifen (Nolvadex), or alternatively block the production of estrogen with an aromatase inhibitor (anastrozole or letrozole) [28] over courses of several years. Monoclonal antibodies, or other immune-modulating treatments, may be administered in certain cases of metastatic and other advanced stages of breast cancer.

Chemotherapy is predominately used for stage 2–4 disease, being particularly beneficial in estrogen receptor-negative (ER–) disease. They are given in combinations, usually for 3–6 months. One of the most common treatments is cyclophosphamide plus doxorubicin or advanced ones are cyclophosphamide with docetaxel or methotrexate, or fluorouracil along with Trastuzumab (Herceptin), a monoclonal antibody to HER2 for non-hormone treatments for triple negative breast cancer [29, 30]. Radiotherapy is normally given after surgery to the region of the tumour bed and regional lymph nodes to destroy microscopic tumour cells that escaped during surgery. It may also have a beneficial effect on tumour microenvironment. Radiation therapy can be delivered as external beam radiotherapy or as brachytherapy (internal radiotherapy) [31].

Breast cancer cell lines (BCC)

An understanding and current knowledge on breast cancer, drug delivery and treatment is based on *in vivo* and *in vitro* studies carried out with many cancer cell lines particularly MCF-7 cell lines. The cancer cell lines can be easily cultured under laboratory conditions, provide an unlimited source of homogenous free of contaminating other cells particularly stromal cells. The first of its kind, BT-20, primary cell culture was established in 1958 and despite of continuous work in this area, the number of permanent cell lines developed was very less. Further, attempts to develop primary cell culture from breast tumours were unsuccessful and suffer with many draw backs. The poor efficiency of cell culturing was often due to technical difficulties associated with the extraction of viable breast tumour cells

from their surrounding stroma and other tissues. Most of the available breast cancer cell lines are isolated in 1970 mainly from pleural effusions of metastatic tumours. Pleural effusions provide large number of dissociated viable tumour cells with no contamination by fibroblasts and stromal cells. Some important breast cancer cell lines with the important properties are listed in table 1.2 [32-40].

Table 1.2 Breast cancer cell lines with some important properties

Sl. No.	Cell line	Primary tumour	Origin	ER	PR	ERBB2 amplification	Mutated TP53	Tumourigenic in mice	Ref
1	600MPE	IDC		+	-				[39]
2	AU565	AC				+			[39]
3	BT20	IDC	primary				+	+	[40]
4	BT474	IDC	primary	+	+	+	+	+	[41]
5	BT483	IDC		+	+				[39]
6	BT549						+		[39]
7	Evsa-T	IDC	MS		+		+		[42]
8	Hs578T	CS	Primary				+		[43]
9	MCF-7	IDC	MS(PE)	+	+			With E2	[33]
10	MDA-MB-231	IDC	MS (PE)				+	+	[44]
11	SkBr3	IDC	MS (PE)			+	+		[45]
12	T-47D	IDC	MS (PE)	+	+		+	+ With E2	[46]

IDC: Invasive ductal carcinoma, **AC**: Adenocarcinoma, **MS**: Metastasis **PE**: pleural effusion **CS**: Carcino sarcoma.

MCF-7 Cells

MCF-7 is a breast cancer cell line isolated from a 69 year old Caucasian (white American) woman, Frances Mallon and she was a nun in the convent of Immaculate Heart of Mary in Monroe, Michigan with the name of Sister Catherine Frances. MCF-7 is named after Michigan Cancer Foundation-7 **(**now known as the Barbara Ann Karmanos Cancer Institute), in Detroit where the cell line was established in 1973 by Herbert Soule and co-workers [37]. MCF-7 cells were the source of much of current knowledge about breast cancer [41], along with two other breast cancer cell lines, T-47D and MDA-MB-231, account for more than two-thirds of all abstracts reporting as per the Medline survey [42]. Earlier to MCF-7, the cell line obtained from breast cancer was capable of surviving only for few months. MCF-7 cells are invasive breast ductal carcinoma, with the luminal epithelial phenotype. Origin of cells was from pleural effusion with estrogen, progesterone receptor positive and responsive to estrogen for proliferation. The cells were capable of developing tumours with estrogen primed mice cells [43-45].

Lung cancer

Lung cancer is the development of tumours due to uncontrolled cell growth in lungs. When left untreated, the growth spreads beyond the lung with metastasis into nearby tissue and also to other parts of the body. Most of the cancers in lung are small-cell lung carcinoma (SCLC), non small cell lung carcinoma (NSCLC) and carcinomas derived from epithelial cells. The common symptoms include coughing with blood, weight loss and shortness of breath, fever, fatigue, chest pain, bone pain, superior venacava obstruction, difficulty in swallowing [46]. The most common cause of lung cancer is a long-term smoking (80–90 %) [47], while non-smokers account for only 10–15 % of lung cancer that includes genetic factors, asbestos, air pollution and second hand smoke. Lung cancer is usually diagnosed by X-ray, computed

tomography (CT scan) confirming with a biopsy of samples [48]. Treatment and long-term outcomes depend on the type of cancer, the stage (degree of spread), and the person's overall health, measured by performance status. Lung cancer is initiated by activation of oncogenes or inactivation of tumour suppressor genes or due to exposure to carcinogens, mutations in the *K-ras* proto-oncogene or EGFR or chromosomal damage or mutations of *p53* tumour suppressor gene, and other genes often mutated or amplified are c-MET, NKX2-1, LKB1, PIK3CA, and BRAF [49-52].

According to histological type the lung cancers are classified as carcinomas (malignancies that arise from epithelial cells) and the two broad classes are non small cell carcinoma (NSCLC) and small cell lung carcinoma (SCLC). The three main subtypes of NSCLC are (a) adenocarcinoma (40 % originates in peripheral lung tissue associated with smoking), (b) squamous-cell lung carcinoma (30 % occur close to large airways) and (c) large cell lung carcinoma (9 % cells are large, with excess cytoplasm, large nuclei and conspicuous nucleoli [47]. In SCLC (strongly associated with smoking), the cells contain dense neurosecretory granules (vesicles containing neuroendocrine hormones) [53], occurs in larger airways (primary and secondary bronchi) [46], cancers grows and spread quickly in early course of the disease. Lung cancer staging helps in the assessment of degree of spreading of cancer for prognosis and potential treatment of the disease [47].

The NSCLC staging uses the TNM classification. This is based on the size of the primary tumour, lymph node involvement, and distant metastasis, through stages 0, IA (one-A), IB (one-B), IIA (two-A), IIB (two-B), IIIA (three- A), IIIB (three-B) and IV (four), which assists with the choice of treatment and estimation of prognosis [54]. In SCLC the stage is either 'limited stage' which is confined to one half of the chest and within the scope of a single tolerable radiotherapy or as 'extensive stage' as a more widespread disease [47]. Based on the imaging by CT scans or PET scan and also biopsy the two general staging is practiced for

both NSCLC and SCLC and they are clinical and surgical staging and clinical staging is performed prior to definitive surgery [55, 56].

