Roles of CCN5 in regulating progression and therapeutic sensitivity of breast cancer

By
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ABSTRACT

Breast cancer is one of the deadliest malignancies worldwide and also in the United States. Patients with triple negative breast cancer (TNBC), where the cancer cells do not express nuclear hormone receptors and human epidermal growth factor receptor 2 (HER2), have worse survival rate compared to the patients with luminal subtypes of cancer. Here, we have shown that Cysteine-rich 61-Connective Tissue Growth Factor-nephroblastoma-overexpressed 5 (CCN5) induces growth arrest of TNBC cells *in-vitro* and in xenograft tumors. Our studies show that after being secreted into the extracellular matrix, CCN5 binds to the α6β1 integrins of the cells leading to inhibition of the PI3K-AKT signaling pathway. This leads to stabilization and nuclear localization of FOXO3A resulting in transcriptional activation of the cyclin-dependent kinase inhibitor P27KIP1. Also, we found that the CCN5-induced PI3K-AKT inactivation leads to stabilization and nuclear accumulation of P27KIP1 resulting in cell cycle arrest of TNBC cells. Next, we have shown that CCN5 protein can induce expression of estrogen receptor-α (ER-α) in mammary epithelial cells. We found that mammary epithelium-specific overexpression of CCN5 in transgenic mice leads to an increase in ER-α expression and that this impact of CCN5 is not restricted to the normal cells. CCN5 treatment leads to an expression of functional ER-α in the TNBC cells, both *in-vitro* and in xenograft models, and sensitizes these cells to tamoxifen, commonly used for endocrine therapies. Mechanistically, transcriptional activation of ER-α by CCN5 is also mediated by FOXO3A stabilization via PI3K-AKT inhibition. Lack of ER-α expression in TNBC cells or loss of ER-α activation after endocrine treatment of luminal cancers makes these breast cancer cells resistant to tamoxifen and other endocrine therapies. Evidently, CCN5-mediated restoration of ER-α and its downstream signaling cascades renders the TNBC cells sensitive to tamoxifen. As these tumors mostly lack CCN5 expression, we anticipate that restoration of CCN5 expression might be able to provide breakthroughs in the treatment of these tumors. Finally, we discuss the effects of CCN5 expression on yet another aggressive breast cancer subtype, characterized by HER2 overexpression. Mammary-specific expression of CCN5 in HER2 overexpressing mice delays tumor progression significantly and reduces the tumor burden. Initial observations indicate that CCN5 induces expression of $P16^{INK4A}$ and $P19^{ARF}$, resulting in cell cycle arrest of the tumor cells. Collectively, these studies suggest that CCN5 restoration can be beneficial for the better management of breast cancer progression.

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CONTENTS

CHAPTER I: General Introduction

ARCHITECTURE OF HUMAN BREAST: AN OVERVIEW

A healthy adult female breast, like most other organs in the human body, consists of both epithelial and stromal elements. However, unlike most of the other organs, the female breast is subject to significant changes in its composition and architecture depending on the pubertal stage and lactation status.

Human female breasts have a comprehensive ductal network that connects the functional units of the breast, the milk-producing lobular acini, to the exterior via the nipple. The lumen of the branching ducts and the lobules are lined by columnar and cuboidal epithelial cells, which are the epithelial element of the human mammary gland **(Figure I.1)**. The stromal component makes up most of the breast volume in the non-lactational state and is composed of adipose and fibrous connective tissues, the ratio of which varies from individual to individual **(Figure I.2)**.

At the prepubertal age, beneath the nipple lies a small number of branching ducts, the epithelial component, which are rudimentary at that stage and does not further develop in males. The proliferative growth and branching of the ductal epithelium are slow at this stage. At puberty, the growth and branching of the ducts, as well as mass of stromal components, increase significantly in the female breast. At the post-pubertal stage, the cells at the terminal ends of the ducts proliferate and give rise to bud-like structures each of which develops into secretory glands during pregnancy.

An adult human female breast, irrespective of the age and body weight, is composed of 15-20 large lactiferous ducts running from individual lobes, named a tubulo-acinar gland. Each of these lactiferous ducts drains-out to the exterior through different openings in the nipple **(Figure I.1)**. Within the mammary lobes, the lactiferous ducts branch and sub-branch into segmental and sub-segmental ducts which finally branch into terminal ducts. Each of these

terminal ducts further branches and form a cluster of acinar (glandular) structures which together forms a single lobule. These lobules have loose fibrous connective tissue interspersed within, which is distinctly different from the denser extralobular stroma. Each lobe in an adult breast has 20-40 such lobules **(Figure I.2)**. As a terminal duct forms acinus and a cluster of these acini form a lobule, the entire lobule along with both the parts of the terminal duct inside and outside of the lobule is known as a Terminal Ductal Lobular Unit (TDLU)(Hayes, 2000) **(Figure I.1)**.

In a resting non-pregnant non-lactating adult breast, larger ducts which are lined with columnar epithelium and stratified cuboidal epithelium are more prominent compared to the acini which are lined with cuboidal epithelium **(Figure I.1)**. At the onset of pregnancy and during lactation, the number of acini increases drastically as the epithelial cells at the terminal ends of the ductal tree start to proliferate under hormonal influence.

Both in the ductal and lobular parts, the epithelial lining is composed of two layers of epithelial cells, namely the luminal epithelial cells (immediate lining of the lumen) and the myoepithelial cells, which are contractile and lies in between the luminal epithelial layer and the basement membrane **(Figure I.1)**. At the post-menopausal stage, involution of the epithelial ductal and lobular network leads to a significant change in the breast tissue architecture leaving the fibrous connective tissue to occupy most of the tissue mass.

BREAST CANCER: A GLOBAL DISEASE

Breast cancer remains as one of the deadliest malignant disease affecting almost every family either directly or indirectly throughout the world. A very similar situation also exists in the Western countries including the United States. Approximately 12.4% of US women or one in every eight US women have a lifetime risk of developing invasive breast cancer. By the end of the current year, approximately 250,000 new cases of breast cancer are anticipated to get reported (R. L. Siegel, Miller, & Jemal, 2017). Despite all the research and treatment advances, 40,000 women are predicted to succumb to this deadly disease (R. L. Siegel et al., 2017). However, the 5-year survival rate for breast cancer is close to 90 percent at present, and the number of deaths due to breast cancer has been steadily decreasing (National Cancer Institute; R. L. Siegel et al., 2017). Timely screenings and tests for this deadly disease have enabled the physicians across the USA and the world to diagnose breast cancer at earlier stages leading to an improvement in the 5-year survival rate of the disease. Regular mammograms for screening every woman after her 40 years of age and breast MRIs for screening in the population with genetic susceptibility to breast cancer owing to a familial history of BRCA gene mutations have led to a gradual decrease of breast cancer mortality (National Cancer Institute).

BREAST CANCER: ORIGIN AND STAGES OF PROGRESSION

Breast cancer, in most of the cases, arises from the ductal and alveolar epithelial cells of mammary gland epithelium. The epithelial cells lining the ductal and alveolar lumen undergoes cycles of proliferation depending on the changing hormonal influences in the human body. These include menstrual cycles, pregnancy, and lactation status. This process renders the proliferating epithelial cells susceptible to mutations which might lead to unrestricted proliferation and

eventual transformation to neoplastic cells leading to invasion followed by metastatic growth in the distant organs such lungs, liver, brain, and bones.

Based on morphological and pathobiological progression, breast cancer is classified into *pre-neoplastic and neoplastic* lesions. These include-

- HYPERPLASIA: The initial pre-malignant stage is known as *hyperplasia* where only hyperproliferation of ductal and lobular epithelial cells can be noticed.
- ATYPICAL HYPERPLASIA: The next stage in the sequence of the progression of breast cancer is *atypical ductal hyperplasia (ADH) or atypical lobular hyperplasia (ALH)* where the proliferating cells under pathologic examination appear to be different from the typical epithelial cells usually with hyperchromatic nuclei.
- CARCINOMA *IN SITU:* The ductal carcinoma in-situ (DCIS) or lobular carcinoma in-situ (LCIS), as the nomenclature suggests, refer to the stage where the abnormally proliferating ductal epithelial cells and lobular epithelial cells have not encroached into the surrounding stroma. At this stage, the cells are limited within the confines of ductal boundaries of myoepithelial cells and basement membrane. The DCIS stage is considered as a non-malignant stage (Stage 0). Whether patients with DCIS should be treated has been a matter of debate among the clinicians as only a fraction of DCIS patients eventually develop invasive breast cancer and had a morbidity rate of 1-2% (Burstein, Polyak, Wong, Lester, & Kaelin, 2004; Ceilley et al., 2004). Depending on the architectural patterns, clinicians classify DCIS into solid, comedo, cribriform, papillary and micropapillary patterns. A significant fraction of DCIS lesions shows similar intra-lesion heterogeneity as invasive carcinomas (Allred et al., 2008).

- INVASION: Eventually, the neoplastic cells invade the surrounding stroma, disrupting the myoepithelial cell lining and the basement membrane matrix surrounding the ducts. At this point, it is classified as invasive ductal carcinoma. As the disease progresses, a population of the cancer cells which have invaded into the stroma might intravasate into the lymphatic vessels and blood vessels, gaining access to distant organs to form metastatic secondary tumors **(Figure I.3)**.

BREAST CANCER STAGING: Breast cancer is most commonly staged by the clinicians per 'TNM' system staging. It follows the criteria of - tumor size (T), lymph node positivity (N) and metastasis status (M). Lymph node positivity (N) signifies the extent of spread of cancer cells to the lymph nodes and metastasis status (M) signifies if the tumor cells have spread or metastasized to distant organs. Breast cancers are broadly staged into Stage 0 to Stage IV depending on the TNM scores. Stage 0 signifies the DCIS stage ($T_{is}N_0M_0$) while stage IV represents metastatic tumor ($T_{any}N_{any}M_1$ - as once evidence for metastasis is confirmed, the tumor size and lymph node positivity do not bear significance clinically).

BREAST CANCER: SUBTYPES AND HETEROGENEITY

The clinicians have classically subdivided breast cancers into three very broad subtypes per expression status of nuclear hormone receptors [estrogen receptor-α (ER-α and progesterone receptor (PR)] and receptor tyrosine kinase HER2 (or ERBB2). The subtypes are - endocrine receptor-positive type (expressing ER-α, PR or both), HER2 positive type (HER2 expression) and triple negative type (TNBC), which expresses none of the receptors (ER, PR or HER2). This classical subtyping has been popular with the clinicians mainly as each of the subtypes of tumors required three broadly different approaches to treatment **(Figure I.4, upper panel).**

Insights from molecular subtyping of breast cancer

Recent advances in molecular and genetic profiling of breast cancer have provided many insights about the breast cancer types and have led to the emergence of sub-classes of breast cancer (Perou, 2011; Perou et al., 2000; Prat & Perou, 2011; Sorlie et al., 2001). The characteristics of these subtypes might be indicative of different cell types of origin (Perou et al., 2000).

One key example is sub-classification of the ER-α positive subtype into two distinct groups, which has been widely accepted by the clinicians. Microarray studies of multiple breast tumor samples revealed that ER-α positive tumors mostly are characterized by the relatively high expression of many genes expressed by breast luminal cells which are further corroborated by the expression of luminal cell keratins 8/18 (Perou et al., 2000). The gene expression analyses have shown that the tumor cells of luminal cluster are characterized by high expression of transcription factors ER- α, GATA binding protein 3 (GATA-3), X-box Binding Protein 1 (XBP1), and Forkhead Box A1 (FOXA1) (as well as luminal cytokeratin 8 and 18) (Perou et al., 2000). Subsequent studies from the same group established that Luminal type of breast tumors can at least be sub-classified into Luminal A and Luminal B subtypes (Sorlie et al., 2001). The Luminal A tumors are usually positive for ER-α and PR and negative for HER2. These tumors are generally of lower pathological grade, and the cells are mostly highly differentiated. The Luminal B tumors are ER-α positive with or without PR and HER2 expression. Luminal B tumors express the same luminal cell gene signature at a lower level and show higher expression of proliferation genes (e.g., Ki67) than luminal A tumors (Hoadley Katherine A., 2014).

The other two major subtypes are (i) basal-like subtype and (ii)HER2 overexpressing subtype. Basal-like tumors mostly lack the expression of HER2 or the endocrine receptors and largely overlap with the classical triple negative group of breast tumors. However, gene

expression profiling demonstrated not all TNBC are basal-type, and not all basal-like tumors are triple negative. Thus, it indicates that molecular stratification of clinically identified triple negative tumors might be highly useful (Prat et al., 2013). High expression of basal cytokeratin 5 and 17, a signature of normal breast basal/myoepithelial cells, is a characteristic of the basal-like subtype (Perou et al., 2000). The most recently identified tumor group per molecular classification is the claudin-low subtype which is highly related to basal tumors regarding molecular signature and are triple negative clinically. The claudin-low tumors have a distinct signature of a subset of gene expressions with low expression of cell junction proteins like claudins and E-cadherin. These tumors have been found to be enriched with mesenchymal and tumor-initiating cell gene signature (Perou, 2011).

HER2 overexpressing or HER2 enriched tumors, as suggested by the name mostly overexpress HER2 receptor tyrosine kinase protein and often show amplification of this locus. Though a fraction of HER2 enriched tumors shows expression of endocrine receptors, most of these tumors have high pathological grade and are negative for ER-α expression (Hoadley Katherine A., 2014).

COMMON AND RECURRENT GENETIC ALTERATIONS: The extensive volume of data generated by The Cancer Genome Atlas Network on DNA copy number and somatic mutations in 792 patients of breast cancer was used to identify the frequency of each genetic alteration across all the cancer subtypes (Cancer Genome Atlas, 2012). Genomic profiling of breast cancer subtypes has shown that each breast cancer subtype has a specific set of recurring genetic alterations. Mutations of tumor suppressor P53, 'the guardian of the genome,' are mostly found in basal-like, HER2 positive and luminal B subtypes of breast tumors, while these mutations are primarily absent in luminal B subtype of cancer.

TP53 mutations have been the only recurrent mutations in basal-like tumors with an incidence rate of more than 10%. Carriers of BRCA1 germline mutations have been shown to be predisposed to developing basal-like tumors (Hoadley Katherine A., 2014). Thus, an association with the mutations of tumor suppressor P53 and with hereditary mutations of BRCA1 could be an essential reason for basal-like tumors with high incidence rate among the African-American population (Huo et al., 2009) and tumors of younger patients. Basal-like tumors are relatively enriched for low-level copy number gains and losses (Hoadley Katherine A., 2014).

Luminal A tumors have been shown to have the highest number of recurrently mutated genes. The long list of genes which are frequently mutated (more than 5%) includes transcription factors [like GATA3, NCOR1, FOXA1, TP53], cell-junction proteins [CDH1], cellular kinases and phosphatases [AKT1, PIK3CA, MAP3K1, PTEN]. However, the total number of mutations and copy number changes is lowest in this subtype suggesting that these alterations are likely to be driver mutations. Phosphatidylinositol 3-kinase, catalytic, α polypeptide (PIK3CA) mutations are most frequent in the luminal A subtype (Hoadley Katherine A., 2014; Saal et al., 2005). High-level DNA amplification has been shown to be very frequent in luminal B subtype of tumors including the regions on chromosome 8q and 11q containing the MYC oncogene and CCND1 (Cyclin D1) genes respectively. Amplifications of these genes might play a vital role in the higher proliferation rate of these tumors. Recurrent mutations are found in PIK3CA, GATA3, PTEN, and TP53 genes (Hoadley Katherine A., 2014).

As mentioned before, high-level amplification of the locus 17q harboring HER2/ERBB2 gene has been reported to be common in HER2 positive tumors. Tumors of the Her2 subtype have highest single nucleotide mutation rate, but a small number of recurrently mutated genes TP53 (71%) and PIK3CA (35%) (Cancer Genome Atlas, 2012).

Therapeutic strategies and responses for breast cancer subtypes

Molecular subtypes of tumors are predictive of clinical outcome. Individuals with luminal A subtype have a more favorable outcome while individuals with HER2 enriched and basal-like tumors generally have the worst consequences (Sorlie et al., 2001). Studies have also shown that these different molecular subtypes have distinct responses to therapy and thus different therapeutic approaches are taken to treat each of these subtypes. Luminal A and B subtypes of tumors are typically responsive to endocrine therapy [aromatase inhibitors (e.g., Anastrozole) and estrogen receptor modulators (e.g., Tamoxifen)]. However, luminal A tumors are less sensitive to chemotherapy while luminal B tumors have a higher pCR (pathological complete response) rate to neoadjuvant chemotherapy (administered before primary treatment of surgery or radiation to shrink tumor size). Luminal B tumors are also more sensitive to adjuvant chemotherapy (administered after primary treatments to control relapse) (Rouzier et al., 2005). Targeted therapy with trastuzumab, a monoclonal antibody against HER2 with a combination of chemotherapy is the most effective therapeutic strategy for HER2 positive cancers (Hayes et al., 2007). Very recently, combination therapy of palbociclib (an inhibitor of cyclin-dependent kinases CDK4 and CDK6) with targeted endocrine therapies for HER2 negative, hormonal receptor-positive advanced breast cancers have been approved by FDA (National Cancer Institute). The basal-like subtype has drawn a lot of attention from the researchers because unlike the other subtypes the basal-like subtype does not have any effective targeted therapy. The clinicians have to trust on systemic chemotherapeutic regimens, which mostly target cell divisions mechanisms like DNA replication and microtubule polymerization (Hayes, 2000).

Intratumor and intertumoral heterogeneity of breast cancer

Breast tumors like most of the tumors of other organs show both intratumor and intertumoral heterogeneity. Intratumor heterogeneity arises from the presence of cancer cells with variable phenotypes in a single tumor. The cells in a tumor typically show different degrees of basal-like and luminal features with gene signatures of luminal and basal cells of breast epithelium. This heterogeneity might arise from the intrinsic heterogeneity of the breast epithelial cells and the type of cells initiating the tumor (Polyak, 2011). However, it has also been shown that a particular cell population in a tumor might lead to multiple clone types owing to the accumulation of genetic alterations. Intertumoral heterogeneity arises from the presence of different cell types within tumors at varying frequencies (Polyak, 2011) **(Figure I.4, lower panel)**.

At the clinical level, the tumors are studied by pathologists for expression of hormone receptors or HER2, and a tumor is classified into a subtype depending on expression status of the major population of the cells, and a treatment therapy is designed. Thus, basal-like tumors mostly have cancer cells with a basal-like phenotype, whereas luminal tumors are composed mostly of luminal breast cancer cells. However, that might lead to a growth advantage of a rare clone of cells (Marusyk et al., 2014) which might be unaffected by the therapy and might lead to tumor relapse, with the relapsing tumor having an entirely different character. Thus, tumor heterogeneity is a considerable problem to clinicians as each tumor is a mixture of cell-types which might need different therapeutic intervention and no tumors are alike regarding cell type frequencies.

CCN FAMILY OF PROTEINS: AN OVERVIEW

The CCN family of proteins is comprised of six highly conserved homologous secreted proteins. The CCN acronym used to name the family owes its origin to the first three family members namely, cysteine-rich angiogenic inducer 61 (CYR 61, also known as CCN1), connective tissue growth factor (CTGF, also known as CCN2) and nephroblastoma overexpressed (NOV, also known as CCN3). The other three members of the family are WNT inducible signaling pathway proteins named WISP1 (or CCN4), WISP2 (or CCN5) and WISP3 (or CCN6) (S. K. Banerjee & Banerjee, 2012; Jun & Lau, 2011) **(Figure I.5)**. Each of the CCN family members had multiple names as multiple groups discovered the members independently while working with different biological systems. The various names are listed in the table below. Later, these proteins were identified to be either the same or other similar proteins sharing structural identity with CYR61, CTGF, and NOV (Holbourn, Perbal, & Ravi Acharya, 2009). A consensus was reached in the meeting of the International CCN society in 2000, and a unified nomenclature for the CCN proteins (CCN1-CCN6) was first described in the literature in 2003 (Brigstock et al., 2003). CCN proteins have been shown to be secreted from cells and localized in the extracellular matrix around the cells. Extracellular localization of the CCN proteins and their ability to modulate multiple cellular functions have led the investigators to classify the CCN proteins as matricellular proteins, a subset of extracellular matrix proteins which primarily plays regulatory roles rather than performing structural functions (Jun & Lau, 2011).

Structural similarities and differences of CCN proteins

All the six members of CCN family of proteins have similar modular structures with an amino-terminal secretory signal peptide. CCN5 (also known as WISP2) being an exception, all the other five CCN members have identical modular structures with four conserved domains. The

domains following the signal peptide in the amino-terminal end are the insulin-like growth factor binding protein (IGFBP) domain, the Von Willebrand factor C (VWC) domain, the thrombospondin type 1 repeat (TSR) domain and the cysteine knot carboxy-terminal (CT) domain (Holbourn et al., 2009; Jun & Lau, 2011). The domains of the CCN family proteins are named according to the proteins to which the domains bear sequence homology and structural similarity (Holbourn, Acharya, & Perbal, 2008; Holbourn et al., 2009). CCN5 lacks the fourth CT domain and thus has a lower molecular weight compared to other family members **(Figure I.5, lower panel)**. Individual exons encode each domain of the CCN family proteins, a common strategy found in multi-modular human proteins, which bears evolutionary proof for exon shuffling resulting in diverse protein products from reshuffled building blocks. Thus, a typical CCN protein prototype is encoded by a transcript carrying five exons followed by a long 3' untranslated region (3'UTR) (Kubota, Hattori, Nakanishi, & Takigawa, 1999). All the CCN proteins as suggested by the name of its first member cysteine-rich angiogenic inducer 61 (cyr61) are rich in cysteine residues. 38 conserved cysteine residues are spread across the four domains of the CCN proteins with exceptions to CCN5 and CCN6. CCN5 lacks the ten cysteine residues of the fourth CT domain, and CCN6 lacks the four cysteine residues in its VWC domain (Holbourn et al., 2008; Holbourn et al., 2009; Jun & Lau, 2011; Pennica et al., 1998). Each CCN molecule has a flexible variable linker or hinge region that separates the molecule into two halves, the amino-terminal half comprised of the IGFBP and the VWC domain and the C-terminal half composed of the TSP and the CT domain (Holbourn et al., 2009; Jun & Lau, 2011).The primary structural design and the arrangement of the domains are conserved in the CCN family proteins. However, there are significant sequence homologies between each of the

members, and all the members are unique which leads to a functional divergence (S. K. Banerjee et al., 2016; Holbourn et al., 2008) **(Figure I.5, lower panel)**.

Table representing diverse nomenclature of CCN family proteins:

CCN NOMENCLATURE	ALTERNATIVE NAMES USED IN LITERATURE
CCN1	CYR61, CTGF-2, IGFBP10, IGFBP-rP4, CEF10
CCN2	CTGF, IGFBP8, IGFBP-rP2, HBGF-0.8, ecogenin, FISP12, Hcs24
CCN3	NOV, NOVH, IGFBP9, IGFBP-rP3
CCN4	Wisp-1, Elm-1
CCN5	Wisp-2, CTGF-L, CTGF-3, HICP, Cop-1
CCN6	Wisp-3

Distinct biological roles of CCN family proteins

The high degree of homology and similar structure of the CCN proteins initially indicated that the proteins might have similar and redundant functions. However, it has been established that the members of the CCN protein family serve both distinct and overlapping biological roles (Holbourn et al., 2008; Jun & Lau, 2011) **(Figure I.5 lower panel)**. These highly conserved proteins have been shown to bind many of the same receptors owing to the conserved receptor binding sites and in many cases function through similar mechanisms to regulate a common set of biological processes. But, the specific physiological effects exerted by the CCN family proteins differ depending on the family member, interacting molecules, and the responding cell.

Multiple studies established that CCN proteins localized in extra-cellular matrix support cell adhesion and spreading in different cell types. The process of cell adhesion is mediated by the cell-surface integrin receptors which lead to other cellular responses like cell migration, proliferation and altered expression of specific downstream genes. For example, the human skin fibroblasts interact with CCN1 and CCN2 through α6β1 integrins and heparan sulfate proteoglycans (HSPGs). This interaction results in cell-adhesion and activation of adhesion-dependent cellular events including the rapid formation of focal adhesion complexes, actin cytoskeleton reorganization, the formation of filopodia and lamellipodia (Chen, Chen, & Lau, 2001).

As mentioned before the effects of the CCN proteins on cell proliferation and migration are cell-specific and context dependent. In a study on fibroblasts, it has been shown that the CCN1, CCN2 and CCN3 proteins enhance DNA synthesis that has been induced by other mitogenic growth factors. Interactions of the CCN proteins with the αVβ3 integrins have been implicated in this process (Chen & Lau, 2009). Similarly, in chondrocytes and osteoblasts, CCN2 promotes DNA synthesis though it has been shown to induce a G1 cell cycle arrest in mesangial cells (Jun & Lau, 2011). CCN1, CCN2, and CCN3 also stimulate migration in the fibroblasts and the endothelial cells and promote invasiveness of certain cancer cells (Chen & Lau, 2009). Interestingly, though CCN1 and CCN2 have been shown to enhance proliferation and migration of vascular smooth muscle cells (Fan, Pech, & Karnovsky, 2000; Grzeszkiewicz, Lindner, Chen, Lam, & Lau, 2002), CCN3 and CCN5 inhibit these processes (Jun & Lau, 2011; Lake, Bialik, Walsh, & Castellot, 2003).

Adhesion of the endothelial cells to the extracellular matrix mediated by CCN1, CCN2 or CCN3 through αVβ3 integrin supports cell survival (Chen & Lau, 2009). But these CCN

proteins as cell adhesion substrates in fibroblasts can also promote apoptosis by interacting with α6β1 integrin (Todorovic, Chen, Hay, & Lau, 2005). It has been shown that CCN1, CCN2, and CCN3 proteins can facilitate tumor necrosis factor (TNF) mediated apoptosis. These proteins can enhance the apoptotic activity of other TNF family cytokines such as the FAS ligand and TNF-related apoptosis-inducing ligand (Chen & Lau, 2010; Chen, Mo, & Lau, 2001; Jun & Lau, 2011). However, though under a different context and in different cells, other members of CCN family proteins have been shown to inhibit TNF mediated apoptosis (Jun & Lau, 2011). CCN1 can induce cellular senescence in fibroblasts by acting as a cell adhesion molecule through its binding to α6β1 integrin and cell-surface HSPGs. Studies also show that CCN1 activates the RAC1-dependent NADPH oxidase 1 to induce reactive oxygen species (ROS) production, leading to activation of the cellular tumor antigen p53 and the retinoblastoma-associated protein (pRb), resulting in senescence (Jun & Lau, 2010b). It has been proved that CCN1-induced senescence of the fibroblasts functions as a critical mechanism for limiting fibrosis during wound healing (Jun & Lau, 2010a, 2011).

CCN proteins are established as potent angiogenic inducers, as some of the members of the CCN family promote proliferation, chemotaxis and induce tubule formation in the endothelial cells. This effect is mediated through direct binding of CCN proteins to αVβ3 integrins (Babic, Kireeva, Kolesnikova, & Lau, 1998; Jun & Lau, 2011; Kubota & Takigawa, 2007; Maity et al., 2014). CCN proteins have also been shown to regulate angiogenesis by influencing expression level and activities of vascular endothelial growth factors (VEGFs). Regulation of angiogenesis by the CCN proteins lead to the critical roles played by these proteins in embryonic development and tumorigenesis (Inoki et al., 2002; Kubota & Takigawa, 2007).

CCN proteins have been shown to directly bind to Bone Morphogenetic Proteins (BMPs) and influence differentiation of osteoblasts and chondrocytes, thus playing a critical role in skeletal development (Jun & Lau, 2011). A study on CCN2 protein first indicated that the CCN proteins could interact with members of the BMP and TGFβ family, regulating the binding affinity of BMPs and TGFβ for their respective receptors (Abreu, Ketpura, Reversade, & De Robertis, 2002). However, studies indicate that the roles played by CCN proteins in this context are also diverse and oppose each other. It has been observed that CCN2 promotes differentiation of chondrocytes and osteoblasts favoring the development of bone and cartilage (Kubota & Takigawa, 2007). But, independent studies have also shown both CCN2 and CCN3 can bind to BMP2 and negatively regulate its functions in promoting chondrogenic and osteogenic differentiation (Maeda et al., 2009; Minamizato et al., 2007). Interestingly, CCN4 can bind to BMP2 enhancing its function in osteogenesis (Ono, Inkson, Kilts, & Young, 2011).

The functions of CCN proteins, as discussed earlier, represent only a few of the diverse roles played by the family. Most of the CCN functions depend on integrin binding and diversifies depending on the types of integrins expressed by the cell type. Also, binding of CCN proteins to cell-matrix and growth factors in the cell environment leads to alteration of the cellular responses. Thus, these direct and indirect influences exerted by the CCN proteins play a crucial role in modulating the outside-in cell signaling mediated by cell surface receptors including integrins.

Identification of CCN5 (Wisp2) gene

The rat orthologue of CCN5 (WISP-2), rCop-1 was first identified in Rat Embryonic Fibroblasts (REFs) as a new member of the CCN family of genes in the year 1998. The study found a significant correlation between loss of expression of the CCN5 with cell transformation by inactivation of P53 and concomitant expression of a constitutively active H-Ras. Thus, this study suggested that CCN5 may be a negative regulator of tumorigenesis (R. Zhang et al., 1998). In the same year, CCN5 (WISP-2) and CCN4 (WISP-1), was identified in a genetically engineered mouse model. These studies indicated that CCN5/WISP-2 is a Wnt inducible gene (Pennica et al., 1998). The study also showed expression of these genes in the adult human tissues and mapped human CCN5 gene in the long arm of the chromosomal 20 at cytogenetic location 20q12-q13.1. Moreover, this study also found a significant downregulation of CCN5 expression in about 80% of the human colon tumor tissues (Pennica et al., 1998). Subsequently, two independent studies reported that the CCN5/WISP2 is a serum and estrogen-inducible gene (Inadera et al., 2000; Zoubine, Banerjee, Saxena, Campbell, & Banerjee, 2001).

The transient expression of CCN5 has been detected in fetal lung, adult skeletal muscle, colon, ovary, and breast (S. K. Banerjee & Banerjee, 2012; S. K. Banerjee et al., 2016; Das et al., 2017). CCN5 has been implicated in having an important role in carcinogenesis, with relevance to human breast disease (S. K. Banerjee & Banerjee, 2012; S. K. Banerjee et al., 2016; Das et al., 2017). Most studies show that, CCN5 expression correlate inversely with the aggressiveness of cancers in breast (S. K. Banerjee & Banerjee, 2012; Ferrand, Stragier, Redeuilh, & Sabbah, 2012; Fuady et al., 2014), pancreas (G. Dhar et al., 2007), salivary gland (Kouzu et al., 2006), gallbladder (Z. Yang et al., 2014) and gastric tissue (Ji et al., 2015). This observation suggests

tumor suppressor/anti-invasive activity of CCN5 (S. K. Banerjee & Banerjee, 2012; S. K. Banerjee et al., 2016; J. W. Russo & Castellot, 2010).

The CCN5 expression is detected in both normal and non-invasive breast cancer cell lines and tissue samples (i.e., ADH and DCIS) (S. K. Banerjee & Banerjee, 2012; Das et al., 2017). CCN5-overexpressed breast cancer cells are less aggressive compared to CCN5-under expressed or negative breast cancer cells (S. K. Banerjee & Banerjee, 2012; S. K. Banerjee et al., 2016; Das et al., 2017). CCN5 expressing breast cancer cells (e.g., MCF-7, BT-474, ZR-75-1, T-47D) are always ER-α positive, while CCN5-negative cells are mostly triple-negative (Human cell lines: MDA-MB-231, MDA-MB-468, HCC-70, BT-20, MCF-DCIS and Mice cell lines: MVT-1 and 4T1) (S. K. Banerjee & Banerjee, 2012; S. K. Banerjee et al., 2016; Fritah, Redeuilh, & Sabbah, 2006), and are enriched with tumor-initiating cells (TICs)/cancer stem cells. Thus, at least in breast cancer, CCN5 can be considered a good prognostic marker (Das et al., 2017). However, further supporting data are required to establish the hypothesis, and thus, the current studies are the milestones to achieve these goals.

Signaling pathways regulating CCN5 expression

Since the discovery of the CCN5 protein, multiple studies have focused on the signaling pathways that directly or indirectly influence CCN5 expression. Apart from the evidence for direct transcriptional regulation of CCN5 by Wnt and estrogen-mediated signaling; indications for P53 mediated repression and influences of growth factor signaling have also been shown by different independent studies as mentioned earlier.

WNT SIGNALING MEDIATED REGULATION OF CCN5 EXPRESSION: Previous studies, which was first identified CCN5 gene in a mouse mammary tumor model, indicated that CCN5

is the second family members of Wnt-1-induced signaling proteins, and thus coined the name of this protein was WISP-2 (Pennica et al., 1998). Although the mechanism of regulation of CCN5/WISP-2 expression is unclear, a study demonstrated direct transcriptional regulation of CCN5 by β-catenin in fibroblast cells (Tanaka, Morikawa, Okuse, Shirakawa, & Imai, 2005). Moreover, physiological activators of Wnt signaling have also been shown to induce CCN5 expression in different cell lines. Upregulated expression of the Wnt-1 protein in the hepatocellular carcinoma cell line Huh-7 and treatment of osteoblast cell line with a small molecule inhibitor of GSK-3β enzyme caused an increase in CCN5 mRNA transcription. Therefore, we can anticipate that CCN5 regulation by Wnt1 could be mediated through GSK-3β/ β-catenin pathway.

ROLE OF ESTROGEN MEDIATED SIGNALING IN EXPRESSION OF CCN5: Role of estrogen-mediated regulation of CCN5 expression was evident from early studies carried out independently in different labs (S. Banerjee et al., 2003; Inadera, Dong, & Matsushima, 2002; Inadera et al., 2000; Saxena, Banerjee, Sengupta, Zoubine, & Banerjee, 2001; Zoubine et al., 2001). In differential expression studies, it was noticed that CCN5 is significantly overexpressed in estrogen receptor-positive breast cancer cell lines compared to other normal and cancer cell lines of the breast (Saxena et al., 2001; Zoubine et al., 2001). Also, it was shown that treatment of ER-α-positive cells with estrogen leads to increase in mRNA transcript and protein levels of CCN5 (S. Banerjee et al., 2003; Inadera et al., 2002; Inadera et al., 2000). Addition of 17β-estradiol to MCF-7 cells in culture increases expression of CCN5 mRNA and protein in a dose-dependent and time-dependent manner which can reach maximum levels by 72 h of treatment (S. Banerjee et al., 2003; Inadera et al., 2002; Inadera et al., 2000). The hormones that interact specifically with ER-α can induce the expression of CCN5. Several other hormones and steroids

(e.g., dexamethasone and tri-iodothyronine) do not affect CCN5 expression, whereas it has been shown that different xenoestrogens can induce CCN5 expression (Inadera et al., 2002). Also, upregulation of CCN5 expression by estrogen was entirely blocked by the pure anti-estrogen inhibitor, ICI 182,780 (S. Banerjee et al., 2003; Inadera et al., 2000). Incidentally, one of these early studies extended this observation to a pathologic context by showing a strong positive correlation between expression of CCN5 protein and estrogen receptor alpha (ER-α) positivity in human breast cancer sample (S. Banerjee et al., 2003). Furthermore, studies have shown that CCN5 gene promoter has estrogen response elements (EREs). ER-α which is the direct mediator of estrogen action binds to the CCN5 promoter in an estrogen-dependent fashion (Fritah et al., 2006).Progesterone was also shown to induce CCN5 protein in ER-positive human breast cancer cells (S. Banerjee et al., 2003).

ROLE OF SIGNALING PATHWAY CROSSTALKS IN CCN5 EXPRESSION MCF-7 breast cancer cells treated with the Protein Kinase A activator, CT/IBMX (cholera toxin plus 3-isobutyl-1-methylxanthine) exhibits an increase in CCN5 mRNA level. However, the same study established that treatment of MCF-7 cells with 12-O-tetradecanoyl phorbol-13-acetate (TPA), a protein kinase C (PKC) activator, completely prevented CCN5 mRNA induction by estrogen although it did not affect another estrogen-responsive gene, pS2 (Inadera, 2003). It has been speculated that the effects of PKA signaling on CCN5 expression might be exerted through decreased expression of the microRNA, miR449, which has been bio-informatically shown to be able to target CCN5 mRNA (Iliopoulos, Bimpaki, Nesterova, & Stratakis, 2009; J. W. Russo & Castellot, 2010). However, in a contradicting study, another PKC activator phorbol 12-myristate 13-acetate (PMA) has been shown to upregulate CCN5 expression through the involvement of the MAPK mediated signaling pathways (Sengupta et al., 2006).

Activation of MAPK pathway and PI3K-AKT mediated signaling pathway have been implicated to play a role in the regulation of CCN5 expression. Epidermal growth factor (EGF) induces expression of CCN5 mRNA in MCF-7 cells in a dose- and time-dependent manner and can act synergistically with estrogen. The effect was possibly mediated through activation of the PI3K, and MAPK signaling pathways as the use of inhibitors (wortmannin and U0126 respectively) against the mentioned pathways abrogated the effect of EGF treatment on CCN5 expression (S. Banerjee, Sengupta, Saxena, Dhar, & Banerjee, 2005). A similar set of studies also established that IGF-1 could induce CCN5 mRNA expression in a dose and time-dependent manner and knockdown of CCN5 abrogate the ability of IGF-1 to stimulate MCF-7 cell proliferation (K. Dhar, Banerjee, Dhar, Sengupta, & Banerjee, 2007). However, the IGF-1 mediated stimulation of CCN5 expression was shown to be dependent on activated MAPK pathway only, as opposed to EGF mediated activation, where both MAPK and PI3K mediated signaling were shown to play roles. Both EGF and IGF-1 mediated expression of CCN5 was dependent on estrogen-mediated signaling as the effects were abrogated by treatment with anti-estrogen ICI 182-780.

REPRESSIVE ROLE OF P53 PROTEIN LEVEL ON CCN5 EXPRESSION: An inverse relation between the expression level of CCN5 and P53 proteins has been indicated in multiple studies involving cancer cell lines and tissues. Pancreatic adenocarcinoma samples showed a significant inverse relationship between the expression level of P53 and CCN5 (G. Dhar et al., 2007). Similarly, ectopic expression of P53 mutants in the ER-α positive breast cancer cell lines MCF-7 and ZR-75-1, downregulated CCN5 mRNA expression, eliciting a possible repressive role played by P53 on CCN5 expression (G. Dhar et al., 2008). Notably, an increase of P53 protein level is frequently associated with stabilizing mutations of P53 where the mutants play a dominant negative role leading to oncogenic transformation.

ROLE OF HIF-2α IN CCN5 EXPRESSION: Hypoxia-inducible factor-2α (HIF-2α) has been implicated in the regulation of CCN5 expression in multiple studies (Aprelikova, Wood, Tackett, Chandramouli, & Barrett, 2006; Fuady et al., 2014; Stiehl et al., 2012). CCN5 was identified as one of the few genes in MCF7 which was preferentially regulated by HIF-2α rather than HIF-1α under hypoxic conditions. It was also shown that transcription factor ELK-1 binds in the promoter region of CCN5 at a position close to the hypoxia response elements (HREs) and directly interacts with the HIF-2α protein in driving CCN5 expression (Aprelikova et al., 2006). Another study also claimed that HIF-2α/AREG/WISP2-expressing tumors were associated with luminal tumor differentiation and are expected to show a better response to standard treatments and thus validated their report of more prolonged patient survival in breast cancer with high levels of HIF-2α (Stiehl et al., 2012). Another study also showed that high WISP-2 tumor levels were associated with increased HIF-2α expression and a better prognosis. Also, a gain of aggressive phenotypes of breast cancer cells can be phenocopied by silencing HIF-2α expression (Fuady et al., 2014).

Regulation of cellular functions by CCN5

As mentioned in a previous section in this chapter CCN proteins have been shown to modulate multiple cellular functions through their interactions with the cell surface receptors (mostly integrins) and their direct binding capability with ECM components and other growth factors. CCN5 though lacking the CT domain can influence an array of these cellular functions as elucidated by multiple studies performed in different cell lines.

- PROLIFERATION: Overexpression of CCN5 or CCN5 treatment in culture media has been shown to induce growth arrest in vascular smooth muscle cells (VSMCs); and also

in human uterine normal myometrial and uterine leiomyoma tumor cells (Delmolino, Stearns, & Castellot, 2001; Lake et al., 2003; Lake & Castellot, 2003; Mason, Lake, Wubben, Nowak, & Castellot, 2004). Thus, CCN5 was widely referred to as a growth-arrest-specific gene by these groups. A similar anti-proliferative effect of CCN5 was noticed in studies with human umbilical vein endothelial cells (HUVECs) and murine pre-adipocyte cell line (Inadera, Shimomura, & Tachibana, 2009; J. W. Russo & Castellot, 2010).As reported by multiple studies and discussed in the next chapter here, CCN5 expression and treatment with CCN5 leads to inhibition of proliferation of triple negative breast cancer cell line MDA-MB-231 both in in-vitro and in-vivo xenograft models (Fritah et al., 2008; Haque et al., 2015).

- MOTILITY AND MIGRATION: Cellular motility and migratory capabilities of cells hold keys for invasive cellular properties. It has been implicated to play a role in multiple disease progression including cancer. CCN5 has been known to play a critical role in inhibiting cellular motility in numerous cell culture studies. In rat vascular smooth muscle cells and smooth muscle cells derived from human uterine myometrial tissue, alteration of CCN5 expression level shows a negative correlation between CCN5 expression and cellular motility (Lake et al., 2003; Lake & Castellot, 2003; Mason et al., 2004). Similarly, CCN5 has been extensively studied as an anti-invasive gene in breast cancer in multiple laboratories (S. K. Banerjee & Banerjee, 2012; Fritah et al., 2008). Studies with triple negative breast cancer cell line MDA-MB-231 has shown evidence for the capability of CCN5 to induce an adverse effect on cellular motility. Ectopic expression of the CCN5 protein in MDA-MB-231 cells and knockdown of CCN5 expression in MCF7 cells lead to an increase in cellular motility and migration in-vitro

(S. Banerjee et al., 2008; Fritah et al., 2008). Similarly, treatment of MDA-MB-231 cells with MCF7 cell conditioned medium (rich in secreted CCN5) led to a lesser motility of the MDA-MB-231 cells as exhibited by Boyden chamber assay. However, such an anti-motility effect on MDA-MB-231 cells was not noticed when treated with conditioned medium of CCN5 ablated MCF7 cells (S. Banerjee et al., 2008; Fritah et al., 2008). Also, another study published in the same year showed that pre-treatment of mutant P53 transfected MCF7 cells with recombinant CCN5 protein leads to a decrease of cellular motility in a Boyden chamber assay (G. Dhar et al., 2008).

