CHAPTER 6

Summary and conclusion

Mammary gland development is a multistep complex process involving the role of steroid hormone E_2, P4 produced by ovaries/placenta and PRL hormone produced by pituitary. Mammary glands are modified sweat glands develop at puberty from epidermis lies in superficial fascia, in female by the action of ovarian hormones (E_2 and P4) and reaches to fully functional state during pregnancy by the action of placental hormones (PRL). Prolactin of anterior pituitary somatomammotrophin of placenta and corticoid hormones of adrenals also plays a role in the development of gland. At parturition, the abrupt decreased levels of circulating E_2 and P4 (as both placenta and corpus luteum degenerates), mammary gland characterized with lactation and discharge milk into ducts by hormonal action of PRL and corticosteroids. More than 90% of cancers starts in milk ducts, the ductal carcinoma are common among breast cancer patients. Ductal carcinomas are characterized with ER positive cells, and hence most of the patients respond to hormonal replacement therapy, the TMX treatment. While >10 % milk gland the lobular carcinoma or due to other cells or tissues. Therefore, in our studies we used ER positive MCF-7 cells.

The estrogen and its receptor (ERs) play key roles not only in development of normal mammary gland but also in the onset and progression of breast cancer. ERs, (ERα and ERβ) are ligand-activated transcription factors regulate the transcription of many genes by directly binding to ERE present in the promoter regions, while in indirect pathways, liganded ERs are tethered into DNA by interacting with other transcription factors. Transcription factors like SP1 or AP-1 or ERs probably involved in cross talks that regulates and converge different kinase pathways. AP-1 plays a critical role in regulation of breast cancer cell proliferation and ERα and AP-1 cross talk and may play an important role in breast cancer etiology and

progression of the disease. AP-1 the family of Jun and Fos proteins that bind as homo or heterodimers to similar DNA motifs, although related structurally, they exhibit distinct properties. The number of possible subunit combinations creates enormous functional diversity. Individual members of the AP-1 family as dimmers may be engaged in totally different cell fates, proliferation to apoptosis, transformation to tumour progression and survival to differentiation. In addition to the combinatorial diversity of AP-1 factors, they integrate diverse signaling pathways activated by different modulators/activators/extracellular cues. The synthesis of AP-1 subunits, their turnover, and their activation states are controlled by many signaling pathways. The rapid diverse responsiveness of AP-1 family of transcription factors during proliferation/MCF-7 cells in disease status may help in identification of markers and the prognosis of the disease. Different AP-1 factors expression pattern is a stage and tissue specific and may be fundamental to the process of oncogenesis. Though literature on AP-1 is enormous, to our knowledge the expression pattern of mRNAs of all the AP-1 factors in MCF-7 cells by the action of E_2, PMA the activator of PKC and Fo the activator of PKA is not being addressed. Hence it becomes imperative to study the role of E_2 on the expression pattern of different AP-1 factors that may help to dissect the individual AP-1 subunits role in development of progression breast cancer. In the present study we made an attempt to study the role of E_2 and effect of TMX on the expression pattern of AP-1 subunits in MCF-7 cells during proliferation. In our studies we also used E_2 negative non-breast cancer cell lines to analyze, whether the same set of AP-1 factors are induced by the same treatment with the mitogens/PKA and PKC modulators during proliferation.

Our studies on the expression of mRNA transcripts of ERα and ERβ confirms the presence of ER, however preferentially expressed ERα over ERβ transcripts, suggesting that the MCF-7 cells as estrogen responsive cells, while A549 cells are non estrogen responsive cells as they do not express ER. The results of our study also show that E_2 being considered as mitogen