Although, many countries banned the industrial and domestic carcinogens, tobacco smoking is still widespread. Ban on tobacco smoking prevents the lung cancer to larger extent. Educating the children and avoid both smoking and passive smoking could be the cost effective means of preventing the disease [57]. Treatment for lung cancer includes palliative care, surgery, chemotherapy, and radiation therapy. Early stage of detection and removal of a tumour by surgery followed by radio therapy reduce the risk of recurrence. However, the survival rates are very less with the advanced stage of lung cancer disease [58].

A549 cell line was first developed by D. J. Giard, et al. in 1972, through the culturing of cancerous lung tissue in the explanted tumour of 58-year-old Caucasian male. They are adenocarcinomic human alveolar basal epithelial cells. These cells are squamous and responsible for the diffusion of water and electrolytes, across the alveoli of lungs. Under *in vitro* conditions the cells grow as monolayer attaching to the culture flasks [59]. They are able to synthesize lecithin and contain high level of desaturated fatty acids, which are important to maintain the membrane phospholipids in cells. A549 cell line are widely used as an in vitro model for a type II pulmonary epithelial cell for drug metabolism and as a transfection host cells. Some of the important liver cell lines used in research are listed in table 1.3.

Table 1.3 Important human lung cancer cell lines

Name	Species	Source	Disease
HCC2935	Human	Lung	Adenocarcinoma
NCI-H23	Human	Lung	Adenocarcinoma, non-small cell
NCI-H835	Human	Lung	Carcinoid
UMC-11	Human	Lung	Carcinoid
NCI-H720	Human	Lung	Carcinoid, atypical
A549	Human	Lung	Carcinoma
A-427	Human	Lung	Carcinoma
NCI-H596	Human	Lung	Carcinoma, adenosquamous
SW 1573	Human	Lung	Carcinoma, alveolar cell
NCI-H1688	Human	Lung	Carcinoma, classic small cell lung cancer

Estrogen Receptors

Estrogen plays a key role in the development and maintenance of the normal sexual and reproductive function in humans. The potent estrogen synthesized in the body is Estradiol-17β (E_2). E_2 is a well-known morphogen plays a critical role in the growth and development of uterus [60], mammary gland [61], ovary [60], prostate [62], brain [63] and lungs [64] Estrogen receptors (ERs) are well known transcription factors and involved in many signal transduction pathways [65].

The estrogen receptors (ERs) are transcription factors belong to superfamily of nuclear receptors (NR). ERs are distinguished from other transcription factors by their ability to control expression of genes by ligands, steroids, retinoid, thyroid hormone, vitamin D, fatty acids and other small hydrophobic molecules binding to their ligand sites. NRs bring about effects or influence the wide range biological processes involved in development, homeostasis, proliferation and differentiation.

Estrogens are steroid hormones induce cellular changes through several mechanisms (Fig. 1.1). Important mechanism of estrogen action is binding of estrogen to its receptor (ER) a transcription factor and transactivation of gene expression. In the first classical mechanism, estrogen diffuses into cell, binds to its receptors ER and later these E2-ER complex moves to nucleus, binds specifically to estrogen response element (ERE) sequences of promoter and directly activates gene expression. In the second E2-ER complex may act indirectly through protein-protein interactions with activator protein-1 (AP-1) or specificity protein-1 (SP-1) binding the promoter region of estrogen responsive genes, in the first one ER–ERE complex directs the recruitment of co-regulatory proteins (co-activators or co-repressors) to the promoter, activates increased or decreased synthesis of mRNA transcripts and associated protein production, with a physiological response. This classical, or “genomic,” mechanism

of action typically requires several hours. However, in contrast, estrogen acts more quickly within seconds or minutes via "non-genomic" mechanism of action. This is either, through the ER located in or adjacent to the plasma membrane, or through the other non-ER plasma membrane associated estrogen binding proteins (Fig. 1.1), resulting in cellular responses. Important non-genomic mechanism of action includes increased intracellular levels of Ca^{2+} or synthesis of NO, or activation of kinases involved in cell signaling. The binding and activation of ERs to ERE of the genes depends on dimer formation of ERs. There are 2 types of ERs, the ERα (NR3A1) and ERβ (NR3A2). ERα gene is found on the chromosome 6 band located at 6q25.1. The gene contains eight exons and code for 595 amino acids [66].

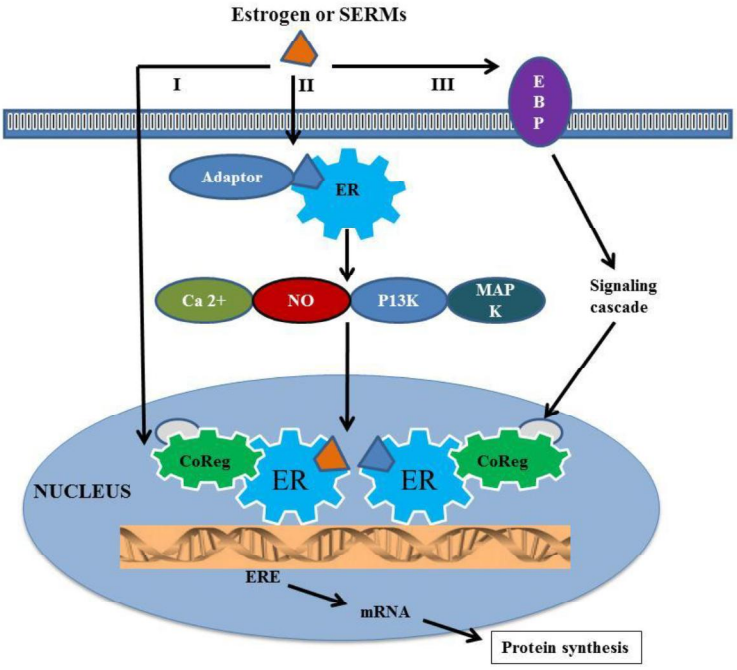

Fig. 1.1A model of estrogen action

In the "classical" pathway of estrogen action (i), estrogen or other selective estrogen receptor modulators (SERMs) bind to the estrogen receptor (ER), a ligand-activated transcription factor that regulates transcription of target genes in the nucleus by binding to estrogen response element (ERE) regulatory sequences in target genes and recruiting co-regulatory proteins (CoRegs) such as co-activators. Rapid or "non-genomic" effects of estrogen may also occur through the ER located in or adjacent to the plasma membrane (ii), which may require the presence of "adaptor" proteins, which target the ER to the membrane. Activation of the membrane ER leads to a rapid change in cellular signaling molecules and

stimulation of kinase activity, which in turn may affect transcription. Lastly, other non-ER membrane-associated estrogen-binding proteins (EBPs) may also trigger an intracellular response (iii).