Both cellular phenomena, proliferation, and motility, are key hallmarks of the cancer cells. Thus, restriction of aberrant proliferation and motility of cancer cells can be two critical roles of CCN5 protein that can be implemented in designing new direction of therapeutics.

CCN5 as a micromanager of breast cancer progression

As indicated in the previous sections of this chapter, the role of CCN5 in breast cancer progression has been studied extensively in-vitro which suggests that CCN5 might play a critical negative role in breast cancer pathogenesis. Studies indicate that loss of CCN5 might be an essential step in gaining of plasticity, motility and invasiveness of breast cancer cells and multiple genetic insults including gain of P53 mutations have been implicated in loss of CCN5 expression (S. Banerjee et al., 2008; S. K. Banerjee & Banerjee, 2012; G. Dhar et al., 2008; Fritah et al., 2008). The expression of CCN5 was observed to be high in precursor lesions of human breast cancer at stages of Atypical Ductal Hyperplasia (ADH) and Ductal Carcinoma In-Situ (DCIS) while its expression gradually decreases to undetected or minimal level from moderately differentiated to poorly differentiated invasive breast cancer samples (S. Banerjee et

al., 2008; S. Banerjee et al., 2003). In a similar study, a negative correlation between CCN5 staining and P53 staining in human breast cancer sections was reported (G. Dhar et al., 2008). As previously discussed, modulation of CCN5 level in breast cancer cells influences the proliferative capacity and the motility of the cells in-vitro (S. Banerjee et al., 2008; Das et al., 2017; Fritah et al., 2008). Expression of matrix metalloproteinases (MMPs) has been implicated in playing a pivotal role in gaining invasive potential of breast cancer cells. Ablation of CCN5 expression has been shown to upregulate the expression level matrix metalloproteinases, MMP2 and MMP9 in MCF7 cells (S. Banerjee et al., 2008). One of the most widely studied phenomena implicated in gaining of invasive potential of breast cancer cells is epithelial to mesenchymal transition (EMT). Though debated, most of the researchers believe and have been successful in partially proving that cancer epithelial cells lose their epithelial characteristics of apicobasal polarity, basement membrane adhesion and cell-cell junctions in the course of time to express a more motile mesenchymal phenotype. This phenotype helps the cells to sculpt through the matrix and invade surrounding tissues. Independent studies have shown that downregulation of CCN5 expression in MCF7 cells leads to decrease of expression of markers for the epithelial phenotype (e.g., E-cadherin) (S. Banerjee et al., 2008; Ferrand et al., 2014). Conversely, treatment of MDA-MB-231 cells leads to upregulation of epithelial marker expression while a robust decrease of mesenchymal markers was noticed (Das et al., 2017). Two studies provided further insights into the mechanisms of CCN5 mediated regulation of EMT.

It was indicated in one study that CCN5 negatively regulates the miR-10b levels in breast cancer cells. miR-10b has been shown to play a critical role in the initiation of metastasis (Ma, Teruya-Feldstein, & Weinberg, 2007). It was shown in the study that depletion of CCN5 in breast cancer cells leads to stabilization and activation of HIF-1α (hypoxia-inducible factor-1α)

which results in Twist-1 mediated (J. Yang et al., 2004) expression of miR-10b (Haque et al., 2011). Another study implicated the role of CCN5 in negative regulation of TGF-β signaling pathway. It was shown that downregulation of CCN5 expression in breast cancer cells leads to a robust upregulation of expression of TGF-β receptor-II (TGF- β-RII). The study concludes from its observations that CCN5 might act as a direct transcriptional repressor of TGF- β-RII gene by binding to its promoter region, which leads to repression of TGF-β signaling mediated EMT of cancer cells (Sabbah et al., 2011).

In very recent studies, loss of CCN5 expression has been implicated in a gain of stem cell-like characteristics of breast cancer cells *(Das et al., 2017; Ferrand et al., 2014)*. Cancer stem cells are believed to be a subpopulation of cancer cells which have been sighted as one of the key reasons for tumor relapse. Though the origin of the cancer stem cells remains highly debated, it has been argued that stem-like cancer cells can arise from the epithelial cells upon accumulation of oncogenic mutations. It has been shown that loss of CCN5 in MCF7 breast cancer cells can promote the emergence of a cancer stem cell-like phenotype as the cells showed a high expression of CD44 and increased activity of aldehyde dehydrogenase activity, which are signatures of breast cancer stem cells. Also, inoculums of a lower number of CCN5 negative cells were capable of tumor formation after injection into the mammary fat pad of immunodeficient mice (Ferrand et al., 2014). It was shown in another study, that CCN5 expression was minimal in the subpopulation of MCF7 cells which showed enrichment of gene expression of stem cell-like signature. This population of cells exhibited a lower expression of the epithelial marker proteins and a higher expression of mesenchymal markers which however was altered after ectopic expression of CCN5. It was also shown that, compared to the CCN5 positive population, the CCN5 negative MCF7 cells possess higher tumorigenic potential when

injected subcutaneously in nude mice. However, ectopic expression of CCN5 in the CCN5 negative subpopulation of cells ablated higher tumorigenicity of those cells (Das et al., 2017).

OVERALL OBJECTIVES AND SIGNIFICANCE OF THE STUDY

At present, there are immense gaps in our understanding of the mechanisms by which CCN5 destroys TNBC growth and metastasis. This Ph.D. dissertation has addressed these deficiencies carefully and, provided new mechanistic insights into the function of CCN5 in preventing or delaying the growth and aggressive phenotypes of TNBC. Since CCN5 downregulation is detected in various cancers, its study will be relevant to other cancers as well. Of note, because restoring CCN5 prevents TNBC growth and progression with no or minimal side effects, small molecule drugs can be designed to restore CCN5 in TNBC for therapeutic utility.

Based on the premise, we ***hypothesized that CCN5 could induce cell-cycle arrest in TNBC cells and HER2 positive tumors***. In the following chapters, in support of the hypothesis, we have proved using in-vitro cell line studies and in-vivo mice model studies that CCN5 expression can play a critical role in delaying tumor progression by inducing arrest at cell-cycle checkpoints. We have also proved that CCN5 induces expression of ER-α in normal and malignant breast epithelial cells which leads to the therapeutic sensitivity of the cells. Thus, in the immediate next chapter (second chapter) we have elaborated the mechanism by which CCN5 induces cell cycle arrest in triple negative cancer cells. In the third chapter, we extended our hypothesis from the observations of the first chapter and elucidated the mechanism by which CCN5 induces expression of ER-α in breast epithelial cells both in-vitro and in CCN5 transgenic

mice model. Finally, in the fourth chapter, our transgenic mice studies unravel a novel mechanism by which CCN5 delays the progression of tumor formation in HER2 transgenic mice.

FIGURES

Fig.I.1

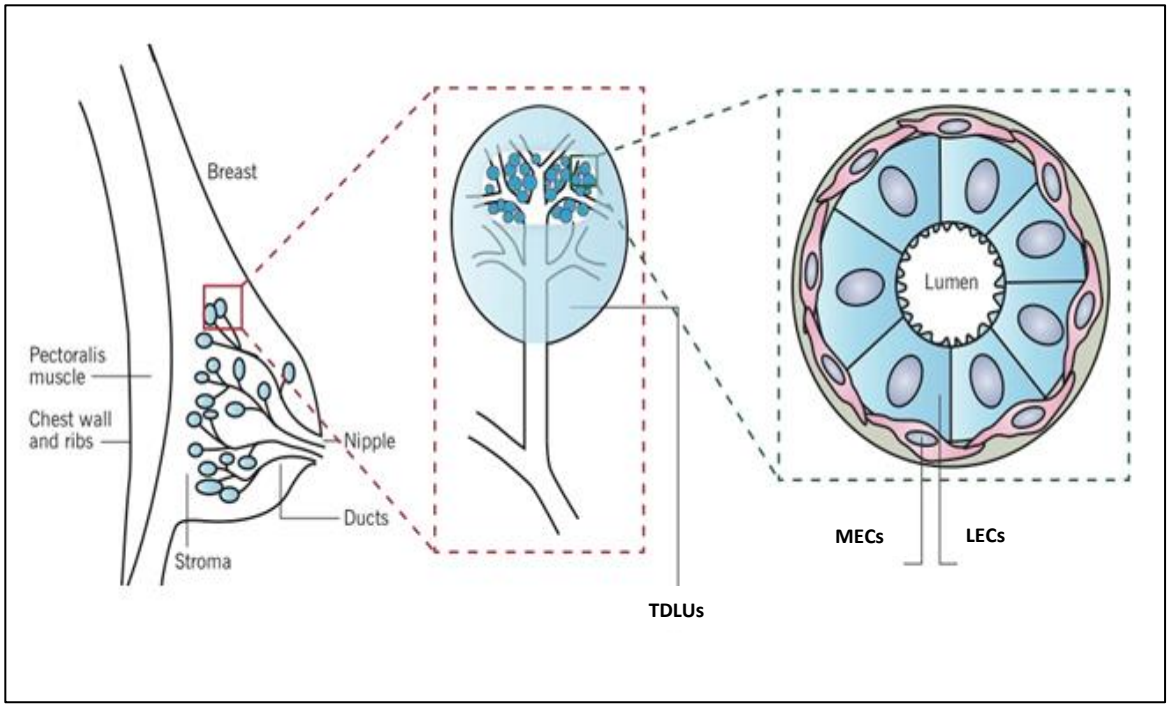


Fig.I.1: Breast cancer structural arrangement and epithelial bilayer: The human breast ductal network is comprised of 10-20 main ducts that drain out through individual openings in the nipple. The ducts branches into subsegmental ducts that ends into Terminal Ductal Lobular Units (TDLUs). Each of the ducts and the alveoli is lined by two layers of epithelial cells – Luminal epithelial cells (LECs), which lines the lumen and myoepithelial cells, between the LEC layer and the basement membrane. (Used with permission from Eric Wong and Sultan Chaudhry. McMaster Pathophysiology Review, www.pathophys.org.)(E. Wong, 2012).

Fig.I.2

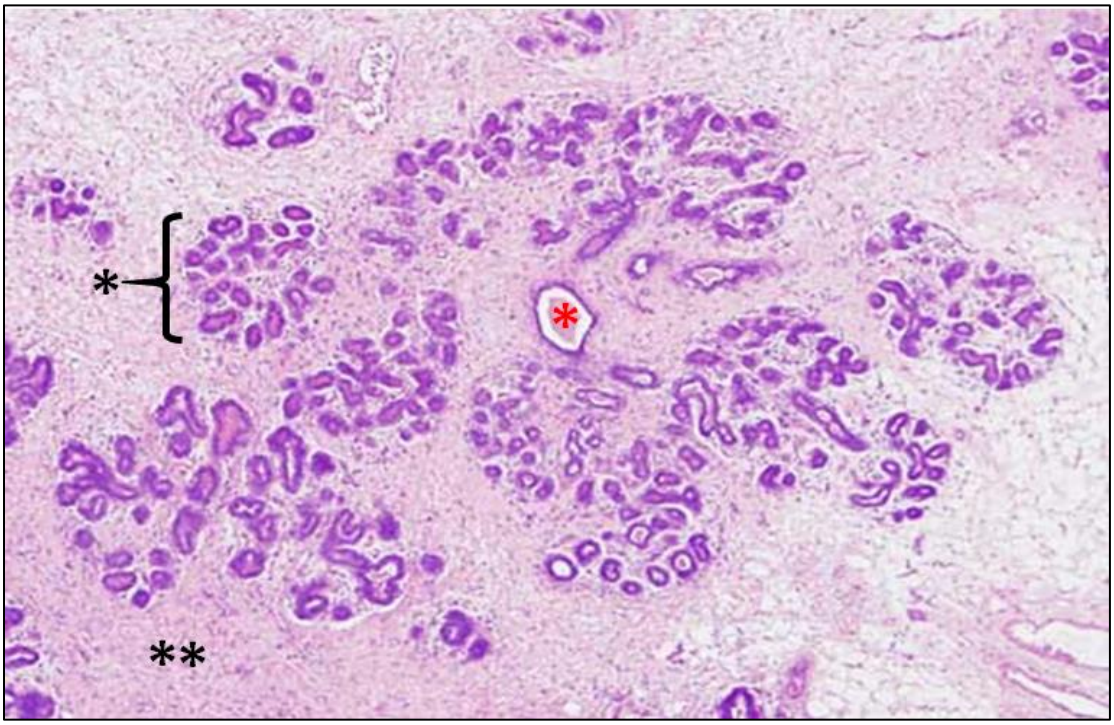


Fig.I.2: Histologic appearance of human breast ductal and lobular architecture: Hematoxylin and eosin staining of an adult human breast tissue (non-lactating). (*)- denotes a single lobular unit (**)- indicates the fibrous connective tissue interspersed between lobules (*)- denotes cross sectional view of a mammary duct. The original image has been used from websource www.breastpathology.info (Thomas, 2006).

Fig.I.3

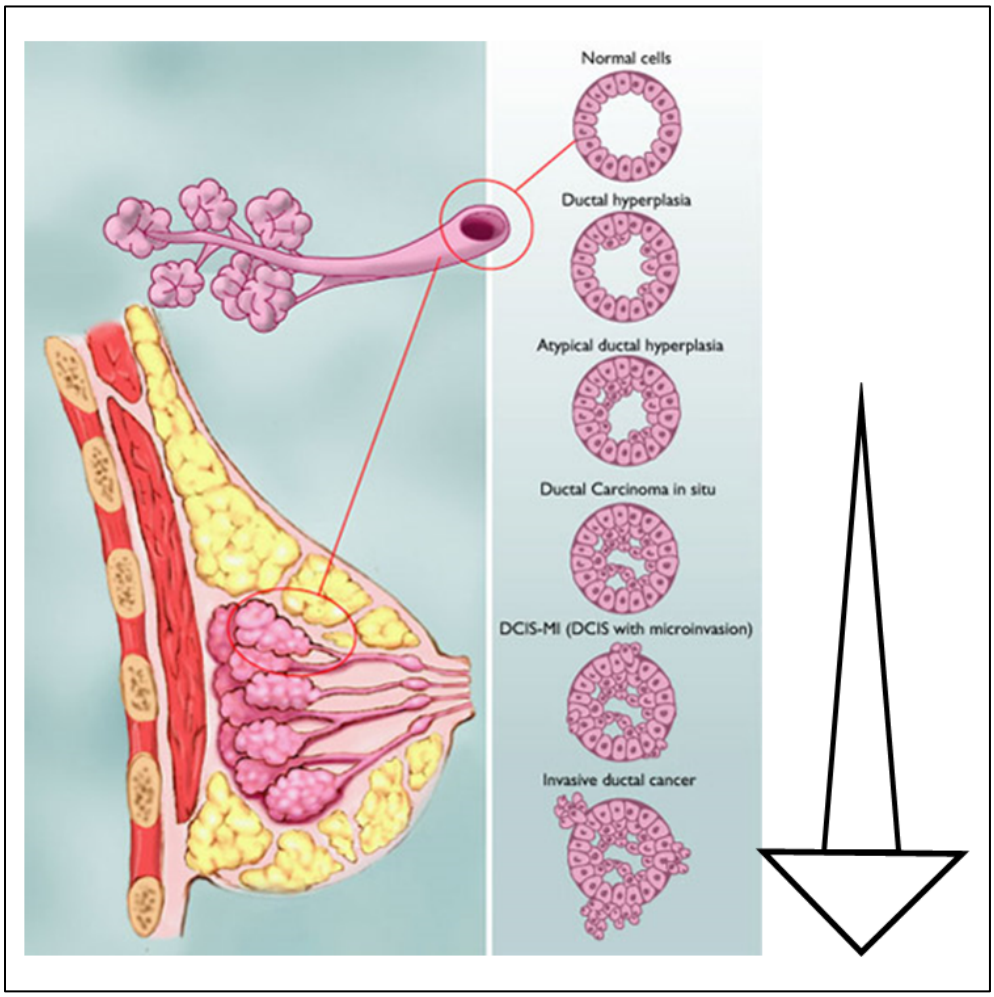
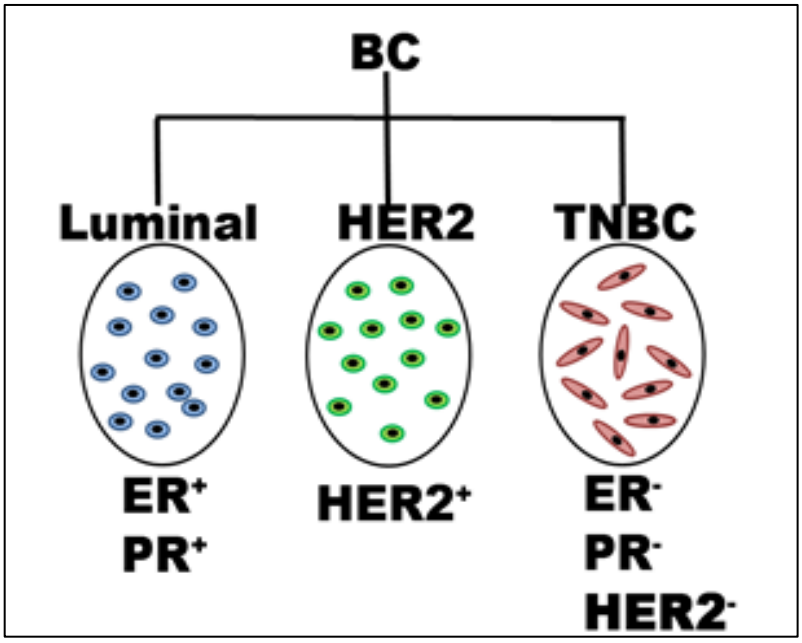


Fig.I.3: Progression of breast cancer: The figure depicts the gradual progression of breast cancer in a breast ductal epithelium from hyperplasia to invasive carcinoma. The arrow denotes the gradual progression of breast carcinoma from DCIS stage to Invasive cancer. Original figure source has been derived from webpage www.breastcancer.org (Breast Cancer.org, 1999)

Fig.I.4



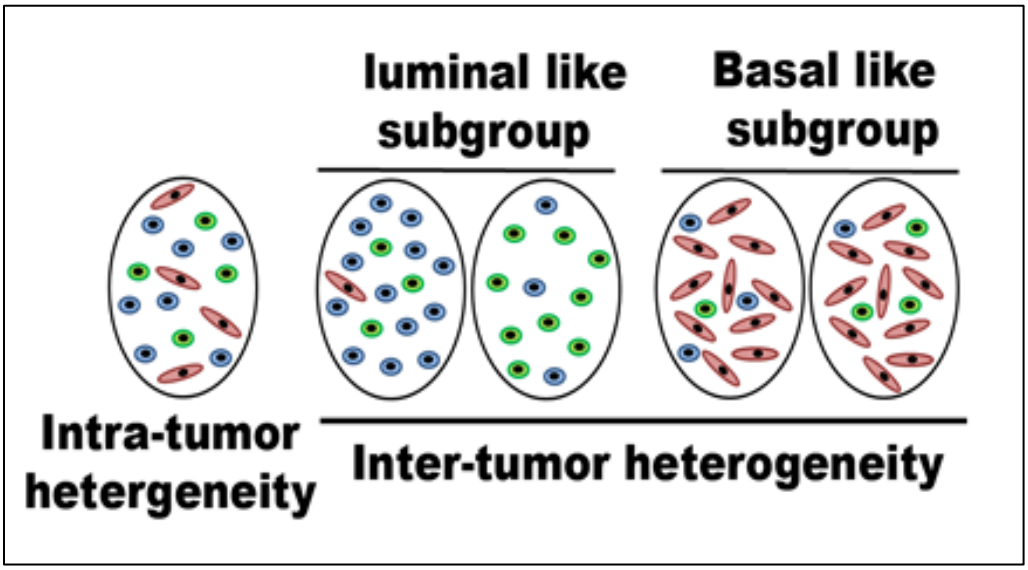


Fig.I.4: Clinical subtypes of breast cancer and heterogeneity: The figure (upper panel) illustrates the three clinical subtypes of breast cancer (lower panel) depicts intra-tumor and inter tumor heterogeneity of cell types.

Fig.I.5

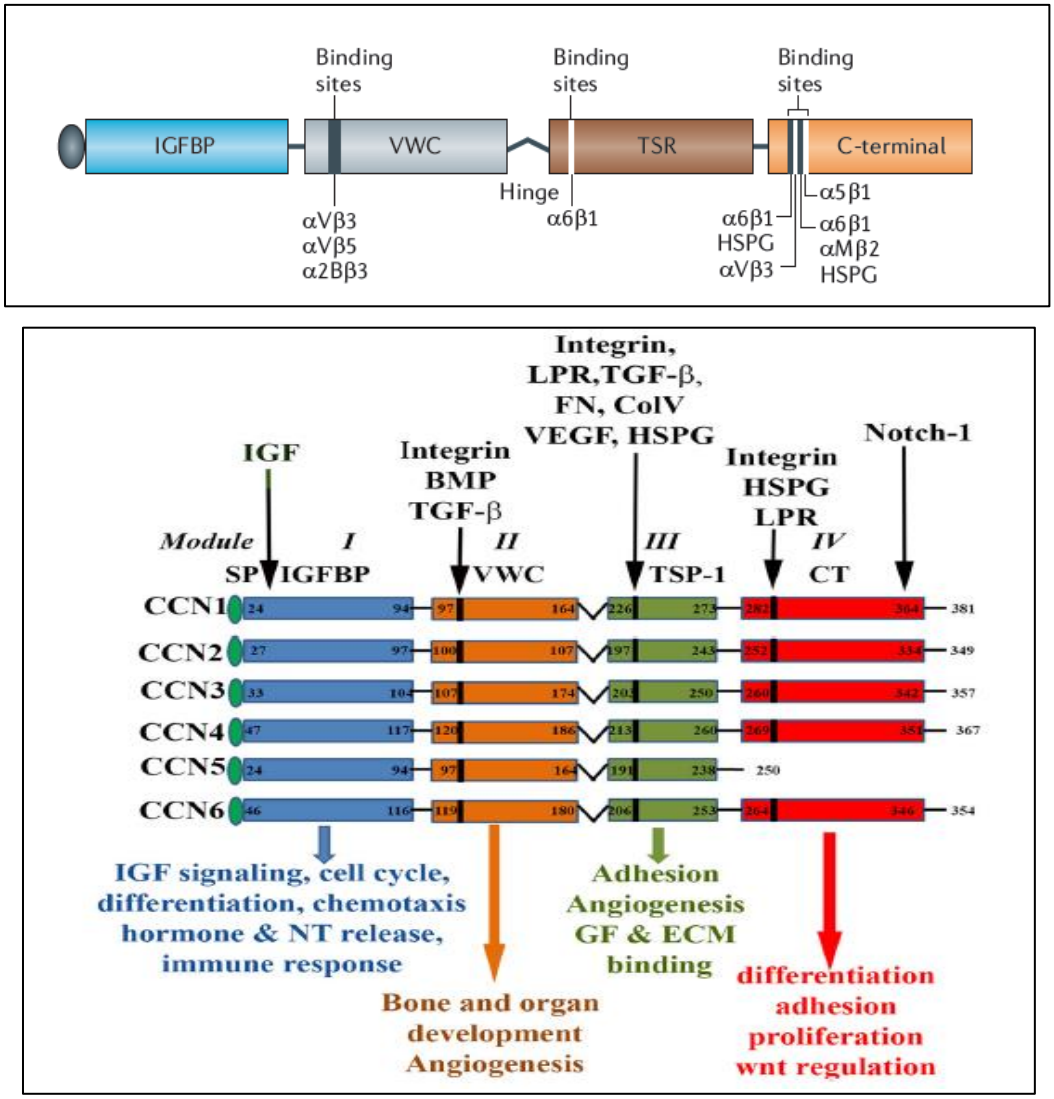


Fig.I.5: Domain structure of a typical CCN protein (upper panel) and individual CCN members (lower panel): The figure (upper panel) shows the domains of a typical CCN protein with the conserved integrin binding sites and corresponding integrin types (Jun & Lau, 2011). The figure (lower panel) also depict domain arrangement of individual CCN members with amino acid positions, the direct interacting partners and down-stream actions (S. K. Banerjee et al., 2016). Both figures used from the cited articles with permissions. [IGF-Insulin-like growth factor; SP-Signal Peptide; BMP-Bone morphogenetic protein; FN-Fibronectin; LRP-Lipoprotein receptor related protein; FN-Fibronectin; HSPG-heparan sulfate proteoglycan; GF-growth factors; TGFβ- tumor growth factor-β; VEGF- Vascular endothelial growth factor.; ECM-extra cellular matrix.]

CHAPTER II: Role of CCN5 in promoting growth arrest of Triple Negative Breast Cancer (TNBC) cells.

INTRODUCTION

KIP1/CIP1 proteins as cyclin-dependent kinase inhibitors

Cell cycle progression in normal non-cancerous cells is a multistep process. It is finely tuned by the coordinated activities of different cell-cycle regulator protein-complexes. These regulatory proteins are mostly composed of cyclin family of proteins, whose actions are dependent on their ability to bind their corresponding cyclin partners to form an active complex leading to the ramifying downstream influences on the effector proteins (Besson, Dowdy, & Roberts, 2008). Another group of proteins named cyclin-dependent kinase inhibitors (CKIs/CDKIs) bind to the CDK proteins to inhibit their catalytic activities leading to cell cycle arrests at definite cell cycle stages (Besson et al., 2008) and thus act as brakes to the cell cycle machinery. The cells without the CDKI activities lead to uncontrolled CDK-cyclin complex formation and actions leading to uncontrolled cell cycle progression associated with common human cancers (Coqueret, 2003). There are two major families of CDK inhibitors (CKIs) namely INK4 family and KIP/CIP family of proteins who serve a critical function of regulating the cell cycle progression by their association with cyclin-dependent kinases (CDKs). The INK4 family members (P16INK4A, P15INK4B, P18INK4C, and P19INK4D) bind to CDK4 and CDK6 and block their association with D-type cyclins leading to inhibition of the kinase activity of CDK4 and CDK6. In contrast to the INK4 family members, CKIs from the CIP/KIP family (P21CIP1, P27KIP1, and P57KIP2) have capabilities to bind to all cyclin–CDK complexes and primarily inhibit the kinase activity of CDK2 and CDK1 (Otto & Sicinski, 2017). Growth inhibitory signals are known to block the G1-S transition of cells by upregulating the expression of INK4 and CIP/KIP family members.

P27KIP1, a non-classical tumor suppressor

P27 also known as KIP1 protein belongs to the KIP/CIP family of proteins and has been one of the most widely studied CKI protein since it's identification. Being encoded by the CDKN2B gene in human, P27 protein is related to another cyclin-dependent kinase inhibitor P21(Toyoshima & Hunter, 1994). The P27Kip1 protein has been reported to be localized in both the nucleus and cytoplasm of a cell. The nuclear component of P27Kip1 participates in all cell cycle phases preventing the activities of all cyclin–CDK complexes as needed without dissociating them. It is well established that downstream effectors of multiple signaling pathways and various microRNAs regulate the expression level of P27Kip1 for cell cycle regulation in normal and malignant cells (Chu et al., 2008; Nourse et al., 1994; Polyak et al., 1994; Sherr & Roberts, 1999). The protein P27Kip1 is widely considered as a tumor suppressor protein (Bloom & Pagano, 2003; Chu et al., 2008; Slingerland & Pagano, 2000). Moreover, various mouse model studies have further established the tumor suppressor function of the P27Kip1 protein (Besson, Assoian, & Roberts, 2004; Chu et al., 2008). Although from a functional perspective view, P27Kip1 protein has been classified as an atypical tumor suppressor protein, there have been contradictory findings. Multiple studies have found evidence that directs to the fact that in some instances P27Kip1 plays a decisive role in tumorigenesis in various organs including the colon, esophagus, prostate, and breast (Besson et al., 2004). It has been found out from further studies that the cellular localization of P27Kip1 plays a vital role in its function and cytoplasmic accumulation of the protein is liable for tumorigenesis (Baldassarre et al., 1999; Chu et al., 2008; Sgambato, Cittadini, Faraglia, & Weinstein, 2000). Accumulation of cytoplasmic P27Kip1 might just lower the nuclear concentration of P27Kip1 and put a check in its tumor suppressive function, or cytoplasmic P27Kip1 might directly exert oncogenic functions or both. These

functions may suppress cellular apoptosis and increase cell motility thus leading to the pro-tumorigenic role of P27Kip1 (Asada et al., 1999; Blagosklonny, 2002; Motti et al., 2005).

Regulation of P27KIP1 protein level by SCF-Skp2

A family of molecular adaptors, the F-box proteins function as substrate-recognition subunits of SCF-type (Skp–Cullin–F-box type) 'E3' complexes, which ligate proteins to ubiquitin chains, allowing recognition for proteasomal degradation(K. I. Nakayama & Nakayama, 2005). The prototypical SCF complex contains an F-box protein (which is p45SKP2, i.e., S-phase kinase-associated protein 2 in the SCF-SKP2 complex), SKP1, CDC53/CUL-1 and RBX/ROC1 **(Figure II.1a)**. The ability of Fbox proteins to recognize a substrate largely depends on specific phosphorylation levels of the substrate(Amati & Vlach, 1999). SCF-Skp2 E3 ligase enzymatic activity holds the key to the proteolytic turnover of multiple proteins involved in cell cycle control and transcriptional regulation of cell cycle (Kossatz et al., 2004). The half-life and stability of P27Kip1 are regulated by the ubiquitination and degradation process through the S-phase kinase-associated protein 2 (Skp2)-containing SCF (SCF-Skp2) complex. Previously, it has been shown that Skp2 associates explicitly with a P27 carboxy-terminal peptide that is phosphorylated at Thr187, **(Figure II.1a,b)** but not with the non-phosphorylated peptide. This indicates that CDK-dependent phosphorylation of P27kip1 on Thr 187 (T187) is critical for Skp2-mediated degradation of P27kip1**(Figure II.1a,b)** (Carrano, Eytan, Hershko, & Pagano, 1999; Tsvetkov, Yeh, Lee, Sun, & Zhang, 1999).

Role Akt pathway in FOXO3a mediated regulation of P27KIP1 expression level

FOXO family of transcription factors belong to the Forkhead family of transcriptional regulators characterized by a conserved DNA-binding domain termed the 'forkhead box.' Among the 19

subgroups of Forkhead family, FOXO subgroup has four members namely, FOXO1, FOXO3, FOXO4, and FOXO6. The FOXO family of transcription factors is directly affected by the activity of PI3K pathway as those are one of the major group of direct substrates of the protein kinase Akt. Direct Akt mediated phosphorylation of FOXO factors at three key conserved regulatory sites (Thr32, Ser253, and Ser315 in the FOXO3a sequence) triggers rapid relocalization of FOXO proteins from the nucleus to the cytoplasm (Greer & Brunet, 2005). Thus, subcellular localization of FOXO3a is directly dependent on PI3K/AKT activity. In absence of active PI3K/Akt signaling FOXO3a transcription factors are localized in the nucleus, where they cause cell cycle arrest, by upregulating a series of critical target genes, one of which is P27Kip1 **(Figure II.2)** (Greer & Brunet, 2005; Nakao, Geddis, Fox, & Kaushansky, 2008; S. Zhang et al., 2013). In the absence of active PI3K/Akt signaling phosphorylated FOXO3a proteins bind to 14-3-3 proteins, which results in the export of FOXO3a from the nucleus into the cytoplasm leading to their sequestration from the target genes like P27Kip1, and eventual proteasomal degradation **(Figure II.2)**. Thus, spatial regulation of FOXO3a transcription factor inhibits cell cycle progression at the G1/S transition through controlling the transcription of P27Kip1(Greer & Brunet, 2005).

MATERIALS AND METHODS

Cell culture condition

In these studies, ER-positive MCF-7 cell line and MDA-MB-231 triple negative breast cancer cell lines were used. These cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were grown in DMEM containing penicillin and streptomycin (100 units/mL) and supplemented with 10% fetal bovine serum (FBS). The cells were cultured at 37^{o}C in a humidified chamber with 5% CO_2. Most of the experiments were performed with 60% confluency cells.

Cloning and Transfection

CCN5-specific shRNA or mismatched shRNA and CCN5 cDNA were cloned in the pSilencer 1.0-U6 (Ambion Inc, Grand Island, NY, USA) and pZsGreen1C1 (Clontech Laboratories, Mountain View, CA, USA) plasmids, respectively. shRNA sequence was designed to target CCN5 mRNA transcript. CCN5 cDNA was reverse transcribed with specific primers from MCF7 RNA and cloned in pS-1.0-U6 according to the manufacturer's instructions. For transfection experiments, breast tumor cells were transfected with the plasmids using the lipid micelle-based Lipofectin reagent following the transfection protocol provided by the vendor (Invitrogen, Grand Island, NY, USA). Electroporation of siRNAs against P27Kip1 and FOXO3a was accomplished using Neon Transfection System (Invitrogen, Grand Island, NY, USA) following cell line specific protocol provided by the vendor.

Cell proliferation assays

MDA-MB-231 cells (vector or CCN5 construct transfected) were grown in the absence of serum for 72 h and then 10% fetal bovine serum was added, and the cells were grown for different time

points as required for the experimental design. 10,000 cells per well were plated onto 96-well tissue culture plates. For the study of the effect of hrCCN5 on MDA-MB-231 proliferation, cells were treated with hrCCN5 for 48h after seeding. To perform the crystal violet assay, at experimental time points media from the wells with the cells were drained and washed with PBS thrice. Following that Crystal violet stain is added to each well and incubated for 20 mins. Next, crystal violet is washed off in standing water till no free satin remains. Lastly, the plate is dried, and the crystal violet stained cells are solubilized using 10% acetic acid and incubated for 30 mins. Colorimetric measurement is made at a 600nm wavelength with the help of a spectrometer and reading from each well is considered as the final cell density in corresponding well.

Tumor xenograft studies

All animal studies were performed with the approval of the IACUC of Kansas City VA Medical Center. For subcutaneous tumor growth, 8-week old female nude mice (N = 5) were injected with 2×10^6 cells subcutaneously. The cells for each injection were suspended in 50 microliters of PBS buffer and mixed with equal volume of Matrigel (Corning, NY, USA) and kept on ice until injection. Injections were made subcutaneously at the hind limb of each mice. The length (L) and width (W) of each tumor were measured by calipers twice per week. Tumor volume (TV) was calculated by the formula $TV = 0.5xLxW^2$. Finally, the tumors were harvested at time point according to the needs of experiments.

Nuclear-cytosolic fractionation

Nuclear and cytoplasmic protein lysates were prepared according to the manufacturer's instructions with brief modifications, Semi-confluent (~60%) MCF-7 and MDA-MB-231 cells were scraped into cold PBS and collected by centrifugation at 500 × *g* for 5 min. The cells were

washed once with PBS and centrifuged, and the cell pellet was re-suspended into ice-cold cytoplasmic extraction reagent 1 (CER1) and incubated on ice. After 10 min, ice-cold CER II was added to the tube. Samples were spun at 16,000 × *g* for 5 min to generate the cytosolic fraction (supernatant). The insoluble (pellet) fraction was re-suspended in ice-cold nuclear extraction reagent (NER). The pellet was homogenized by vortexing and was centrifuged at 16,000 x g for 10 min to generate nuclear fraction (supernatant).

Western Blot analysis

Equal amounts of protein were subjected to 7.5-10% SDS-PAGE, and the gel-fractionated proteins were transferred to nitrocellulose membranes (Bio-Rad) and reacted with specific antibodies. Blots were developed using ECL kits (Pierce, Rockford, IL) and bands were detected and analyzed using Carestream Molecular image station (Carestream Health, Inc. Woodbridge, CT, USA).

Immunofluorescent staining

MDA-MB-231 cells were grown in two-well chamber slides containing DMEM supplemented with 10% serum in the presence or absence of recombinant CCN5 protein (100 ng/ml) for 48 h. The cells were first washed with PBS followed by fixation with with 2% paraformaldehyde. Then, the cells were permeabilized with 1 × -Triton X (or with methanol), washed with PBS and blocked with 5 % goat serum blocking solution (Invitrogen, Grand Island, NY, USA) for 10 min. Finally, the cells are incubated with the anti P27Kip1 antibody (Cell Signaling Technologies, Danvers MA, USA) at a dilution of 1:300 in PBS, followed by a final incubation with the FITC-conjugated secondary antibody (1:1000 in PBS for one h) along with nuclear DAPI staining. The

expression of P27Kip1 was visualized by confocal microscopy (Leica Microsystems, Buffalo Grove, IL, USA).

Cycloheximide treatment for protein stability assay

MDA-MB-231 cells were seeded at a confluency of 60 percent of growth surface area and were treated with Cycloheximide (Sigma, St. Louis, MO, USA) solution at a final concentration of 25µM in complete DMEM supplemented with 10% FBS. The treatment was continued for 8hrs and protein was extracted from the cells at each time point of 0, 1, 2, 4 and 8hrs for western blotting.

AKT inhibitor (LY294002) treatment

MDA-MB-231 cells were seeded at a confluency of 60 percent of growth surface area. Inhibitor of PI3KAkt activity, LY294002 was used to treat the semi-confluent cells at a final concentration of 10mM in complete DMEM supplemented with 10% FBS. The treatment was continued for 8hrs and protein was extracted from the cells in lysis buffer supplemented with phosphatase inhibitor cocktail.

RESULTS:

Effect of CCN5 overexpression and recombinant CCN5 treatment on TNBC (MDA-MB-231) cell viability and cell-cycle

Crystal violet based cell viability assays were performed in both induced CCN5 overexpressing MDAMB231 cells and in the presence of human recombinant CCN5 protein (hrCCN5) to evaluate any possible role of CCN5 protein on cell-survival and proliferation of triple-negative breast cancer cells MDA-MB-231. MDA-MB-231 cell lines with stable transfection of the only GFP-overexpressing vector [MDA-MB-231CCN5$^{(-)}$] and with GFP fused CCN5-overexpressing constructs[MDA-MB-231CCN5$^{(+)}$] were grown under a serum-deprived condition for 72h and subsequently were cultured in complete media (with 10% FBS) for 24-72hrs timepoints. The results from the crystal-violet staining of the cells demonstrate that ectopic expression of CCN5 induces growth inhibition of MDA-MB-231 cells significantly in a time-dependent manner **(Figure II.3a)**. Similar crystal violet assays were performed in presence different doses of hrCCN5 (0-1000ng/ml) in cell culture medium to determine the effect of human recombinant CCN5 protein (hrCCN5) on MDA-MB-231 cell viability. A dose-dependent growth inhibition of MDA-MB-231 cells was observed when cells were grown in the presence of hrCCN5 for 48 h **(Figure II.3b)**. Further insight into the CCN5 induced growth arrest of MDA-MB-231cells was brought in with flow-cytometry based cell-cycle analysis of control and 48h of CCN5 treated MDA-MB-231 cells. The cell numbers in the G0/G1 phase was significantly increased with a concomitant reduction of cell numbers in S and G2/M phases in the CCN5-treated cells as compared with the control **(Figure II.3c)**. These results indicate that CCN5-induced inhibition of MDA-MB-231 cell growth was due to cell cycle arrest at a stage before the restriction point.

Effect of CCN5 on tumor progression in TNBC (MDA-MB-231) xenograft model

Once the CCN5-mediated growth inhibitory effect on MDA-MB-231 cells was established under in-vitro conditions, the study was extended to an in-vivo xenograft model experiment. MDA-MB-231-xenograft tumors were established with both MDA-MB-231CCN5$^{(+)}$ cells and MDA-MB-231CCN5$^{(-)}$ cells. The effect of CCN5 overexpression, on tumor growth progression, was evaluated by monitoring the tumor volume increase in both groups of the mice. The nude (nu/nu) mice inoculated with MDA-MB-231CCN5$^{(+)}$ cells displayed reduced tumor volume increase relative to MDA-MB-231CCN5$^{(-)}$ cell-generated tumors. After 3 weeks of tumor cell inoculation the control group of mice had a significantly larger volume of tumor as compared to the experimental group of mice with CCN5 expressing cells **(Figure II.3d)** Throughout the study period that extended for a month the tumors from MDA-MB-231CCN5$^{(+)}$ cells maintained a significantly lower volume as compared to the control tumors and a growth arrest was noted in those tumors.

Role of P27Kip1 in CCN5-induced growth inhibition of TNBC cells

MDA-MB-231 cells were transfected with short interfering RNA (siRNA) specific for P27Kip1 to block the expression of P27Kip1 to test the status of the P27Kip1 protein in CCN5-induced growth inhibition of MDA-MB-231 cells. Firstly, the extent of knockdown was evaluated in the protein extracts from scrambled siRNA transfected, and P27Kip1 siRNA transfected MDA-MB-231 cells by Western Blotting **(Figure II.3e),** and subsequently, the effect of hrCCN5 protein on cell proliferation was tested. As expected, hrCCN5 protein significantly reduced the proliferation of scrambled-transfected MDA-MB-231 cells, whereas this inhibitory effect of CCN5 was decreased significantly in P27-siRNA-transfected cells **(Figure II.3e lane4)** as compared to the

scrambled-siRNA transfected cells treated with hrCCN5. This observation indicates that cell growth inhibition by CCN5 is mediated by P27Kip1 and could be prevented by blocking P27Kip1 expression in MDA-MB-231 cells.

Role of CCN5 on P27Kip1 expression and its subcellular localization

As it was observed that CCN5 reduces the growth rate of MDA-MB-231 by arresting the cell cycle at the G0/G1 stage through P27Kip1 regulation, it was tested whether the presence of CCN5 plays a role in the regulation of the P27Kip1 protein expression. Firstly, the relative expression of the P27Kip1 protein in MDA-MB-231 cells was tested in the presence of different concentrations of hrCCN5 and for various time points. It was found that the levels of P27Kip1 protein were significantly elevated in CCN5-treated cells in a time and dose-dependent manner **(Figure II.4a)**. Second, to determine whether CCN5 plays any role in the distribution of P27Kip1 in the nucleus and cytoplasm of breast cancer cell lines, the nuclear and cytosolic fractions were isolated from extracts of CCN5-positive MCF-7 cells, CCN5-silenced MCF-7 cells, CCN5-negative MDA-MB-231 cells and CCN5-overexpressed MDA-MB-231 cells. The status of P27Kip1 protein expression in individual fractions was measured by western blotting. Results showed a decreased nuclear-to-cytoplasmic ratio in CCN5-silenced MCF-7 cells as compared to the CCN5-positive MCF7 cells, whereas this ratio was increased in CCN5-overexpressed MDA-MB-231 as compared with CCN5-negative MDA-MB-231 cells **(Figure II.4b).** In a similar experiment, the influence of hrCCN5 on the nuclear-to-cytoplasmic ratio of P27Kip1 in MDA-MB-231 cells treated and untreated with hrCCN5 was tested and observed to be increased in treated cells **(Figure II.4c)**. These results were further confirmed by immunofluorescence analyses, which indicated that hrCCN5 treatment increases the P27Kip1 protein level in the nucleus when compared with the untreated cells **(Figure II.4d)**. Collectively,

these studies reveal that presence of CCN5 in the micro-environment leads to an enrichment of P27Kip1 in the nucleus of aggressive TNBC cells.