increased the cell number in a dose dependent manner. The significant increase in cell number (32%) was observed at, as low as 50 nM concentration of E_2. However, the E_2 found to have no effect on the proliferation of A549 estrogen non responsive cells. Study using TMX confirms the proliferative effect of E_2 in MCF-7 cells. TMX decreases the cell viability of MCF-7 cells in a dose dependent manner and maximum of significant decrease of 29% cell viability was observed at 0.8 and 1 μM concentration of TMX. Studies on the differential expression of mRNAs of AP-1 factors in MCF-7 cells, suggested that, although Jun and Fos family members mRNA transcripts were expressed at different levels, E_2 induced c-Jun mRNAs significantly by two folds and c-Fos, Fra-1 transcripts significantly by 1 and 1.5 folds respectively. However significant decrease in mRNA transcripts of c-Jun by 60%, c-Fos by 40% and Fra-1 by 60% by TMX was observed, confirms that these genes are regulated by estrogen. Further, TMX also decreased the gene expression of Jun-D by more than 70 % and Fra-2 by 40 % suggesting that these genes are also regulated by E_2. However, expression of FosB was negative both in control and E_2 treated cells confirms the observation made by others that FosB expression might be necessary for normal proliferation and differentiation of mammary epithelial cells while reduced FosB expression might be involved in transformation and tumourigenesis of breast. E_2 induce the cyclin D1 and E1 significantly and marginal increase of CDK4 mRNA transcripts, while TMX decreased the cyclin D1 transcript by more than 50% and marginal decrease of CDK4 transcripts compared to control, suggested that E_2 regulate the expression of cyclin D1 and CDK4 probably through c-Jun and c-Fos protiens. The MCF-7 cells treated with E_2 or TMX , the Bcl-2 an anti-apoptotic gene expression was found to be increased by more than 30 % and marginal decrease by TMX confirms the mitogenic effect of E_2.

Studies carried out on various cancer cell types established that cAMP acts as both activator as well as inhibitor of cell cycle proliferation. The important target of cAMP is the mitogen-

activated protein kinase MAPK-ERK-cascade. 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 show the requirement for PKA. The conflicting reports prompted us to study the role of cAMP in the proliferation of MCF-7 cells and its effect on AP-1 factors.

Protein kinase C (PKC) is a multigene family of related serine/threonine kinases present at the crossroads of many signal transduction pathways and are implicated in a wide range of G protein-coupled receptors and other growth factor-dependent cellular responses. Many PKCs are activated by tumour-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA), which anchors PKC in their active conformation to membrane. 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 the cascade many mitogenic signals converge at a later point and probably AP-1 factors.

Earlier study using immature rat granulosa cells suggested that follicle stimulating hormone (Fo/cAMP), rapidly but transiently induced c-Jun/c-Fos/Fra-2, while Jun-D and Fra-2 were induced by LH (Fo) in transiently differentiated luteal cells. Other mitogens like DAG or hormones that increase intracellular cAMP may have similar effects on the expression pattern of AP-1 factors. However, less is known about the role of PKA and PKC in the signal transduction pathways that regulate 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 adenylcyclase and Phorbol ester (PMA) an analogue of 1, 2 diacyl glycerol (DAG) that activates PKC on the proliferation and expression pattern of AP-1 factor mRNAs in the breast cancer cells.

In our study MCF-7 cells treated with PMA show significant increase in cell proliferation (32 %), however Fo a protein kinase A activator does not show any effect, while inhibited the PMA induced proliferation. In A549 cells Fo and PMA found to have no effect on proliferation, but Fo along with PMA decreased the proliferation by 20 % suggested the integration of PKA and PKC pathway in non-breast cancer cells and needs to be investigated. MCF-7 cells treated with PMA like E_2 induce the c-Jun, c-Fos and Fra-1 transcripts significantly. Fo decreased the expression of c-Jun, PMA induced Fra-1 expression and also c-Fos mRNA levels. Further, immunofluorescent studies of PMA treated MCF-7 cells show induced expression of c-Jun and c-Fos proteins compared to control correlates and reflects well with the increased expression of c-Jun and c-Fos mRNA transcripts by PMA. Western blot analysis confirms that Fo decreased c-Jun protein levels compared to control as well as PMA induced levels suggesting that PMA and Fo have opposing effects on the expression pattern of AP1 factors in MCF-7 cells. Analysis of expression of cell cycle regulators and apoptosis mRNA transcripts suggested that in MCF-7 cells, Fo show significant inhibitory effect on cyclin D1, CDK4, ERα, anti-apoptotic Bcl2 and PKCε expression, and marginal inhibitor effect on ERβ , p53. While PMA have little or no effect on above genes, except it has significant stimulatory effect on cyclin E1 and marginal effect on CDK4 expression. Fo also show decreased CDK4 expression induced by PMA. However, both Fo and PMA has no effect on pro-apoptotic Bax mRNA levels. Above results suggested that PMA proliferative effect is not through ERα and but both ERα and PMA proliferative effect is through AP-1 factors (c-Jun, c-Fos and Fra-1). Fo has opposing effects on the expression pattern of above AP-1 factors.