ERβ gene is present on the chromosome 14 and located at 14q22-24 [67]. The protein ERβ is slightly smaller than ERα containing of 530 amino acid residues [68]. Both ERα and β contains 6 domains (A-F). A/B domain of ER protein contains a constitutive estrogen independent transcriptional activation function, AF1 (Fig. 1.2) [69] and phosphorylation of Ser^{118} is required for AF1 activity. Hinge region (D) is involved in estrogen mediated transcriptional repression [70], E is a ligand binding domain, required for stable dimerization of receptor and a second estrogen inducible transcriptional activation function-AF2, AF1 and AF2 can act independently and synergistically to enhance transcription [69]. Between ERα and ERβ the highest homology exist in the DNA biding domain (95 %), moderately conserved in hormone-binding domain (58 % identity). While, A/B domains are poorly conserved (20 %) [71].

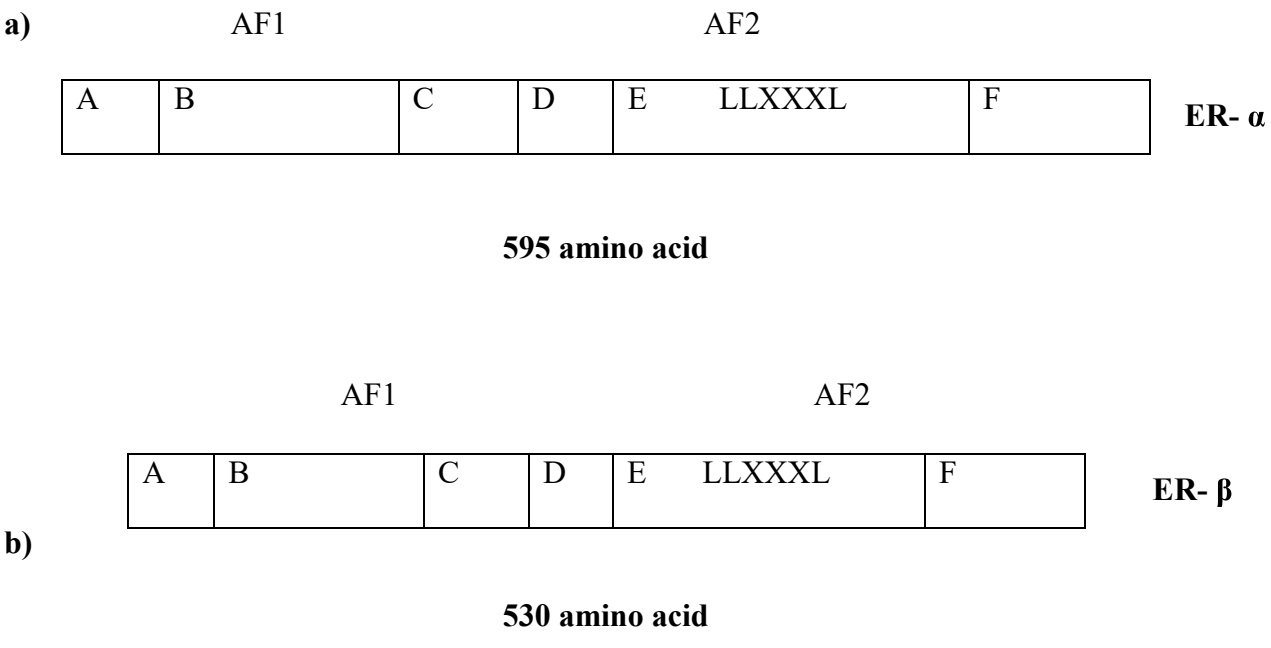

Fig.1.2 Structure and functional domains of ERα and ERβ

Domains A and B: Transcription activation; C: DNA binding and receptor dimerization; D: Nuclear localization signal; E: ligand binding domain, co activator binding, transcription

Activation, receptor dimerization; F: Probably contributes to transactivation but function is unknown (Adapted from [71]).

The overall sequence similarity of ERα and ERβ is 47 %, there is little or no homology between their AF-1 domains. ERs being transcription factor large number of reports highlighted that ERs are involved in the mammary gland development and function. In addition ERs also control the development of bone, reproductive cells, tissues and brain. ERs in particular ERα is involved in metastatic cascade suggested the possibilities for novel therapeutic targeting of specific ERα signaling components that mediate migration, invasion, and epithelial to mesenchyme transition. It is proven that ERs, which have distinct tissue expression pattern [72] in both humans and rodents, and gene-targeted animal models lacking these receptors exhibit distinct phenotypes and provide some of the most definitive experimental models for evaluating the role of the ER in disease and normal physiology [60]. ERα and ERβ as encoded by 2 separate genes (ESR1 and ESR2) and numerous mRNA splice variants exist for both the receptors in normal and diseased conditions.

Approximately 60−70 % of all the breast cancers are ER positive and hence anti-estrogens like TMX can be used in the treatment [73]. Many patients, who initially respond to hormonal therapy, later develop resistance. ER plays a pivotal role in the development and progression of breast cancer and also in the treatment. Mechanism of action involved in the activation and regulation of the ERs is important in estrogen therapy and role of ERs signal transduction are the key area of research to develop new therapeutic modality for treatment.
A large portion of metastases retain their ER*α* when the primary tumours are ERα positive. A recent study using breast cancer cells provide evidence that ERβ expression was associated with less cell migration. Mechanistic studies indicated that ERβ affects integrin expression and clustering and consequently modulates adhesion and migration of breast cancer cells [74].

Estrogen and AP-1 factors

ERs are characterized with closely related structure and functions contain evolutionarily conserved functionally distinct domains, helps in binding DNA with activation and expression of spectrum of genes. ERs regulate transcription of genes through direct and indirect interactions with DNA. In direct mode of estrogen action E_2–ER complex bind to estrogen responsive elements (EREs) in *cis*-regulatory genome sequences and activate the transcription of genes [75]. However, in the indirect mode of action liganded ERs are tethered to DNA by interacting with other transcription factors like, SP-1 or AP-1 or any other transcription factors or activators [76, 77]. The molecular details of the later interactions and how these relate to transcriptional activation of different genes in breast cancer development remains unclear. Because of limited studies only few genes such as collagenase [78], human insulin-like growth factor I [79] and the human choline acetyltransferase gene [80] are characterized to contain AP-1 binding sites in their promoter regions and shown to be regulated by ERα via AP-1 site. However, recruitment of ERα to AP-1 binding to regulatory regions of many target genes and the potential molecular interplay between these transcription factors and subsequent signaling pathways needs to be characterized.

AP-1 plays a critical role in regulation of breast cancer cell proliferation and cell growth via Cyclins and E2F factors [81]. The ERα and AP-1 cross-talk found to be important in breast cancer etiology and progression of the disease. In ERα positive breast cancer cell lines (MCF-7) AP-1 activity was found to be up-regulated and associated with TMX resistance and increased invasiveness of cells [81].