CCN5 enhances stability of P27Kip1 protein in TNBC cells

The P27Kip1 protein levels and functions in cells are also governed by post-translational modifications of the protein in the cell which regulates the stability of the protein. To determine whether CCN5 has any role in maintaining the protein stability of P27Kip1 MDA-MB-231 cells were treated with a protein synthesis inhibitor, cycloheximide (CHX), in the presence or absence of hrCCN5 for different time points. Following the blocking of P27Kip1 protein translation by CHX treatments, P27Kip1 protein status was determined in the whole cell protein extracts. In CCN5 untreated MDA-MB-231 cells after CHX treatment, the P27Kip1 protein level started decreasing after one hour and declined to a minimal level by 4hrs. of treatment **(Figure II.5a)**. In the presence of hrCCN5, the initial protein level of P27Kip1 was higher than the control cells and even after 8hrs. Of CHX treatment the protein level of P27Kip1didnot decreases significantly **(Figure II.5a)**. In a related experiment, it was shown that not only CCN5 treatment of MDA-MB-231 cells augments P27Kip1 expression level, but the elevated level of P27Kip1 protein is also maintained even after 48hrs of withdrawal of CCN5 from the culture medium **(Figure II.5b)**. These results indicate that the presence of CCN5 protein augments the P27Kip1 protein stability in MDA-MB-231 cells.

CCN5 influences SKP2 mediated degradation of P27Kip1 protein in TNBC cells

Treatment with hrCCN5 significantly reduced the protein level of Skp2 in MDA-MB-231 cells in a time-dependent manner as found out by western blot (**Figure II.6a)**. Observation of an increase in P27Kip1 stability post CCN5 treatment and western blot showing a decline of Skp2

protein level in MDA-MB-231 cells after CCN5 treatment indicate that upregulation of P27Kip1 by CCN5 through improvement of P27Kip1 protein stability could be carried out by blocking the Skp2 production and thus it's enzymatic activity. As phosphorylation of P27Kip1 at T187 is critical for Skp2 mediated degradation of P27Kip1, the effect of CCN5 treatment on T187 phosphorylation levels in MDA-MB-231 cells was observed in CCN5 treated cell extracts and it was discovered that CCN5 treatment drastically reduces the T187 phosphorylation levels **(Figure II.6b)**. Collectively, these studies indicate that CCN5 suppresses both Skp2 expression and P27Kip1 phosphorylation at T187 in MDA-MB-231 cells and thus protects P27Kip1 from degradation.

Upregulation of P27Kip1 by CCN5 is mediated through the stabilization of FOXO3a transcription factor via inhibition of PI3K/AKT pathway

FOXO3a (Forkhead Box O 3a), a transcription factor has been shown to play a significant role in controlling the transcription of P27Kip1 in various cells. As an upregulation of P27Kip1 transcription with decreased cell survival and G1 arrest was observed after treating MDA-MB-231 cells with hrCCN5, the expression level of FOXO3a protein in CCN5-treated MDA-MB-231 cells was determined. Immunoblot analyses revealed an upregulation of FOXO3a protein level in CCN5-treated MDA-MB-231 cells in a time-dependent manner **(Figure II.7a)**. In addition to an upregulation of FOXO3a expression level, it was also noticed that CCN5 treatment led to an increase in localization of FOXO3a in the nucleus as compared to the untreated cells **(Figure II.7b)**. As nuclear transport and cytosolic-nuclear localization of FOXO3a is subject to the phosphorylation of Ser318, Ser253 and Thr32 residues in FOXO3a protein, the role of CCN5 treatment on phosphorylation levels of the mentioned residues was observed by western blotting.

A decrease in the phosphorylation level at Ser318 and Ser253 was noticed in CCN5 treated MDA-MB-231 extracts **(Figure II.7c)**.

It has been already established from previous studies and mentioned earlier that PI3K/AKT pathway activity directly regulates the phosphorylation levels of FOXO3a protein leading to its cytoplasmic accumulation and degradation. Given the importance of the role of AKT activity and capabilities of CCN family proteins to interact with cellular integrins, the status of the activated phosphorylated form of AKT in MDA-MB-231 cells after CCN5 treatment was checked. Western blotting against the phosphorylated form of AKT at Ser473 (p-AKT) showed a significant decrease of p-AKT levels in CCN5-treated cells as compared with untreated cells, whereas the expression level of total AKT protein was unchanged in both treated and control groups **(Figure II.7d)**. A marked decline of Cyclin D1 and Bcl-2 protein expression level in CCN5-treated cells was also observed **(Figure II.7d)** as both proteins are critical downstream targets of the PI3K/AKT pathway and are regulated by FOXO3a mediated transcription.

Next, to discern a link between CCN5-induced inactivation of AKT and induction of FOXO3a and P27Kip1, experiments were designed to investigate whether an AKT inhibitor exerts a similar effect as hrCCN5 and augments FOXO3a and P27Kip1 expression in MDA-MB-231 cells. It was found that levels of p-AKT and p-FOXO3a (S253) and p-P27Kip1 (T187) were significantly reduced whereas the total P27 level was markedly elevated in the presence of the PI3K/AKT inhibitor, LY294002 **(Figure II.8)**. No effect of AKT inhibitor treatment was observed on the levels of total FOXO3a protein. Then it was determined whether the CCN5 treatment mimics the AKT inhibitor's action in breast cancer cells and it was found that overexpression of CCN5 in MDA-MB-231 cells mimic the Akt inhibitors' effect on MDA-MB-

231 cells **(Figure II.8)**. To investigate whether FOXO3a is a critical intermediate molecule that needs to be stabilized by CCN5 to exert its regulatory effect on P27Kip1 protein levels in MDA-MB-231 cells, FOXO3a expression was silenced by siRNA specific for FOXO3a in MDA-MB-231 cells, and P27Kip1 levels were detected in these cells before and after hrCCN5 treatment. It was observed that though hrCCN5 enhances both FOXO3a, as well as P27Kip1 protein levels in control siRNA-transfected cells, this effect of CCN5, was nullified when FOXO3a expression was abolished from the cells through RNAi-based silencing. **(Figure II.9)** Collectively, these studies indicate that CCN5-induced upregulation of P27Kip1 is mediated through the regulation of FOXO3a.

DISCUSSION

The antitumorigenic role of CCN5 in breast cancer, though established in multiple studies, is not yet completely understood (S. K. Banerjee & Banerjee, 2012). In this section, mechanisms of P27Kip1 regulation by CCN5 in triple negative breast cancer line MDA-MB-231 cells has been elucidated. CCN5 mediated signaling has been shown to contribute to the increased protein level, activity and nuclear relocalization of P27Kip1 leading to cell growth inhibition and possibly invasion suppression in aggressive TNBC cells. The expression level of P27Kip1, its subcellular distribution and corresponding prognostic value in breast cancer have been studied for decades. Although the prognostic significance of P27Kip1 in breast cancer is debatable there has been consensus on the fact that heterogeneous distribution of P27Kip1 protein plays a key role in aberrant cell cycle progression in cancer cells (Barbareschi et al., 2000; De Paola et al., 2002; Tan et al., 1997; Troncone et al., 2004; Viglietto et al., 2002). In studies performed in our laboratory, in corroboration with previous studies, the heterogeneous distribution of P27Kip1 was noted in human breast samples. Though both high and low levels of P27Kip1 expression were detected in infiltrating ductal adenocarcinoma samples, and the localization of P27Kip1 was observed to be predominantly in the cytoplasm. In pre-neoplastic lesions such as atypical ductal hyperplasia (ADH) and ductal carcinoma in-situ (DCIS), the P27Kip1 expression is very high and was mostly located in the nucleus. Interestingly, a positive correlation as observed between the nuclear localization of P27Kip1 and CCN5 expression in both human breast tissue samples and breast cancer cell lines (Haque et al., 2015). The dichotomous function of P27Kip1 depending on its subcellular localization in the nucleus and cytoplasm in cancer cells creates a debate as for whether to classify it as a tumor suppressor or an oncogene (Besson et al., 2008; Blagosklonny, 2002; Sgambato et al., 2000; Viglietto et al., 2002) . The growth-arresting activity

of P27Kip1 depends on its nuclear localization whereas aberrant shifting to cytoplasm impairs the growth-arresting function (Baldassarre et al., 1999; Jiang, Zhao, & Verfaillie, 2000; Viglietto et al., 2002).Studies in this section test the hypothesis that P27Kip1 protein level and trafficking could be regulated by CCN5 mediated signaling in breast cancer cells leading to the manifestation of the growth-inhibitory action of CCN5. The findings of this section involving, CCN5 depleted and overexpressing breast cancer cell lines and treatment of hrCCN5, provide evidence that CCN5 can act as an upstream regulator of P27Kip1 expression level and localization. Previous studies on P27Kip1, in various normal and cancer cells, suggest that P27Kip1 protein levels can be increased in a cell mainly by enhancement of its stability (half-life) (Slingerland & Pagano, 2000) through the regulation of ubiquitination. and eventual degradation via a ubiquitin ligase complex, SCF-Skp2 (Carrano et al., 1999; Kossatz et al., 2004; K. Nakayama et al., 2004; K. I. Nakayama & Nakayama, 2005; Spruck et al., 2001). Furthermore, P27Kip1 levels have been shown to be directly regulated at the transcriptional level, and localization of FOXO3a transcription factor plays a vital role in this event.(Greer & Brunet, 2005; S. Zhang et al., 2013). The observations listed in this section indicates that upregulation of P27Kip1 in MDA-MB-231 cells via CCN5 can be mediated both by increased stability of P27Kip1 and transcriptional activation involving stabilization and nuclear localization of FOXO3a transcription factor. The stability of P27Kip1 could be maintained by CCN5 through repressing Skp2, a key adaptor molecule necessary for binding of the SCF-Skp2 complex to P27Kip1. Also, CCN5 can stabilize P27Kip1 by blocking the phosphorylation of P27Kip1 at T187 which is necessary for substrate recognition of SCF-Skp2 enzyme complex, resulting in prevention of Skp2-mediated degradation of P27Kip1**(Figure II. 1a,b)** (Carrano et al., 1999).In parallel, it has been shown that CCN5 upregulates P27kip1 mRNA expression in

MDA-MB-231 cells through the increase of expression level as well as increased nuclear translocation of FOXO3a. The changes in stability and subcellular localization of FOXO3a are the results of the phosphorylation status of specific serine and threonine residues which are direct substrates of Akt kinase activity (Greer & Brunet, 2005; Nakao et al., 2008; S. Zhang et al., 2013). Because CCN5 blocks Ser318 and Ser253 phosphorylation in MDA-MB-231 cells via inhibiting AKT activity, the nuclear translocation of FOXO3a by CCN5 could be mediated through repressing the AKT induced phosphorylation of S318 and S253 of FOXO3a **(Figure II.2)**. However, interestingly, though not studied here, previous studies have shown that Akt can directly phosphorylate P27Kip1 protein at T157 which leads to cytoplasmic localization of P27Kip1 thus precluding its capability to induce cell cycle arrest (Shin, Rotty, Wu, & Arteaga, 2005; Viglietto et al., 2002). In addition to Skp2 expression regulation leading to stability of P27Kip1 and direct transcriptional activation via FOXO3a, CCN5 might reduce cytoplasmic localization of P27Kip1 by inhibiting its direct phosphorylation of P27Kip1 at T157 by Akt. This regulation of P27Kip1 was found to be a direct mechanism regulated by Akt that sustains proliferation of breast cancer cells.

CCN5 like other CCN family members after being secreted localizes outside the cell and thus is likely to exert its function via cell-surface receptors. It is widely established that various functions of CCN family proteins are mediated through integrin receptors (Haque et al., 2012; Jun & Lau, 2011). Also, previously it has been shown that integrins differentially regulate the proliferation of CD34+ normal and leukemic cells through the regulation of P27Kip1 (Jiang et al., 2000). Studies investigating roles of CCN family proteins suggest a context-dependent role of integrin receptors in various tissue types. Results from an extension of these studies in this section indicate that CCN5 exerts its effect through α6 and β1 integrins to regulate P27Kip1

expression. The conclusion was derived from the observation that the treatment of α6 and β1 integrin antibodies block CCN5-induced upregulation of P27Kip1 mRNA and protein in MDA-MB-231 cells. Also, CCN5 protein has been shown to be directly interacting with the integrins (Haque et al., 2015). Overall, to exert the growth arrest function, CCN5 enhances P27Kip1 expression as well as its nuclear translocation through the regulation of Skp2 and AKT/FOXO3a molecular signaling pathways (**Figure II.10)**. Thus, inducing activation of CCN5 in aggressive breast cancer cells has the potential to affect growth arrest of these aggressive cells when applied alone or in conjunction with other effective therapies.

FIGURES:

Fig.II.1a

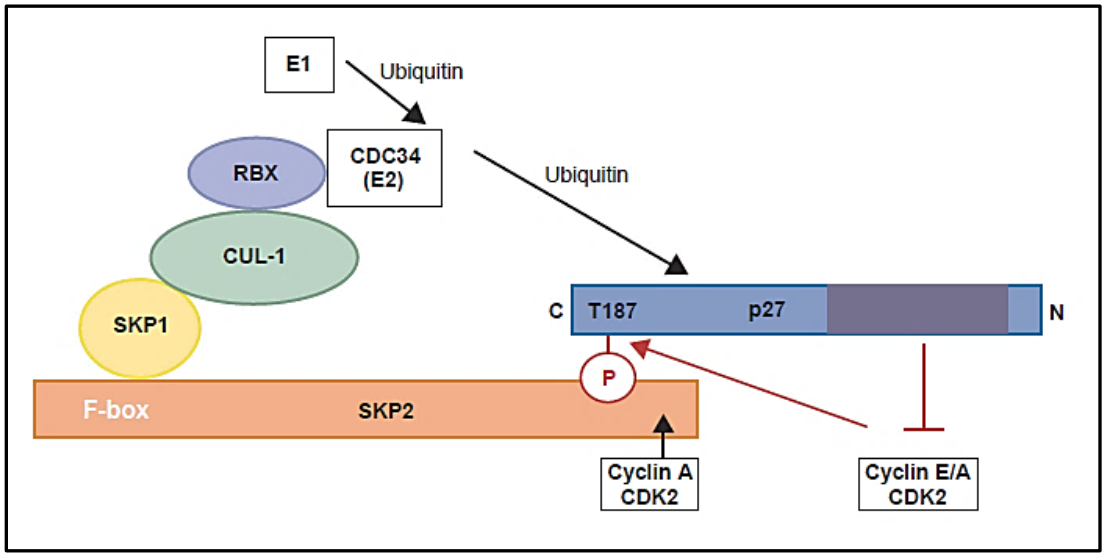


Fig.II.1a: This schematic figure shows binding of SCF-SKP2 ubiquitin ligase complex composed of SKP1, CUL-1, RBX and SKP2 proteins to p27Kip1 with T187 phosphorylation, critical for recognition of p27 as a substrate for ubiquitination and degradation. The phosphorylation of p27Kip1 is carried out by Cyclin-CDK2 complex. (Amati & Vlach, 1999). Figure used from the article with permissions.

Fig.II.1b

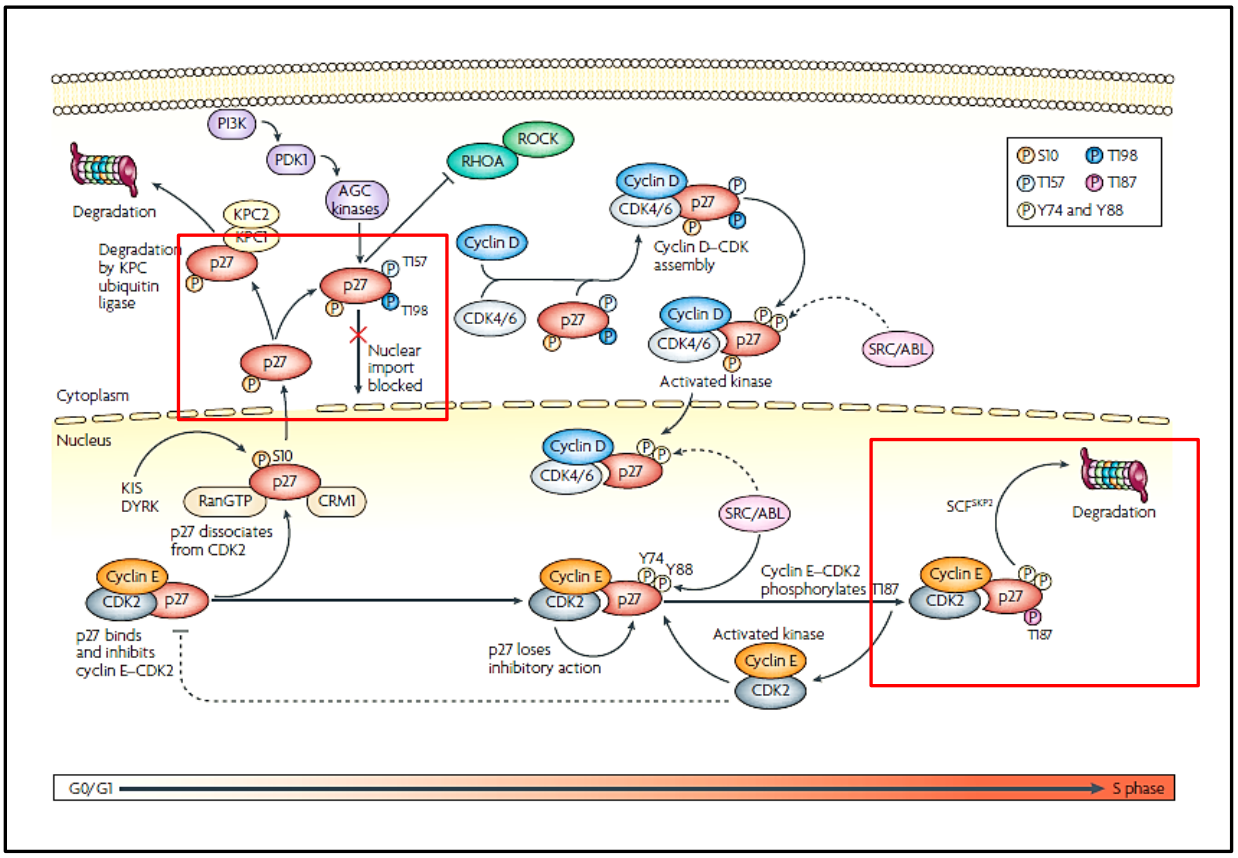


Fig.II.1b: Regulation of p27Kip1 by signaling pathways: In early G1, phosphorylation at S10 increases leading allowing CRM1-dependent nuclear export and KPC-mediated proteolysis of cytoplasmic p27Kip1. ABL and SRC phosphorylate p27 in early G1 which results in activation of cyclin E–CDK2 leading to T187 phosphorylation of p27 and proteolysis through SCFSKP2. In early to mid G1, PI3K activity phosphorylates P27 at T157 blocking nuclear import and promoting the assembly of cyclin D–Cdk–p27 complexes. (Chu, Hengst, & Slingerland, 2008). Figure used from the cited article with permissions.

Fig.II.2

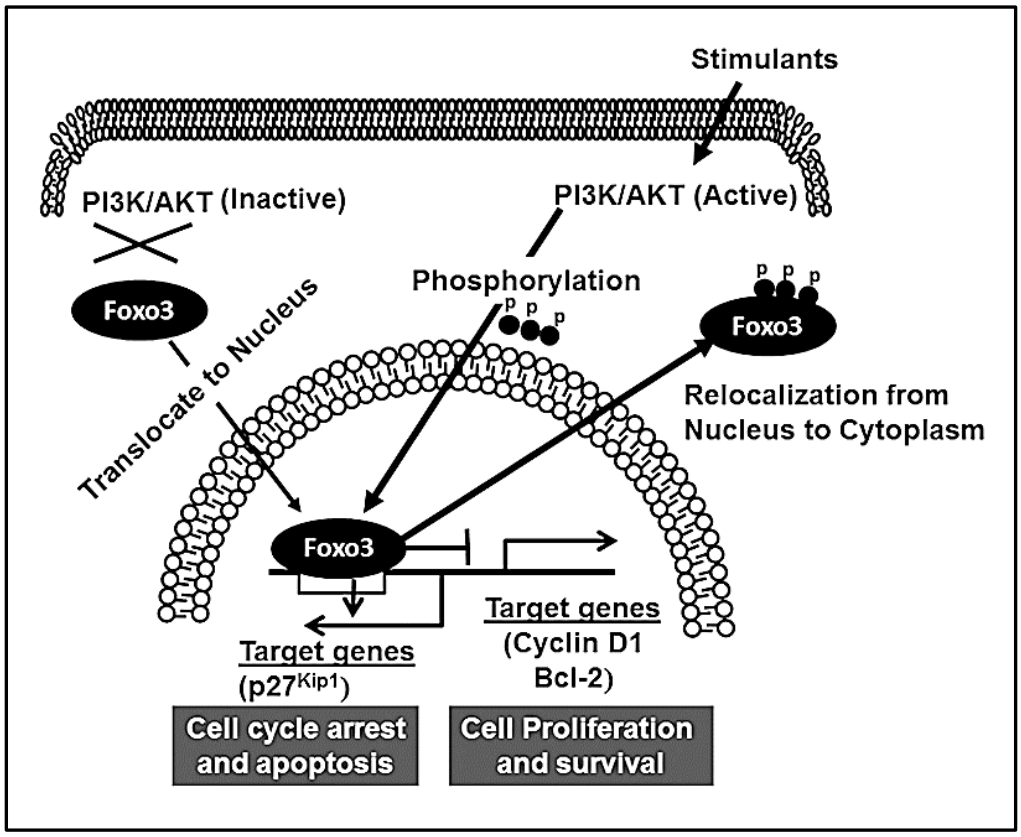


Fig.II.2: Regulation of p27Kip1 by Akt: Active Akt signaling leads to phosphorylation of FOXO3a transcription factors resulting in their cytoplasmic relocalization, sequestration from DNA binding leading to decreased p27Kip1 expression. Inactivated or repressed Akt signaling leads to nuclear localization of FOXO3a leading to p27Kip1 transcriptional activation (Haque et al., 2015).

Fig.II.3a

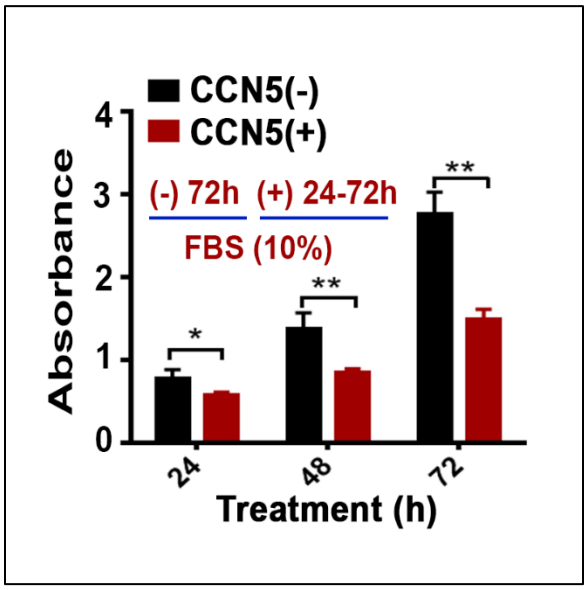


Fig.II.3b

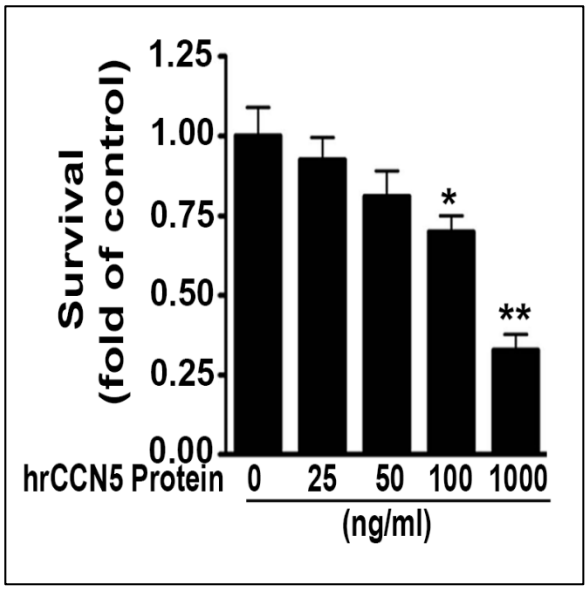


Fig.II.3: Effect of CCN5 on TNBC cell growth: (a) Cell survival assay was performed by crystal violet staining. All quantitative data are presented as mean±s.e.m. of at least eight sets of experiments. *P<0.03 vs corresponding CCN5(−), **P<0.001 vs corresponding CCN5(−). P-value was generated by two-tailed t-test. (b) Crystal violet cell survival assay with MDA-MB-231 cells, treated with different concentration of hrCCN5 protein for 48 h. All quantitative data are presented as fold ±s.e.m. of at least eight sets of experiments. *P<0.01 vs untreated cells, **P<0.001 vs untreated cells. P-value was generated by two-tailed t-test (Haque et al., 2015).

Fig.II.3c

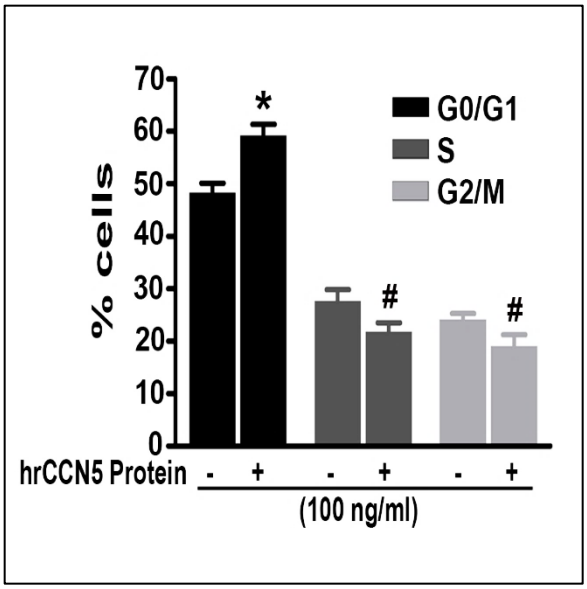


Fig.II.3d

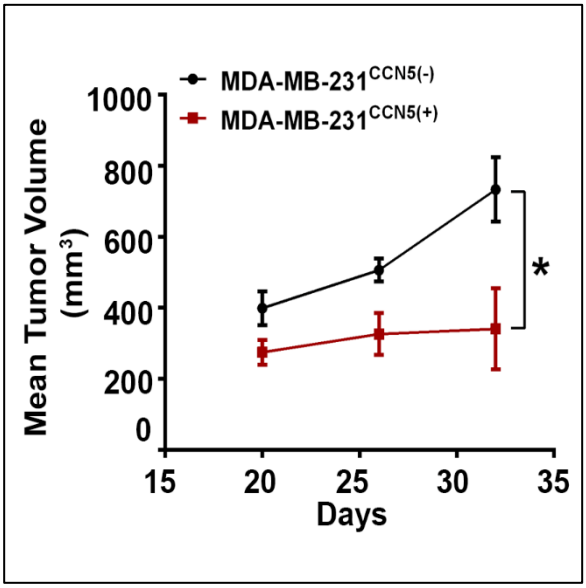


Fig.II.3: Effect of CCN5 on TNBC cell growth: (c) Flow-cytometric cell-cycle analysis of MDA-MB-231 cells treated with vehicle or hrCCN5 protein(100ng/ml). All quantitative data are presented as mean ±s.e.m. of at least three sets of experiments. *P<0.01 vs untreated cells; #P<0.03 vs untreated cells. P-value was generated by two-tailed t-test. (d) Measurement of the size of xenograft tumors of MDA-MB-231CCN (+) or MDA-MB-231CCN5 (−) cells subcutaneously into the female nude mice (N= 5 per group). All quantitative data are presented as mean ±s.e.m. *P<0.006. P-value was generated by two-way ANOVA (Haque et al., 2015).

Fig.II.3e

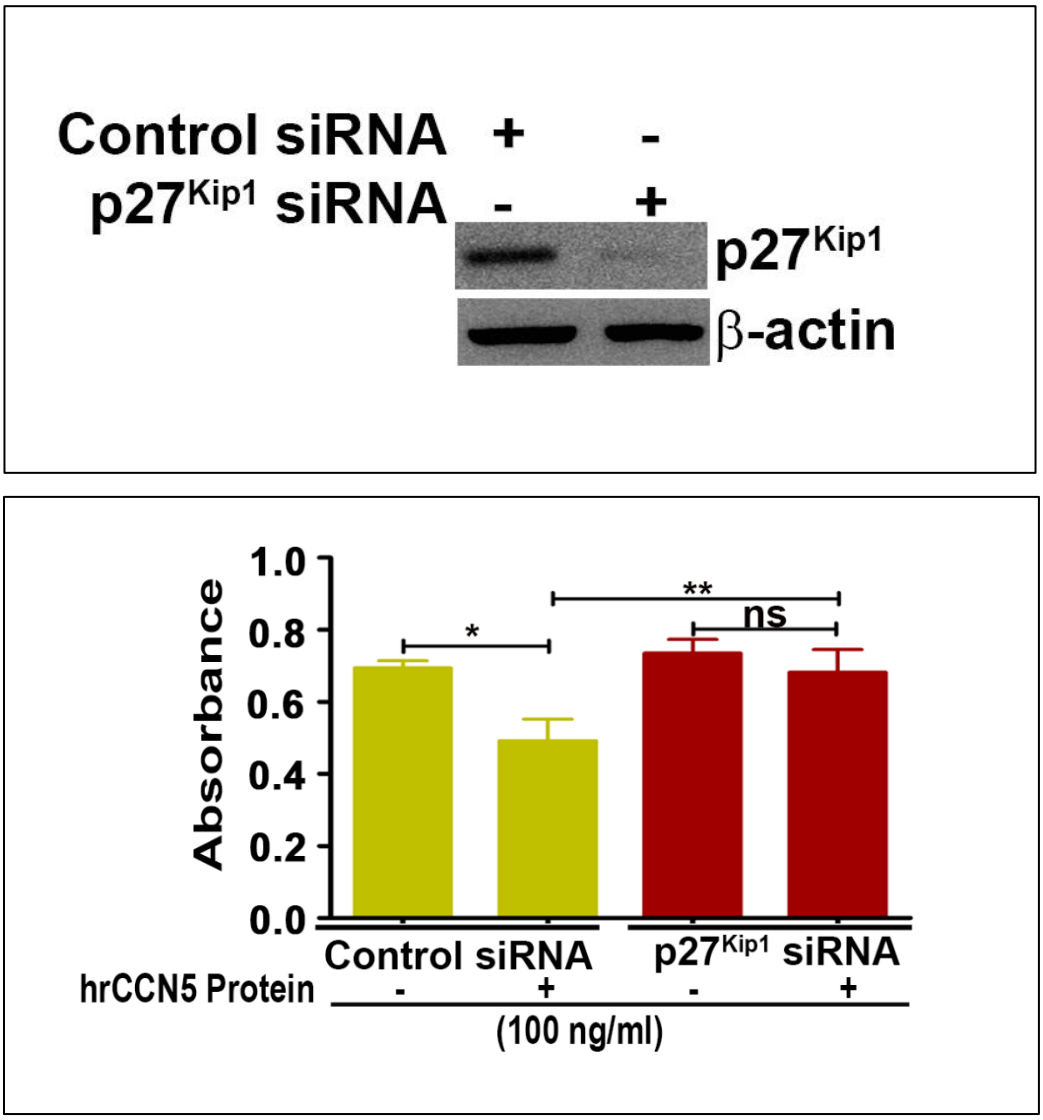


Fig.II.3e: Role of p27Kip1 in CCN5 mediated cell growth inhibition: Cell growth inhibition by hr.CCN5 was measured with p27Kip1 or scrambled-siRNA transfected MDA-MB-231 cells. Crystal violet cell viability assays were performed. The western blot illustrates the efficiency of p27Kip1-siRNA (upper panel). All quantitative data are presented as mean ±s.e.m. of at least three sets of experiments. *P<0.001vs CCN5-untreated control siRNA (lane 1) and **P<0.014 vs CCN5-treated control siRNA (lane 2). P-value was generated by two-tailed t-test (Haque et al., 2015).

Fig.II.4a

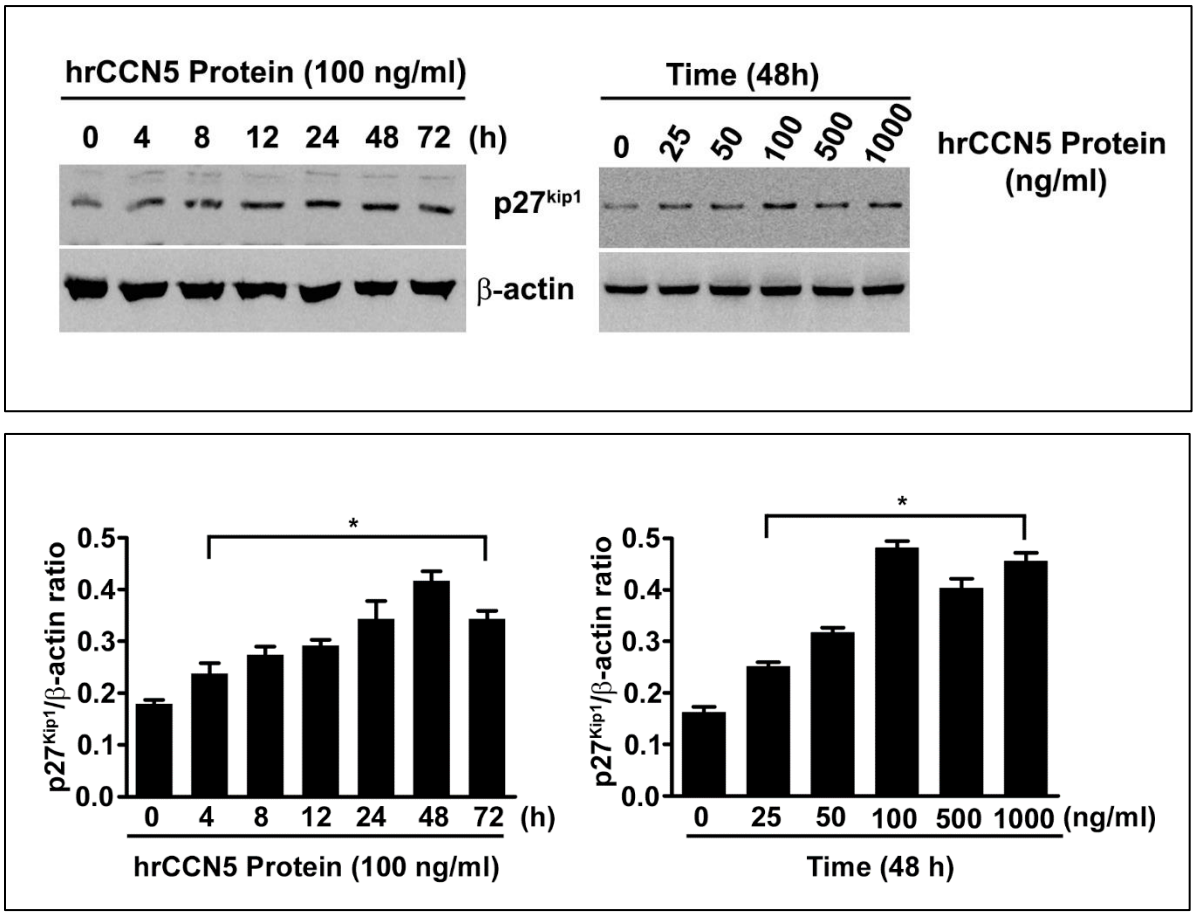


Fig.II.4a: Effect of CCN5 on p27Kip1 expression: Western blots demonstrating p27Kip1 protein levels in MDA-MB-231 cell lysates treated with vehicle or hrCCN5 protein in a time- and dose-dependent manner. The images are representatives of three independent experiments. Bar graph represents quantitative analysis of relative p27Kip1 protein level. All quantitative data are presented as mean ± SEM of at least three sets of experiments. *P < 0.001 vs untreated. P value was generated by two-tailed t test (Haque et al., 2015).

Fig.II.4b

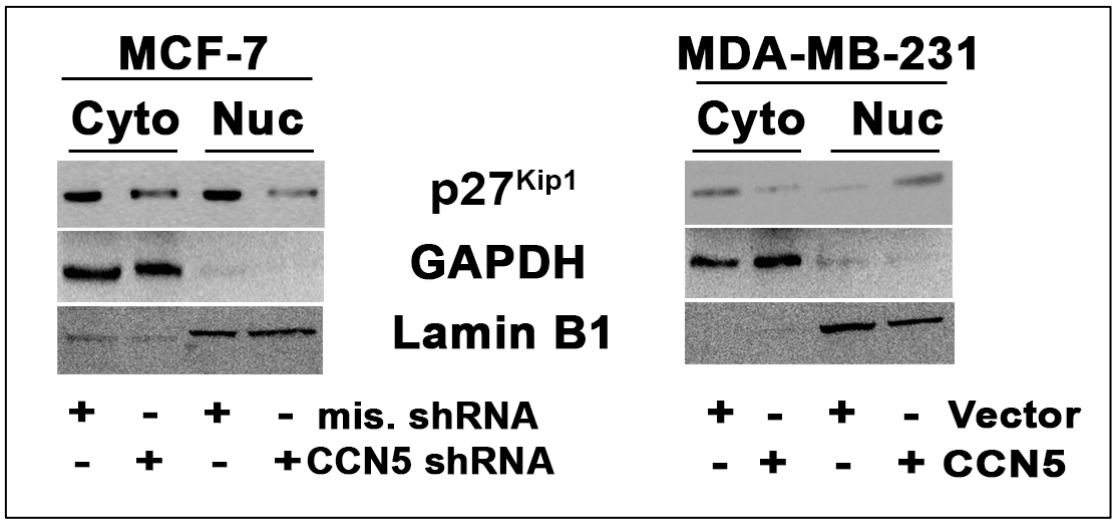


Fig.II.4c

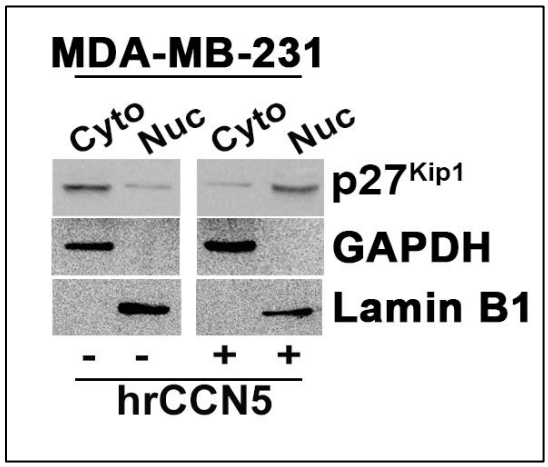


Fig.II.4: Effect of CCN5 on p27Kip1 localization: Western blots demonstrating relative cytosolic and nuclear p27Kip1levels in CCN5-depleted MCF-7 cells (b, left panel), CCN5 overexpressed MDA-MB-231 cells (b, right panel) and hrCCN5-treated (100 ng/ml for 48 h) or vehicle-treated (control) MDA-MB-231 cells (c). Blots against GAPDH, Lamin B1 and β-actin are used as quality and loading controls (Haque et al., 2015).

Fig.II.4d

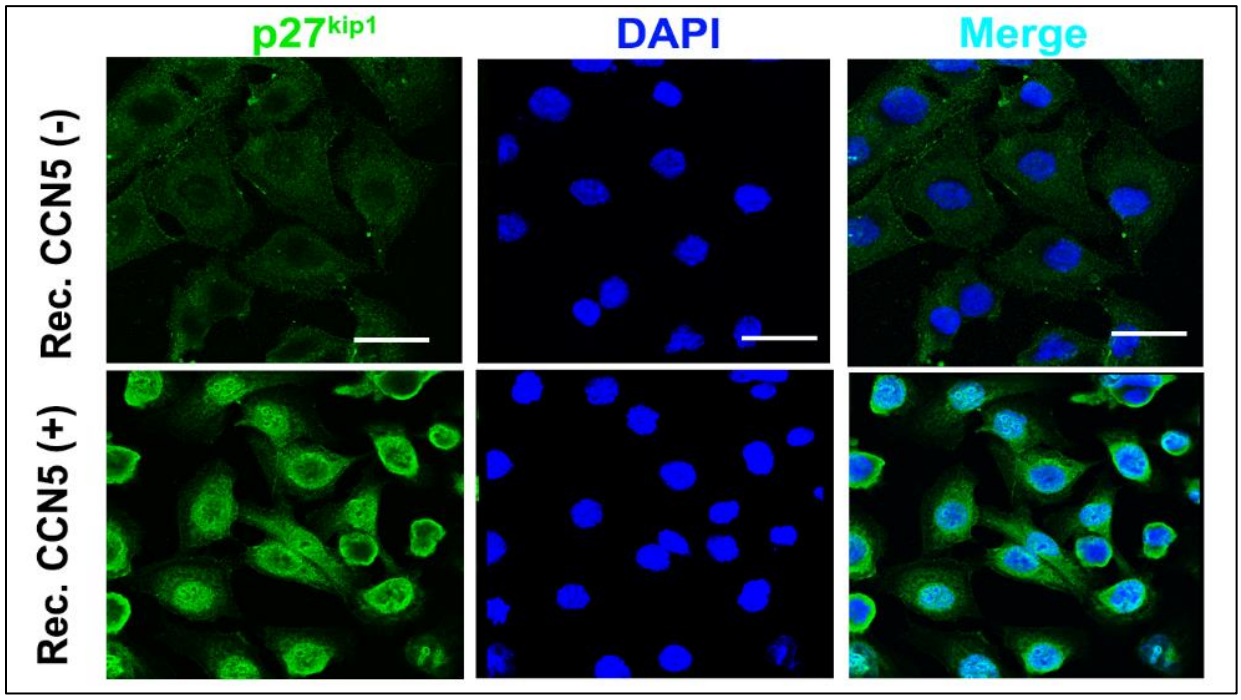


Fig.II.4: Effect of CCN5 on p27Kip1 localization: (d) Immunofluorescence images from confocal microscopy of MDA-MB-231 cells treated with hrCCN5 protein or vehicle control for 48 h, against p27Kip1 antibody. P27Kip1 immunostaining is shown in green, and nuclei, counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) are shown in blue. Scale bar represents 100 μm (Haque et al., 2015).