Cell division is a highly regulated process and can be broadly divided into G1, S, G2 and M phases. Different cyclins interact with CDKs, act and activate different phases, in contrast, CDK inhibitory proteins (INK4), kinase inhibitory proteins (KIP) inhibit and thereby keep the check on the cell cycle. Deregulation of cell cycle control is one of the initial events in

the development of most of the cancers. Among CDKs the CDK4 is expressed in a variety of normal cells at G phase and is often over expressed in many human tumours. CDK4 a Ser/Thr protein kinase found to play an important role in regulating retinoblastoma protein function. Many cancer drugs have been characterized and extensively used for the cancer treatment, but they lack specificity and most of the time patients develop multidrug resistance. Hence, the selective blocking of CDK4 expression by specific CDK4 antisense technology could be an attractive strategy for targeting different types of tumours. A549 cells treated with normal antisense oligos show significant decrease in cell proliferation and cell number compared to control and scrambled oligos. CDK4-atisense treatment also show decreased transcription of cell cycle regulator, Cyclin D1, anti-apoptotic gene Bcl-2 by more than 30 % while, induced the pro-apoptotic genes Bax by 35 % and caspase-8 by 3 folds confirms the role of CDK4 in proliferation. MCF-7 cells treated with different phosphorothioate antisense oligos (designed against transcription initiation, ATP binding region and cyclin binding regions) also show decreased viability confirming the role of CDK4 in cell cycle

To understand the direct role of AP-1 factors (c-Jun and c-Fos) in MCF-7 cell proliferation, the mRNA expression was blocked by first generation antisense oligo nucleotides and investigated the expression of cell cycle regulators and genes involved in apoptosis. The c-Jun and c-Fos AS-ONs significantly decreased the cell viability compared to control and scrambled oligos treated cells suggested the probable involvement of c-Jun and c-Fos in MCF-7 cells proliferation. MCF-7 cells treated with c-Jun antisense oligo's nucleotides (AS-ONs) show decreased expression of mRNA transcripts by more than 50 % compared to control and scrambled ONs. These results confirm the involvement c-Jun and probably the ratio of c-Jun/c-Fos dimers is required for proliferation of breast cancer cells.

Recent study (Dahalman-Wright et al. 2012) show that PKIB (protein kinase A inhibitor) as a novel ERα /AP-1 target molecule subject to cross talk by E_2 and Jun/Fos protein. PKIB found to be involved in proliferation and has binding sites for ERα and AP-1 in potentially regulatory regions. They confirmed that E2 or TPA the PKC activator induced PKIB expression. The ChiP experiments confirmed the recruitment of ERα and AP-1 to the intron 1 of the PKIB suggesting the convergence of the signals on the same regulatory regions inducing the PKIB gene expression. Use of Si RNA pool targeting PKIB, effectively decreased the expression of PKIB and knock down of PKIB significantly reduced the BrdU incorporation and cell proliferation. All the above observations suggested that PKIB an important molecule probably involved in cell proliferation and cancer development. Our study also shows that E2 through ERα activates the transcription of c-Jun, c-Fos and Fra-1. PMA through PKC induce c-Jun, c-Fos and Fra-1 mRNA levels. AP-1 factors (c-Jun, c-Fos and Fra-1) induce the proliferation of cells through the activation of E2F1 retinoblastoma pathway. At the same time AP-1 factors induce the PKIB expression which has inhibitory effect on PKA. Our study also confirms that Fo the activator of PKA decreases the levels of mRNAs of c-Jun, c-Fos and Fra-1 and has an inhibitory effect on cell proliferation. Fig 6.1 summarizes the mechanism of E_2 and PKC action in stimulation of cell cycle through AP-1 factors and opposing effect of PKA in cell proliferation. The important players in E2 and PKC regulated cell proliferation and cell cycle are c-Jun, c-Fos and Fra-1 acting through PKIB. The target of AP-1 together with CDK4 is the activation of E2F1 and subsequently its activation of downstream target genes that are critical for ER and PKC regulation activation of proliferation in MCF-7 cells. Fig 6.2 shows the mechanism of activation of E2F1 the target of AP-1 and CDK4. Finally the Fig 6.3 summarizes our work the opposing effects of PKA and PKC in MCF-7 cell proliferation.