1.1 AP-1 factors

Activator protein-1 (AP-1) is one of the first mammalian sequence specific transcription factors recognized [82, 83] and the first one to be isolated as a transcription factor that binds the DNA sequences found in the promoter regions of genes. AP-1 factors are stimulated by phorbol esters such as 12-*o*-tetradecanoylphorbol-13-acetate (TPA) [82]. The AP-1 belongs to the superfamily of transcription factor made up of Jun (c-Jun, Jun-B and Jun-D) and Fos (c-Fos, Fos-B, Fra-1 and Fra-2) the major proteins. Activating transcription factors (ATF2, LRF1/ATF3, and B-ATF) and Jun dimerization partners (JDP1 and JDP2) are also minor AP-1 factors including least studied Maf subfamily (c-Maf,MafB,MafA,MafG/F/K and Nrl) proteins [84].

AP-1 is a dimeric transcription factor comprising of protiens from several families whose common denominator is the possession of basic leucine zipper (bZIP) domain. The bZIP domain is essential for dimerization, DNA binding and activation. Jun proteins can both homo and heterodimerize, where as Fos proteins cannot homodimerize, but heterodimerize with Jun proteins and binds DNA. The Jun–Jun or Jun–Fos heterodimers bind preferentially to a heptamer consensus sequence known as TPA responsive element (TRE) (5′-TGA(C/G) TCA-3′) whereas Jun–ATF dimers bind with higher affinity to another consensus sequence known as cyclic AMP responsive element (CRE) (5′-TGACGTCA-3′). The Jun/Fos AP-1 complex regulates the expression of many genes binding to TRE element present in the upstream region. The Jun/ATF dimers regulate the CRE genes include aromatin, inhibin including c-Fos and Fra-2 AP-1 factors. The broad combinatorial possibilities provided by the large number of AP-1 proteins determine its binding specificity and affinity and consequently, regulates the spectrum of different genes [85]. Studies show that the Jun/Fos heterodimers are more stable and have a stronger DNA binding activity than Jun homodimer and the functional activation of AP-1 factors in mammalian cells requires phosphorylation [86], which is a pre requisite for binding to DNA.

The sequences to which AP-1 dimers bind may differ as they interact with structurally unrelated NFAT (nuclear factor of activated T-cell), or Ets or Smad proteins, and thus, differ in the expression and regulation of wide range of genes [87]. Thus studies show the transactivated AP-1 factors are implicated in the regulation of variety of cellular processes, such as proliferation, transformation, growth, survival, cell migration, apoptosis and including cancer development [85].

AP-1 transcription factors are expressed in most cell types and are activated by wide range of specific kinases such as protein kinase A (PKA), protein kinase C (PKC), cell cycle controller (cdc2) and mitogen activated protein kinases (MAPK) [88]. These kinases are activated by incredible number of external and internal stimuli like growth factor stimulation, UV light, oxidative stress, tumour promoters or oncogenes over expression or activation of many genes [89]. The expression of both c-Jun and c-Fos is rapidly increased in many cell types in response to epidermal growth factor (EGF) suggested that AP-1 complex is necessary for proliferation. Studies on breast cancer cells also show that, IGF, EGF, estrogens and retinoids, modulate the AP-1 transcriptional activity [90], which further demonstrate that ER and AP-1factors interact to regulate the expression of certain estrogen responsive genes [91]. Activation of AP-1 factors contribute to tumour cell invasion leading to TMX resistance in breast cancer [92]; AP-1 also found to play a critical role in cell proliferation and transformation in fibroblast [93, 94]. Transformed fibroblast cells by v-Fos are constitutively invasive under *in vitro* condition and express genes CD44, krp1, ezrin, mts-1, MMPs and TSC-36 responsible for invasiveness [95-97]. Thus, AP-1 transcription factors may be a critical cell cycle regulator of gene expression in response to mitogen and the activation of oncogenic signal transduction cascades, probably through the upstream dominant oncogenes such as Ha-ras and c-src [98].

1.2 Structure of AP-1 factors

c-Jun

The best characterized AP-1 component is c-Jun. Proto-oncogene (c-Jun) was originally isolated from avian sarcoma virus 17 in 1987 as a cellular homolog of the retroviral oncogene v-Jun [99], and further it was reported that c-Jun is a major component [83] and is a nuclear protein expressed in many cell types at low levels. Its expression is up-regulated by growth factors, cytokines, and UV irradiation. c-Jun is fairly conserved among different species. Human c-Jun is a 3.1 kb proto-oncogene, lacks introns [100]. The c-Jun protein is composed of 334 amino acids and having three domains that are well conserved among the different Jun and Fos family members; the C terminus leucine zipper (bZIP) domain, the basic region and N terminus transactivation domain (Fig. 1.1). The bZIP, domain containing two parallel α-helices form a coiled structure in C-terminus and is responsible for the dimerization of AP-1 proteins [101]. The characteristic feature of the leucine zipper is a periodic repeats of leucine located at every seventh amino acid forming interacting hydrophobic ridges in the dimer. The conserved positively-charged basic region is located immediately towards N-terminus to leucine zipper mediates DNA binding [102, 103]. The DNA-binding domain contains nuclear localization signal (NLS), which is identical in both v-Jun and c-Jun [104]. Within the transactivation domain towards N-terminus (S and T) are MAPK/ERK1/2 phosphorylation sites. The N-terminus of c-Jun also contains δ-domain, which is the docking site for JNK (c-Jun N-terminal kinase) that mediates ubiquitin dependent degradation of c-Jun [105]. Expression of c-Jun protein is induced by many external or internal stimuli. The stimuli may be cytokines, growth factors, steroids, mitogens, environmental stress, bacterial and viral infections, and oncogenes. The activated of AP-1 factors results in the formation of Jun/Jun or Jun–Fos dimers that binds to DNA with highest affinity to TRE elements of AP-1 activated genes. Dimers also bind to cAMP response element (CRE) but with lower affinity.

Jun–ATF dimers bind preferentially to CRE and their by expression of c-Jun is induced. Phosphorylation c-Jun protein influence the activity of AP-1 dimers protein affects DNA-binding, property, stability and ability to interact with other proteins, including transactivation of signals. Normally c-Jun is phosphorylated at serines (S63 and 73) and threonines (T91 and 93) present within transactivation domain (Fig. 1.3). Many kinases such as JNK isoforms, MAPK-dependent phosphorylation, ERK1/2 [106-108] activates c-Jun. Activation of c-Jun requires phosphorylation of serine 63 and 73 residues, and dephosphorylation of at least one at C-terminal end. It has been reported that c-Jun is phosphorylated by glycogen synthase kinase 3 (GSK3) at threonine 239 and serine 249 located proximal to DNA-binding domain, inhibits the binding of c-Jun to DNA [109]. Thus phosphorylation of c-Jun at S63 and 73 stimulates transcriptional activity by recruiting co-activator CREB-binding protein (CBP). CBP binds to N-terminal activation domain of c-Jun and connects the phosphorylated activation domain to the basal transcriptional machinery [110] of gene and brings about transactivation.