Fig.II.5a

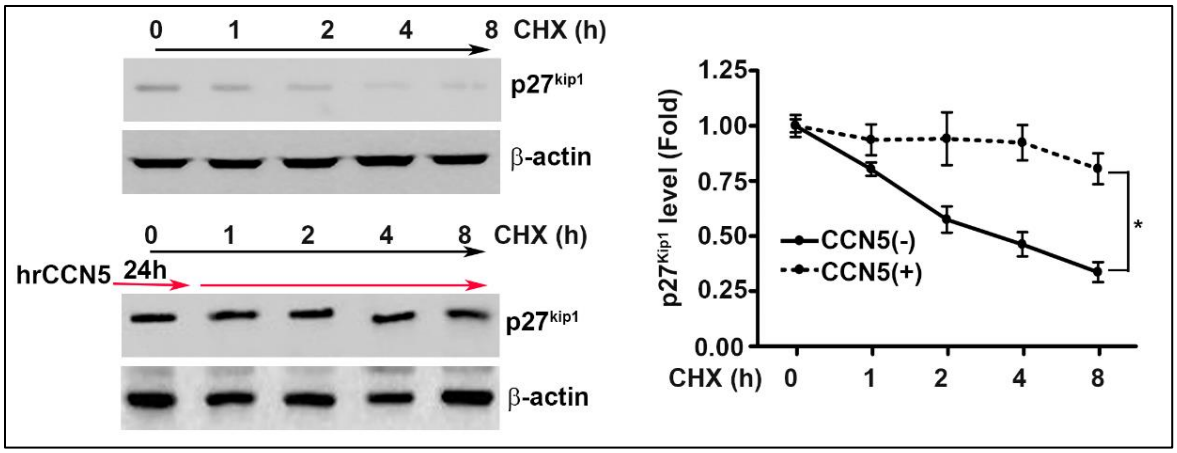


Fig.II.5b

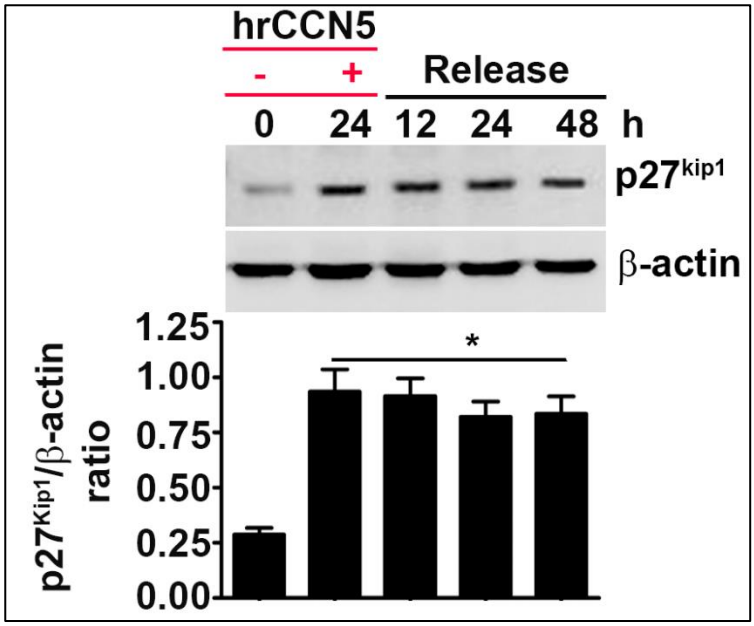


Fig.II.5: Stabilization of p27Kip1 in TNBC cells exposed to recombinant CCN5. (a) Western blots from lysates of MDA-MB-231 cells which were either exposed to cycloheximide (25 µg/ml) or pre-treated with hrCCN5 protein (100 ng/ml) for 24 h followed by a combination treatment of hrCCN5 and cycloheximide (lower panel). The images are representative of three independent experiments and all quantitative data are presented as mean ± s.e.m. *P<0.001. P-value was generated by two-way analysis of variance. b) Western blots demonstrating relative p27Kip1 protein levels in MDA-MB-231 cell extracts, after withdrawing hrCCN5 treatment (100 ng/ml) at indicated time points. All quantitative data are presented as mean ± s.e.m. of at least three sets of experiments. *P<0.001 vs untreated. P-value generated by two-tailed t-test (Haque et al., 2015).

Fig.II.6a

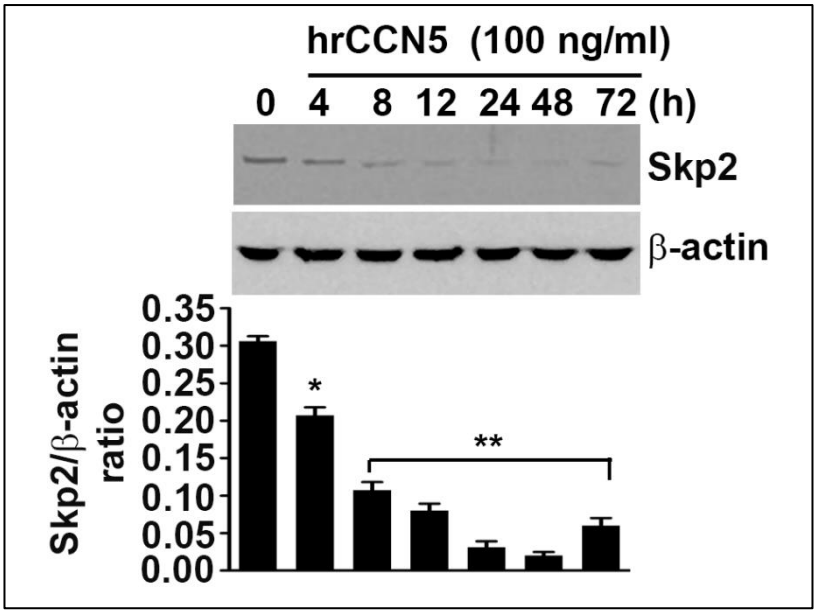


Fig.II.6b

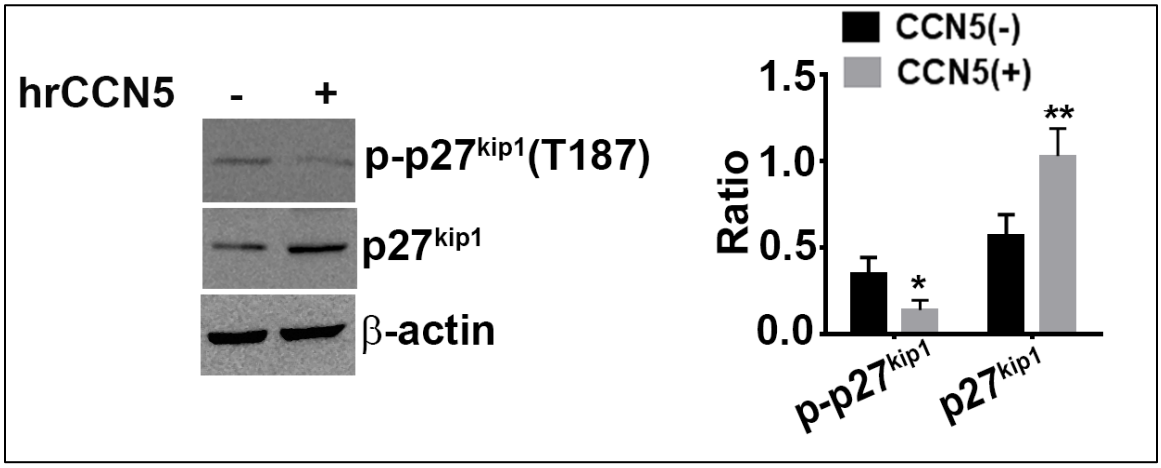


Fig.II.6: Expression level of Skp2 and phosphorylation levels of p27Kip1 after hr.CCN5 treatment : (a) Western blots demonstrating relative Skp2 protein level in vehicle- and hrCCN5 (100 ng/ml)-treated MDA-MB-231 cells. Fold changes were normalized to β-actin, a loading control. All quantitative data presented as mean± s.e.m. *P<0.001 vs control, **P<0.0001 vs control. P-values were determined with two tailed t-test. (b) Western blots demonstrating relative p-p27Kip1(T187) protein level in MDA-MB-231 cells treated with hrCCN5 (100 ng/ml). Fold changes were normalized to β-actin and all quantitative data presented as mean ±s.e.m. *P<0.01 vs CCN5(−), **P<0.003 vs CCN5(−). P-values were determined with two-tailed t-test (Haque et al., 2015).

Fig.II.7a

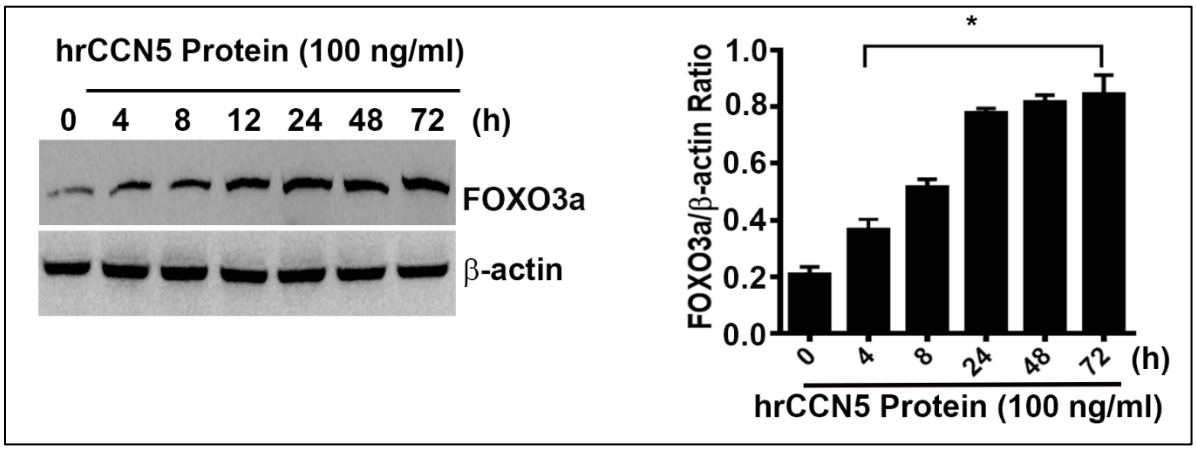


Fig.II.7b

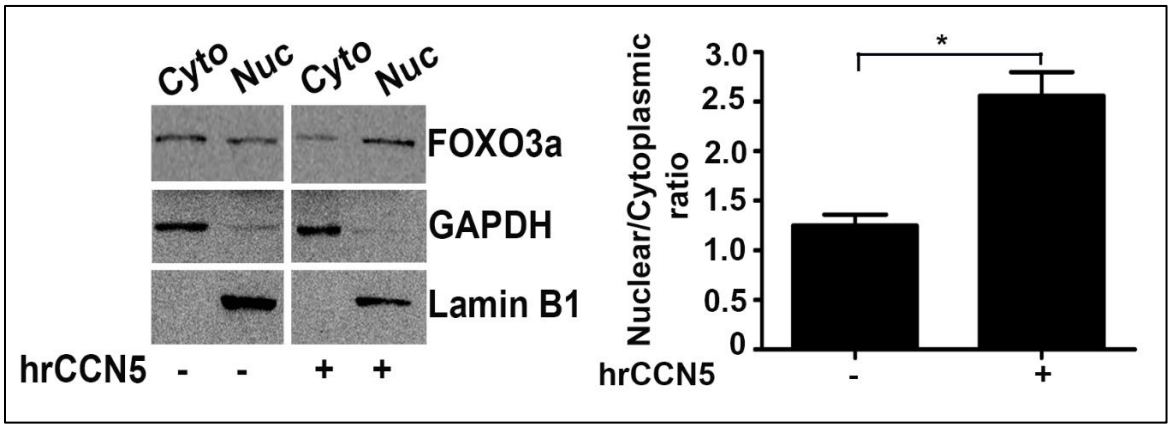


Fig.II.7: FOXO3a regulation by CCN5 in breast cancer cells. (a) Relative FOXO3a protein level in hrCCN5 protein (100 ng/ml)-treated (time-dependent) MDA-MB-231 cell extracts were assessed by western blotting. Fold changes were normalized to β-actin and all quantitative data presented as mean±s.e.m. *P<0.001 vs CCN5 untreated (0 h). P-values were determined with two-way analysis of variance. (b) Relative FOXO3a protein levels in cytosolic (Cyt) and nuclear (Nuc) fractions of untreated or hrCCN5 (100 ng/ml)-treated MDA-MB-231 cells were assessed using western blotting (left panel). GAPDH and Lamin B1 are used as quality and loading controls. Fold changes were normalized to respective loading controls and all quantitative data (nuclear-to-cytoplasmic ratio) are presented as mean ±s.e.m. of three sets of experiments (right panel). *P<0.01 vs untreated cells. P-values were determined with two-tailed t-test (Haque et al., 2015).

Fig.II.7c

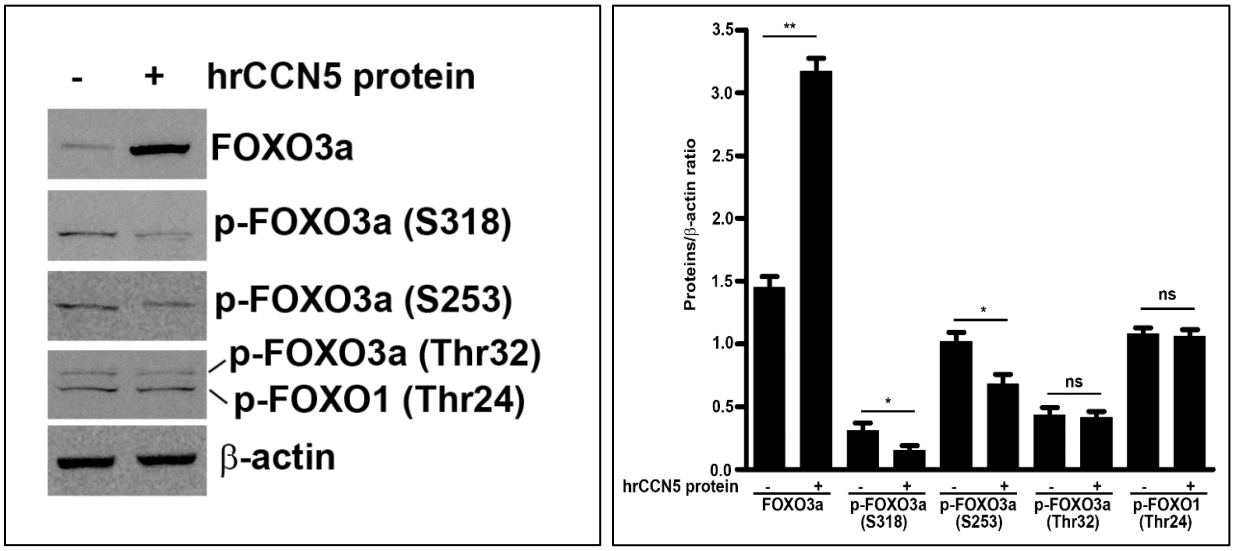


Fig.II.7d

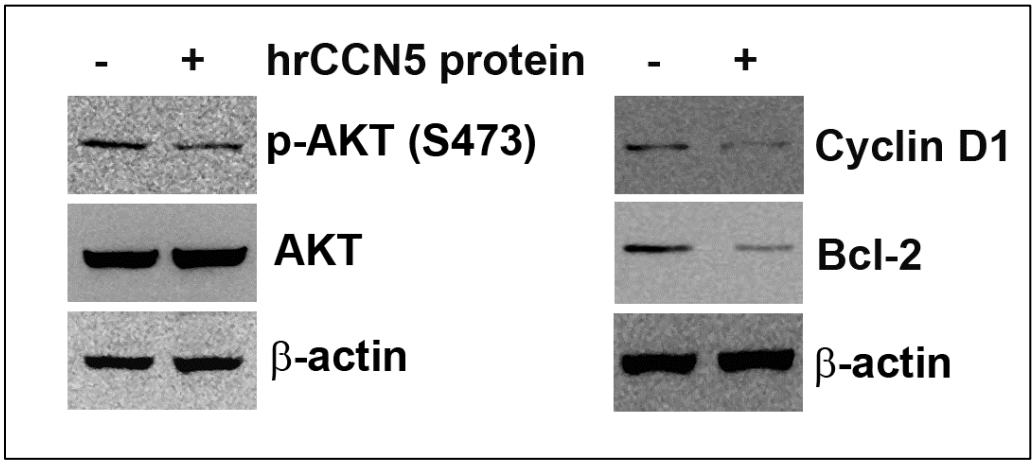


Fig.II.7: Regulation of FOXO3a and Akt phosphorylation by CCN5 signaling. (c) Representative images of western blots demonstrating relative levels of FOXO3a and different phosphorylated forms of FOXO3a in vehicle- and hrCCN5-treated MDA-MB-231 cell extracts. Bar graph represents quantitative analysis of relative levels of FOXO3a and different phosphorylated forms of FOXO3a. Fold changes were normalized to β-actin. All quantitative data are presented as mean ± SEM of at least three sets of experiments. *P<0.02 vs corresponding untreated, **P< 0.001 vs corresponding untreated. P value was generated by two-tailed t test. (d) Representative images of western blots demonstrating relative levels of p-AKT, AKT (left panel), CyclinD1 and Bcl2 (right panel) in vehicle- and hrCCN5 (100 ng/ml)-treated MDA-MB-231 cell extracts (Haque et al., 2015).

Fig.II.8

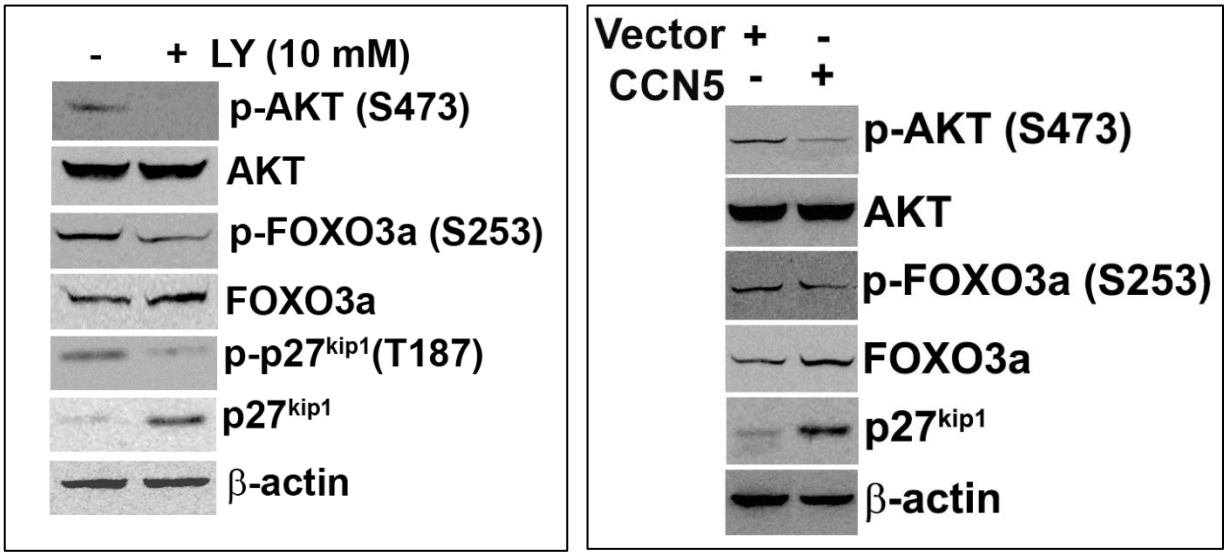


Fig.II.8: Akt inhibitor treatment mimics CCN5 overexpression: Relative levels of phosphorylation in AKT, FOXO3a and p27Kip1 in MDA-MB-231 cells treated with an AKT inhibitor LY294002 (10mM) or vehicle for 3 h were determined using western blot analysis. Fold changes were normalized to β-actin, a loading control (left panel). Relative levels of phosphorylation in AKT, FOXO3a and expression level of p27Kip1in vector alone- or CCN5-vector-transfected MDA-MB-231 cell extracts were determined using Western blot analysis. Images are representative of three independent experiments (Haque et al., 2015).

Fig.II.9

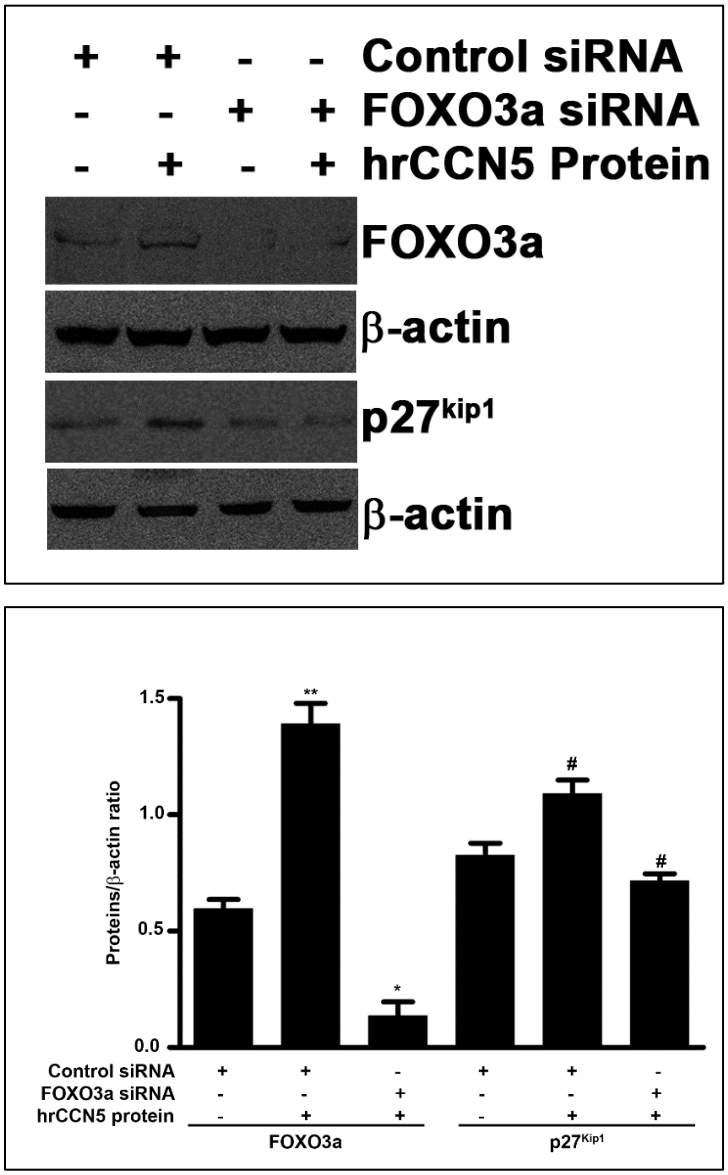


Fig.II.9: CCN5 mediated upregulation of p27Kip1 is FOXO3a dependent: Representative images of western blots demonstrating relative levels of FOXO3a and p27Kip1 in FOXO3a-depleted MDA-MB-231 cells treated with hrCCN5. Bar graph represents quantitative analysis of relative levels of FOXO3a and p27Kip1 in FOXO3a-depleted MDA-MB-231 cell treated with hrCCN5. Fold changes were normalized to β-actin. All quantitative data are presented as mean ± SEM of at least three sets of experiments. *P<0.0001 vs control siRNA. **P<0.001 vs control siRNA and #P<0.003 vs control siRNA. P value was generated by two-tailed t test (Haque et al., 2015).

Fig.II.10

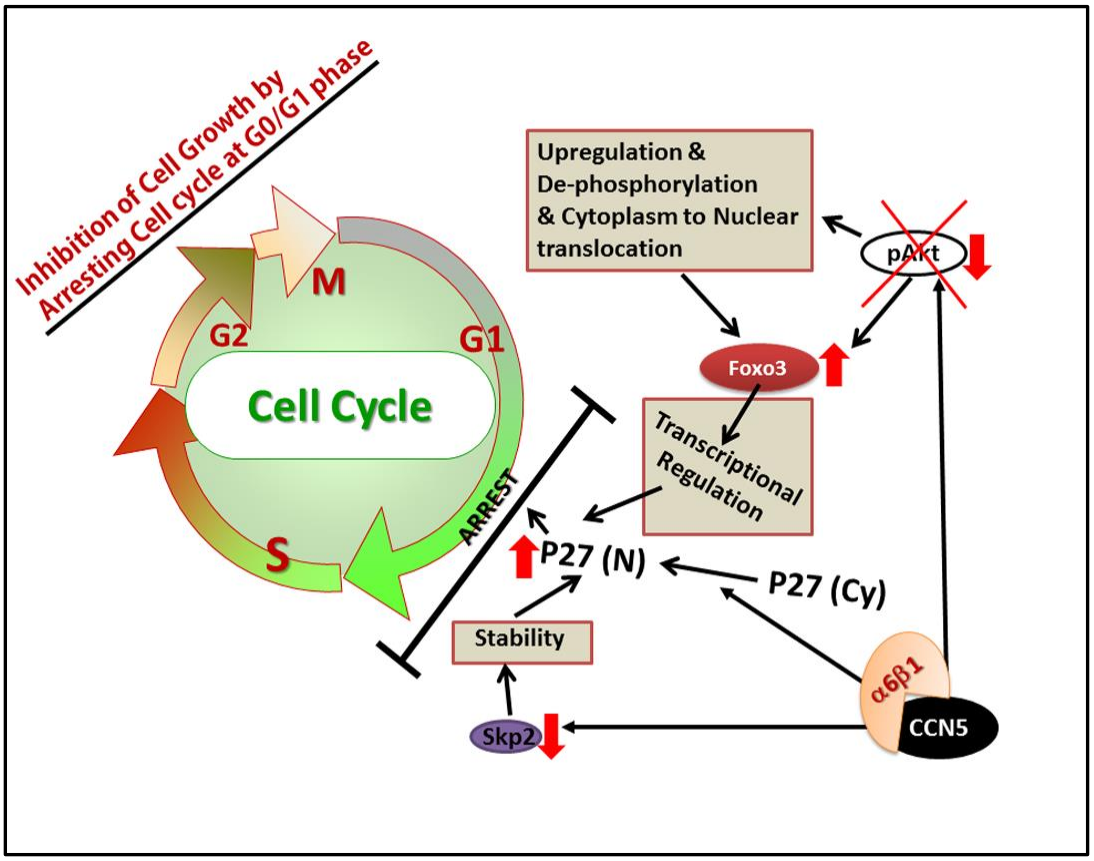


Fig.II.10: CCN5 signaling network for p27Kip1-dependent TNBC cell growth arrest: Schematic representation of CCN5 mediated transcriptional regulation of p27Kip1 by nuclear localization of FOXO3a and nuclear localization and stabilization of p27Kip1(through downregulation of Skp2 and dephosphorylation at T187) and leading to cell cycle arrest. CCN5 binds to integrins α6β1to exert its inhibitory effect on Akt phosphorylation(inhibition) which in turn leads to the aforesaid transcriptional activation (through Foxo3 dephosphorylation) and nuclear localization of p27Kip (through dephosphorylation at T157) (Haque et al., 2015).

CHAPTER III: Role of CCN5 in inducing expression of Estrogen Receptor -α in normal breast epithelial cells and triple-negative breast cancer cells and sensitization of the cancer cells to tamoxifen.

INTRODUCTION

Estrogen Receptor -A member of Nuclear Hormone Receptor family

Nuclear hormone receptors were discovered from the studies that aimed to investigate the cellular mediators of lipophilic, cell membrane permeable hormones (Mangelsdorf et al., 1995). It was long established that these hormones along with their cellular mediators played diverse physiological roles (Mangelsdorf et al., 1995). An array of ligands was shown to bind directly and specifically to their corresponding receptor protein partners. The expression level of the receptors and their distribution in different tissues led to tissue-specific accumulation and activity of these ligands. Since the discovery, a large superfamily of nuclear receptors of about 150 members has been identified both in vertebrates and invertebrates. Ligands for some of these receptors have been recognized while the others are still putative orphan receptors as their ligands have not been yet discovered (Mangelsdorf et al., 1995). The receptors for estrogen, progesterone, retinoic acid, vitamin D and thyroid hormone are few common widely studied examples of nuclear hormone receptor. Nuclear hormone receptors are defined as 'a family of hormone-activated transcription factors that can initiate or enhance the transcription of genes containing specific hormone response elements'(MacGregor & Jordan, 1998).

Estrogen receptor was first discovered in rat uterus, with the help of radioactively labeled estrogen in 1962 by Jensen and Jacobson, who also demonstrated organ-specific retention of estrogen (MacGregor & Jordan, 1998). Estrogen receptor was identified as a member of the conserved superfamily of nuclear receptors and was one of the first RNA polymerase II transcription factors to be cloned (S. Green et al., 1986; Greene et al., 1986; Mangelsdorf et al., 1995). Eventually, in 1996 with the discovery of a second receptor for estrogen, two distinct receptors for estrogen namely, Estrogen receptor-α (ER-α) and Estrogen receptor-β (ER-β), have

been shown to exist (S. Green et al., 1986; Kuiper, Enmark, Pelto-Huikko, Nilsson, & Gustafsson, 1996). Both estrogen receptors play distinct roles in estrogen-mediated signaling in different physiologic and pathologic context.

Estrogen Receptor-α (ESR1) gene structure and promoter organization

Human estrogen receptor gene (ESR1) spans across a length of 300kb of the 'q' arm of the chromosome 6 (Gosden, Middleton, & Rout, 1986). The complex genomic organization of the ESR1 gene and its promoter region has been extensively studied, and a consensus nomenclature of the promoter region has been widely accepted (Kos et al., 2001). The promoter region of ESR1 extends for 150kb while the protein coding region extends for another 140kb. The protein coding region is comprised of 8 exons and seven introns. The protein-coding exons are named numerically one to eight (1-8). The transcription of the ER-α gene is carried out from 7 possible promoter regions comprised of upstream seven exons which are named alphabetically (A-F and T). Alternative usage of the promoter and RNA splicing leads to multiple transcript variants of ER-α. However, all the splice variants are generated using a conserved splice acceptor site located at +163 position in exon1 upstream of the translation start site at +223 position. Thus, all the splice variants of ER-α mRNA have a different 5-′UTR region but encode the same protein of size 66kDa **(Figure III.1a)**.

Estrogen receptor protein structure and dynamic changes upon estrogen and anti-estrogen binding

Both the estrogen receptors ER-α and ER-β have similar domain structure with six functional domains which are characteristic signature of the nuclear receptor family of proteins **(Figure.III.1.b)**. The N-terminal domain is known as activation function domain 1 (AF1 or A/B domain). It is poorly conserved in ER-α and ER-β leading to differential binding with other

transcription factors(Nilsson & Gustafsson, 2002).The next domain is DNA binding domain (DBD or C domain) which is highly conserved and carries two zinc finger binding motifs helping in direct recognition and binding of cis-estrogen response elements (EREs) in target gene promoter. Next to the DNA binding domain is the hinge region (D domain) which has a Nuclear Localization Sequence (NLS) and the sites for post-translational modifications of the protein (Sentis, Le Romancer, Bianchin, Rostan, & Corbo, 2005; C. Wang et al., 2001). The C-terminal region of the protein (E/F domains) have overlapping ligand binding domain (LBD) and activation function domain 2 (AF2). The ligand binding domain also carries dimerization sequence for ER-α. The AF2 domain is critical for ligand-dependent binding of other transcription co-factors. The ligand binding domain shows structural changes in the presence of ligands that regulate ER-α activity. One mobile α-helical arm of the ligand binding domain named H12 changes position depending on the presence of an agonist or antagonist. Upon binding of an agonist like estrogen, the arm position allows binding of co-activators to an exposed hydrophobic groove of an AF2 domain. Anti-estrogens displace the H12 arm in a way that it blocks the binding of co-activators to the AF2 domain (Brzozowski et al., 1997) **(Figure .III.1.d)**. Thus, in the presence of ligands, the ligand binding domain regulates the AF2 mediated co-activator binding and transcriptional activity (White, 1998).

Estrogen receptor classical and non-classical transcriptional activities

In the absence of ligands, ER-α exists as monomers and remains bound to chaperone proteins (e.g., Hsp90, Hsp70) forming heteromeric complexes (Pratt & Toft, 1997). After binding, ligand ER-α undergoes a conformational change and dissociates from the chaperone complex. These activated ER-α proteins form dimers in the nucleus to carry out the downstream transcriptional

activities. In the nucleus, ER-α can drive target gene transcription by both classical and non-classical genomic pathways.

In case of a classical genomic function, the ER-α dimers bind directly to the Estrogen Response Elements (EREs) present at the upstream promoters of the estrogen-responsive genes (Griekspoor, Zwart, Neefjes, & Michalides, 2007; Sanchez, Nguyen, Rocha, White, & Mader, 2002; White, 1998). ER-α binding to the EREs is followed by the recruitment of coactivators and components of the RNA polymerase II transcription initiation complex for transcriptional activation.

In case of a non-classical nuclear function, the ER-α binds to other DNA-bound transcription factors thus regulating the downstream gene transcription indirectly. Few popular examples are binding of estrogen bound ER-α to the AP1 (Activation Protein 1) and SP1 (Specificity Protein 1) transcription factors leading to the transcriptional activation of the corresponding downstream genes (Bjornstrom & Sjoberg, 2005; Kim, Barhoumi, Burghardt, & Safe, 2005). The ability of ER-α to bind to other transcription factors helps it to transcriptionally regulate even the genes which lack ERE sites in their promoter. Estrogen receptor-mediated estrogen signaling leads to downregulation of pro-apoptotic factors, transcriptional repressors, cell cycle inhibitory proteins and upregulation of genes related to cell cycle progression and DNA synthesis.

Regulation of estrogen receptor-α expression in breast cancer cells

A significant proportion of all the breast cancer patients have ER-α positive tumors where most of the cancer cells profoundly express ER-α. The cell proliferation in these tumors is driven by ER-α mediated transcription of cell cycle progression genes. The expression level of ER-α decides the estrogen sensitivity and endocrine therapy responsiveness of the breast tumors.

Estrogen receptor expression can be regulated - at transcriptional level by the cis-promoter elements and trans-factors; at post-transcriptional level by alteration of RNA stability (Ing, 2005; Pinzone, Stevenson, Strobl, & Berg, 2004) and regulation of translation efficiency (Ing, 2005); and at post-translational level by ligand (Laios et al., 2003; Laios et al., 2005; Long & Nephew, 2006), cofactor and phosphorylation (Valley et al., 2005) dependent regulation of protein stability and degradation.

Transcriptional regulation of ER-α expression

The proximal promoters A and B have been studied widely as these were the first regulatory sites of ER-α regulation to be discovered (Kos et al., 2001). Expression from these two promoters has been established to be a significant player for ER-α overexpression in breast cancer. The majority of ER-α promoter activity resides within the promoters A and B, between -245 bp and +212 bp relative to the first described transcription start site at exon 1 (S. Green et al., 1986). The minimal promoter encompassing the mentioned region has multiple conserved transcription factor binding sites (e.g., GC box for binding of Sp1 and Sp3 binding,).

It has been shown that ER-α can regulate its expression from the minimal promoter by interacting with the Sp1 transcription factors bound to the GC box in the minimal promoter (deGraffenried, Hilsenbeck, & Fuqua, 2002; deGraffenried, Hopp, Valente, Clark, & Fuqua, 2004). A wide variety of transcription factors regulate expression of ER-α from the minimal proximal promoter as well as from the distal promoter elements. ERBF1- transcription factor has been shown to drive ER-α transcription from promoter C (Tanimoto, Eguchi, Yoshida, Hajiro-Nakanishi, & Hayashi, 1999). p53 can also activate the ER-α transcription from the minimal promoter interacting with factors that bind the -70 to -40 bp region of promoter A (Angeloni et al., 2004).

In multiple studies, it has been shown that transcriptional regulation of ER-α expression can be dictated by chromatin remodeling. A transcription factor AP2γ (ERF-1) induces DNase1 hypersensitive sites at the promoter A of ER-α gene in human mammary epithelial cells (and possibly also at promoter B and C in ER-α overexpressing breast cancer cells).Thus, these phenomena lead to trans-activation of ER- α expression (McPherson, Baichwal, & Weigel, 1997; McPherson & Weigel, 1999; Schuur, McPherson, Yang, & Weigel, 2001).

ER-α transcription has been shown to be regulated through chromatin remodeling in breast cancer, by direct binding of pRb2/pl30 complex within the promoter A. It engages a multi-protein complex differentially recruiting specific chromatin modifying enzymes including histone methyltransferase SUV39H1, DNMT1 (DNA methyltransferase 1), HDAC1(Histone De-acetylase 1) and HAT (Histone Acetyl Transferase) p300. It has been proposed that., in MCF-7 cells, the high activity of p300 within the pRb2/pl30 complex induces the activation of ER-α transcription from promoter A by maintaining an open state chromatin (Macaluso, Cinti, Russo, Russo, & Giordano, 2003; Macaluso, Montanari, & Giordano, 2005; Macaluso et al., 2007). In the ER-negative MDA-MB-231 cells, the pRb2/pl30-based multiprotein complex represses ER-α gene transcription by chromatin condensation through the recruitment of DNMT1 and concomitant dislocation of HAT p300. Both DNMT1 and HDAC1 are established as important factors involved in the epigenetic silencing of many genes including ER-α.

Transcriptional regulation of ER-α by FOXO3a transcription factor and possible role of CCN5

The transcription factor FOXO3a has also been implicated in the regulation of ER-α gene transcription. As discussed in detail in the previous chapter, FOXO3a, a member of the mammalian Forkhead Box (FOX) family of transcription factors has been shown to regulate

expression of multiple genes implicated in cell cycle progression. In a study using breast cancer cell lines, it has been identified that the nuclear localization of FOXO3a, correlates with ER-α expression(Guo & Sonenshein, 2004). The study using Chromatin Immuno-Precipitation and reporter assays have shown that FOXO3a can directly bind and regulate transcription of ER-α from promoter B of the gene. As the previous chapter describes, it has been established that -CCN5 through modulation of the PI3K-AKT pathway influences phosphorylation status of FOXO3a, leading to nuclear localization and stabilization of FOXO3a (Haque et al., 2015). In multiple studies, our lab has noticed a direct correlation between ER-α and CCN5 expression which was an expected observation given the established fact that, CCN5 is an estrogen-responsive gene under direct transcriptional regulation of ER-α (S. Banerjee et al., 2003; Fritah et al., 2006; Inadera et al., 2000). However, conversely other published studies have shown, and in multiple experiments, our lab has noticed that ER-α expression level can be influenced by presence and expression level of CCN5 in breast cancer cells (Fritah et al., 2008). All these collective preliminary observations led us to investigate the possible role of CCN5 in the expression of ER-α in normal and cancerous breast epithelial cells.

Estrogen receptor-α (ER-α) mediated roles of estrogen in breast physiology and cancer

Since its discovery estrogen receptor-α (ER-α), the estrogen-dependent transcription factor, encoded by the ESR1 gene has been widely studied. These studies established its essential role in the estrogen-dependent development of the female reproductive system, reproductive functions, neuroendocrine functions, cardiovascular functions and breast carcinogenesis (Emmen & Korach, 2003; Ferguson & Davidson, 1997; Korach et al., 2003; Nilsson & Gustafsson, 2002).

A subset of non-proliferating epithelial cells expresses ER-α in rodent and human normal mammary gland tissues (R. B. Clarke, Howell, Potten, & Anderson, 1997; J. Russo, Ao, Grill, & Russo, 1999; Seagroves, Lydon, Hovey, Vonderhaar, & Rosen, 2000). However, it has been shown in studies conducted in mice that ER-α absolutely critical for the growth and morphogenesis of the adult mammary gland (Mallepell, Krust, Chambon, & Brisken, 2006). Consequently, studies from the same group suggested that the ER-α-mediated paracrine signaling pathways through activation of amphiregulin expression (Ciarloni, Mallepell, & Brisken, 2007; LaMarca & Rosen, 2007) promote proliferation of the neighboring ER-α-negative epithelial cells and morphogenesis in the mammary gland (Mallepell et al., 2006).

Role of estrogen in breast cancer was first indicated in studies that date back to the end of the 19th century. In 1896, British physician Beatson discovered that ovariectomy resulted in tumor regression in premenopausal women with metastatic breast cancer (MacGregor & Jordan, 1998). Soon after this discovery, another study reported that about one-third of all of the breast cancer patients show tumor regression after ovariectomy (MacGregor & Jordan, 1998). This background knowledge, after the discovery of estrogen receptors, translated to the development of the first ER assays in the 1960s (by Jensen and his colleagues) as a predictive test. The test was designed to determine whether and which breast cancer patients would respond to estrogen ablation by ovariectomy (in premenopausal women) or adrenalectomy (in postmenopausal women). This test, in a way, laid the foundation for modern endocrine therapies for ER-α positive breast cancers.

Though most of the typical mammary epithelial cells do not express ER-α, the majority (~75%) of human breast cancers and precursor lesions express high levels of ER-α (Allred, Brown, & Medina, 2004). In a transgenic mouse model study, it has been shown that ER-α overexpression

in mammary epithelial cells is associated with the precursor lesions (Frech et al., 2005). Moreover, higher ER-α expression was observed in the mammary epithelial cells of female populations who are at higher risk for breast cancer as compared to the populations at relatively lower risk (Lawson et al., 1999).To our interest studies have noticed that deregulation, dysfunction or suppression of ER-α involve in tumor aggressiveness, metastasis and possibly hormone resistance (Harrell et al., 2006).

Endocrine therapy strategies and use of tamoxifen as an effective treatment drug

As mentioned previously in this chapter, it has been observed for a long time that estrogen ablation by ovariectomy prevents the progression of estrogen receptor-positive breast cancer, which paved the way for modern endocrine therapy strategies. Approximately 80% of the breast cancers are ER-α positive and are dependent for their growth on estrogen and functional ER-α (Ali & Coombes, 2002; Kohler et al., 2015). Endocrine therapies which are widely practiced clinically involves three classes of drugs – a) Aromatase Inhibitors (AIs), b) Selective estrogen receptor modulators (SERMs) and c) Selective estrogen receptor downregulators (SERDs).

a) Aromatase Inhibitors (AIs): AIs act by preventing the formation of estrogen from androgens in postmenopausal women through inhibition of the aromatase enzymes. Anastrozole (Arimidex®), letrozole (Femara®) and exemestane (Aromasin®) are the 3 FDA-approved AIs for the treatment of ER-α-positive breast cancer in postmenopausal women. Adjuvant therapy (therapy used to treat breast cancer patients after primary treatments mostly surgery and radiation therapy) with an aromatase inhibitor in early stage breast cancer for post-menopausal patients reduces the risk of recurrence and improves survival. It acts better compared to an adjuvant tamoxifen (SERM) treatment (Early Breast Cancer Trialists' Collaborative, 2015). The aromatase inhibitors are approved and used to treat postmenopausal women as initial therapy for

metastatic or locally advanced hormone-sensitive breast cancer or women with advanced breast cancer whose disease has worsened after treatment with tamoxifen (Mauri, Pavlidis, Polyzos, & Ioannidis, 2006).

b) Selective Estrogen Receptor Modulators (SERMs): SERMs are named so as these drugs bind to estrogen receptors in such a way that they can potentially both block estrogen activity (as estrogen antagonists) in some tissues and mimic estrogen effects (as estrogen agonists) in other tissues. Examples of SERMs approved by the FDA for treatment of breast cancer are tamoxifen (Nolvadex®) and toremifene (Fareston®).