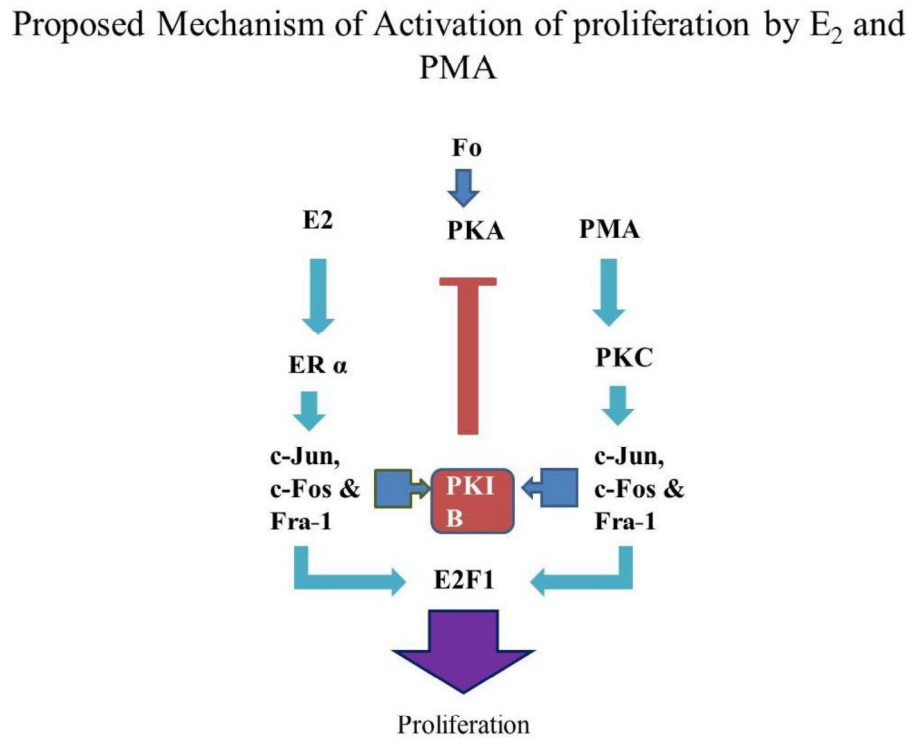

Fig. 6.1 Proposed Mechanism of Activation of proliferation MCF-7 cells by E_2 and PMA