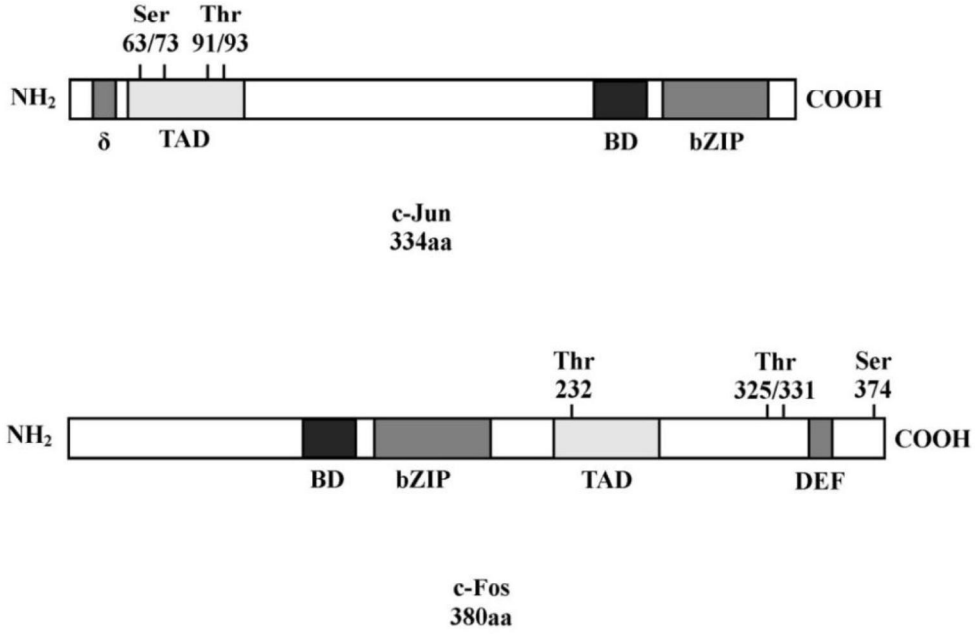

Fig. 1.3 Structure and functional domains of c-Jun and c-Fos

1.3 JunB

Similar to other Jun family transcription factors, JunB also plays a role in regulating the gene expression in response to growth factors, steroids and other stimuli. Binding specificity of Jun-B is more like c-Jun because of having very similar primary structure and DNA-binding specificity except for transactivation domain. JunB lacks the N-terminal serine residues, serine 63 and 73, which are critical for MAPK-dependent phosphorylation [111]. However, threonine 102 and 104 are phosphorylated by JNK and these sites are similar to threonine 91 and 93 of c-Jun [112, 113]. The intensity of transcription activation and specifications are greatly different between c-Jun and JunB. The c-Jun is an efficient activator of collagenase promoter, which contains a single TRE-binding site, while JunB inhibit the activation of this promoter. However, JunB activate constructs containing multiple TREs [114]. The c-Jun and JunB have been shown to act antagonistically in controlling cell transformation, differentiation and expression of AP-1-dependent target genes. In rat embryonic fibroblasts JunB induce transformation, when co-expressed with activated c-Ha-ras, but it is significantly less active than c-Jun. Hence the activities of c-Jun and JunB differ in their capacities and their impact on biological process and act opposite to each other. Recent studies on JunB expression in MCF-7 cell during proliferation suggest that, JunB expression is unaltered [115] and studies are in agreement with breast cancer cells MDA-MB231 cells where JunB expression is not significantly varied [116]. Further investigation using JunB knock out mouse model suggested that JunB effects and plays a major role in extra embryonic tissue, placenta , yolk sac, testis, heart development [117, 118].

1.4 JunD

JunD the third member of the Jun family has similar primary structure in DNA-binding and phosphorylation sites as c-Jun. JunD is the most ubiquitously expressed among AP-1

proteins [119, 120]. Opposing roles and effect for c-Jun and JunD have been found to c-Jun as a major cell cycle regulator and cell proliferator while JunD have opposing effect. However, JunD-deficient fibroblasts also show as reduced proliferation, indicate that JunD regulates cell cycle progression as both positive and negative modulator and it depends on cellular types and context [121, 122]. However, the over expression of JunD in fibroblasts suppresses the proliferation and antagonizes Ras-mediated transformation [123]. One of the studies show that JunD expression correlates well with the transition of proliferating granulosa cells to terminally differentiated, non-dividing luteal cells [124]. JunD has been shown to directly interact with menin, the product of the tumour suppressor gene MEN1. Menin interaction inhibits the transcriptional activity of JunD by inhibiting both ERK-1/2 and JNK-dependent phosphorylation of JunD [125]. In addition, JunD expression is increased during osteoblast differentiation, but menin suppresses osteoblast maturation possibly by inhibiting the differentiation actions of JunD [126]. Above studies suggested that JunD is mostly involved in differentiation, while c-Jun and JunB probably have a major role in cell proliferation.

1.5. c-Fos

One of the most important transcription factors of AP-1 family, the c-Fos was originally identified in FBJ (Finkel, Biskis, Jinkins) and FBR (Finkel, Biskis, Reilly) in murine sarcoma viruses [127]. The c-Fos protein also binds to same TRE sequence in DNA as c-Jun [128]. c-Fos is considered as an immediate early proto-oncogene with rapid and transient transcriptional activation by mitogenic stimuli [129]. Like c-Jun, c-Fos is also involved innumerous cellular processes such as proliferation, differentiation, transformation, and apoptosis including development.

The mammalian c-Fos gene (4 kb) codes for a protein of 381 amino acids [130]. Similar to Jun, Fos proteins share a hydrophobic bZIP region (Fig. 1.3) that mediates protein–protein

interactions, and a region containing basic amino acids that mediates DNA binding. Fos family members form heterodimer with Jun proteins, with varying affinities [131] while it cannot form homodimer with themselves. c-Fos also contains a more recently discovered ERK-docking site, the DEF domain, in the C-terminus region [132]. The c-Fos mRNA and protein are very unstable. The c-Fos protein is degraded by ubiquitinization and also by ubiquitin-independent mechanism by two distinct C- and N-terminus regions called as destabilizers. The c-Fos protein is normally expressed at low or undetectable levels in many cell types. However, it is rapidly and transiently induced in response to various stimuli, such as growth factors, environmental and physical stress. Similar to c-Jun, c-Fos is also associated with a variety of biological processes, from cell-cycle progression, cell differentiation and cell transformation including tumourigenesis [133]. High level of c-Fos protein found to involve in the development of bone, nervous system, and in differentiation of hematopoietic cells, the megakaryocytes [134, 135]. Tumourigenic properties of c-Fos have been demonstrated by over expression of the protein, which transforms chondroblasts and osteoclasts to osteosarcomas [136]. In addition, one of the studies reported that c-Fos is critical for MCF-7 breast cancer cell growth [137]. The regulation of c-Fos mostly occurs at mRNA level. The activity of the c-Fos promoter is modulated by many extracellular signals, which acts through *cis*-inducible serum responsive elements (SRE) [138]. Phosphorylation of many kinases like ERKs, MAPKs, in addition, phosphorylated CREB stimulates c-Fos transcription possibly by collaborating with SRE factors, SRF and TCF [139].