Tamoxifen (**Figure.III.1.c)** has been used for more than 40 years now as the first line treatment of early and advanced ER-α-positive breast cancers in both pre- and post-menopausal women (Jordan, 2007). Tamoxifen is activated by its active metabolites including 4-hydroxytamoxifen in the body, which outcompetes estrogen in binding the Ligand Binding Domain of ER-α (Ring & Dowsett, 2004). This phenomenon results in the blocking of the Activation Function 2 region (AF2) of the receptor and inhibition of ER-α- mediated transcription through recruitment of co-repressors such as NCoR and SMRT (K. A. Green & Carroll, 2007). However, tamoxifen exhibits beneficial and estrogen-like effects in tissues like bones, thus preventing osteoporosis in post-menopausal women (Jordan, 2007). Also, the partial-agonist activity of tamoxifen in the uterus has been shown to increase the risk of endometrial cancer (Fisher et al., 1998).

Tamoxifen is FDA-approved for adjuvant hormone therapy of premenopausal and postmenopausal women with early-stage ER-α positive breast cancer as these women who receive at least five years of tamoxifen therapy after surgery has reduced risks of breast cancer recurrence. (Untch & Thomssen, 2010) . Both the FDA-approved SERMs for breast cancer treatment, tamoxifen and toremifene are used to treat metastatic breast cancer. Multiple large

clinical trials have shown that five years of tamoxifen treatment, reduced the risk of developing invasive breast cancer by about 50% in postmenopausal women who were at increased risk (Cuzick et al., 2015; Vogel et al., 2006). Tamoxifen was the first drug to be approved by FDA for breast cancer prevention after successful clinical studies(Fisher et al., 1998).

c) Selective Estrogen Receptor Downregulators (SERDs): Pure-anti-estrogen drugs such as FDA-approved fulvestrant (Faslodex®), have been found to be effective for treatment of tamoxifen non-responsive tumors. SERDs like fulvestrant bind to ER-α with much higher affinity than tamoxifen and downregulate the cellular levels of the receptor by targeting them for degradation *via* the 26S proteasomal complex. Fulvestrant prevents receptor dimerization and blocks the nuclear localization of the receptor. Most importantly, fulvestrant has no estrogen agonist activity since it blocks both the AF1 and AF2 sites of the receptor, resulting in complete downregulation of ER-α mediated transcription(Osborne, Wakeling, & Nicholson, 2004). FDA approves Fulvestrant for postmenopausal women with metastatic ER-α positive breast cancer that has relapsed and spread after treatment with other anti-estrogen therapies including tamoxifen(Howell, 2006).

Mechanisms of endocrine therapy resistance

Tamoxifen treatment in approximately 70% of ER-positive breast cancers result in tumor remission and improve the overall survival. However, all patients with metastatic disease and almost 40% of patients with early-stage breast cancer receiving adjuvant tamoxifen therapy eventually have a relapse of cancer. The cancer cells acquire resistance to tamoxifen despite continued expression of ER-α or loss of ER-α expression (Ali & Coombes, 2002; R. Clarke, Tyson, & Dixon, 2015; Ring & Dowsett, 2004). De-novo resistance to tamoxifen can be noticed in ER-α negative tumors, but interestingly a significant fraction of ER-α positive tumors also

show resistance to tamoxifen from the beginning of treatment. As reviewed in several publications, different mechanisms that could contribute to tamoxifen resistance have been proposed including - ER-α mutation, increased prevalence of ER-α protein variants, alteration of the expression and activity of ER-α co-factors and of cell cycle regulators, as well as reduced intra-tumoral levels of metabolically activated tamoxifen (Ali & Coombes, 2002; R. Clarke et al., 2015; Nass & Kalinski, 2015). Besides these, two widely studied mechanisms for tamoxifen resistance are - cross-talk of ER-α mediated signaling with the MAPK and AKT mediated signaling pathways activated by the receptor tyrosine kinases (HER2/EGFR and IGF-IR) (Osborne, Shou, Massarweh, & Schiff, 2005) and loss of ER-α expression (Ali & Coombes, 2002; R. Clarke et al., 2015; Kurebayashi, 2003; Nass & Kalinski, 2015).

Loss/lack of ER-α expression and its significance in endocrine therapy resistance: Lack of ER-α expression in 30% of the breast tumors and loss of ER-α expression in initially ER-α positive tumors after prolonged hormonal treatments are major hurdles for the clinicians in the treatment of breast cancer. Both adaptive mechanisms and selective mechanisms have been proposed as the reason behind ER-α loss. While adaptive mechanism suggests that the ER-α expressing cells evolve and gradually stop expressing the receptor, the selective mechanism argues that the tumors being heterogeneous mass of cells, the ER-α negative cells are selected over the ER-α positive ones after prolonged endocrine treatment (Allred et al., 2004). The underlying molecular mechanisms that lead to ER-α loss is still an enigma (Kurebayashi, 2003). Loss of critical transcription factors driving ER-α expression like ERBF-1 has been suggested as a possible reason for ER-α loss (Yoshida et al., 2000).

An inverse relationship exists between EGFR/HER-2 expression and ER-α level in breast cancer. Overexpression of these growth factor receptors is associated with decreased sensitivity

to endocrine therapy and poor prognosis (Ciocca et al., 1992; Konecny et al., 2003). It has been established that sustained growth factor signaling mediated by the tyrosine kinase receptors and activated cell survival pathways (MAPK and PI3K pathways) lead to decrease and loss of ER-α expression (Creighton et al., 2010; Massarweh & Schiff, 2006; Oh et al., 2001; Saceda et al., 1996) which eventually results in endocrine non-responsiveness.

It has been described in an elegant study that suppression of hyperactivated PI3K-AKT pathway leads to stabilization and nuclear localization of the transcription factor FOXO3a which activates ER-α expression from promoter B (Guo & Sonenshein, 2004). This study and the findings discussed in this chapter establish a link between the activated cell survival pathways and downregulation of ER-α transcription.

Epigenetic silencing of the ER-α gene has also been proposed as one of the mechanisms for lack or loss of ER-α expression. Recruitment of a transcriptional repression complex including DNMT1 (causing methylation of CpG islands in ER-α promoter) and HDAC1 (inducing chromatin condensation by deacetylation of histones) in the proximal promoters of the ER-α gene might cause ER-α silencing in ER-α-negative breast cancer cell lines. Hypermethylation of CpG islands in the promoter region of the ER-α gene is directly correlated with lack of ER-α expression in some ER-α-negative breast cancer cells. Also, in some studies, ER-α expression was recovered by treatment with DNMT1 and HDAC inhibitors (Sharma, Saxena, Davidson, & Vertino, 2006; X. Yang et al., 2001).

MATERIALS AND METHODS

Chemicals reagent and antibody sources

All the chemicals and drugs including 4-hydroxytamoxifen (Tam or 4OH-Tam), 17β-estradiol (E2) and tamoxifen citrate were purchased from Sigma-Aldrich (St Louis, MO, USA). CCN5 human recombinant protein (hrCCN5) was obtained from PeproTech (Rocky Hill, NJ, USA). Doxycycline (Dox) was purchased from Takara Bio (Mountain View, CA, USA). Antibodies for western blot analysis, immunofluorescence, immunohistochemical staining were obtained from following vendors: Anti-ERα (Cell Signaling, Danvers, MA, USA; and Abcam, Cambridge, MA, USA), anti-pER-α (Cell Signaling), Anti-CCN5 (Abcam), anti-Akt (Cell Signaling), anti-p-Akt (Cell Signaling), mouse Anti-FLAG/DDK (Origene, Rockville, MD, USA), mouse anti-β-actin (Sigma), anti-Integrin α6 (Millipore, Billerica, MA, USA) and anti-Integrin β1 (Millipore). These companies provided the authentication certificates for all these chemicals, drugs and antibodies.

Experimental animals

Wild-type, FVB/N mice, were obtained from Taconic Biosciences (Hudson, NY, USA) and were housed in animal care facilities of the Kansas City Veterans Administration Medical Center (KCVAMC). All the transgenic mice strains used for the in-vivo studies, mentioned in this chapter, were generated and maintained in the FVB/N background. MMTV-rtTA transgenic mice (carrying reverse tetracycline transactivator expressing transgene under control of Mouse Mammary Tumor promoter) were obtained as a generous gift from Dr. Lewis Chodosh's laboratory in University of Pennsylvania. Tet-op-CCN5/GFP conditional transgenic mice (carrying transgenes CCN5 and GFP under a tetracycline-regulated operator) were generated at the University of Kansas Medical Center Transgenic facilities under the guidance of Dr. Melissa

Larson. Doxycycline-inducible mammary specific conditional transgenic mice were obtained from mating between the MMTV-rtTA transgenic mice and the Tet-op-CCN5/GFP transgenic mice at the animal care facilities of KCVAMC.

Construction of the targeting vector to generate Tet-op-CCN5-GFP mice

The objective of this procedure was to generate a mouse strain carrying the human CCN5 gene under the control of a Tet-operator (tetracycline controlled promoter). The first aim was to clone the coding sequence of the human CCN5 gene (765 bp) at the multiple cloning site (MCS) positioned downstream of the Tetracycline-regulated promoter in the pTRE-Tight BI-AcGFP-1 vector (Takara Bio, Cat#631066) **(Figure III.3a.ii)**.

The pTRE-Tight BI-AcGFP-1 vector construct carries a modified Tetracycline Response Element sequence (TRE) that provides a tighter control of gene expression and eliminates any chance of leaky transgene expression in the absence of the inducer (tetracycline). The tetracycline operator (TRE) sequence is flanked by two minimal CMV (cytomegalovirus) promoters which drive bi-directional gene expression. This vector also carries a green fluorescence protein gene sequence (Ac-GFP1) under control of one of the CMV promoters, and thus the expression of Ac-GFP1 is regulated by the TRE. Downstream of the other CMV promoter, lies the Multiple Cloning Site (MCS) which can be engineered to introduce a transgene according to study's need. Thus, once the transgene has been cloned into the construct, both the GFP and the inserted transgene will be under control of the same tetracycline operator (TRE) sequence **(Figure III.3a.iii)**.

The PRK5-hCCN5 vector was primarily obtained from Dr. Pennica (Genentech Inc., San Francisco, CA, USA). CCN5 cDNA fragments were amplified by PCR using forward and reverse primers with KpnI, and NheI restriction sequences tagged respectively. The PCR

amplified fragments were cloned into the multiple cloning site of the vectors at the KpnI and NheI restriction sites. The recombinant clones were validated by restriction digestion analysis and PCR with primers against human CCN5.

The founder mice line was generated by injecting the linearized construct into fertilized oocytes that were harvested from super-ovulated mice of FVB/N background. Finally, all the progenies (Tet-op-CCN5) were screened for the presence of the transgene using PCR analysis with the transgene-specific primer sequences (mentioned in section- 'Tail DNA extraction and genotyping'). All founders were genetically identical (FVB/N) as they were of fully inbred genetic background.

Generation of MMTV-rtTA/Tet-op-CCN5-GFP conditional bi-transgenic mice and doxycycline treatment to validate inducible CCN5 expression

Tet-op-CCN5-GFP-transgenic mice were bred with heterozygous MMTV-rtTA mice to generate MMTV-rtTA/Tet-op-CCN5-GFP-conditional transgenic mice. Tail DNA genotyping (described below) of the mice litters were performed at the age of 3 weeks to screen for the progeny mice carrying both MMTV-rtTA and Tet -op-CCN5-GFP transgenes.

After the genotype confirmation, we determined whether CCN5 is expressed conditionally in a mammary epithelial-specific and doxycycline-dependent manner. To determine the time and dose of doxycycline treatment optimal for the CCN5 induction, the drinking water of the mice were replaced, with 10% sucrose solution (and left overnight) carrying doxycycline ranging from 2 to 4 mg/ml concentration, twice a week. The control mice were fed with only 10% sucrose solution. The doxycycline treatment was carried out for two different time periods (45 and 90 days). At the endpoint, mammary tissues were harvested for validation of CCN5 overexpression.

Tail DNA extraction and genotyping

At three weeks of age individual mice from mice, litter was numbered, and a tail tip tissue up to 3mm of length was excised with sterile scissors. 50µl of The QuickExtract DNA Extraction Solution (Epicentre Bio) was added to each of the tail tissue samples, and the tissues were finely minced. Tail DNA extraction was carried out following the QuickExtract DNA Extraction Protocol provided by the vendor. Briefly, the minced tissue suspension was vortexed for 15 seconds and incubated at 65ºC for 6 minutes. The suspension was again vortexed for 15 seconds and then incubated at 98ºC for 2 minutes. After a quick spin, the supernatant was collected, and one µl of the sup was used for PCR amplification of the transgenes.

Primer sequences used for genotyping are as follows:

(1) CCN5 Forward: 5'-TCGAGGTAGGCGTGTACGGT-3'

(2) CCN5 Reverse: 5'-TATGTTTCAGGTTCAGGGG-3'

(3) rtTA Forward: 5'-TGCCGCCATTATTACGACAAGC-3'

(4) rtTA Reverse: 5'-ACCGTACTCGTCAATTCCAAGGG-3'

Cell lines, cell- culture conditions, and transfection

MDA-MB-231, MCF-7, and ZR-75-1 cell lines were procured from American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% FBS and 1% Penicillin-Streptomycin solution (Gibco,Thermo Fisher Scientific, Waltham, MA, USA) . HMECs were obtained from LONZA (MD, USA) and maintained in MEBM (mammary epithelial basal medium) with vendor provided growth factor combinations. All the cell lines were grown in a humidified incubator with 5% CO2 at 37 °C.

Transfections used for ectopic overexpression and shRNA mediated gene silencing were performed with the help of Neon Transfection System (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturers instruction. Cells were trypsinized and seeded on the preceding day of transfection in a manner that the cells reach a confluency of 70% on the day of transfection. Next day, the cells were trypsinized, washed with sterile DPBS, counted and then suspended in Buffer R (resuspension buffer). $1X10^6$ cells were mixed with plasmid DNA (5–10 μg) or siRNA (100nM concentration). The cell suspension was then electroporated for transfection at a vendor specified (specific for each cell line) voltage pulse using Neon Transfection System. For si-RNA mediated gene knockdown studies, validated si-RNA oligos were purchased from Cell Signaling Technologies (Danvers, MA, USA).

Cell viability study using crystal violet assay

Cell viability assay was performed per our previously published method. The breast cancer cells were seeded in quadruplicates in 96-well cell -culture plates. Approximately 60–70% confluent serum-deprived MCF-7 cells were treated with the CCN5 antibody (500 ng/ml) for a period of 48 h and followed by treatment with E2 (10 nM) and Tam (10 μM) alone or together for another 48 h. MDA-MB-231 cells were treated with Tam (10 μM) and hrCCN5 (250 ng/ml) together for 48 h. Cellular viability was measured using crystal violet-based assay (as described earlier in the previous chapter). The viability studies were also carried out in CCN5-shRNA transiently transfected MCF-7 cells following a similar protocol.

RT-PCR analysis

Total RNAs were extracted from mouse breast tissue samples using the Trizol reagent (Invitrogen, Thermo Fisher Scientific) following an extraction procedure as suggested by the vendor. An equal amount of RNA samples (1μg) were used for reverse transcription with the

help of a PCR based reverse transcription RNA amplification kit (Perkin Elmer, Waltham, MA, USA). PCR amplifications were carried out with human CCN5, mouse ER-α and mouse GAPDH (for loading control) -specific primers. The sequences of the primers were:

human CCN5 Forward: 5′CCTACACACACAGCCTATATC-3′

human CCN5 Reverse: 5′CCTTCTCTTCATCCTACCC-3′

mouse ER-α Forward: 5′TTCTCCCTTTGCTACGTCAC-3′

mouse ER-α Reverse: 5′ATCGCTTTGTCAACGACTTC-3′

mouse GAPDH Forward: 5′CTGCTGTCTTGGGTGCATTGG-3′

mouse GAPDH Reverse: - 5′CTCGGCTTGTCACATCT–3′

Immunofluorescent staining

Cells were fixed with methanol for a period of 20 min followed by a step of permeabilization with 0.1% Triton X-100/PBS for 5 min. Next, the samples were blocked with a ready-to-use blocking solution (Histostain kit, Invitrogen). Finally, the cells were incubated with mouse-anti-FLAG/DDK and rabbit anti-ER-α antibody overnight at 4 °C. Cells were then labeled using fluorescent conjugated anti-rabbit IgG (Alexa Flour 488, Molecular Probes, Eugene, OR, USA), and the nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Lastly, the cells were mounted with antifade mounting reagent (Molecular Probes) and examined under a fluorescence microscope (Leica).

Probe preparation and in situ hybridization

The DIG-labeled PCR-based probe preparation and in-situ hybridization were carried out following the protocol described in previously published reports (S. Banerjee et al., 2003; Stephenson, Banerjee, Saxena, Cherian, & Banerjee, 2002). Briefly, 5-µm paraffin sections were deparaffinized, hydrated and digested with proteinase K for 10 min followed by fixation in 1%

formaldehyde in Phosphate Buffered Saline (1X). Then the slides were thoroughly washed with RNase-free ddH_2O for 5 min. The sections were then incubated in a humidified chamber overnight at 37 °C with the DIG-labeled PCR-generated probe against CCN5 (250 ng/ml). The slides were washed three times with PBST (PBS with 0.1% Tween-20). Finally, alkaline phosphatase-conjugated anti-DIG antibodies were incubated for one h. The complexes were detected with the substrate chromogen combination of BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (Nitro Blue Tetrazolium chloride). The sections were counterstained with nuclear fast red stain.

Western blot analysis

For analysis of protein expression level using western blots, cells were harvested in a lysis buffer and subjected to SDS-PAGE electrophoresis and western blotting as described in the previous chapter. After incubation with primary antibodies, the blots were incubated with HRP-conjugated secondary antibodies. Finally, the blots were incubated with Super Signal Ultra Chemiluminescent (Pierce, Thermo Fisher Scientific) substrate and chemiluminescence signals were imaged and measured using Kodak ID Image Analysis Software Version 3.6 (Carestream, Rochester, NY, USA).

Luciferase reporter Promoter assay

The MDA-MB-231 cells were transfected with promoter reporter vector, pLightSwitch_LR (Active Motif, Carlsbad, CA) carrying the promoter sequence of human ESR1 (ER-α) gene or the sequence carrying human Estrogen Response Element (ERE) cloned into the multiple cloning site (MCS) of the vector, at a position immediate upstream to the Renilla Luciferase reporter gene. The transfected MDA-MB-231 cells were cultured in the presence or absence of hrCCN5

protein in OptiMEM media. The concentration of CCN5 in the medium was 250 ng/ml. A set of cells were treated with the antibodies against different integrin receptors, and the rest were left untreated. The difference of luciferase activities between each experimental sample was measured using Lightswitch™ luciferase assay system (Active Motif) following the protocol provided by the manufacturer.

In vivo treatment with CCN5 and Tamoxifen:

MDA-MB-231 xenograft model was used for this in-vivo study. Briefly, MDA-MB-231 cells (1×10^6 per injection) were resuspended in 50% Matrigel and were injected subcutaneously in right hind limb of the nude mice (N = 5 per experimental group). Once the average tumor volume reached 80–100 mm^3, mice were randomized into four groups and treatment was started. Tamoxifen (2.5 mg/mouse) was administered orally three times a week, and hrCCN5 (2 mg/Kg) was injected twice a week intratumorally for a time period of 27 days. For combinatorial treatment, both Tamoxifen and hrCCN5 were given concomitantly. Tumor growth, RTV, %TGI, and body weight of the mice were measured using Studylog® measurement tools and software (Study log Inc, San Fransisco, CA, USA) three times a week. The tumor volume (TV) was estimated using the formula: TV (mm^3) = L (length) x W^2 (width) x 1/2 The RTV was calculated using formula: RTV = Vd/Vo, where Vd is the tumor volume of each day and Vo is the initial tumor volume as recorded at the beginning of the treatments. The %TGI (percentage tumor growth inhibition) was estimated at the final day of the study by using the formula: %TGI = [1-(RTV treated group)/ (RTV control group) × 100].

RESULTS

CCN5 augments ER-α expression in normal human mammary epithelial cells (HMECs) and ductal epithelial cells of mouse mammary glands under in vitro conditions

In-vitro experiments were designed to test our hypothesis that presence of CCN5 could influence ER-α expression. We tested whether treatment of human recombinant CCN5 (hrCCN5) is sufficient to upregulate the expression and activity of ER-α in normal mammary ductal epithelial cells. To examine the effect of hrCCN5 on the expression of ER-α and pER-α in the normal human mammary epithelial cell line (HMEC), the cells were treated with hrCCN5 at a dose of 250 ng/ml for 48 h. The hrCCN5 treatment was done either in the presence or absence of a CCN5 neutralizing antibody (500 ng/ml). We observed in our western blots that both ER-α and pER-α levels were significantly increased in hrCCN5-treated HMECs compared to untreated cells **(Figure III.2a)**. Further, we noticed that the effect of the hrCCN5 on ER-α expression was reduced in the cells which were treated with CCN5-antibody along with hrCCN5 **(Figure III.2a)**.

Our next aim was to determine whether the addition of hrCCN5 in the mouse mammary gland culture media could also enhance the levels of pER-α in mammary ductal epithelial cells. Whole mammary explants were cultured under in-vitro conditions in presence and absence of hrCCN5 in the culture medium. The treatment of the whole mammary gland explants was carried out for seven days. We observed an increased signal for immune-reaction against the p-ER-α protein in the ducts/lobules and ductal epithelial cells of the whole mammary explants, treated with hrCCN5 (250 ng/ml) in the culture medium **(Figure III.2b)**.

Collectively, these results indicate that presence of CCN5 in the culture medium can influence the expression of ER-α in both human and mouse normal mammary epithelial cells and this influence is possibly exerted on the epithelial cells in a paracrine fashion.

Validation of doxycycline-regulated overexpression of CCN5 in mouse mammary epithelial cells in CCN5 overexpressing conditional transgenic model

The relevance of the in-vitro findings that indicated towards a role of CCN5 in inducing expression of ER-α was also tested in-vivo. A doxycycline(Dox)-inducible -CCN5 overexpressing transgenic mouse model (MMTV-rtTA/Tet-On-CCN5-GFP) was developed. This conditional transgenic strain was obtained from the breeding of two separate transgenic strains of mice - a) MMTV-rtTA mice line and b) a novel Tet-On-CCN5-GFP mice line **(Figure III.3a.i)**.MMTV-rtTA, as suggested by the nomenclature expresses, reverse tetracycline inducible transactivator protein (rtTA), which can bind to tetracycline response elements (TREs). However, rtTA can recognize tetracycline response elements (TREs) and drive downstream gene-transcription only after the rtTA protein is bound to tetracycline or doxycycline (a modified form of tetracycline). In MMTV-rtTA transgenic mouse line, the transgene is such designed that the transactivator protein (rtTA) expression is under control of a mammary epithelium-specific MMTV (mouse mammary tumor virus) promoter. Thus, the protein is expressed specifically in the mammary epithelial cells of MMTV-rtTA mice **(Figure III.3a.iii).**

Tet-On-CCN5-GFP mice strain carries two bidirectional transgenes, CCN5 and GFP that is under control of a tetracycline-inducible operator carrying TRE sites transcription from which can be activated by rtTA factors bound to tetracycline or doxycycline. Thus, the mice from MMTV-rtTA/Tet-On-CCN5-GFP bi-transgenic line are expected to express CCN5 protein specifically in mammary epithelial cells after doxycycline treatment **(Figure III.3a.iii)**.

We validated the CCN5 overexpressing mouse model for expression of CCN5 specifically in the mouse mammary glands upon doxycycline (Dox) treatment. Mice were selected from MMTV-rtTA/Tet-On-CCN5/GFP transgenic lines and were either treated with Dox (2 mg/ml in 10% sucrose solution) or with only vehicle (10% sucrose solution). After 30 days of Dox treatment, age-matched and estrous stage-matched control, and Dox-treated mice were sacrificed. RT-PCR analysis of RNA extracted from mouse mammary glands exhibited a significant increase in the level of CCN5 transcript in Dox-treated mice **(Figure III.3b.i)**. *In-situ* hybridization against CCN5 transcript and immunohistochemical staining against CCN5 protein were performed in the sections of mammary tissues harvested from the control and Dox-treated mice. In both the cases, a significantly higher level of reaction against CCN5 was observed in Dox-treated mice tissues as compared to control mice **(Figure III.3b.ii, Figure III.3c.i)**. The mammary ducts/lobules of Dox-treated mice also exhibited expression of GFP, a marker for successful activation of the transgene gene by Dox-treatment, as the transgene also had a Dox-inducible GFP under the control of Tet- operator **(Figure III.3c.ii).**

Conditional overexpression of CCN5 results in expression of ER-α in mammary epithelial cells in transgenic mouse model

We tested the level of ER-α expression in the mammary ductal epithelial cells of the control (vehicle treated) and experimental mice (Dox-treated) after Dox treatment for different time points (1–3 months). RNA isolation followed by RT-PCR analysis showed that Dox-induced upregulation of CCN5 resulted in a dramatic increase in mRNA level of ER-α in mice mammary glands in Dox-treated mice (**Figure III.4a**). Consistent with the RT-PCR observation, immunohistochemical analyses of sections from control and experimental mouse mammary tissues showed a significant increase in the number of CCN5- and ER-α-positive ducts and

lobules in the mammary glands of Dox-treated transgenic mice **(**10/10) compared to the glands of the control mice **(Figure III.4b)**. Collectively, these data indicate that forcibly induced CCN5 expression upregulates ER-α expression in mouse normal mammary epithelial cells.

CCN5 promotes ER-α expression in human breast cancer cells

To test whether CCN5 influences ER-α expression and activity in breast cancer cells, the effect of CCN5 ablation by CCN5shRNA (cloned in pENTR/ H1/TO) on ER-α expression in ER-α-positive breast cancer cell lines MCF-7 and ZR-75-1 was investigated. In parallel, the impact of ectopic expression of CCN5 (upon transfection of pCMV6-CCN5-DDK) on ER-α expression and activity in ER-α-negative MDA-MB-231 breast cancer cell line was also investigated. Both experiments indicated that CCN5 is a positive regulator of ER-α in breast cancer cells. We observed that knocking down CCN5 expression drastically reduces the ER-α expression in MCF-7 cells and ZR-75-1 cells **(Figures III.5a.i and III.5a.ii)**, while CCN5-DDK (CCN5-Flag) overexpression induces ER-α expression in MDA-MB-231 cells **(Figures III.5b)**.

CCN5 influences ER-α expression at the transcription level in breast cancer cells by interacting with the integrins α6 and β1

We performed the following experiment to understand the possible mechanism by which CCN5 induces ER-α expression. To elucidate the effect of CCN5 treatment, if any, on ER-α transcription, a luciferase reporter based promoter assay was designed. We transfected a luciferase reporter plasmid (pLightSwitch_Prom) carrying the human ER-α (ESR1) promoter sequence into MDA-MB-231 cells. The cells were grown for 48 h after transfection and were treated with hrCCN5 (at a concentration of 250 ng/ml) or vehicle alone for another 48 h. We observed increased luciferase activity in ESR1-prom-transfected cells which were treated with hrCCN5 as compared to ESR1-prom transfected untreated cells or vector-transfected cells.

(Figure III.6, lane 2 or 3 vs. 4). This observation suggests that CCN5 treatment can induce ER-α expression at the transcriptional level. However, the hrCCN5-untreated ESR1 promoter -transfected cells **(lane 3)** also exhibited a statistically significant increase of luciferase activity compared to empty reporter vector-transfected cells **(lanes 1 and 2).** We speculated that there might be unknown serum component which could be responsible for this increase in luciferase activity.

In the previous studies, it has been already established that CCN family proteins including CCN5 exert their influences on various cellular activities through their direct binding to integrins including integrins α6, β1- and β3 (Haque et al., 2015; Jun & Lau, 2011). We tested whether the CCN5-induced ER-α promoter activity is mediated through integrins. ER-α promoter-transfected MDA-MB-231 cells were treated with blocking antibodies against the α6, β1 and β3 integrins for 24 h followed by treatment with hrCCN5 protein. We found that treatment with antibodies against the integrins α6 and β1 significantly impaired the hrCCN5 induced effect on ER-α-promoter activity, while treatment with the blocking antibody against integrinβ3 was unable to interfere in the CCN5-mediated induction of ER-α. The treatment of integrin antibodies alone had no detectable effect on ER-α-promoter luciferase activities.

Regulation of ER-α expression by CCN5 can be mediated by PI3K-Akt-Foxo3a-signaling

It has been reported in previous studies that transcription factor FOXO3a can act as a regulator of ER-α gene transcription (Guo & Sonenshein, 2004). This FOXO3a mediated regulation of ER-α can be repressed by an activated PI3K/Akt-signaling pathway (Guo & Sonenshein, 2004). Recently, as discussed here in the previous chapter, our studies have shown that CCN5 enhances FOXO3a protein level by suppressing Akt signaling in MDA-MB-231 cells (Haque et al., 2015).

We performed experiments to test if CCN5-induced upregulation of ER-α is dependent on FOXO3a protein level in breast cancer cells. The MDA-MB231 cells were transfected with a FOXO3a-siRNA that abrogates FOXO3a expression to about 70% of the expression level as compared to scrambled siRNA transfected cells.Transfected cells were grown either in the presence or absence of hrCCN5 for 48 h. Western blotting for ER-α with the cell extracts revealed that though CCN5 treatment increased the ER-α level **(Figure III.7a, lane 2)**, knockdown of FOXO3a expression by siRNA nullifies the CCN5 mediated effect on the ER-α expression level **(Figure III.7a, lane 3)**.

We observed a similar effect in the normal mammary epithelial cell line (HMEC). In a very similar experimental design, HMEC cells were transfected with FOXO3a siRNA and scrambled siRNA before hrCCN5 treatment. Immunofluorescence staining against ER-α was performed, and it was observed that CCN5 treated cells transfected with FOXO3a siRNA exhibited almost no staining for ER-α. However, a signal for ER-α staining was detectable in scramble siRNA transfected cells treated with CCN5 **(Figure III.7b)**. These studies, collectively, suggest that CCN5-induced upregulation of ER-α is mediated by the transcription factor, FOXO3a.

Earlier studies have shown that FOXO3a expression level is negatively regulated by activated PI3K-Akt signaling as discussed herein the previous chapter. Previously published studies from our lab has established that CCN5 treatment reduces Akt phosphorylation levels in MDA-MB-231 cells leading to FOXO3a stabilization (Haque et al., 2015). We can argue that suppression of Akt activity by CCN5 leads to increase in ER-α expression via FOXO3a stabilization in MDA-MB-231 cells.

ER-α induced by CCN5 in MDA-MB-231 cells is functionally active

We performed an estrogen-response element (ERE) luciferase reporter assay in-vitro, in the presence or absence of estrogen (E2) in MDA-MB-231 cells. This experiment aimed to determine whether the ER-α, expressed after CCN5 treatment of cells, is functionally active. ER-α binds directly to the conserved sequences known as the estrogen response elements (ERE) in the promoter region of a gene and carries out its transcriptional activity leading to downstream gene expression.

First, MDA-MB-231 cells were transfected with the pLightSwitch_Prom vector carrying ERE upstream to a luciferase reporter gene. The cells were then treated with either E2 (10 nM) or hrCCN5 alone or with a combination of both for 48h. Finally, luciferase activity from each experimental group was measured as a readout of ER-α activity. E2-treatment significantly enhances luciferase activity in the hrCCN5-treated cells **(Figure III.8a lane 3)** as compared to vehicle-treated **(lane 1)** and only estradiol-treated cells **(lane 4)**. This study indicates that the hrCCN5-induced ER-α is functionally active as it can form E2-ER complexes and bind to EREs of DNA leading to gene transcription.

Interestingly we observed that the luciferase-activity also markedly elevated in hrCCN5-treated cells in the absence of E2-treatment **(lane 2)**. We can argue that estrogen-independent activation of ER-α possibly caused due to phosphorylation of ER-α by CCN5 might have promoted ER-α-ERE binding in-vitro in the absence of the ligand.

Phosphorylation of ER-α plays an effective role in the regulation of the functional activity of ER-α, and it is mediated by the controlling ER-α-ERE binding that modulates the expression of the downstream target genes. We examined the effect of the ectopic expression of CCN5 in MDA-MB-231 cells or the shRNA-based depletion of CCN5 in MCF-7 cells, on the phosphorylation

level of ER-α at Ser104/106. The phosphorylation status of ER-α at Ser104/106 is considered as a marker for ER-α activity. We found that the level of phospho-ER-α (Ser104/106) was significantly increased in CCN5-transfected MDA-MB-231 cells as compared to the vector-transfected cells. Also, the p-ER-α level is markedly reduced in CCN5-depleted MCF-7 cells as to their corresponding control cells **(Figures III.8b.i and III.8b.ii)**.

Together, these studies prove that the ER-α induced by CCN5 treatment or overexpression in MDA-MB-231 cells are functionally active and are capable of driving transcription of their target genes.

Suppression of CCN5 expression reduces sensitivity of ER-α-positive breast cancer cells to estrogen and tamoxifen

We aimed to investigate the possible effect of CCN5 depletion and inhibition on the response of ER-α positive breast cancer cell lines to estrogen(E2) and tamoxifen. Consistent with previous findings we observed that E2 (10 nM) treatment for 48 h, significantly increased proliferation of ER-α positive MCF7 cells. This pro-proliferative effect of E2 was significantly reduced when the cells, pre-treated with the CCN5-blocking antibody (CCN5Ab) for 48hrs were treated with the same dose of E2 in the presence of CCN5Ab **(Figure III.9a.i)**.

Moreover, we also observed that though not completely, CCN5Ab treatment of MCF7 cells partially reduced the cytotoxic effect of 4-hydroxytamoxifen in these cells. The cells, when treated with CCN5Ab before and during the 4-hydroxytamoxifen treatment (10 µM), responded to a lesser extent as compared to the cells which were not treated with the antibody **(Figure III.9a.i)**. The partial rescue of MCF-7 cells from the catastrophic effect of 4-OH-Tam can also be seen in CCN5 depleted shRNA- CCN5-transfected MCF-7 cells. , Control shRNA and CCN5-shRNA transfected cells were treated with 4-OH-Tam at a dose of 10µM for 48h, and it was

observed that cell growth arrest induced by 4-OH-Tam in CCN5 depleted cells is significantly lower as compared to control cells **(Figure III.9a.ii)**. , Tamoxifen exerts receptor (ER-α)-dependent and independent influence on breast cancer cells to induce growth arrest and apoptosis. Thus, we argue that CCN5 ablation, which interrupts ER-α expression, may only partially promote desensitization to Tamoxifen.

CCN5 treatment sensitizes triple negative MDA-MB-231 cells to tamoxifen

The observations discussed so far in this chapter indicate strongly that CCN5-mediated signaling might play a critical role in ER-α-expression in normal and neoplastic breast cells. We extended our study to test its implication in possible therapeutic strategies against triple negative breast cancer.

Firstly, we investigated the impact of 4-hydroxytamoxifen (at a dose of 10 μM) on MDA-MB-231 cell growth both in the presence and the absence of hrCCN5 (250 ng/ml). The cell viability studies revealed a minimum growth inhibitory effect of Tamoxifen (10 μM for 48 h) on MDA-MB-231 cells. An increased effect on cell growth arrest was observed when MDA-MB-231 cells were treated with 4-hydroxyamoxifen in conjunction with hrCCN5 for 48 h. A growth inhibition of MDA-MB-231 cells was also documented in the experimental sets with the treatment of only hrCCN5 **(Figure III.9b)**. Collectively, the studies suggest that CCN5 boosts the Tamoxifen action in these cells through the activation of ER-α.

The possible therapeutic potential of Tam-hrCCN5 combined treatment of triple negative breast cancer was further explored in an MDA-MB-231 xenograft model. In this xenograft model, while treatment of Tamoxifen alone led to no significant response in tumor growth, combinatorial treatment of hrCCN5 and Tamoxifen exhibited significant inhibition of tumor growth. Relative tumor volume (RTV) was measured for each time point of tumor volume

measurement, and percentage tumor growth inhibition (%TGI) relative to control tumors was calculated. A decrease in the relative tumor volume and the TGI increase of significant level was observed in the CCN5, and Tam treated tumors as compared to the tumors from other experimental groups **(Figure III.10a)**. We performed immunohistochemical staining against ER-α and CCN5 in the sections of the harvested tumors. We detected an increased staining for ER-α in the CCN5 treated tumor sections while no such staining was observed in the tumor sections from the control groups **(Figure III.10b)**. Immunoreaction for CCN5 protein, which is normally absent in MDA-MB-231-tumor xenograft tissues, was detected in tumor samples of treated groups implicating the availability of hrCCN5 protein at the target sites **(Figure III.10b)**. Importantly, no signs of morbidity or body weight loss were detected in these group of animals indicating undetectable or controllable toxicity from CCN5 and tamoxifen combinatorial treatment.

DISCUSSION

The experiments and observations discussed in this chapter provide two significant discoveries. First, these studies prove that CCN5 promotes ER-α expression in normal breast epithelial cells and breast cancer cells. CCN5-mediated induction of ER-α expression, at least in breast cancer cells, takes place at the transcriptional level. Our studies indicate that CCN5 exerts its influence on the breast cancer cells through stabilization and nuclear localization of FOXO3A transcription factor which is a result of integrin-mediated suppression of PI3K-AKT signaling pathway **(Figure III.11)**. Second, we report that ER-α, restored by CCN5, is functionally active and renders triple-negative breast cancer cells MDA-MB-231 sensitive to tamoxifen.

More than 70% of the human breast cancers show high expression of ER-α (Allred et al., 2004), which is an important biomarker for better prognosis as these tumors respond to hormonal therapies such as Tamoxifen treatment. The non-responding tumors either do not express ER-α or show resistance despite ER-α expression. As discussed in the introduction section only two-thirds of the advanced ER-positive breast cancer patients respond to Tamoxifen (Kurebayashi, 2003).A significant proportion of tumors, which respond initially, gradually become hormone-independent and endocrine therapy resistant and lose ER-α expression. Collectively, it is manifested that the disappearance of ER-α in breast cancer cells is one of the vital causes of relapse and aggressive behavior of this disease (Gruvberger et al., 2001; Kurebayashi, 2003; Nass & Kalinski, 2015).

Our findings from in-vitro and mouse model studies indicate a strong link between CCN5 signaling and ER-α expression. The mechanism that we propose, based on these results and our previous work (Haque et al., 2015) is that CCN5 interacts with integrin α6β1 to suppress the PI3K/Akt-signaling which then activates FOXO3a. Activated FOXO3a, in turn, upregulates

activated form of ER-α and enhances the response of estrogen or estrogen antagonist. As discussed in the previous chapter (General Introduction), CCN5 is an estrogen response gene, and this study proposes the possibility of a feedback signaling loop between CCN5 and ER-α. However, we believe that this might be only one of the multi-dimensional mechanisms implemented by CCN5 to exert this effect. Thus, suppression of PI3K-AKT pathway alone with an inhibitor will not be sufficient to replicate the effect of CCN5 treatment. Epigenetic silencing of ER-α gene promoter has been implicated in lack or loss of ER-α expression (Sharma et al., 2006; Yan, Yang, & Davidson, 2001). Interestingly, in a study, it has been shown that CCN5 might directly localize into the cell nucleus and interact with histone deacetylases to affect transcriptional changes (Sabbah et al., 2011). Though the evidence of nuclear localization of CCN5 is sparse, we cannot rule out the possibility that it might also play a similar role in this case in addition to the mechanism we elucidated. Also, it has been shown in multiple studies that activated Akt signaling directly phosphorylates at conserved Serine residues in activation function one domain of ER-α and leads to a reduction of tamoxifen activity (Massarweh & Schiff, 2006; Osborne et al., 2005). Thus, CCN5 mediated suppression of PI3K-AKT pathway might be an additive in inducing tamoxifen sensitivity.

ER-α is required for the Tamoxifen action as an antagonist of estrogen to prevent breast cancer cell growth. The present *in vitro* and *in vivo* studies holds therapeutic significance by providing a mechanism-based rationale for combination therapy of Tamoxifen and CCN5 to treat triple negative breast cancers.

FIGURES

Fig.III.1a

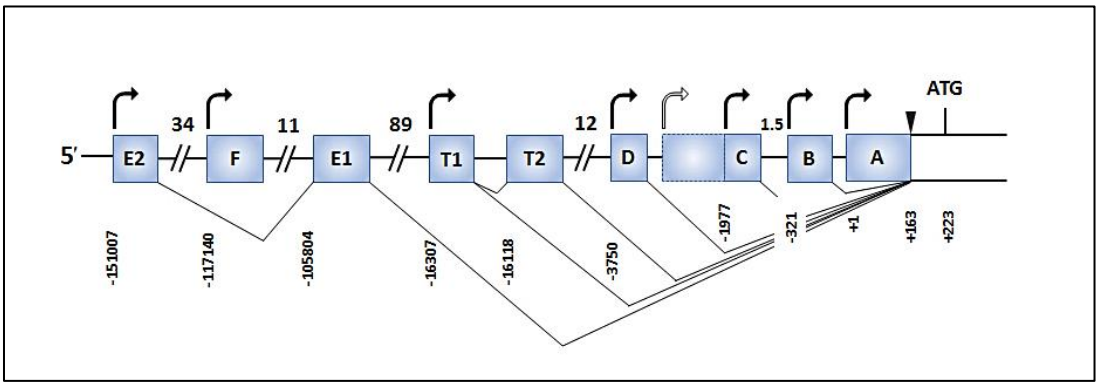


Fig.III.1b

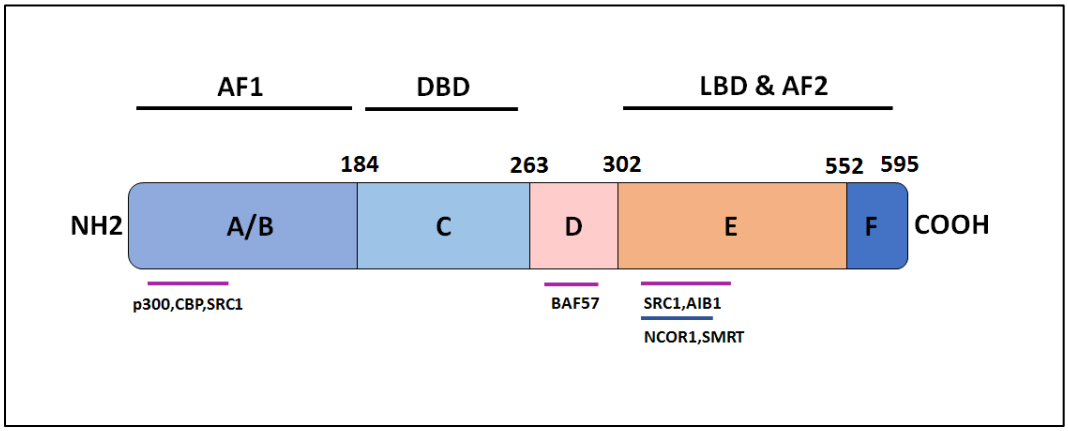


Fig.III.1: a) Genomic organization of ESR1 (ER-α) promoter: Boxes represent upstream exons with names according to the suggested nomenclature. Promoters are depicted as broken arrows. Numbers below exons correspond to the distance from the originally described transcription start site _1 in base pairs. Numbers between exons show the size of major introns in kilobase pairs. Broken lines symbolize observed splicing, and the common acceptor splice site in exon 1 is represented by an inverted triangle. The figure has been adapted from previous publication (Kos, Reid, Denger, & Gannon, 2001).b) Domain structure of ER-α protein depicting the Activation function domains, ligand binding domain and DNA binding domain. Pink line shows binding regions for co- activators. Blue line shows binding regions of co-repressors. The figure has been adapted from previous publication (K. A. Green & Carroll, 2007).