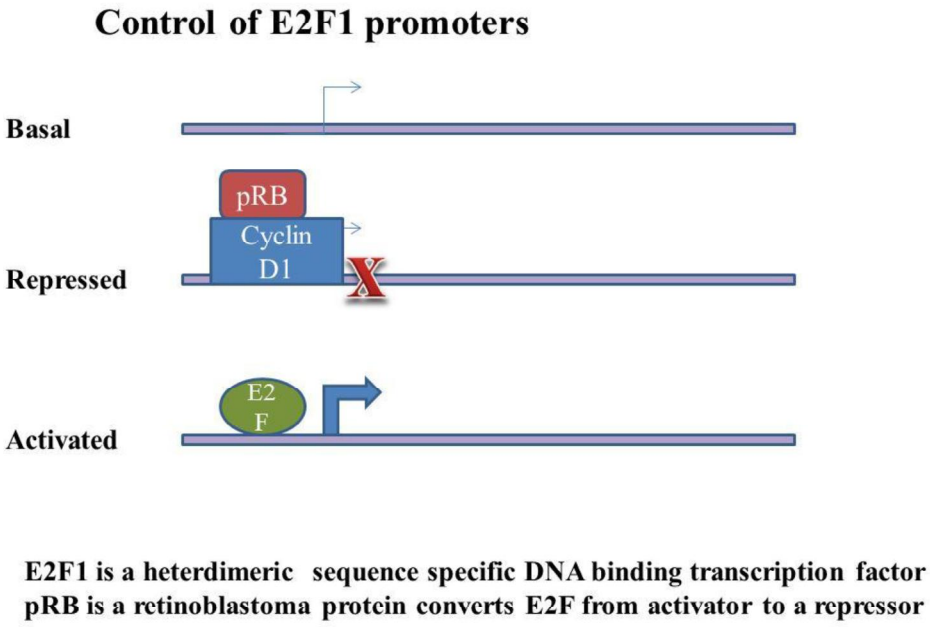

Fig. 6.2 Mechanism of activation of E2F1

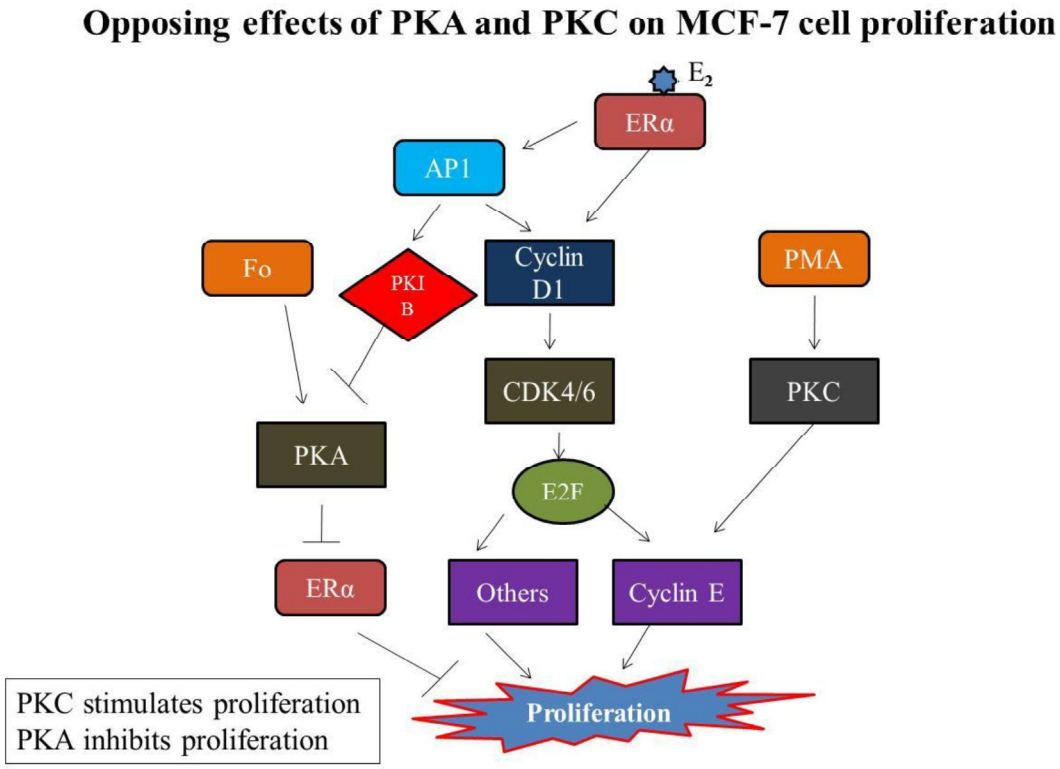

Fig. 6.3 Opposing effects of PKA and PKC on MCF-7 cell proliferation

Conclusion

PKIB together with c-Jun, c-Fos and Fra-1 provides new targets for the development of anticancer drugs. Fra-1 probably and could be used as marker in the diagnosis of breast cancer.