AP-1 protein family members are nuclear proteins bound to DNA constitutively in normal cellular conditions, whereas many of their interacting protein partners are localized in the cytosol. These protein partners are translocated to the nucleus prior to interaction with AP-1 proteins. The casein kinase 2-interacting protein-1 (CKIP-1) functions as a plasma membrane bound protein regulates AP-1 activity. During apoptosis, CKIP-1 is cleaved

through caspase-3-dependent mechanism and cleaved protein translocated to the nucleus. Apoptosis promoted by CKIP-1 forms a positive feedback loop with caspase-3. The C-terminal cleaved fragments reduce c-Fos AP-1 activity and favors apoptosis through enhanced caspase-3 activity [140]. Thus c-Fos is critical for many biological processes and activated by interacting with several transcription factors leads to complicated pathway, at cell, tissue and organ levels. Hence understanding the mechanism c-Fos and other AP-1 factors and their pattern of expression in breast cancer cells are vital.

1.5. Fos-B

Fos-B oncoprotein along with other Fos family members including c-Fos, Fra-1 and Fra-2 [141, 142] play an important role in different cellular processes and functions. Like c-Fos, Fos-B expression is induced in response to serum and other mitogens. Fos-B forms a complex with c-Jun and Jun-B *in vitro* [141]and brings about transactivation of genes. The expression of Fos-B has been localized to neuronal tissue and bone during embryonic development, although no known essential function is identified during embryonic development [143]. Fos-B deficient mice develop normally but have a nurturing defect [144]. In one of the studies Fos-B is found to be highly expressed in normal mammary epithelia, but down-regulated in poorly differentiated breast carcinomas [145] and this is in agreement with our study in MCF-7 cells where Fos-B expression is undetectable [115] suggested its role in transformation.

1.6. Fra-1

Another Fos-family member is Fos related antigen-1 (Fra-1), lacks C-terminal transactivation domain and therefore proposed to be a negative regulator of AP-1activity. Overexpression of Fra-1 has a growth inhibitory effect and induces apoptosis in glioma cells [146]. In contrast, Fra-1 is involved in Ras-induced transformation of NIH 3T3 cells, and stimulates

transformation, increases invasiveness and motility of epithelioid adenocarcinoma cells, reflects that Fra-1 act as a positive regulator [147]. Studies reported that Fra-1 expression levels regulate the proliferation and invasiveness of breast cancer [148]. In contrary to the negative regulation of Fra-1, studies reported that Fra-1 expression is induced in MCF-7 cells with mitogen, indicates that negative regulation of Fra-1 is not essentially valid [115]. In addition, previous studies also reported that Fra-1 expression level modulates regulation of activator protein-1 activity by estradiol in breast cancer cells [149].

1.6. Fra-2

Fos-related antigen 2 (Fra-2) is identified as a serum-inducible gene homologous to other members of Fos family protein, but has significantly lower transforming activity compared to c-Fos protein [142, 150]. The Fra-2 protein is phosphorylated by several kinases (PKA, PKC, cdc2, MAPK), with an increased DNA binding activity [88]. The Fra-2 found to have a unique role in cellular differentiation especially during fetal development. Fra-2 expression has been identified in differentiating epithelia, developing cartilage and in the central nervous system during embryonic development [151]. Similar to Fra-1, the Fra-2 protein lacks the C-terminal transactivating domain which is the characteristic feature of c-Fos and Fos-B and does not stimulate artificial AP-1-responsive promoters *in vitro* [152, 153]. But in spite of these biochemical properties, several recent investigations indicate that Fra-1 and Fra-2 might play an important role in the progression of various human tumours types under *in vivo* conditions [154]. Fra-2 is detectable in breast cancer cell lines and tumour tissues in variable amounts and in different phosphorylation states [116]. In mouse mammary adenocarcinoma cell lines (CSMLO) with different metastatic potential and high level of Fra-1 and Fra-2 mRNA and protein expression was found in metastatic cells, whereas c-Fos and Fos-B were undetectable [147]. In transient transfection studies, the invasive potential of both MCF-7

and MDA-MB231 cells was strongly increased by Fra-1, whereas Fra-2 had a weaker effect on invasion.

AP-1 activities in cancer

Since its discovery in 1987, the AP-1 transcription factor has been extensively studied to elucidate the role of different components expressed during biological processes and functions. Reports suggest that AP-1 factor functions extend beyond the activity of proliferation to other biological processes such as inflammation, differentiation, apoptosis, cellular migration, wound healing, invasion, transformation including metastasis [85, 87, 155]. These biological processes of AP-1 factors are controlled by the phosphorylation using c-Jun N terminal kinase (JNK). JNK in response to environmental stress and cues [156-158] phosphorylate and regulates the activity and expression of other Jun proteins [159, 160]. The JNK pathway is one of the most studied stress pathways with the involvement of AP-1 proteins plays a major role in development of cancer. Cancer appeared to be derived from multi-factorial effects acting at several physiological cell autonomous, micro environmental and systemic levels. At first level, the ability of AP-1 proteins to regulate cellular proliferation and survival is pivotal [133, 155]. In second level the AP-1 helps to develop and support angiogenesis with increasing the ability of tumours to cope up with harsh micro environmental anaerobic hypoxia conditions, i.e. lack of oxygen [161]. The relationship between AP-1 proteins with hypoxia-inducible factor 1α (HIF1α) [162] and its role in metastasis and invasiveness have been extensively studied.

Further, high expression of AP-1 found to induce the epithelial cell adhesion molecule (Ep-CAM) expression in breast cancer invasion. Studies using mammary carcinomas reveal that Fos family AP-1 proteins (c-Fos, Fos-B,Fos-B2,Fra-1 and Fra-2) are important and required for invasion and particularly Fra-1 and Fra-2 play an important role in the progression of

various human tumours under *in vivo* conditions [154]. Thus AP-1 proteins play a major role in the development of cancer and acts as an important regulator of growth and invasion.

Studies using primary culture of granulosa cells of rat ovary, in response to E_2 and FSH treatment, AP-1 factors exhibited a specific expression pattern. There was a rapid induction of Jun-B, c-Fos, and Fra2, the immediate early genes; however Jun-D and Fra-2 genes were induced by LH during differentiation of granulosa cells to luteal cells [124]. The c-Jun and c-Fos AP-1 factors promote tumourigenicity in human prostate cancer and regulates breast cancer cell growth [163]. Different AP-1 factors expression pattern is a stage and tissue specific and may be fundamental to the process of oncogenesis. Hence it becomes imperative to study the role of E_2 on the expression pattern of different AP-1 factors and that may help in understanding the role of individual components of AP-1 in proliferation/transformation and during progression of breast cancer. Some important AP-1 responsive genes involved in different biological processes are listed in table. 1.4.