Fig.III.1c

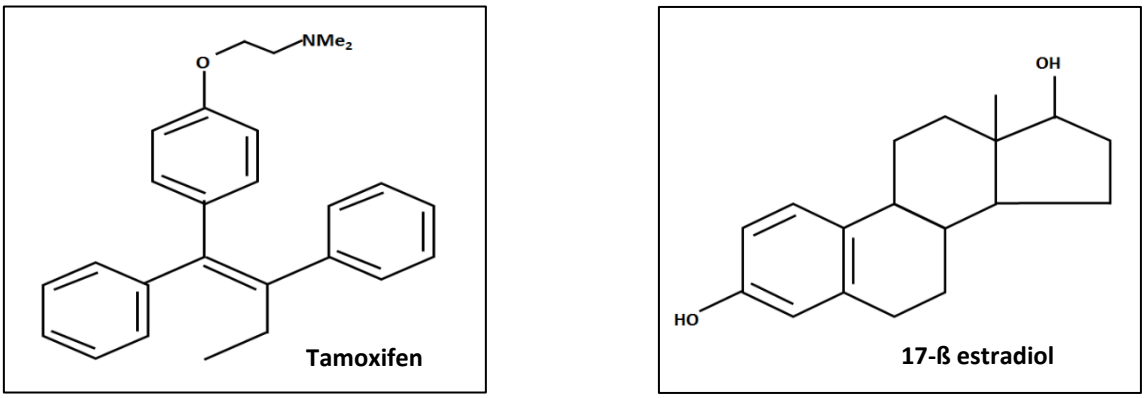


Fig.III.1d

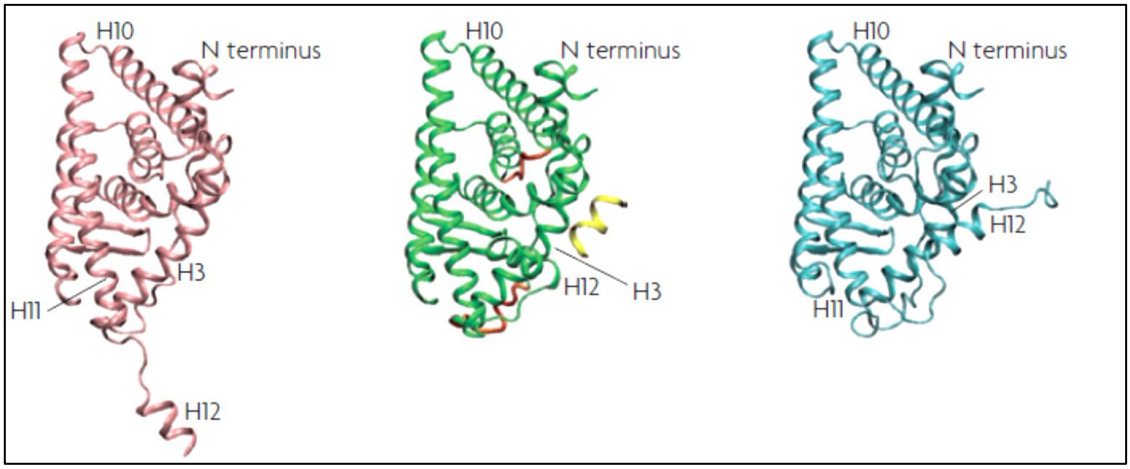


Fig.III.1: c) Chemical structure of estrogen and tamoxifen. d) Flexible H12 helical arm of ER-α in presence of different ligands: The backbone structure of unbound ERα (pink), ERα bound to estrogen (green) and ERα bound to tamoxifen(blue) show the differences in the position of helix 12 which determine co-factor binding properties. The picture has been used from previously published article (K. A. Green & Carroll, 2007). Figure used from the cited article with permission.

Fig.III.2a

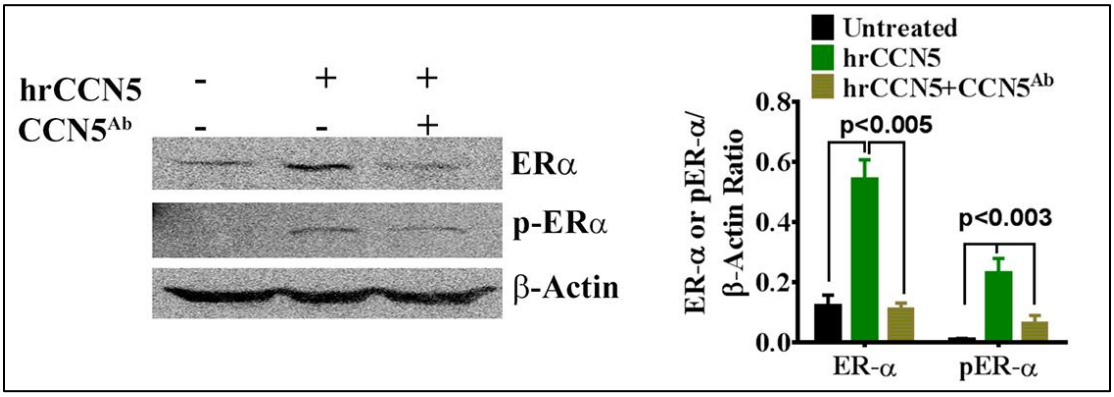


Fig.III.2b

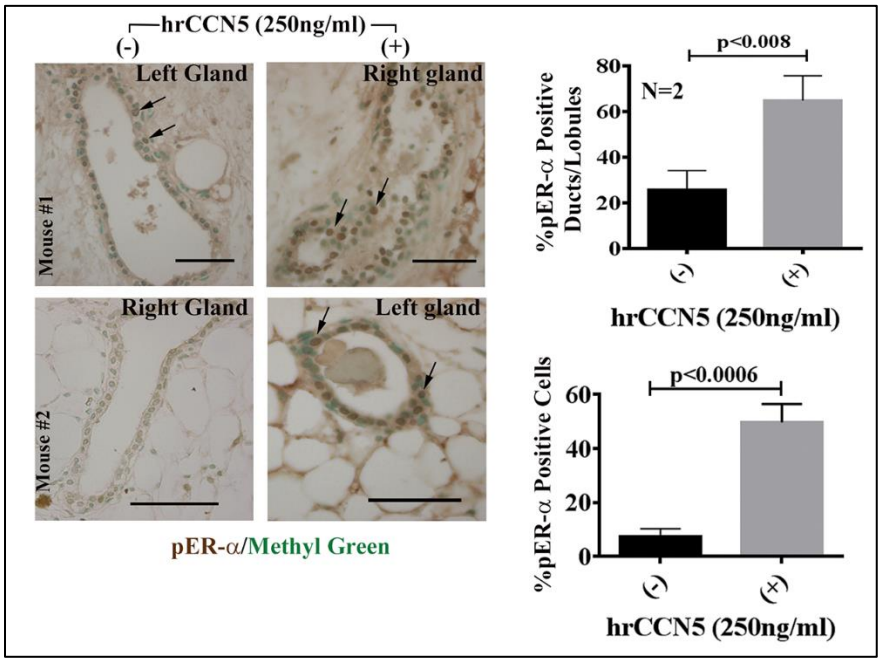


Fig.III.2: CCN5 influences ER-α expression in normal HMECs and ductal epithelial cells of mouse mammary gland (a) Representative western blot and quantification of ER-α and p-ER-α (active form) protein level in HMECs treated with or without hrCCN5 (250 ng/ml) or in combination of hrCCN5 and CCN5 antibody (500 ng/ml) for 48 h. All data represent means ± s.e.m. of three independent experiments. P-values were calculated using one-way analysis of variance and two-tailed unpaired Student's t-test. (b) Immuno-histochemical localization and quantification of pER-α in the ducts and lobules of mouse mammary glands cultured as indicated for 7 days in the presence or absence of hrCCN5 (250 ng/ml). Arrows indicate the ER-α positive ductal cell in glands of different FVB/N mice. Scale bars, 100 μm. Data are presented as mean±s.e.m. P-values were calculated using two-tailed unpaired Student's t-test. (Sarkar et al., 2017).

Fig.III.3a.i

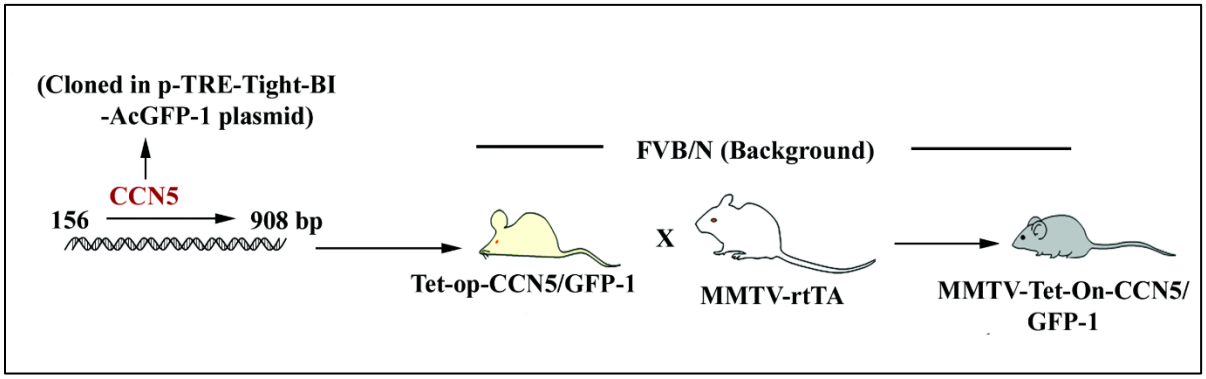


Fig.III.3a.ii

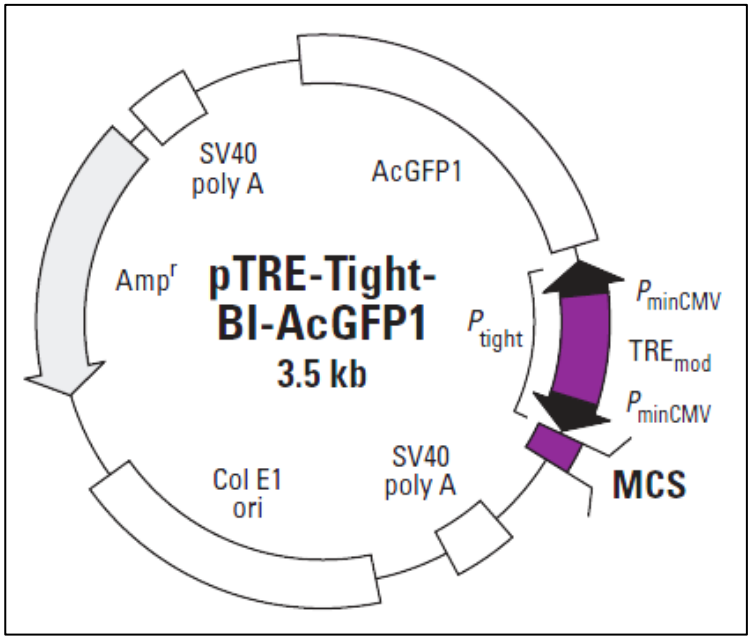


Fig.III.3: (a.i) Schematic flow of generation of MMTV-rtTA/ Tet-op-CCN5-GFP mice from crossing of MMTV-rtTA strain and Tet-op-CCN5-GFP strain of mice (Sarkar et al., 2017). (a.ii) Schematic map of the plasmid p-TRE-Tight-Bi-AcGFP1 showing bidirectional CMV promoters under control of a modified Tetracycline Response Element (TRE).Vector diagram used from Vector Information handbook provided by Takara Bio Inc after necessary permission.

Fig.III.3a.iii

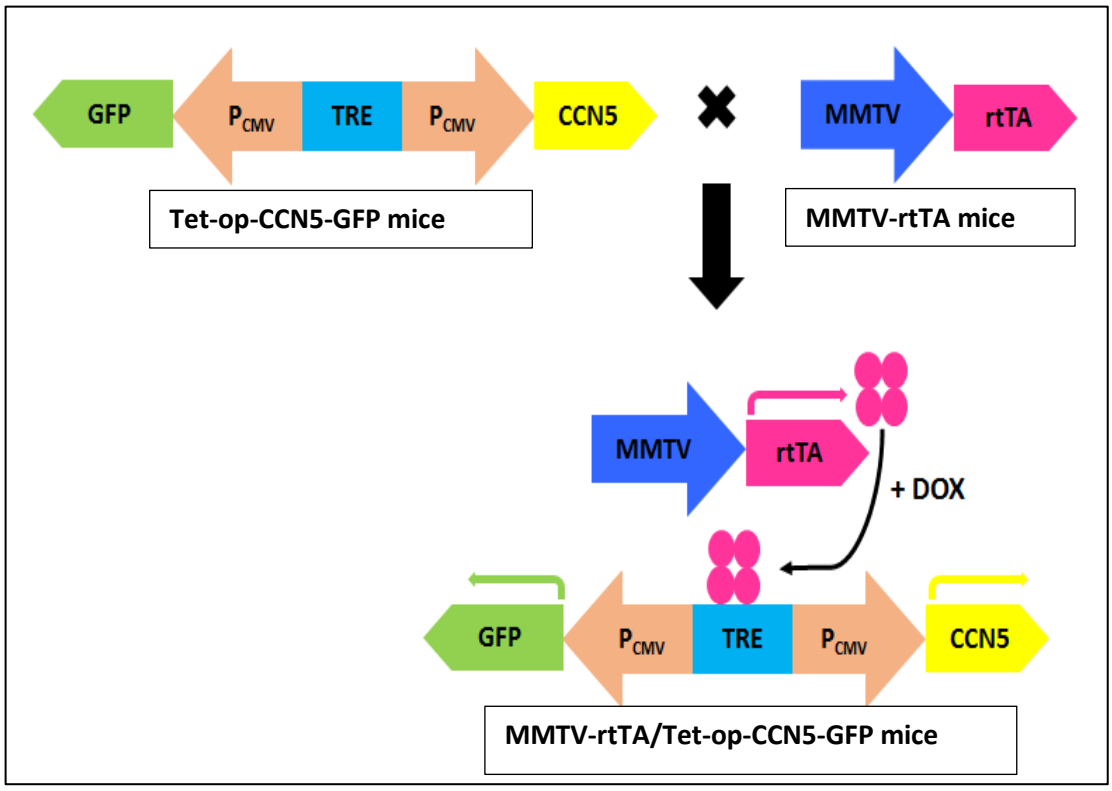


Fig.III.3: (a.iii) Schematic of the doxycycline regulated mechanism of CCN5 and GFP expression in MMTV-rtTA/ Tet-op-CCN5-GFP mice.

Fig.III.3b.ii

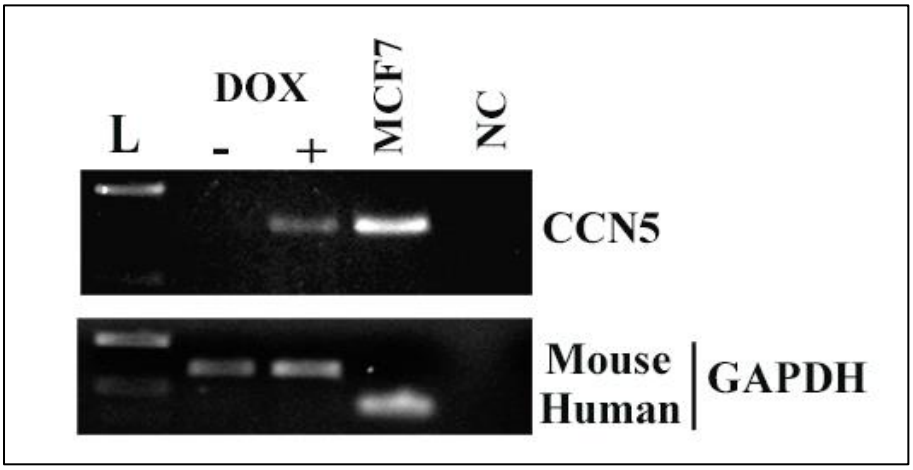


Fig.III.3b.ii

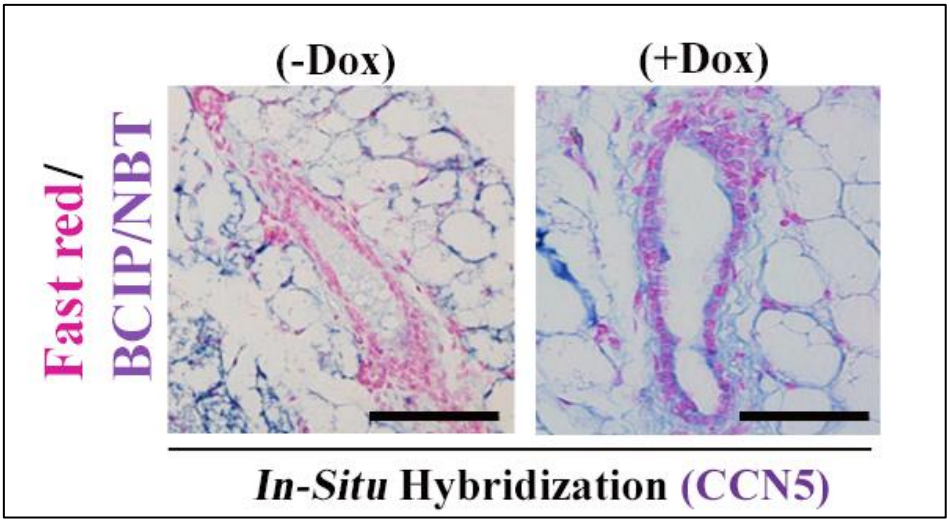


Fig.III.3: Validation of conditional transgenic mice bearing CCN5 and GFP transgenes overexpressed in mammary epithelial cells by doxycycline (Dox) treatment. (b.i) RT-PCR for CCN5 in the RNA harvested from mammary glands from Dox-untreated and treated mice. Lane 1.: ladder 2: untreated mice gland RNA, 2: Dox-treated mice gland RNA and 3: MCF-7 RNA and 4: negative control (no RNA). GAPDH is used as loading controls. (b.ii) Localization of CCN5 mRNA expression (BCIP/NBT) in the mammary ducts from Dox untreated (−Dox) and Dox-treated (+Dox) CCN5-transgenic mice using in situ hybridization. Scale bars, 100 μm. (Sarkar et al., 2017).

Fig.III.3c.i

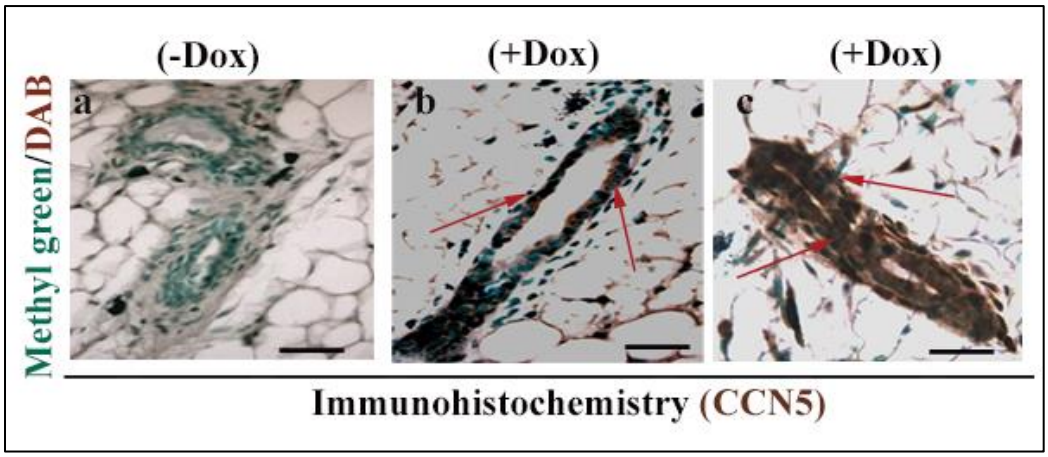


Fig.III.3c.ii

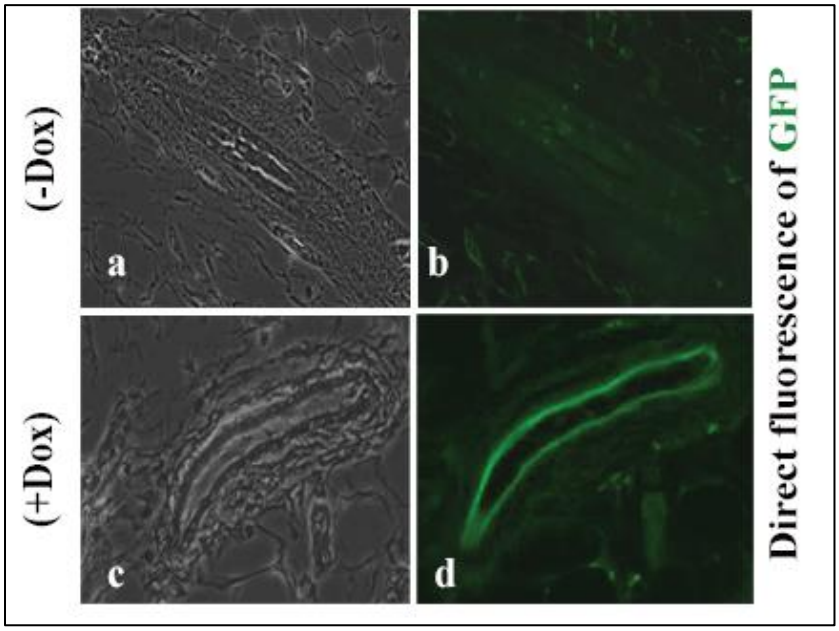


Fig.III.3: Validation of conditional transgenic mice bearing CCN5 and GFP transgenes overexpressed in mammary epithelial cells by doxycycline (Dox) treatment. (c.i) Immunohistochemical localization of CCN5 (DAB) in the ducts of mammary grands from Dox-untreated (a) and Dox-treated (b–c) transgenic mice. Methyl green was used as counter staining. Scale bars, 100 μm. Red arrows indicate the CCN5 expression. (c.ii) Detection of direct fluorescence of GFP in the ducts of mammary glands from Dox untreated (− Dox) and Dox treated (+Dox) CCN5-transgenic mice. (a and c) The examples of bright fields in Dox untreated and treated samples, and (b and d) the examples of GFP-fluorescence in Dox untreated and treated samples (Sarkar et al., 2017).

Fig.III.4a

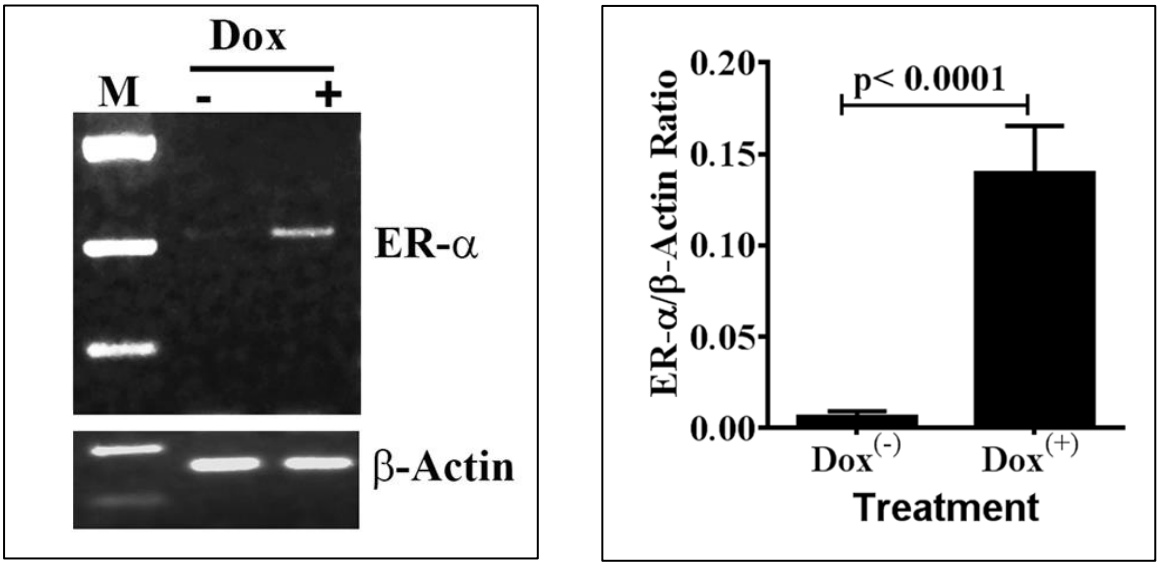


Fig.III.4: Conditional activation of CCN5 promotes ER-α expression in mammary epithelial cells of CCN5 transgenic mice. (a) Representative PCR with reverse transcription and quantification of ER-α in the RNA harvested from mammary glands from Dox-untreated and treated mice. M: molecular markers. (−): untreated gland, (+): Dox-treated. All data represent means ±s.e.m. of three independent experiments. P-values were calculated using two-tailed unpaired Student's t-test (Sarkar et al., 2017).

Fig.III.4b

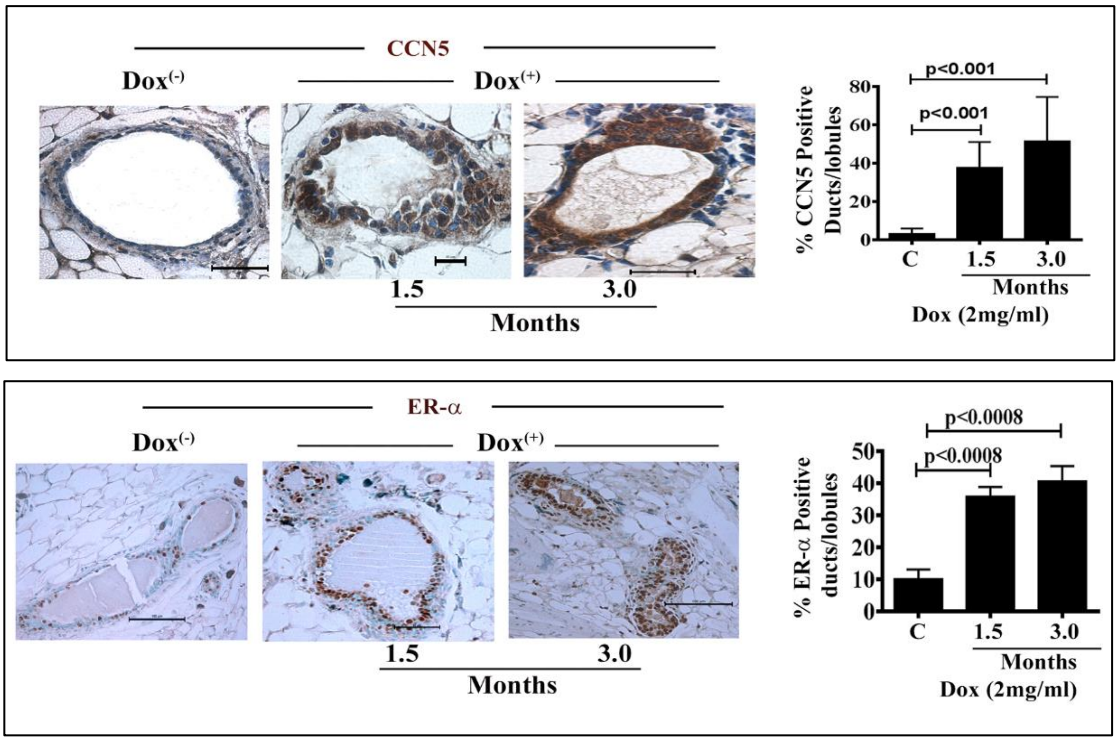


Fig.III.4: Conditional activation of CCN5 promotes ER-α expression in mammary epithelial cells of CCN5 transgenic mice. (b) Immunohistochemical localization of CCN5 protein (upper panel) in the mammary glands of untreated and Dox-treated mice. Scale bars, 100 μm. The bar graph represents the quantitative estimation of CCN5-positive ducts/lobules in Dox- treated and untreated mouse mammary glands. Data are presented as mean±s.e.m. (n=5 mice). Immunohistochemical localization of ER-α (lower panel) in the mammary glands of untreated and Dox-treated CCN5-transgenic mice. Scale bars, 100 μm. The bar graph (represents the quantitative estimation of ER-α-positive cells in the ducts and lobules of Dox-treated and -untreated mouse mammary glands. Data are presented as mean±s.e.m. (n=5 mice) (Sarkar et al., 2017).

Fig.III.5a.i

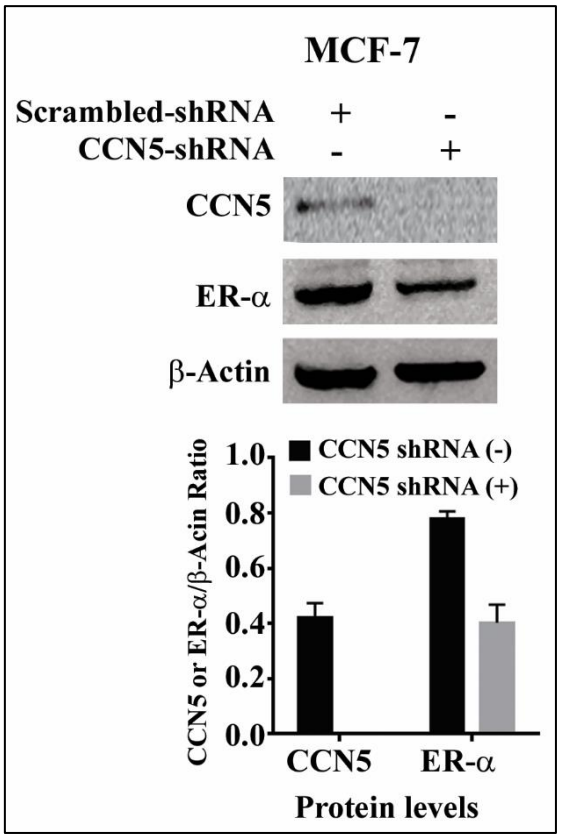


Fig.III.5a.ii

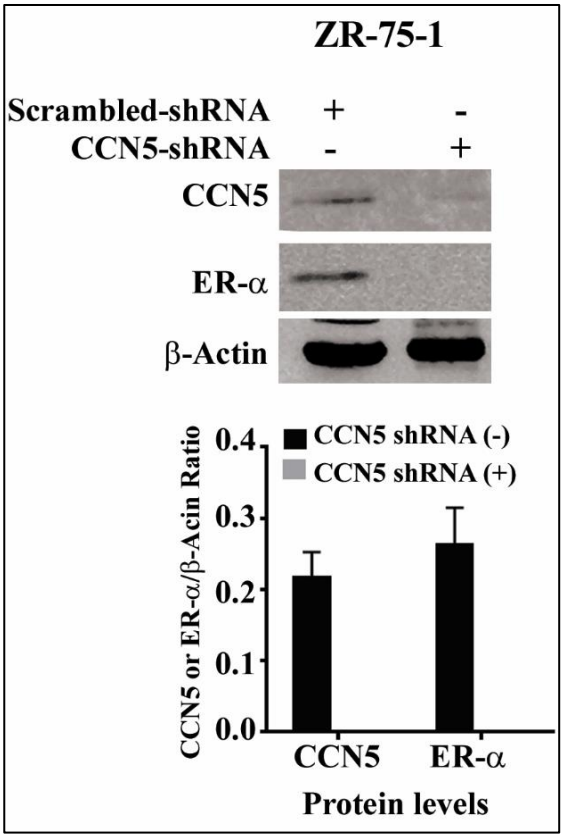


Fig.III.5: CCN5 increases ER-α protein levels in breast cancer cells. (a.i & a.ii) Representative western blots of CCN5 and ER-α in cell lysates of ER-α-positive breast cancer cell lines MCF7 (a.i) and ZR-75-1 (b.i) transfected with scrambled shRNAs or CCN5-specific shRNAs. The bar graph represents the relative protein expression levels of CCN5 and ER-α with respect to β-actin (loading control). Data are presented as mean ±s.e.m. of at least three independent experiments. P-values were calculated using two-tailed unpaired Student's t-test (Sarkar et al., 2017).

Fig.III.5b.i

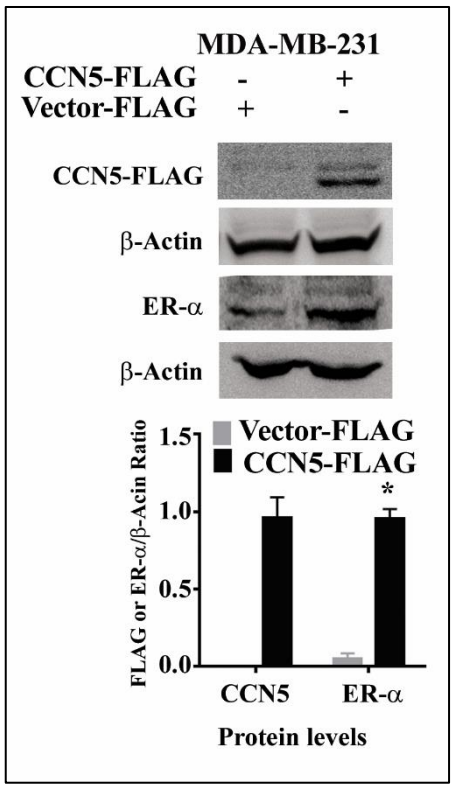


Fig.III.5b.ii

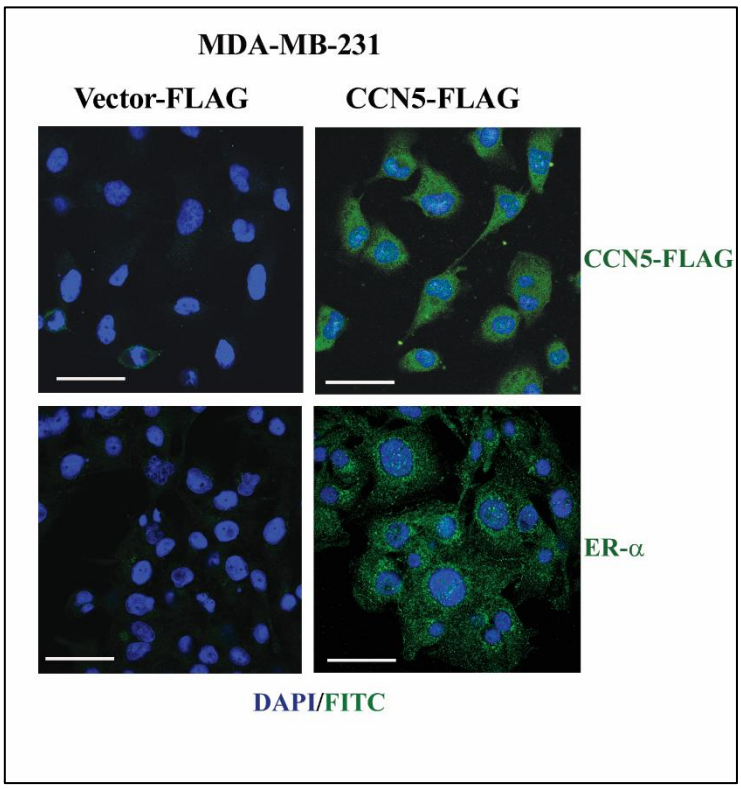


Fig.III.5 :(b.i) Representative western blots of ER-α in cell lysates of CCN5-FLAG (FLAGCCN5) tag transfected or vector (FLAG) -transfected MDA-MB-231 cells. CCN5 levels were detected with anti-FLAG antibody. The bar graph represents the relative protein expression levels of CCN5 and ER-α with respect to β-actin (loading control). Data are presented as mean ±s.e.m. of three independent experiments. P-values were calculated using two-tailed unpaired Student's t-test. (b.ii) Representative photographs of immunofluorescent staining using anti-FLAG tag (upper panel, green) and anti-ER-α (lower panel, green) antibodies to show increase of ER-α expression in CCN5-Flag transfected MDA-MB-231 cells compared to empty vector-transfected cells. DAPI was used to stain the nuclei (blue) and FITC-labeled secondary antibodies (green) are used for staining the antigens. Scale bars, 200 μm.(Sarkar et al., 2017)

Fig.III.6

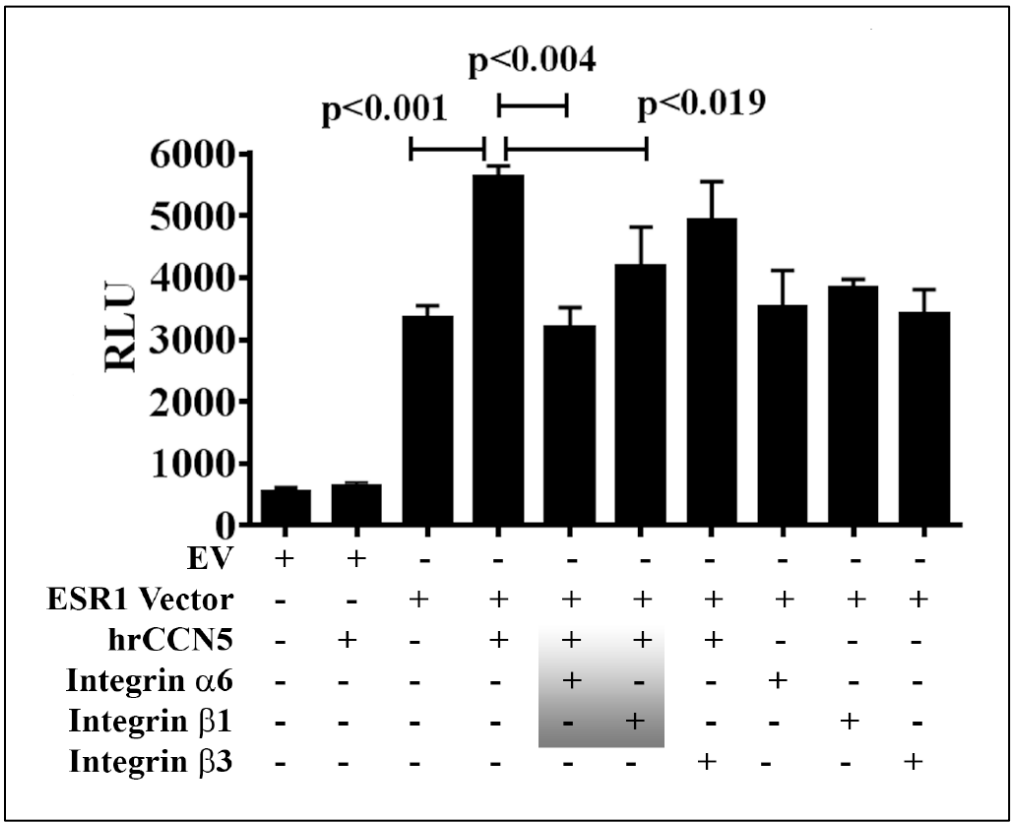
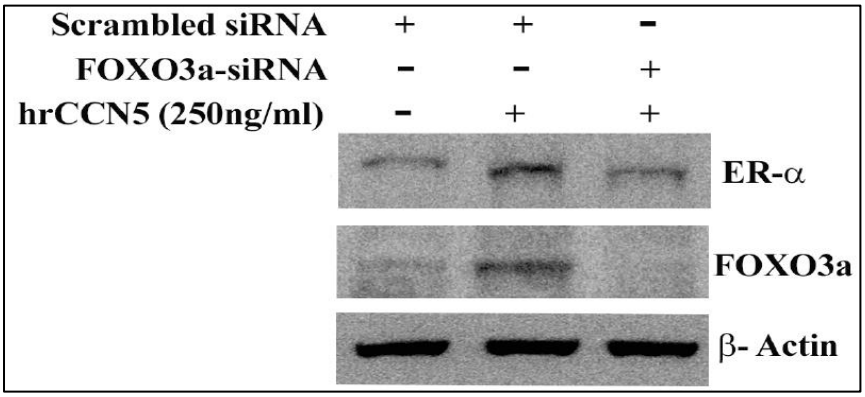


Fig.III.6: Integrins α6β1 and FOXO3a are required for CCN5-mediated regulation of ER-α expression in breast cancer cells.ER-α promoter luciferase reporter assay: MDA-MB-231 cells were transfected with empty construct or ER-α-Luc promoter construct followed by hrCCN5 protein (250 ng/ml) treatment or vehicle alone for 48 h and either in the presence or absence of different neutralizing antibodies of integrins. A luciferase assay was performed following vendor provided protocol. EV: empty vector, and ESR1 vector: ER-α promoter vector. Data are presented as mean± s.e.m. of eight independent experiments. P-values were calculated using two-tailed unpaired Student's t-test. (Sarkar et al., 2017).

Fig.III.7a



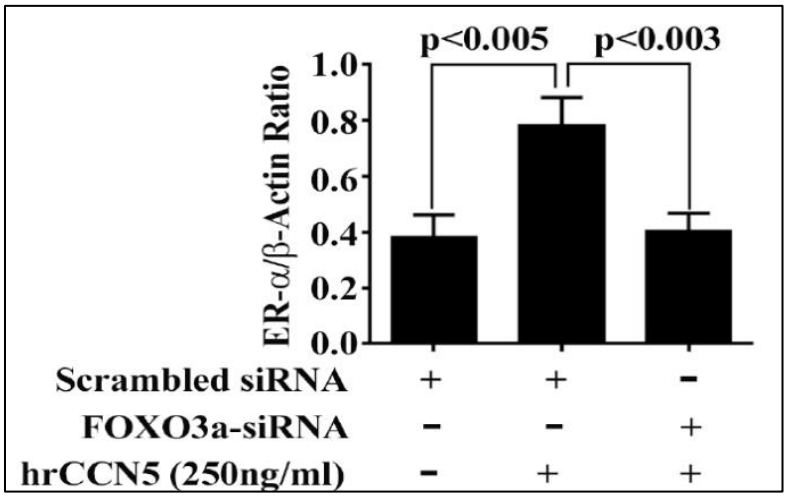


Fig.III.7: (a) Representative western blots and quantification of ER-α in cell lysates of FOXO3a-siRNA-transfected or scrambled siRNA-transfected MDA-MB-231 cells. Data are presented as mean±s.e.m. from triplicate experiments. P-values were calculated using two-tailed unpaired Student's t-test.

Fig.III.7b

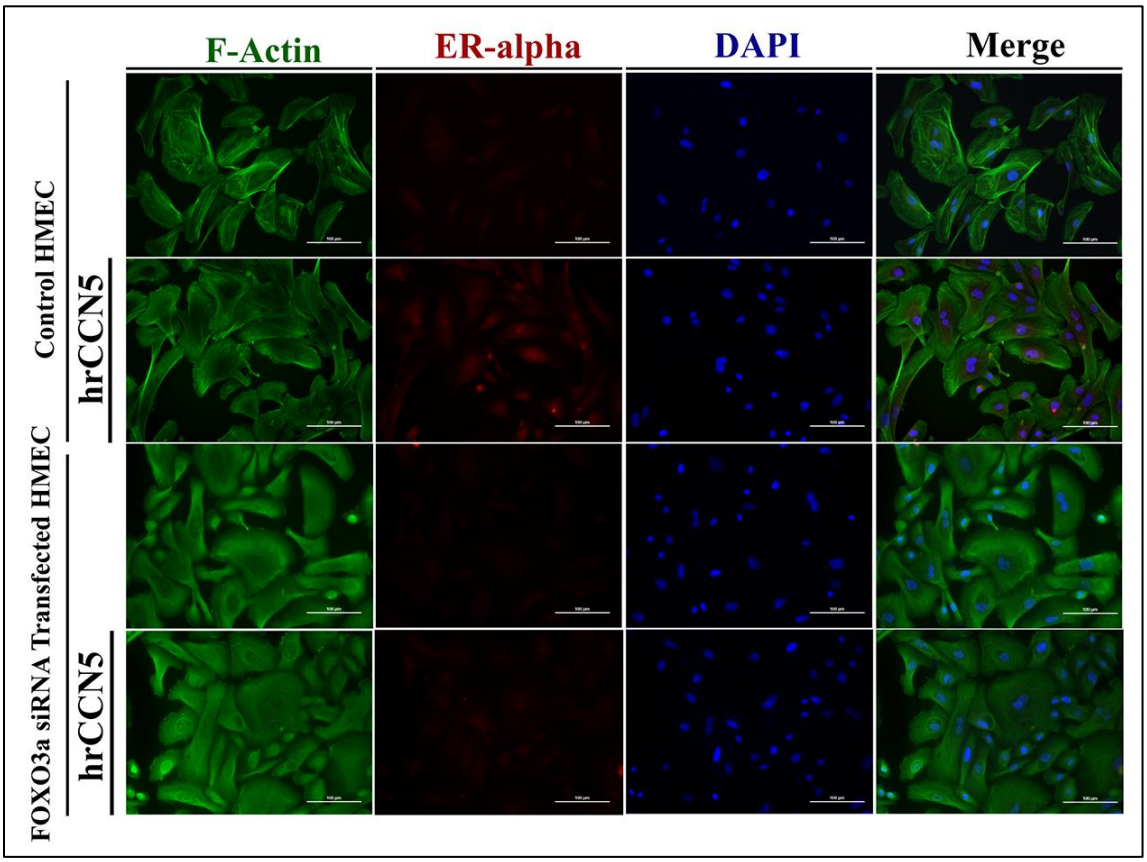


Fig.III.7:(b) Representative images of immunofluorescent staining for the detection of the effect of FOXO3a ablation on ER-α expression in HMECs, cultured in the presence or absence of hrCCN5 (250 ng/ml). Scale bar, 100 μm.(Sarkar et al., 2017).

Fig.III.8a

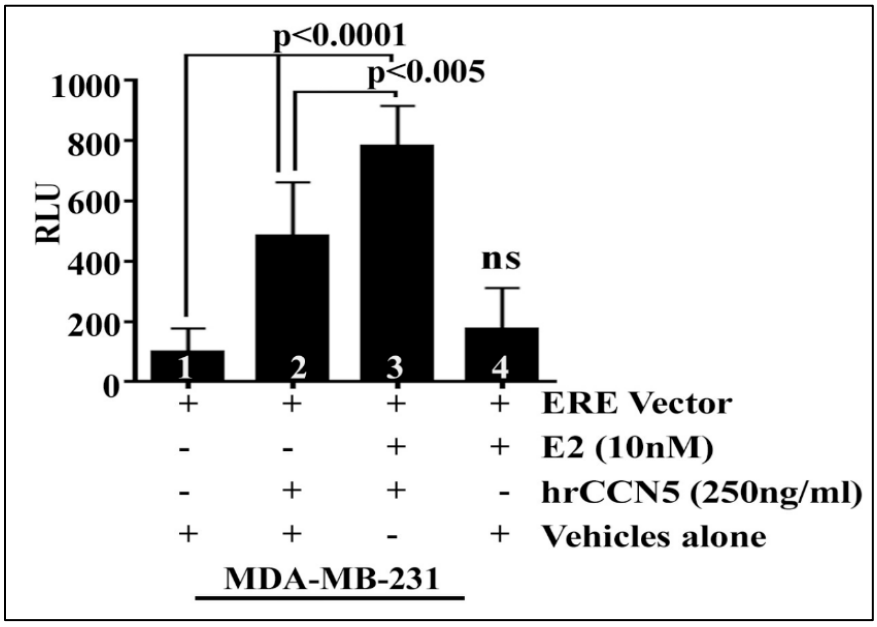
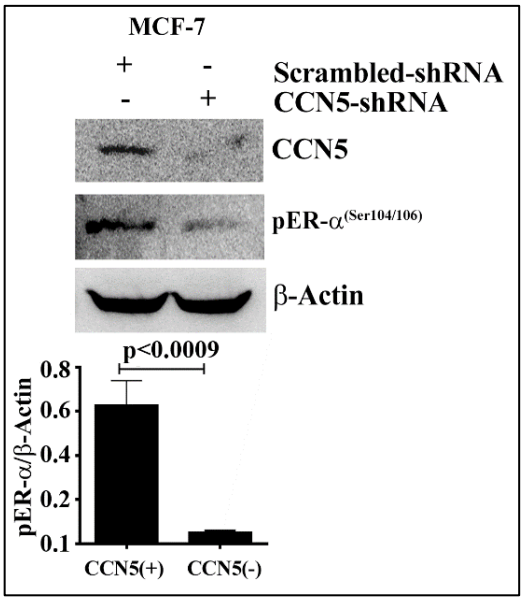


Fig.III.8: CCN5-induced expression of ER-α is functionally active in BC cells. (a) Functional luciferase assay. MDA-MB-231 cells were transfected ERE -luciferase reporter constructs or vector alone followed by E2 or hrCCN5 or combination treatment for 48 h and luciferase activity was measured. Data are presented as mean±s.e.m. of eight independent experiments. P-values were calculated using two-tailed unpaired Student's t-test (Sarkar et al., 2017).

Fig.III.8b.i

Fig.III.8b.ii



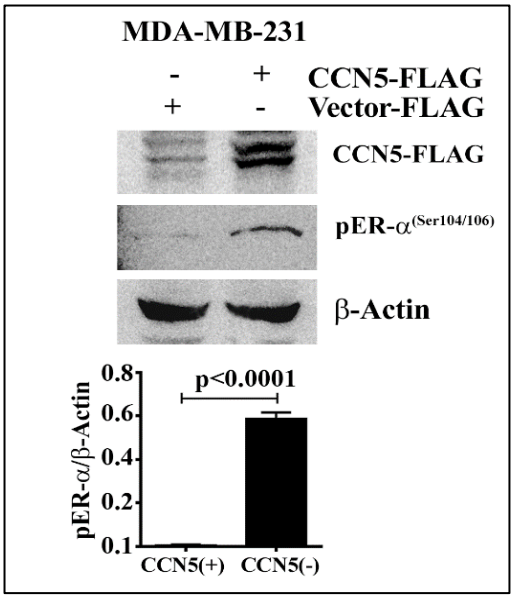


Fig.III.8: CCN5-induced expression of ER-α is functionally active in breast cancer cells. (b.i) ER-α phosphorylation change in MCF-7 cells following CCN5 ablation by shRNA. Representative western blots of CCN5 and phospho-ER-α (Ser104/106) in cell lysates of scrambled shRNA or CCN5-shRNA transfected MCF7 cells (left panel). (b.ii) ER-α phosphorylation change in MDA-MB-231 cells following CCN5 transfection. Representative western blots of FLAG-CCN5 and phospho-ER-α (Ser104/106) in cell lysates of FLAG-vector (FLAG) or FLAG-CCN5-vector (FLAG-CCN5) transfected MDA-MB-231 cells. (right panel). The bar graph represents the relative protein expression levels of p-ER-α with respect to β-actin (loading control). Data are presented as mean ±s.e.m. of at least three independent experiments. P-values were calculated using two-tailed unpaired Student's t-test (Sarkar et al., 2017).

Fig.III.9a.i

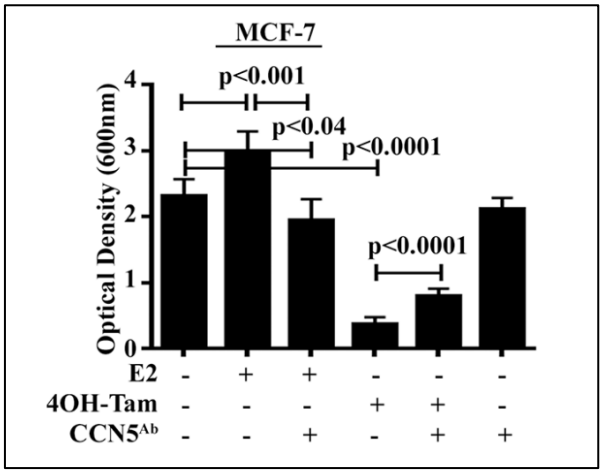


Fig.III.9a.ii

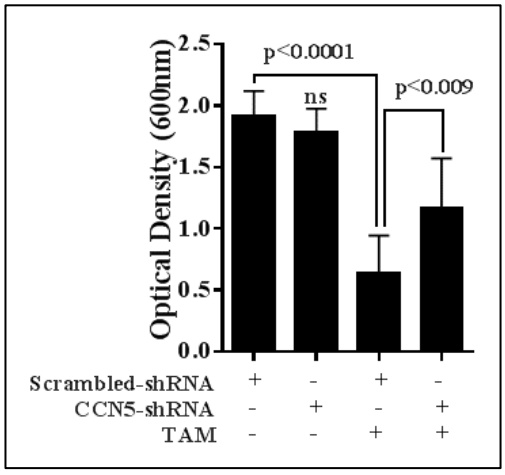


Fig.III.9b

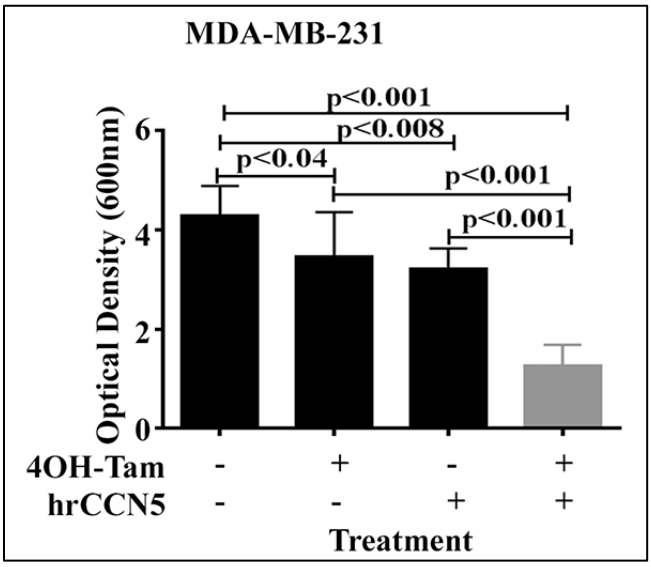


Fig.III.9: CCN5 and Tamoxifen exhibits additive effect on TNBC cell growth in vitro and in vivo. (a,b) Cell viability assay. MCF7 (a.i), and MDA-MB-231(b) cells treated with 17β-estradiol (E2, 10 nM) or 4-hydroxy-Tamoxifen (4OH-Tam, 1 μM) or both in the presence or absence of CCN5 antibody (CCN5Ab, 500 ng/ml) or CCN5 recombinant protein (hrCCN5, 250 ng/ml) for 48 h. Cell viability was determined using crystal violet assay. (a.ii) Similar viability assays were performed with scramble -shRNA and CCN5-shRNA transfected MCF7 cells Data are presented as mean ±s.e.m. of eight independent experiments. P-values were calculated using one-way analysis of variance and two-tailed unpaired Student's t-test (Sarkar et al., 2017).

Fig.III.10a

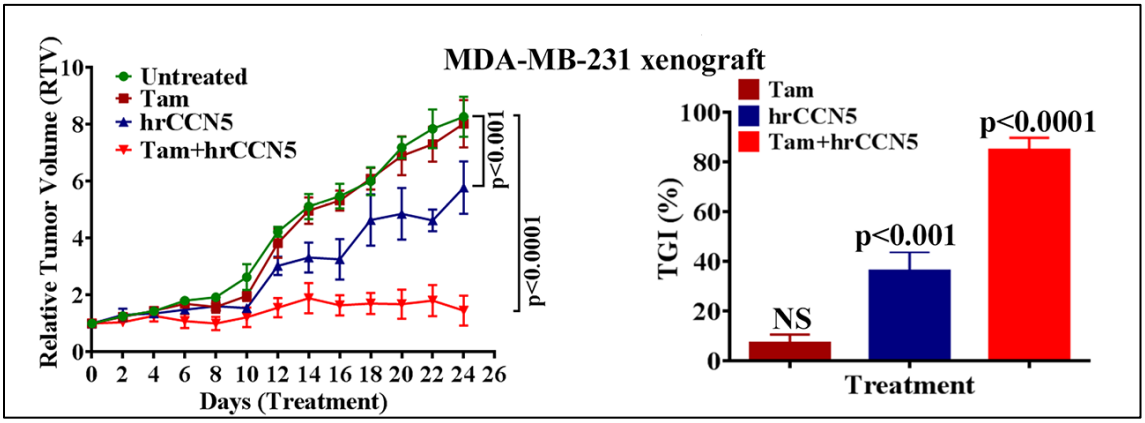


Fig.III.10b

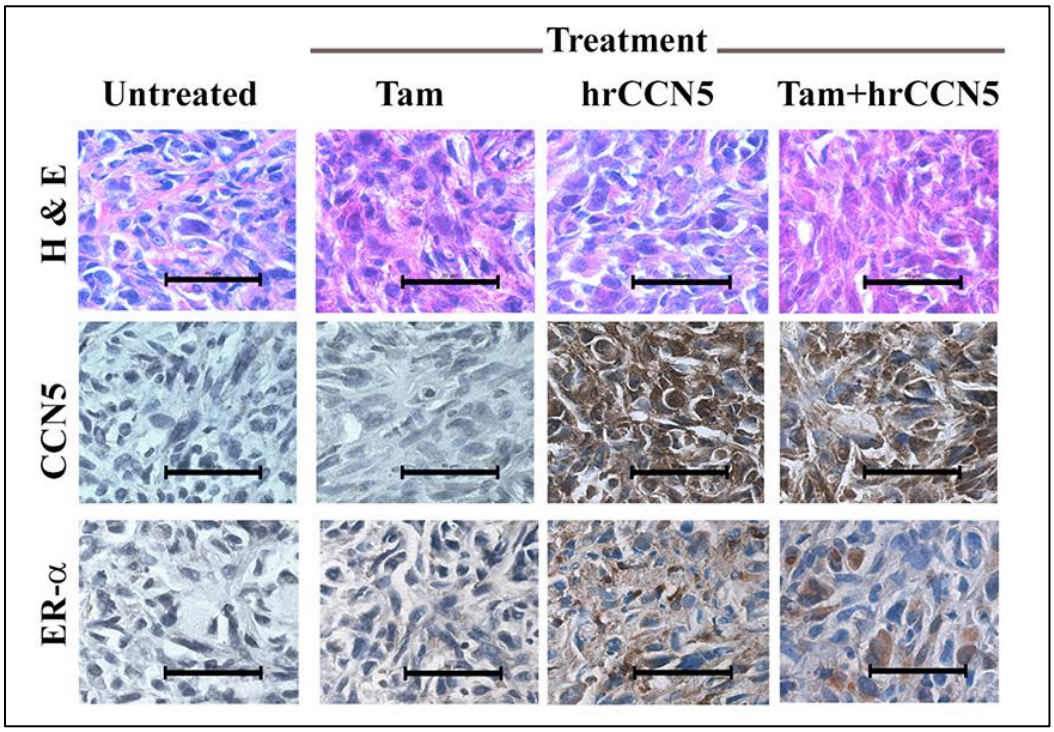


Fig.III.10: CCN5 and Tamoxifen exhibits additive effect on TNBC cell growth in in vivo. (a): Additive antitumor efficacy of CCN5 and Tam in xenograft model. MDA-MB-231 xenografts in female nu/nu mice (n=5) were treated with Tam (oral), hrCCN5 [intratumoral injection] or combination three times a week for 24 days. Growth curve was measured using RTV three times per week and %TGI was measured as end point tumor growth using analysis of variance and two-tailed unpaired Student's t-test. Data are presented as mean±s.e.m. of five animals. (b) Hematoxylin and eosin staining and immunohistochemical localization of ER-α and CCN5 in tumor samples of MDA-MB-231 xenograft model. Scale bar, 100 μm. (Sarkar et al., 2017)

Fig.III.11

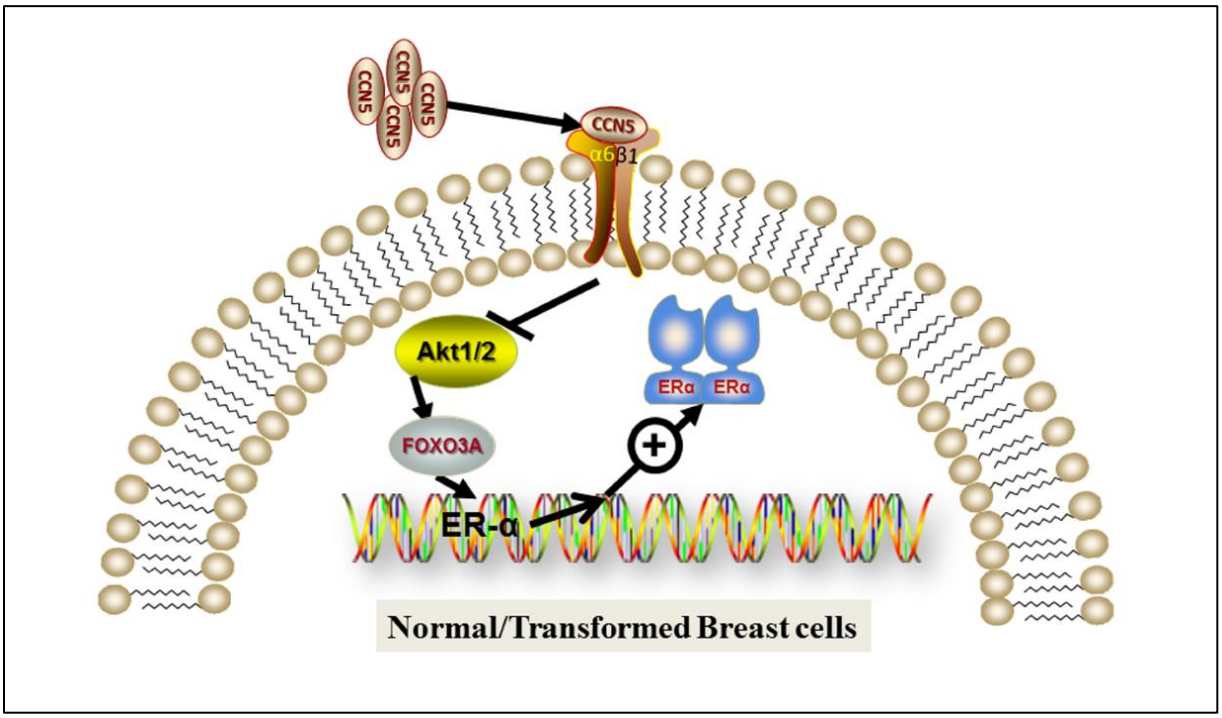


Fig.III.11: Schematic representation of mechanism of induction ER-α expression in ER-α negative cells by CCN5. CCN5 binds to cell surface integrin receptors (α6 and ß1) leading to suppression of PI3K-AKT pathway. This results in stabilization and nuclear localization of the transcription factor FOXO3a which drives transcription of ER-α (Sarkar et al., 2017).

CHAPTER IV: Role of CCN5 in delaying Her2/Neu overexpression mediated tumor progression.

INTRODUCTION

Membrane receptor tyrosine kinases

Tyrosine kinases are a class of enzymes that catalyze the transfer of the γ-phosphate of ATP specifically to the tyrosine residues on protein substrates. Tyrosine kinase-dependent phosphorylation is one of the key covalent modifications of proteins which regulates multiple downstream signaling pathways. Both, transmembrane receptor tyrosine kinases and cytoplasmic non-receptor tyrosine kinases play critical roles in cell signaling pathways. The significance of tyrosine kinase catalytic activity mediated cell-signaling has been underscored by identification of many of these enzymes as oncogenes.

A large family of nonreceptor tyrosine kinases (NRTKs) including the Src kinases, Jak (Janus kinase), and c-Abl (Abelson tyrosine kinase), serve as intracellular components of the signaling cascades. These cascades are triggered by the transmembrane receptor tyrosine kinases and by other cell surface receptors, e.g., G protein-coupled receptors and receptors of the immune system (Hahn & Weinberg, 2002; Hubbard & Till, 2000).

Receptor tyrosine kinases (RTKs) comprise a large superfamily of receptors that are widely used by the cells to interpret and transduce the extracellular signals to the cytoplasm. RTKs are transmembrane glycoproteins and are activated by the binding of their cognate ligands. The members of this superfamily of proteins have a common structural design with an extracellular ligand-binding domain, a membrane-spanning region and a cytoplasmic tyrosine kinase domain (catalytic domain). Ligand binding promotes receptor dimerization, consequently stimulating kinase activity of the cytoplasmic kinase domain and triggering autophosphorylation of specific tyrosine residues which are positioned within the cytoplasmic domain of the receptors. The phosphorylated tyrosine residues serve as docking sites for other cytoplasmic proteins. The

proteins that are recruited to these docking sites are either other proteins with enzymatic activities [e.g., PLC-γ (phospholipase C-γ), PI3K (Phosphatidylinositol-3-kinase), SRC-tyrosine kinase] or are adaptor proteins that in-turn recruits downstream factors involved in intracellular signaling cascades. One example of adaptor-mediated recruitment of signaling proteins, is the recruitment of SOS (Son of Sevenless), a guanine nucleotide exchange factor, by an adaptor protein GRB2 (growth factor receptor-bound protein-2), which leads to activation of RAS and its downstream signaling. Thus, binding of specific ligands to the extracellular domains of the RTKs transmits the extracellular signal by phosphorylating their specific tyrosine residues (autophosphorylation) and also on other signaling proteins that are recruited to the receptor (Hubbard & Till, 2000).

RTKs, depending on the ligand and receptor type, activate numerous different signaling pathways that lead to cell growth, proliferation, differentiation, migration, or metabolic changes. The RTK superfamily includes multiple families of receptors which have distinctly different extracellular domain structures but similar cytoplasmic catalytic domains. The extracellular portion of the RTKs typically contains diverse globular domains such as immunoglobulin (Ig)-like domains, fibronectin type III-like domains, cysteine-rich domains, and EGF-like domains. The diversity of domain types enables these proteins to interact with a diverse array of ligands. The domain organization of the cytoplasmic portion of RTKs is simpler, consisting of a juxtamembrane region (adjacent to the transmembrane helix), followed by the tyrosine kinase catalytic domain and a carboxy-terminal region. The juxta-membrane and carboxy-terminal regions vary in length among RTKs while the tyrosine kinase catalytic domain is mostly conserved in most of the members. Depending on the structural arrangement of the extracellular domains, the RTKs act as receptors for multiple growth factors. These include insulin-like

growth factor (IGF), epidermal growth factor (EGF), transforming growth factor-α (TGF-α), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and nerve growth factor (NGF).

The human EGF receptor (HER) family

The EGF-R (Epithelial Growth Factor-Receptor) family of receptors is comprised of four distinct protein members namely – ERBB1 (EGF-R), ERBB2 (HER2/Neu), ERBB3 (HER3) and ERBB4 (HER4). These four ErbB membrane receptors share a similar structure with two cysteine-rich regions in their extracellular region, and a kinase domain flanked by a carboxy-terminal tail with tyrosine autophosphorylation sites. The ErbB receptors have been shown to homodimerize with themselves and heterodimerize with other members of the ErbB family after binding to the ligands (Hubbard & Till, 2000; Yarden & Sliwkowski, 2001) **(Figure IV.1a)**. The ErbB receptors are located at the basolateral surfaces of in the epithelial cells, which enable them to relay the signals originating from the mesenchyme to the epithelial cells (Yarden & Sliwkowski, 2001).

ErbB receptors bind to a diverse array of ligands including EGF, TGF-α, HB-EGF (heparin-binding EGF), amphiregulin (ARG), epiregulin, β-cellulin and a large family of Neuregulins (NRGs). Some of these ligands bind to the ErbB receptors with a narrow specificity. EGF, ARG, and TGF-α act as specific ligands for ERBB1 (HER1) while NRG3 and NRG4 bind to ERBB4 in a specific fashion (Hynes & Lane, 2005; Yarden & Sliwkowski, 2001). However, in some cases, ligands can bind to more than one distinct receptors in an overlapping fashion. Ligands like epiregulin and β-cellulin can bind to both ERBB1 and ERBB4; while NRG1 and NRG2 bind to both ERBB3 and ERBB4 (Hynes & Lane, 2005; Yarden & Sliwkowski, 2001). ERBB2 (HER2) does not have an identified ligand and is thus named as an ‘orphan receptor.’ However,

cytoplasmic domain of ERBB2 exhibits strong kinase activity (Klapper et al., 1999). Both ERBB3 and ERBB4 bind to Neuregulin family of ligands. ERBB3 which is devoid of any cytoplasmic kinase activity is incapable of autophosphorylation, and its phosphorylation is dependent upon its dimerization with ERBB2 (Graus-Porta, Beerli, Daly, & Hynes, 1997) **(Figure IV.1a)**. Overexpression of ERBB2 leads to a bias in heterodimer formation where one of the partners is ERBB2 thus leading to a broader ligand specificity, and ligands that are better at recruiting ERBB2 can compete and reduce the binding of other less effective ligands **(Figure IV. 1a)**.

After formation of the dimeric complexes, a vast array of phospho-tyrosine-binding proteins associate with the tail of each ErbB molecule. The type of ligand and the heterodimer partners decide the tyrosine residues which are phosphorylated and thus, in turn, regulates the type of intracellular engaging molecules. Though the ErbB members share some overlapping downstream signaling effects, the individual receptor can bind with a unique and distinct set of signaling proteins.

The most commonly affected downstream signaling pathways are; - RAS- and Shc-activated mitogen-activated protein kinase (MAPK) pathway, the PI3K-mediated AKT pathway and the ribosomal protein S6 kinase (p70S6K/p85S6K) pathway, which can be activated downstream to AKT pathway (Hynes & Lane, 2005). The activation of multiple signaling cascades, including the MAPK pathway, protein kinase C (PKC)-mediated pathway and the AKT pathway, leads to activation of distinct transcriptional machinery in the nucleus. These involve a wide range of transcription factors including MYC, SP1 (Specificity protein1) and AP1 (Activator Protein1) (Yarden & Sliwkowski, 2001) **(Figure IV.1b)**.

The output of cellular processes mediated by the ErbB receptors ranges widely from cell proliferation, migration, adhesion, differentiation, and apoptosis. The output varies depending on the cell type, interacting a ligand and the dimerization partners. Although neither ERBB2 nor ERBB3 alone can be activated by a ligand, the ERBB2-ERBB3 heterodimer is the most transforming and mitogenic receptor complex and has also been also shown to increase cell motility on ligand binding (Graus-Porta et al., 1997; Yarden & Sliwkowski, 2001) **(Figure IV.1a)**.

Role of HER (ERBB) family of proteins in cancer:

ERBB1 (EGFR) and ERBB2 (HER2) have been implicated in the development of many human cancers and the cancer patients with alterations in ERBB receptors tend to have a more aggressive disease with a prediction of a poor clinical outcome (Hynes & Lane, 2005).

Potent mitogenic and transforming effects of the ERBB receptors lead to tumorigenesis, which is most cases arise from constitutively activated signaling mediated by the receptors. The constitutive activation of the ERBB signaling can result from - overexpression of the ligands; overexpression of an ERBB member leading to aberrant and more frequent dimerization; and mutations leading to structural alteration of the receptors leading to constitutive dimerization, activation along with recruitment of downstream signaling complex.

The most prominent example of tumor progression owing to overexpression of an ERBB ligand is the role of TGF-α expression in ERBB1 expressing lung, colon and ovarian tumors leading to a poorer prognosis (Yarden & Sliwkowski, 2001). Both overexpression and mutations leading to structural alterations of ERBB1 (EGFR) have been implicated in multiple cancer types. Overexpression of ERBB1, resulting from amplification of the gene locus is very frequently observed in brain tumors, and amplification of the ERBB1 gene occurs in 40% of gliomas

(Hynes & Lane, 2005; Yarden & Sliwkowski, 2001). Overexpression of ERBB1 is associated with higher grade, the higher proliferation of the tumors and reduced survival of the cancer patients. In gliomas, ERBB1 amplification is often accompanied by a common mutation (type III) that causes in-frame deletions within the extracellular domain of the receptor, resulting in a constitutively active receptor by structural rearrangements. Carcinomas of the breast, lung and ovaries have also been reported to express this variant of ERBB1 suggesting broader implications to human cancer (Ekstrand, Sugawa, James, & Collins, 1992; Moscatello et al., 1995; Wikstrand, Reist, Archer, Zalutsky, & Bigner, 1998; A. J. Wong et al., 1992).

Mechanism of ERBB2 (HER2) mediated signaling in breast cancer:

ERBB2 was first discovered at Dr. Robert Weinberg's lab in MIT as a 185 kDa oncoprotein, expression of which was specifically induced by transforming DNA of rat Neuroblastomas (Neu) (Padhy, Shih, Cowing, Finkelstein, & Weinberg, 1982). Soon after, the same lab established 'Neu' as an ERBB related oncogene (Schechter et al., 1984). Successive studies discovered it to be a novel gene located at chromosome 17 in human with homology to the EGFR in the tyrosine kinase domain (Schechter et al., 1985) and with transforming capabilities (Hudziak, Schlessinger, & Ullrich, 1987).

As mentioned in the General Introduction, amplification of ERBB2 locus (17q12) leading to overexpression of the receptor (ERBB2/HER2) is detected in a significant subset of breast tumors (25% of all invasive ductal carcinoma). ERBB2 gene amplification was shown to be associated with an increased risk of relapse and death for patients with early-stage breast cancer (Slamon et al., 1987). After the discovery of the role of ERBB2 overexpression in breast cancer, implications of ERBB2 overexpression in other human cancers such as ovarian, gastric and salivary cancers were suggested (Hynes & Lane, 2005; Hynes & Stern, 1994; Slamon et al.,

1989). Amplification of the ERBB2 gene, leading to its overexpression pushes the equilibrium of the ERBB dimers towards ERBB2 homodimer and heterodimer formation, leading to activation of downstream signaling pathways. Though ERBB2 is unable to bind a ligand of its own, it serves as the preferred heterodimeric partner of ligand-activated ERBB receptors (Tzahar et al., 1996). Also, ERBB2 heterodimers have increased ligand binding affinities and can prolong signaling by delaying the ligand-induced receptor endocytosis (Emde et al., 2012). One of the important heterodimers is comprised of ERBB2 and ERBB3 (HER3) as it has been shown to be endowed with potent oncogenic activities (Wallasch et al., 1995) **(Figure IV.1a)**.

However, ERBB2 overexpression is frequently accompanied by the incidence of truncated forms of the receptor resulting from proteolytic cleavage, alternative translation initiation or alternative pre- mRNA splicing. These truncated forms of ERBB2 are characterized by enhanced oncogenic potential (Emde et al., 2012). A splice variant of ERBB2 with an in-frame deletion of 16 amino acids in the extracellular domain can induce ligand-independent activation of ERBB2 and has been shown to be oncogenic (P. M. Siegel, Ryan, Cardiff, & Muller, 1999).

ERBB2 mediated signaling and significance of G1/S cell cycle checkpoints:

As mentioned in the previous section of this chapter, ERBB receptors lead to potent activation of PI3K-AKT mediated, and RAS-MAPK mediated mitogenic signaling pathways. Thus, ERBB2 overexpression drives cell cycle progression by activation of the mentioned pathways. One of the critical checkpoints in the cell cycle is G1 to S phase transition checkpoint where an individual cell must decide on entering the S-phase by interpreting the extracellular signals. This point is known as restriction point as once past this checkpoint a cell must commit to completing the cycle and engage a program of gene expression and protein regulation required for cell division.

The presence of ERBB2 overexpression-induced mitogenic signaling pathways leads to progression of the cell-cycle irrespective of the presence of an extracellular mitogenic signal.

In a normal cell, the extracellular mitogenic signals lead to activation of PI3K-AKT and RAS-MAPK signaling pathways, which in turn positively regulates transcription of Cyclin D proteins. Persistent mitogenic signaling leads to accumulation of Cyclin D which binds to Cyclin-dependent kinases 4/6 forming an active kinase complex. Transcription of Cyclin D, assembly of Cyclin D-CDK4/6 complex and stability of the complex is directly facilitated by active PI3K-AKT and RAS-MAPK pathways. Accumulation of Cyclin D-CDK4/6 complex in cell nucleus leads to activation of Cyclin E–CDK2 complex which is achieved by the differential binding of P27KIP1 and P21CIP1 proteins to the complexes. The CIP/KIP proteins bind to Cyclin D-CDK4/6 complexes at a higher affinity as compared to Cyclin E-CDK2. The CIP/KIP proteins, however, show low or no inhibitory effect and instead stabilizes the Cyclin D-CDK4/6 complex. At a quiescent stage, the Cyclin D levels are low and accordingly, the Cyclin D-CDK4/6 complex is also low in concentration. At this point, the P27KIP1 protein binds and inhibits the activity of Cyclin E-CDK2 complex. After mitogenic stimulation, as Cyclin D-CDK4/6 complex accumulates, it sequesters the bound P27KIP1 protein, relieving the Cyclin E-CDK2 complex from its inhibitory actions. At this point, both the Cyclin E-CDK2 complex and the Cyclin D-CDK4/6-KIP1 complex collaboratively exerts their kinase activity to relieve cell-cycle inhibitory effects of Retinoblastoma protein (RB) family. At the quiescent stage, the RB protein maintains it active hypo-phosphorylated state and binds to the E2F transcription factors repressing E2F mediated transcription. It also recruits epigenetic repressors (e.g., HDAC) at promoter sites of E2F target genes. After mitogenic activation, the Cyclin-CDK complexes phosphorylate RB. Hyper-phosphorylated RB is inactive and fails to bind E2F transcription factors and recruit

epigenetic repressions at E2F target gene promoters. This repression of E2F targets leads to activation of E2F mediated transcription of its target genes which encodes for proteins needed for S phase as well as Cyclins E and A. ERBB2/ HER2 mediated cell signaling drives the cells constitutively past this checkpoint by activation of PI3K-AKT and RAS-MAPK pathways **(Figure IV.1b)** (Sherr & McCormick, 2002).

Interestingly, the direct significance of E2F transcription factors has been established recently. The studies analyzed a database of 1172 expression data from a variety of mouse models of breast cancer which revealed the high activity of the E2F family of transcription factors (E2F1, E2F2, and E2F3) in the MMTV-Neu mouse model (with a mammary-specific expression of ERBB2/Neu). The study also established that loss of any E2F delayed Neu-induced tumor onset in Neu transgenic mice while the loss of first two E2F proteins correlated with the decreased metastatic potential of the cancer cells. Moreover, HER2 positive patients with high E2F1 activity were shown to have worse outcomes such as distant metastasis-free survival and relapse-free survival (Andrechek, 2015; Rennhack & Andrechek, 2015).

However, cells exert another checkpoint through recruitment of another class of Cyclin D-CDK inhibitor proteins. Four INK4 (Inhibitors of CDK4) proteins (P16INK4a, P15INK4b, P18INK4c, and P19INK4d) specifically inhibit the activity of cyclin D-dependent kinases to prevent phosphorylation of RB family proteins **(Figure IV.1c.i, ii)**. Role of P16INK4a (encoded by the CDKN2A locus) has been established in inducing cellular senescence, and a decrease of P16INK4A levels renders mice more prone to tumor formation after exposure to chemical carcinogens. Loss of INK4a expression in high frequencies in multiple cancer types including breast cancer (30%), pancreatic cancer (80%) underscores the importance of INK4a regulation of G1-S checkpoint (Sherr & McCormick, 2002). Interestingly, a mouse model study directed to

establish the role of Cyclin D-CDK4/6 complex in ERBB2 (Neu) overexpressing mammary tumors, showed that overexpression of a P16 (specific inhibitor of CDK4/6) transgene in Neu transgenic mice blocked tumorigenesis (C. Yang et al., 2004).

The INK4a (CDKN2A) locus encodes a second gene product, from an alternative reading frame that overlaps sequences encoding P16INK4a **(Figure IV.1c.i)**. The alternative reading frame protein (P19ARF in mice and p14ARF in humans) is a potent tumor suppressor that activates p53 mediated cell-cycle arrest and apoptosis. ARF binds directly and inactivates action of MDM2 (HDM2 in human), that binds and facilitates degradation of P53 protein. Thus, ARF stabilizes P53 and enables it to exert its tumor-suppressor action through cell cycle arrest and apoptosis (Sherr & McCormick, 2002) **(Figure IV.1c.ii)**.

Introduction to the transgenic Neu (ERBB2) mice used for this study

The ERBB2 /Neu overexpressing mice model used in this study was established in 1992 in Dr. William Muller's laboratory (Guy et al., 1992). The transgenic mice model expresses an inactivated wild-type rat Neu proto-oncogene as a transgene under direct regulation of Mouse Mammary Tumor Virus (MMTV) promoter. MMTV promoter drives expression of the Neu transgene in a mammary epithelium-specific manner. Though multiple mice models expressing an activated rat Neu transgene (carrying activating mutations) were already established by the time (Bouchard, Lamarre, Tremblay, & Jolicoeur, 1989; Muller, Sinn, Pattengale, Wallace, & Leder, 1988), this mouse model aimed at investigating the role of overexpression of inactivated wild-type Neu in mammary carcinogenesis. The strains of MMTV/activated Neu mice exhibited an early onset of transgene expression in the mammary epithelium of female mice which was associated with the synchronous and rapid development of tumors in the entire mammary epithelium. However, expression of unactivated Neu in the mammary epithelium resulted in the

development of focal mammary tumors after a long latency period. The study describes the generation of 6 transgenic lines, of which the most well-characterized line (N202) is available from the commercial vendor and was used for the studies described here. This particular mouse transgenic line had a tumor latency period of 205 days (50% of mice developed tumor by 205 days), and 72 percent of the mice were shown to have lung metastasis by eight months of age (Guy et al., 1992).

MATERIALS AND METHODS

Generation of MMTV-rtTA/ Tet-op-CCN5-GFP/MMTV-Neu (Tri-transgenic) mice

The MMTV-Neu strain of mice was generated by Dr. William Muller at McGill University, Canada and is distributed under strain name FVB/N-Tg(MMTVNeu)202Mul/J (Stock No.: 002376) by The Jackson Laboratory. Male and female MMTV-Neu mice were purchased from the mentioned vendor. The line of MMTV-Neu strain of mice was established and maintained in the Kansas City Veterans Administration Medical Center (KCVAMC) animal care facilities by breeding the purchased male and female mice of the strain. This mouse strain overexpresses a rat Neu oncogene under MMTV promoter and thus specifically in the mice mammary gland epithelial tissue.

The tri-transgenic mice strain was generated by breeding the bi-transgenic mice carrying the MMTV-rtTA and Tet-op-CCN5/GFP transgenes with the MMTV-Neu transgenic strain **(Figure IV.2)**. The details of the generation and maintenance of the bi-transgenic line have been discussed in the previous chapter. The mice obtained from the cross between the bi-transgenic line and the MMTV-Neu strain were genotyped by tail DNA extraction and PCR following the protocol described before. The primers used for the detection of the MMTV-rtTA and CCN5/GFP transgenes were also same as mentioned in the previous chapter. The Jackson Laboratory provided primer sequences and PCR cycle conditions for detection of Neu transgene. The mice carrying all the three transgenes were selected for further tumor incidence and tumor volume studies. Primer sequences used for the genotyping are as follows:

(1) Neu Forward: 5’-TTTCCTGCAGCAGCCTACGC -3’

(2) Neu Reverse: 5’-CGGAACCCACATCAGGCC -3’

Doxycycline administration to tri-transgenic mice and tumor volume measurement

The tri-transgenic mice (carrying three transgenes: MMTV-rtTA, Tet-op-CCN5-GFP, MMTV-Neu) which were used for further studies were distributed into the vehicle-treated control and the doxycycline-treated experimental groups. Doxycycline was administered orally to the experimental group. The drinking water of the mice were replaced, with 10% sucrose solution (and left overnight) carrying doxycycline at a concentration of 2 mg/ml., twice a week. The control mice were fed with only 10% sucrose solution. The doxycycline treatment of the tri-transgenic mice was started at five months age of individual mice. Each of the control and the Dox-fed experimental mice was checked for the appearance of palpable breast tumor once every week. After the appearance of mammary tumors, the length and width of the tumors were measured with slide-calipers once a week till the study endpoint. The mice were sacrificed approximately at eight months of age or earlier if the tumor burden of the mice exceeded permissible limit.