Table 1.4 AP-1 responsive genes involved in different biological processes

Sl. No.	Biological processes	Genes
1.	Proliferation	Cyclin D1, Rb, p16
2.	Differentiation	ER, Myogen, Involucrin
3.	Hypoxia	CYP212
4.	Angiogenesis	VEGF
5.	Metastasis	CD44, osteopontin, KAI-1
6.	Apoptosis	BCl-2, BCl-Xl, FasL
7.	Invasion	MMP's, uPA, PAI-1, uPAR, TIMP-1

Protein Kinase C

Protein kinase C (PKC) is a super family of serine–threonine kinases and classified into three major groups: classical, novel and atypical. Activation of classical enzymes (cPKCs: α, β and γ) depends on Ca2+ and diacylglycerol (DAG), novel enzymes (nPKCs: δ, ε, η and θ) are activated by diacylglycerol (DAG) and atypical enzyme (aPKCs: μ, ξ and τ) activation is independent of calcium or DAG, but activated by other PKCs [164]. PKCs, the α, β and δ are abundant isoenzymes expressed in various tissues and extensively studied. Activation of different PKC isoenzymes depends on different cellular responses and cross talks between PKC isoenzymes are specific for particular cell types. Normal epidermal differentiation occurs with co-ordinated action of PKC α and δ. The δ enzyme promotes while α inhibits the differentiation process.

Under physiological conditions, PKC activation occurs in response to various growth factors and stimulus. Growth factor mediated phospholipase C (PLC) activation plays a central role in the activation of cPKC and nPKC, PLC generates DAG and inositol triphosphate (IP3) from the hydrolysis of plasma membrane phospholipids. DAG activates both the cPKC and nPKC and IP3 releases Ca^{2+} from intracellular stores, which potentiates the activation of cPKC [165]. Furthermore, series of phosphorylation of substrates regulates PKC activation [166]. Downstream targets of PKC are wide and largely unknown. Although, tumour promoting agents such as PMA binds with high affinity to DAG binding site of PKC promoter and prolonged activation of PKC, not much is known about subsequent activation or depletion of PKC isoenzymes in the cancer development. However, the expression profiles of PKC enzymes depend on cancer cell type and progression. Over expression of PKCα in high grade urinary bladder, prostate, and endometrial cancer, while significant reduction in the expression was observed in breast, colon and basal cell cancers. PKCβ was found to be up-regulated in colon and prostate cancer and down regulated in bladder cancer. Thus the PKC isoenzymes expression profiles are variable and depends upon cancer cell

types and in general conclusions cannot be drawn from the expression levels of PKC isoenzymes in carcinogenesis.

The most important cancer related PKCs direct or indirect targets are thought to be the extra cellularly related kinase 1/2 (ERK 1/2), glycogen synthase kinase-3 beta (GSK-3b), nuclear factor kappa beta (NF-κB) and P glycoprotein [167]. PKCs are involved in regulation of various physiological processes. The short-term activation of PKC is associated with short-term events like secretion and ion-influx, while sustained activation induces long-term effects. The long term effects include cellular process like proliferation, differentiation, apoptosis, migration, or tumourigenesis [168]. PKC isoenzymes have been shown to display variable expression profiles during cancer progression and development [169]. The importance of PKC expression in breast cancer was recently demonstrated and PKC activity has been shown to be higher in breast cancers than in normal breast tissue [170]. Many research groups investigated the role of PKC on the activation of AP-1 and NF-κB pathways in different cells. However, in MCF-7 cells how PKC regulate the expression of different AP-1 factors is not known. Hence our aim of the present study is also to investigate the expression pattern of AP-1 factors regulated by PKC in breast cancer cells.

Protein Kinase A (PKA)

Many hormones, neurotransmitters, growth factors, cytokines bind to G-protein coupled receptors, activate G-protein (α, β and γ subunit complex) by releasing GTP bound α subunit, which later activate membrane bound adenylyl cyclase (AC). AC catalyzes the formation of 3′, 5′-cyclic mono phosphate (cAMP). The cAMP in turn activates cAMP dependent protein kinase A (PKA) and downstream targets. Although, cAMP activates cyclic nucleotide gated channels, guanine exchange factors (Epac 1 and 2), in majority of mammalian cells, cAMP activates PKA. Normally PKA occurs enzymatically inactive tetrameric holoenzyme

consisting of 2 catalytic subunits (c) bound to regulatory subunit (R) dimer. During activation four molecules of cAMP binds co-operatively to two R subunits releasing two active C subunits. Activated free C subunits phosphorylate Ser and Thr residues of targeted protein, which subsequently induce signal transduction and elicit specific cell responses [171]. Receptors that activate PKA through cAMP regulate vast number of cellular responses such as metabolism [172], cell growth and division, gene regulation [173], sperm motility [174] as well as channel conductivity [175]. Due to ubiquitous nature of PKA signaling pathways it underlie strict spatial and temporal control to achieve specificity, A kinase anchoring proteins (AKAPs) play a major role in sequestration of cAMP effectors, ACs, phosphodiesterases (PDEs), PKAs, GTP exchange factors, cyclic nucleotide gated channels (CNGs). Interpretation of signals at discrete cellular locations is achieved by sequestration of these cAMP effectors to particular signaling nodes. AKAPs direct PKA to specific cellular sites with close proximity of downstream substrates. More than 40 AKAPs are expressed in a cell and organelle in specific fashion, which can tether different of set of ACs (10 human isoforms), PDEs (isoforms 1−11 and subsets) protein phosphatases (different isoforms) and other isoforms of effectors will give diversified and specific cAMP/PKA signaling with complexity in different cells. PDEs hydrolyze phosphodiester bond, protein phosphatases (PPs S/T or Y types) terminate PKA signaling by removing phosphate from PKA substrates. Thus AKAPs act as platforms for various effectors and may provide complexity, integration of many pathways and compartmentalized cAMP signaling, including novel targets for therapeutic intervention of PKA signaling associated disease [176].

The effect of gonadotropins on ovarian cell proliferation and differentiation are mediated by changes in intracellular cAMP that activates cAMP-dependent protein kinases (protein kinase A) [177], as well as several other kinases [178]. Known targets of protein kinase A are the cAMP regulatory element (CRE)-binding protein (CREB) and the CREB-binding protein

(CBP)[179]. CREs are essential for transcriptional activation of aromatase [86] and inhibin A, as well as the AP-1 factors, c-Fos[180] and Fra2[181]. AP-1 factors in turn mediate FSH-regulated expression of inhibin-bA by binding to a variant CRE in the promoter of the inhibin-bAgene [182]. Although, Jun–Fos heterodimers bind preferentially to a heptamer consensus sequence called TPA responsive element (TRE) (5′-TGA(C/G) TCA-3′) and Jun–ATF dimers bind with higher affinity to another consensus sequence called cyclic AMP responsive element (CRE) (5′-TGACGTCA-3′) [87]. Thus some members of the AP-1 transcription factor in response to cAMP that may regulate cell proliferation, differentiation and or associated with transformation [183]. These observations suggest that specific AP-1 factors or the combination of specific AP-1 factors may regulate different sets of functions like transformation and tumour formation. Despite the intense research efforts that have analyzed the function of Jun/Fos family in many different cell types, relatively few studies have analyzed how hormones regulate the expression of AP-1 factors in MCF-7 cells and therefore it becomes imperative to know which AP-1 factors are present in breast cancer cells and might be regulated or activated by estradiol-17β following the transformation.

Aim and scope of the present investigation

Main objectives of the study:

- To study the expression of Estrogen receptors (ERα and β) in MCF-7 cells.
- To study the effect of estradiol-17β, Tamoxifen (anti-estrogen), Phorbol-12-myristate-13- acetate (PKC activator) and Forskolin (PKA activator) on the Proliferation of breast cancer cells (MCF-7).
- To study the effect of estradiol-17β on the expression pattern of AP-1 factors in MCF-7 cells.
- To study the role of Protein kinase C and Protein Kinase A on the expression pattern of AP-1 factors in MCF-7 cells.
- Transfection studies of antisense of induced AP-1 factors on cell proliferation.

Breast cancer is the leading cause death in women, originates from breast tissue and most common one is epithelial ductal carcinoma. Estrogen and estrogen receptors (ERs) play key role in the growth and the onset and progression of breast cancer. Most of the breast cancers are estrogen receptor positive. Although, many patients initially respond to TMX an anti-estrogen, later develops resistance to the treatment. Over the years, studies from different laboratories have shown that in addition to estrogen, the growth of human breast cancer is also controlled by insulin like growth factors mediated by tyrosine kinase associated trans-membrane receptors, AP-1 and other transcription factors. Studies using primary culture of granulosa cells of rat ovary, in response to E_2 and FSH treatment AP-1 factors exhibited a specific expression pattern. There was a rapid induction of Jun-B, c-Fos, and Fra-2, the immediate early genes; however Jun-D and Fra-2 genes were induced by LH during differentiation of granulosa cells to luteal cells. The c-Jun and c-Fos AP-1 factors promote

tumourigenicity in human prostate cancer and regulate breast cancer cell growth. Different AP-1 factors expression pattern is a stage and tissue specific and may be fundamental to the process of oncogenesis in breast. Hence, our important aim of the present investigation is to study the role of E_2 on the expression pattern of different AP-1 factors that help in understanding the role of individual components of AP-1 in proliferation/transformation and during progression of breast cancer.

Many research groups working with various cancer cell types established the role of PKA in cell cycle growth and cancer development. PKA found to act as both activator as well as inhibitor of cell cycle proliferation, mediated through mitogen-activated protein kinase (MAPK)-extracellular signal regulated kinase (ERK)-cascade. Hormonal cAMP regulation of the ERK cascade provides an important cross talk between hormones and growth factor signaling. ERK signals through GTPase, Ras activates Raf-1 (MAPKKK) which phosphorylates, activates the MAPKK. MAPKK phosphorylates and activates MEK, which later phosphorylates, activates ERK and induce cell proliferation. The cAMP activates another GTPase of the Ras superfamily the Rap1, whose effects on ERKs seems to act parallel to those of the above action of cAMP inhibiting the ERK and cell growth. The Rap1 acts independently of PKA, regulates ERKs and complicated the action of hormones. Thus, multiple pathways exist from cAMP to either inhibit or activate ERK signaling with both PKA-dependent and independent pathways. Many laboratories reported that cAMP inhibits mitogenic action of vascular endothelial growth factor and fibroblast growth factor in capillary endothelial cells by blocking Raf activation. The cAMP also shown to activate multiple intracellular signaling cascades independent of its activation of PKA; however, most of the studies examining cAMP inhibition of ERKs showed requirement for PKA. The above findings prompted us to study the role of cAMP in the proliferation of MCF-7 cells and its effect on expression pattern of AP-1 factors.

Protein kinase C (PKC) a multigene family related to Ser/Thr kinases present at the crossroads of many signal transduction pathways and is implicated in a wide range of G protein-coupled receptors and other growth factor-dependent cellular responses. Thus multiple signal transduction pathways involving different types of PKCs may be involved in breast cancer growth, as many cancer drugs were shown to be ineffective, because they inhibit at the distal point. However in a cascade many like E_2–ER, cAMP–PKA, DAG–PKC and other mitogenic signals may converge at a point and probably AP-1 factors. Thus AP-1 factors may have differential effects on proliferation, transformation and progression of breast cancer. This prompted us to investigate the role of PKC on the expression pattern of AP-1 factors in MCF-7 cells. In the present study we investigated the role of cAMP a second messenger involved in PKA activation using Forskolin (Fo) an activator of adenyl cyclase and Phorbol ester (PMA) an analogue of 1, 2-diacylglycerol (DAG) that activates PKC on the proliferation and expression pattern of AP-1 factor mRNAs in the breast cancer cells. Further to understand the direct role of AP-1 factors in MCF-7 cell proliferation, the mRNA expression was blocked by antisense oligo nucleotides and investigated the expression of cell cycle regulators and genes involved in apoptosis. CDK4 is expressed in a variety of normal cells and is often over expressed in human tumours. Blocking of CDK4 expression could be an attractive strategy for the treatment of cancers. Hence, in the present study antisense oligos to CDK4 DNA were designed (20-mer), and used for the blocking of CDK4 expression in lung epithelial (A549) cells as well as in MCF-7 cells and investigated the expression of mRNA transcripts of cell cycle regulator genes and apoptotic genes.

Accordingly, in our studies, we used ER positive MCF-7 cells and non-breast cancer ER negative A549 lung epithelial cells as a control. In the first phase following the standardization of culture conditions of cancer cells (MCF-7 cells and A549), the cell viability MTT assay, total RNA isolation, formaldehyde agarose gel electrophoresis and RT-

PCR techniques were standardized. The results of the studies on the role of E_2 and effect of TMX on the expression pattern of AP-1 factors in MCF-7 cells and A549 cells are presented in Chapter 3. Fos-B is not expressed, while, c-Jun, c-Fos and Fra-2 was significantly stimulated by E_2. Further, the effect of E_2 on induced expression of mRNAs of Cyclin-D1 and Cyclin-E1 and anti-apoptotic Bcl-2 gene confirms the proliferative effect of estrogen.

To study the effect of PKA, forskolin was used and to study the effect of PKC, the PMA was used and the results of such a study are presented in Chapter 4. Fo inhibits and PMA activates c-Jun, c-Fos and Fra-1 and found to have opposing effects on the expression pattern mRNAs of AP-1 factors. The same was reflected at protein levels i.e Fo significantly decreased the c-Jun, while PMA significantly induced the expression of c-Jun.

Finally the effect of antisense oligos of AP-1 factors and CDK4 on the proliferation and expression of cell cycle regulators and apoptosis gene is presented in Chapter 5. The results suggested that both c-Jun and c-Fos oligos significantly decreased the proliferation and also expression of c-Jun mRNA transcript by the treatment with antisense oligos.

A general discussion of the results obtained with the proposed mechanism of E_2, PKA and PKC action on AP-1 factors in the light of other studies carried out on MCF-7 cells and other cancer cells are presented in Chapter 6. To conclude c-Jun, c-Fos and Fra-1 provide a new targets for the development of anticancer drug for the breast cancer treatment.