Immunohistochemical staining

The tumors which were harvested from the control and the experimental mice were divided into parts, and a part of each tumor was utilized for immunohistochemistry. The tumor parts were fixed in 10% formalin solution in PBS overnight at 4ºC, embedded in paraffin. Paraffin-embedded tissue sections of 5µm thickness were used for immunohistochemical staining following previously published protocol. Rabbit monoclonal antibody against HER2 (Cell Signaling Technologies, Danvers, MA, USA; Cat # CST 2165) was used to stain the sections for qualitative analysis of HER2 expression in the control and the experimental mice tumor tissues.

Extraction of total RNA from mice mammary gland tissues

Total RNA from mammary tumor tissues were extracted using Trizol Reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and miRNeasy Mini Kit (Qiagen, Germantown, MD, USA). 50mg of tumor tissues were minced with the help of RNase contamination free sterile scissors and then homogenized by repeated passage through the syringes with 20G and 25G needles. The mincing and homogenization steps were performed quickly on ice in the Trizol reagent to minimize RNA degradation. The homogenate was used for RNeasy mini spin column-based RNA extraction following the protocol provided along with miRNeasy Mini Kit. To minimize the risk of genomic DNA contamination on-column DNase digestion was performed using the RNase-free DNase set (Qiagen) as recommended by the vendor.

Mouse 'Breast Cancer' gene array using RT^2 Profiler PCR Array

Total RNA extracted from the control mice tumor tissues and Dox-fed experimental tumor tissues were used for RT-PCR analysis of 84 key genes which are commonly involved in the dysregulation of signal transduction in breast oncogenesis. The Mouse Breast Cancer RT^2 Profiler PCR Array (Qiagen, Cat # 330231 PAMM-131ZA) was used for the analysis. An equal amount of RNAs (500ng per sample) from the control and the Dox-treated samples were used for the cDNA synthesis. RT^2 First Strand Kit (Qiagen), recommended for the PCR Arrays was used for the synthesis, and the vendor provided protocol was followed. The prepared cDNA samples were mixed with 2x RT^2 SYBR Green qPCR Mastermix and nuclease-free water before loading on to the array plate wells. The PCR amplifications were carried out in the BioRadCFX96™ thermal cycler. The ΔC_T values for individual genes were calculated, and the $\Delta\Delta C_T$ values for the quantification of the gene expression changes in experimental samples relative to the control samples were calculated in the online portal of the vendor (Qiagen Data Analysis Center).

Quantitative PCR (qPCR) validation of the genes upregulated in PCR Array

The key cell cycle-related genes (P19ARF, P16INK4A, P21CIP1 and cyclin E) which were observed to be differentially expressed in the array were further validated using independent qPCR analyses. Fresh RNA was extracted from the tumor samples, and 1µg RNA of each sample was used to prepare cDNA using MuLV reverse transcriptase. SYBR-green-based qPCR analysis was performed in Step One plus real-time PCR system (Applied Biosystems, Thermo Fisher Scientific). The sequences of the primers are listed below:

P19 Forward: 5'-GCTCTGGCTTTCGTGAACAT-3'

P19 Reverse: 5'-CGAATCTGCACCGTAGTTGA -3'

P16 Forward: 5'-AACGCCCCGAACTCTTTC -3'

P16 Reverse: 5'-CAGTTCGAATCTGCACCGTA -3'

P21 Forward: 5'-GTCTGAGCGGCCTGAAGAT -3'

P21 Reverse: 5'-TCTGCGCTTGGAGTGATAGA -3'

Cyclin E Forward: 5'-AAGCGAGGATAGCAGTCAGC -3'

Cyclin E Reverse: 5'-TCTGGGTGGTCTGATTTTCC -3'

Mouse GAPDH Forward: 5'-TGTTCCTACCCCCAATGTGT -3'

Mouse GAPDH Reverse: 5'-CCCTGTTGCTGTAGCCGTAT -3'

RESULTS

Elevated HER2/Neu expression in tri-transgenic mice

Immunohistochemical staining against HER2 (Neu) oncoprotein exhibited a robust and comparable staining intensity in both the control and Dox-treated tumor sections. The staining intensity was found to be higher at the cell boundaries in keeping with the transmembrane localization of the HER2 protein. The similar staining intensity for HER2 indicated that both the control set and the Dox-treated set of tumors exhibited comparable levels of HER2 expression, which is the oncoprotein driving the tumorigenesis in the mouse model under study **(Figure IV.3a)**.

Elevated CCN5 expression in Doxycycline-treated tri-transgenic mice

The gene expression quantification from the q-PCR analysis performed with the control and Dox-treated samples exhibited that CCN5 transcript levels in the tumors from Dox-treated mice were significantly higher as compared to the tumors from control mice **(Figure IV.3b)**. This observation validated the Doxycycline regulation of CCN5 expression in the tri-transgenic mouse model.

Reduced tumor burden in Doxycycline-treated CCN5 overexpressing tri-transgenic mice

It was observed that by the study endpoint, at approximately 250 days of age of the mice, both tumor incidence and tumor burden were significantly diminished in Dox-treated CCN5 overexpressing mice in comparison to the control mice. At the time of study endpoint, the Dox-treated mice either did not develop any mammary tumors or the tumors which developed, were of significantly smaller volume as compared to the tumors in the control mice. We observed an increase in the tumor latency period (as the palpable tumor was noticed at a later time point in

Dox-treated mice) and also a decreased tumor growth rate in CCN5-overexpressing tri-transgenic mice, which were manifested in the reduced tumor burden at endpoint **(Figure IV.4a,b)**.

Upregulation of major Cdk-Inhibitors in CCN5 overexpressing HER2 tumors

In the gene expression array studies, we noticed significant changes in the expression status of multiple genes in CCN5 overexpressing tumors relative to the control tumors which were related to cell-cycle regulation **(Figure IV.5)**. These differentially expressed genes included two Cyclin dependent-kinase inhibitor loci (CDKN1A and CDKN2A) along with CCNE1(Cyclin E1). The levels of these two transcripts were significantly upregulated (> 2.5 fold) in the Dox-treated CCN5 overexpressing tumors. The CDKN2A locus generates two transcripts for $P16^{INK4A}$ and $P19^{ARF,}$ and CDKN1A transcription leads to $P21^{CIP1}$ expression. Interestingly, expression of cyclin E was also upregulated (2.8 fold) in the CCN5 expressing tumors.

The results from the breast cancer panel gene array were validated by independent real-time q-PCR assays **(Figure IV.6a,b,c,d)**. As mentioned, the CDKN2A locus transcribes into two mRNA transcripts resulting from alternative splicing and independent primers for both of these transcripts (P16INK4A and P19ARF) were designed for qPCR validation. Our initial results indicate that expression levels of both these transcripts were higher in CCN5 overexpressing tumors harvested from Dox-treated mice. In corroboration of the gene array data, expression of Cyclin E and P21 were also higher in dox-treated tumors.

DISCUSSION

The observations from studies of conditional overexpression of CCN5 in the Neu (ERBB2) overexpressing transgenic mouse model have been listed in this chapter. The preliminary findings indicate that CCN5 might play a role in delaying the tumor onset and tumor progression in ERBB2 (Neu) overexpressing breast tumors. As discussed in previous chapters, and also as previously published (Haque et al., 2015), it has been established that CCN5 can exert an inhibitory effect on PI3K-AKT mediated signaling pathway most likely through its interactions with the integrin receptors. Overexpression of ERBB2 (Neu) receptors in cancer cells have been shown to drive mitogenic signaling primarily through activated PI3K-AKT and RAS-MAPK signaling pathways (Hynes & Stern, 1994; Yarden & Sliwkowski, 2001). Thus, one logical anticipation was that CCN5 overexpression might be able to negatively influence cell proliferation in the Neu overexpressing mammary tumors and delay tumor progression. Interestingly, as mentioned in the observation section, the CCN5 overexpressing mice did show delayed tumor onset and slower progression. Though the activation status of the PI3K-AKT pathway has not been checked, the gene expression array did not show any change in Cyclin D1 expression in CCN5 expressing tumors. The CyclinD1 expression is directly upregulated by activated PI3K-AKT and RAS-MAPK signaling. It might be a possibility that the effect of CCN5-induced suppression of PI3K pathway is compensated by activated MAPK pathway leading to similar expression status of CyclinD1 in control and Dox-treated tumors.

It has been established that ERBB2 overexpression hotwires cell cycle progression through deregulation of G1-S transition checkpoint (Sherr & McCormick, 2002; Yarden & Sliwkowski, 2001). We observed upregulation of transcript levels of two CDK inhibitor (CDKN) loci, CDKN1A and CDKN2A in CCN5 overexpressing tumors. CDKN1A transcript translates into

P21CIP1 protein while alternative reading frames of CDKN2A locus gives rise to two proteins P16INK4A and P19ARF (P14ARF in human). All of these three proteins enforce G1-S transition checkpoints.$P21^{CIP1}$ inhibits CDK2 activity in early G1 phase. P16INK4A acts as an inhibitor to the CDK4/6 mediated phosphorylation and inactivation of retinoblastoma protein (Rb) and induces cellular senescence. P19ARF (P14 ARF) induces wild-type P53 mediated apoptosis or cell cycle arrest in response to aberrant mitogenic stimulation mediated activation of E2F transcription factors. CCN5 induced overexpression of these checkpoint gene transcripts indicates that CCN5 mediated negative regulation of Neu tumor progression might be through reinforcement of G1-S cell cycle checkpoint. For treatment of ERBB2 overexpressing tumors, clinicians rely primarily on monoclonal antibodies against the oncoprotein (e.g., trastuzumab) that prevents its dimerization or on inhibitors designed against tyrosine kinase activity of the receptor (e.g., lapatinib) (Emde et al., 2012). However, tumor cells (at the initial stage or after relapse) have frequently been shown to have deregulated downstream signaling pathways and exhibit resistance to the mentioned therapeutic strategies progressing through uncontrolled proliferation (Emde et al., 2012; Hynes & Lane, 2005). As a response to this situation, multiple clinical trials have been trying to examine benefits of the use of CDK4/6 inhibitors as a therapeutic regimen to be used in conjunction to receptor antibodies or inhibitors (Corona et al., 2017). Recently, a CDK4/6 inhibitor, Palbociclib has been approved by FDA for treatment of patients with endocrine receptor-positive metastatic breast cancer. In this context, observations of this study indicate a possible role of CCN5 in reinforcing CDKN mediated G1-S checkpoint and holds special clinical relevance.

FIGURES

Fig.IV.1a

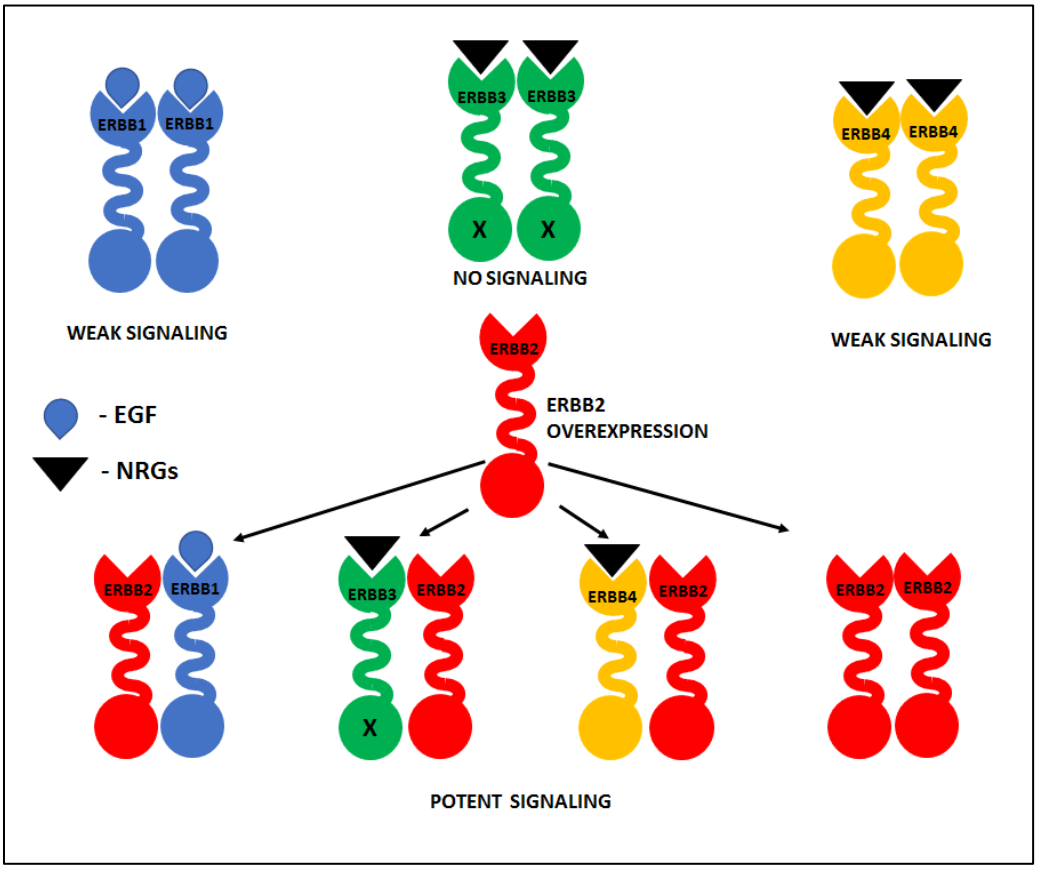


Fig.IV.1a: Overexpression of the ERBB2 (HER2/Neu) leads to heterodimerization with other 3 ERBB partners. ERBB2 does not bind to a ligand while ERBB3 though having specific ligands (Neuregulins) does not have a kinase activity of its own. However, heterodimer of ERBB3 and ERBB2 induces most potent signaling. ERBB2 heterodimers shows slower ligand dissociation, relaxed ligand specificity, slower endocytosis of receptors and prolonged signaling activation. ERBB2 mediated signaling leads to increased cell proliferation, migration and decreased apoptosis. Figure inspired from published article and redrawn (Yarden & Sliwkowski, 2001).

Fig.IV.1b

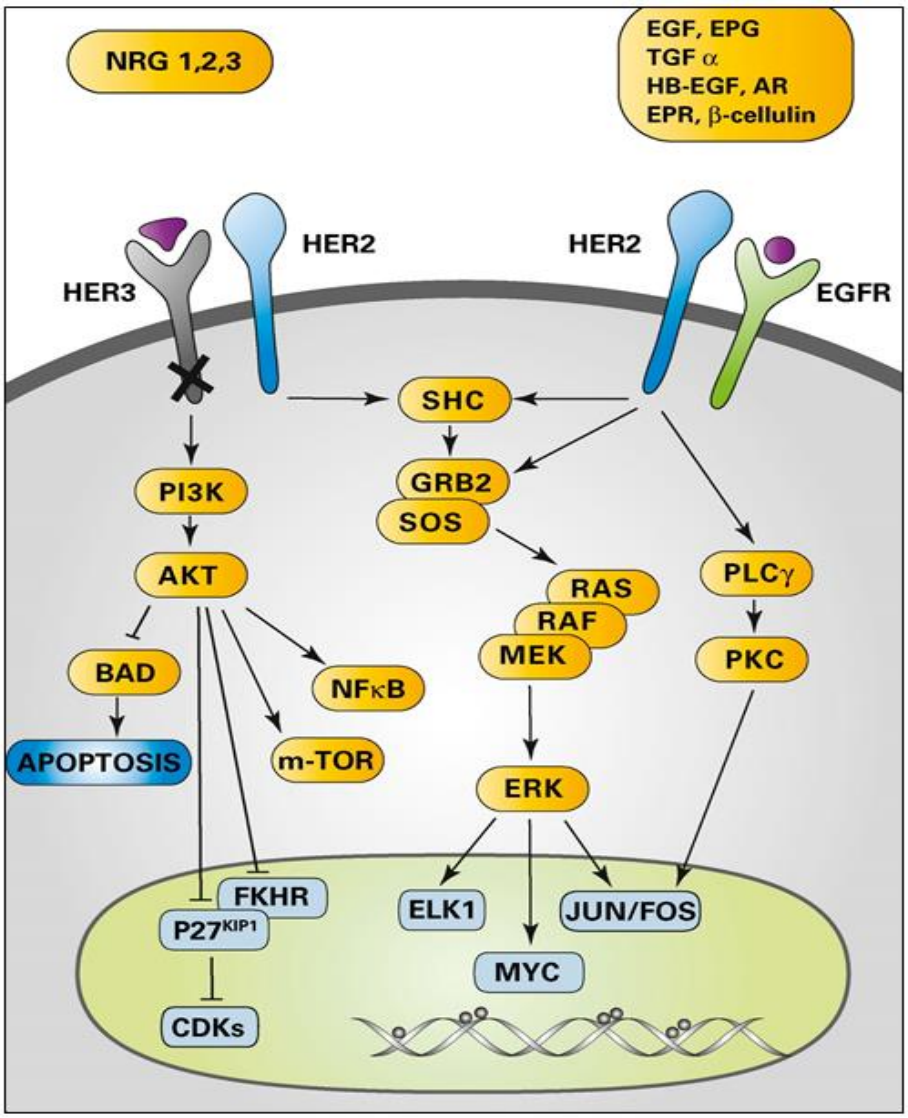


Fig.IV.1b: The figure shows the major signaling pathways and transcription factors activated by signaling through ERBB1(EGFR)-ERBB2(HER2) and ERBB3(HER3)-ERBB2(HER3) heterodimers. Upon ligand binding induced heterodimer formation the intracellular signaling activation majorly takes place through PI3K-AKT and RAS-RAF-MAPK pathways. This eventually leads to prevention of apoptosis; release of $P27^{KIP1}$ and FOXO3a mediated repression of CDKs; mTOR (mammalian Target of Rapamycin) mediated cell growth; activation of transcription factors like AP1(JUN/FOS), MYC which further drives cell proliferation (Emde, Kostler, Yarden, Association of, & Oncology of the Mediterranean, 2012). This figure in its current state has been directly used from cited publication with permission.

Fig.IV.1c.i

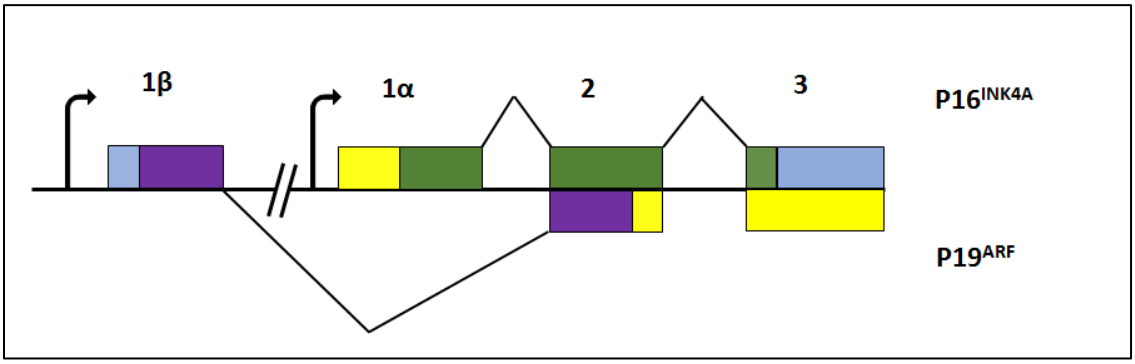


Fig.IV.1c.ii

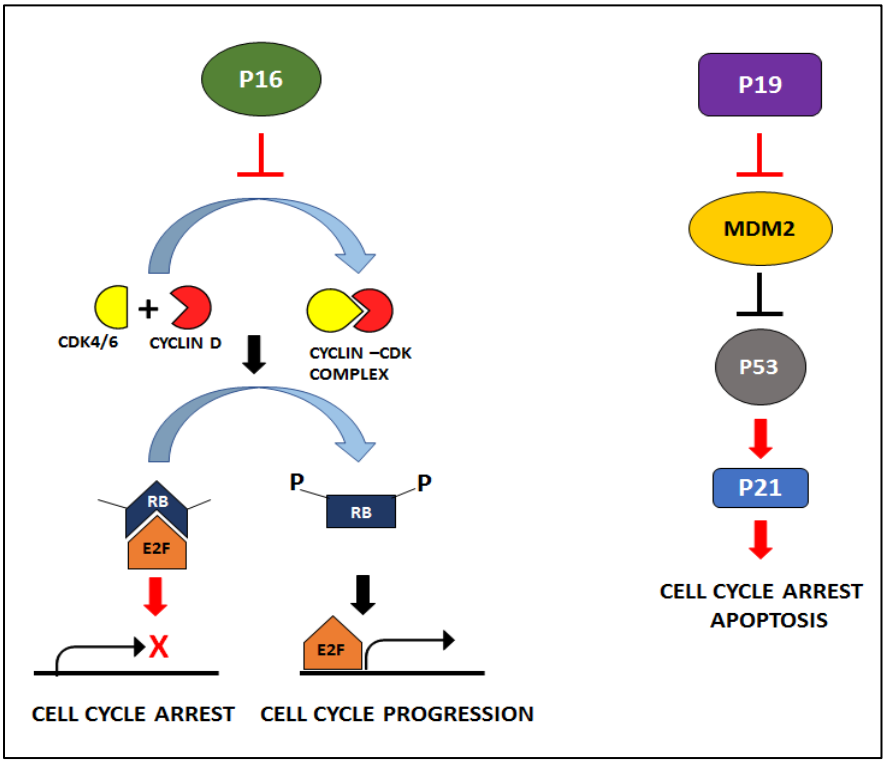


Fig.IV.1c: (i) INK4A locus leads to transcription of two different transcripts leading to translation of two different proteins P16INK4A and P19ARF . Regions of the locus indicated in green and purple translates to P16INK4A and P19ARF proteins respectively. The figure adapted and redrawn from previous publications (Hahn & Weinberg, 2002; Sherr, 2001). (ii) Mechanisms of both the proteins in inducing cell cycle arrest (discussed in detail in text). The figure has been redrawn according to a previously published illustration (Park, Morrison, & Clarke, 2004).

Fig.IV.2

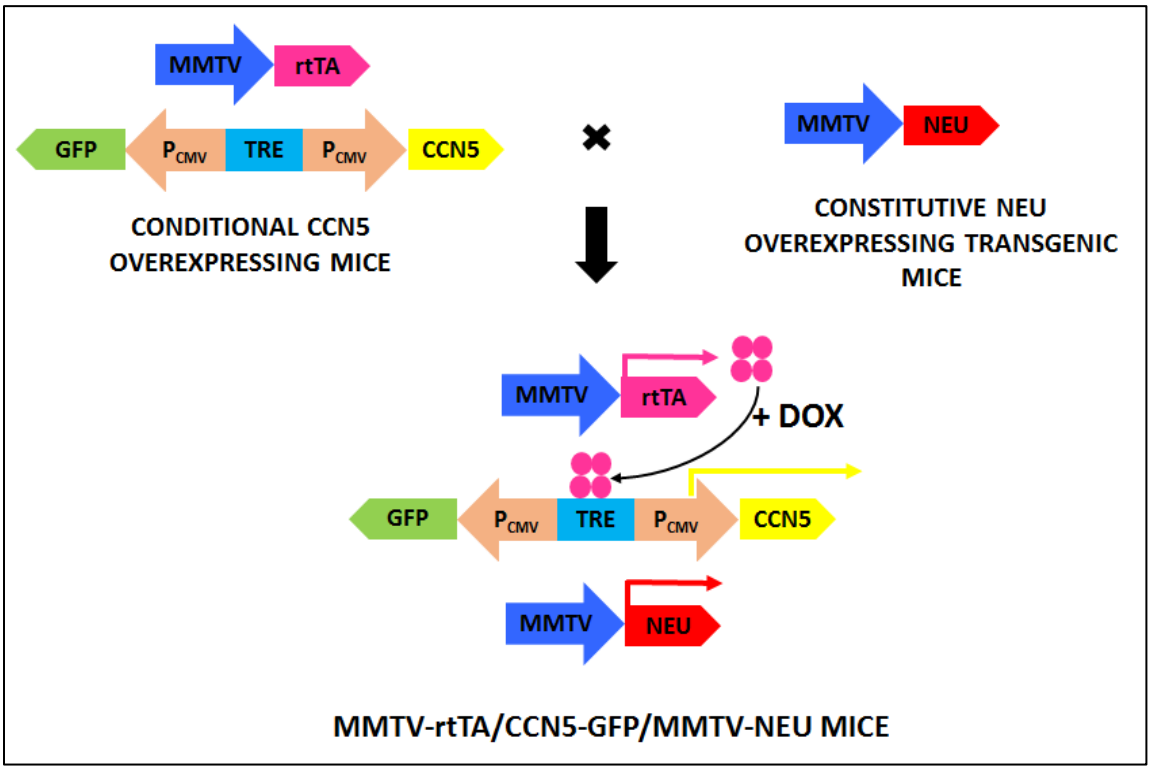


Fig.IV.2: Generation of Tri-transgenic mice line (MMTV-rtTA/CCN5-GFP/MMTV-Neu): Conditional CCN5 overexpressing mice (which has been described in the previous chapter) was crossed with constitutive mammary specific Neu overexpressing mice (MMTV-Neu). Reverse tetracycline trans-activator (rtTA) and Neu are constitutively expressed in mammary epithelium. Doxycycline treatment leads to activation of rtTA which leads to expression of CCN5 in mammary epithelium.

Fig.IV.3a

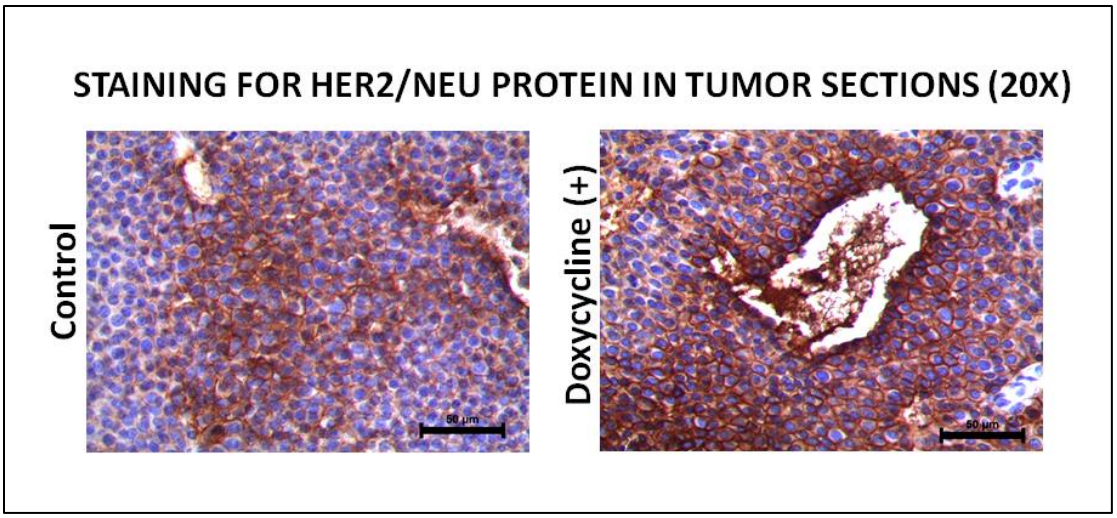


Fig.IV.3b

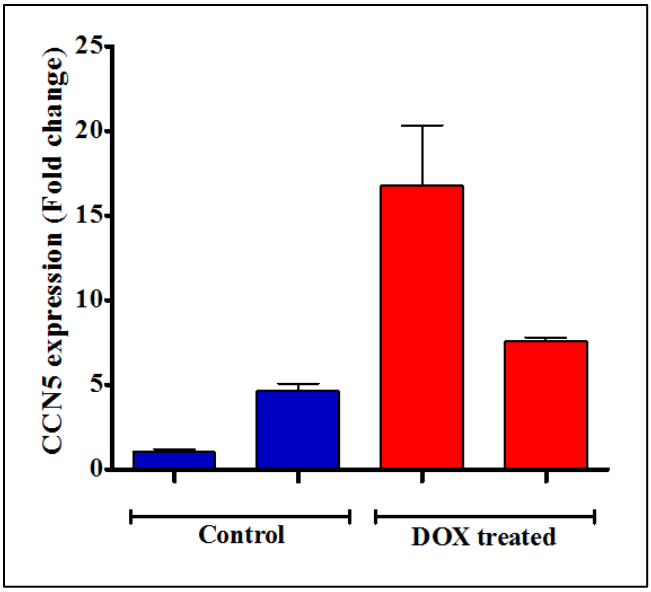


Fig.IV.3: (a) Representative images of comparative immunohistochemical staining against Neu in control and doxycycline -fed tri-transgenic mice which shows comparable staining intensity (b) Quantitative representation of CCN5 transcript expression in tumors of control and dox-treated tri-transgenic mice. Bar graph represents the fold change of CCN5 transcript in Dox treated mice as compared to control littermates. Error bars represent standard deviation from mean value. The Dox-treated mice tumors, even which exhibited lower CCN5 expression, had higher concentration of CCN5 transcript than control tumors.

Fig.IV.4a

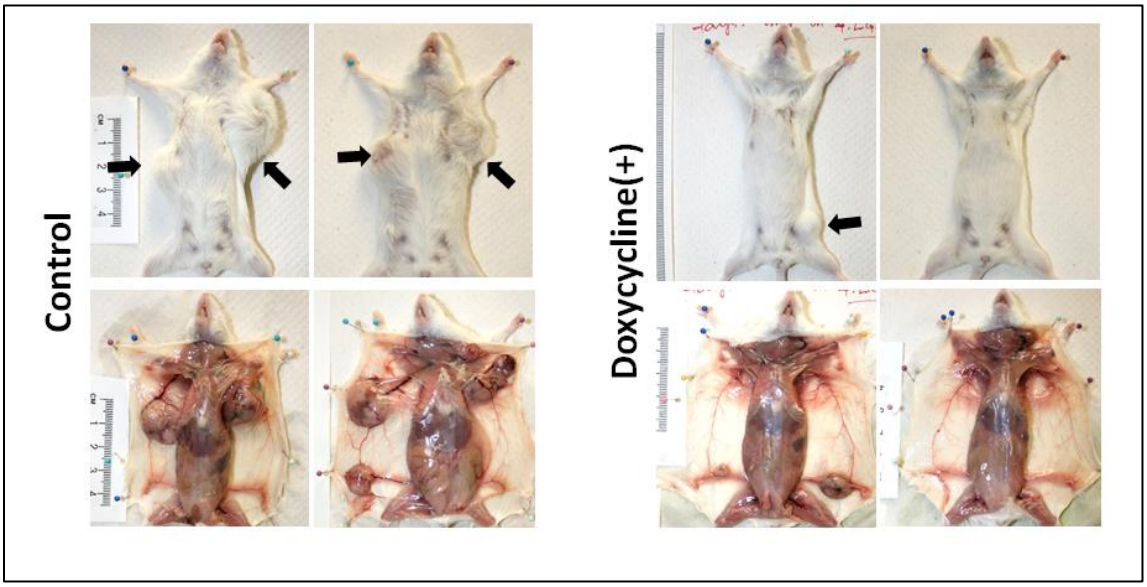


Fig.IV.4b

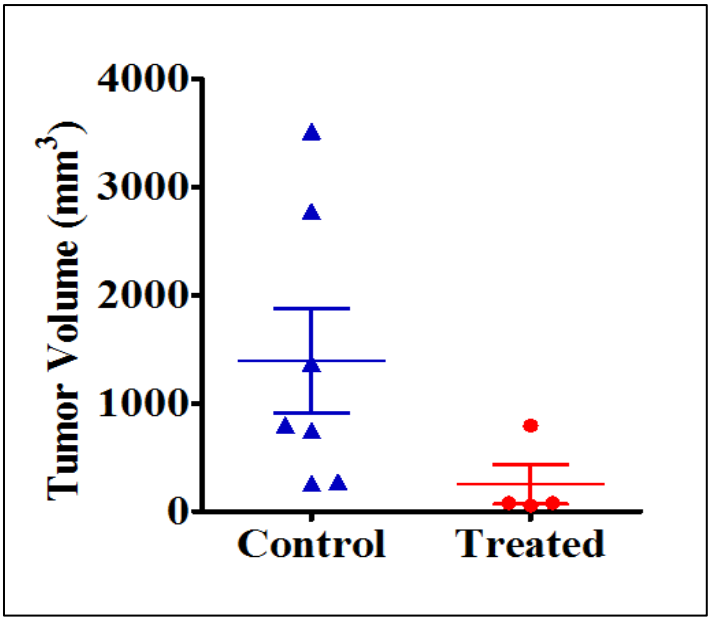


Fig.IV.4: (a) Representative images of control and Dox treated tri-transgenic mice showing smaller tumor volumes and tumor numbers in CCN5 overexpressing dox-treated mice (b) Quantitative representation showing lower tumor burden in Dox-treated mice. Each point represents an individual tumor. The number of tumors in treated mice was lower as compared to control mice. The tumor volumes represent the volume of the tumors measured at the endpoint.

Fig.IV.5

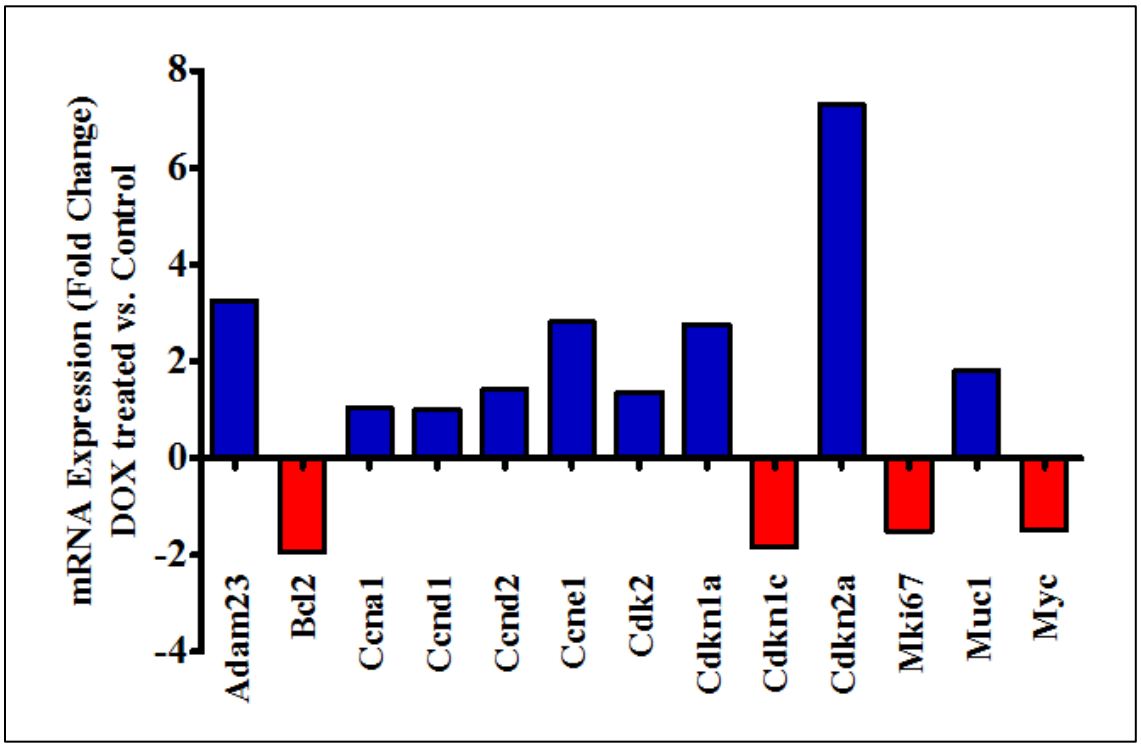


Fig.IV.5: Graphical (bar graph) representation of the fold changes of gene expressions in Dox-treated tumors relative to control tumors as observed from qPCR based gene expression array. Critical cell cycle and cell proliferation related genes and genes which were shown to be upregulated or down-regulated by at least 2-fold have been represented. Transcripts from CDKN2A locus were most differentially expressed (upregulation) in dox treated tumors. Down regulation of proliferation marker Ki-67 and anti-apoptotic BCL-2 in dox-treated mice tumors was noted.

Fig.IV.6a

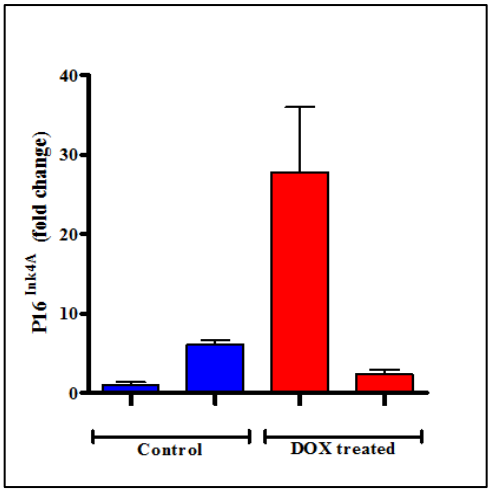


Fig.IV.6b

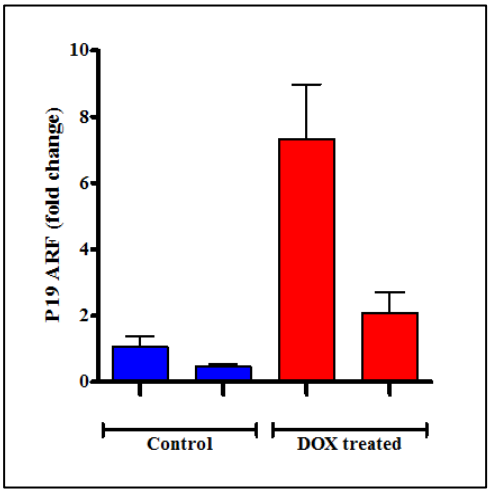


Fig.IV.6c

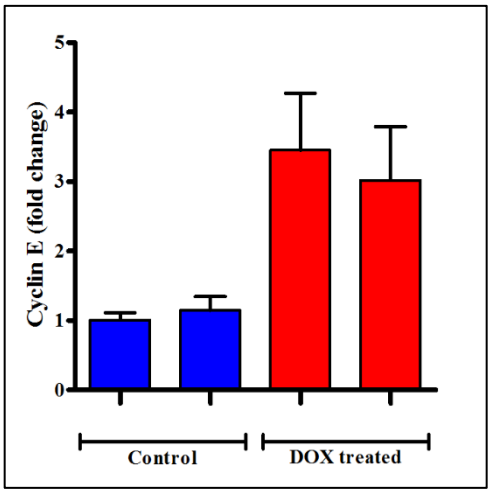


Fig.IV.6d

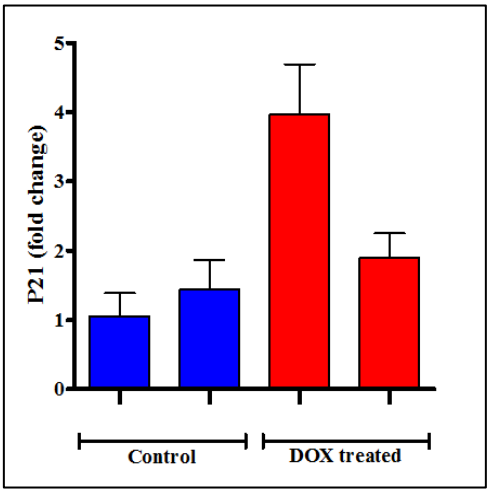


Fig.IV.6: (a-d) Bar graph representation of independent qPCR based validation of expression changes of key cell-cycle related genes observed in gene array. Both transcripts from INK4A (CDKN2A) locus (P16INK4A and P19ARF) were observed to be upregulated in CCN5 expression tumors. Upregulation of Cyclin E transcript and P21 CIP1 transcript was upregulated in Dox -treated tumors. Error bars represents standard deviation from mean value.

CHAPTER V: General Discussion

The diverse physiological and pathological roles of the CCN family proteins (CCN1-CCN6) have been extensively studied by multiple labs, pursuing different research interests.(S. K. Banerjee et al., 2016; J. W. Russo & Castellot, 2010). However, CCN5 has been far less studied as compared to the other members of the family, especially the first two members (CCN1 and CCN2). The roles played by CCN5 in impacting breast cancer progression and the possible signaling mechanisms by which CCN5 exert those roles, has been the central theme of the studies which are described in this dissertation.

After the discovery of *WISP2* (CCN5) gene, most of the preliminary studies were focused on the influence of CCN5 protein on cellular proliferation, motility, adhesion, and differentiation. However, these studies did not provide much information on the cell-signaling pathways that are impacted by CCN5. Also, the studies were largely limited to the smooth muscle cells and other mesenchymal cells (e.g., osteoblasts and chondrocytes) (J. W. Russo & Castellot, 2010). But interestingly, rCOP1 (the rat homolog of CCN5) was identified for the first time as a gene, whose loss of expression was a key step to the transformation of the Rat Embryonic Fibroblasts (REFs) (R. Zhang et al., 1998). In another study, which led to the independent identification of CCN5 (or WISP2, as named by this group), decreased expression of CCN5 was observed in the colon cancer tissues as compared to the normal tissues (Pennica et al., 1998). Hence, since discovery, CCN5 was poised to be an important candidate for further research on cancer progression. Soon after, multiple studies established that CCN5 is highly expressed in the estrogen receptor (ER) positive breast cancer cells and is under the direct transcriptional regulation of estrogen receptor-α (ER-α) (S. Banerjee et al., 2003; Inadera et al., 2002; Inadera et al., 2000). Since then, CCN5 has been established as a ‘micromanager’ of breast cancer as most of the studies point towards CCN5-mediated negative regulation of breast cancer progression (S.

Banerjee et al., 2008; S. K. Banerjee & Banerjee, 2012; Das et al., 2017; Ferrand et al., 2014; Fritah et al., 2008; Haque et al., 2011).

The key findings described in this dissertation provide a deeper insight into the roles played by CCN5 in impacting breast cancer cell signaling. The first section of the study (Chapter II) establishes a role of CCN5 in inducing growth arrest of the triple negative breast cancer cells. CCN5 has been recognized as a growth arrest specific (*gas*) gene for the ability to induce cell cycle arrest and senescence (Lake et al., 2003; Lake & Castellot, 2003) but these studies did not investigate the influence of CCN5 on epithelial cancer cells. Rather, it was shown that CCN5 was necessary for the estrogen receptor (ER-α) positive breast cancer cell proliferation, induced by estrogen and growth factors like IGF-1 and EGF (S. Banerjee et al., 2005; K. Dhar et al., 2007).

Role of integrin-mediated action of CCN5 in the suppression of the PI3K-AKT pathway in breast cancer cells is one of the important observations of the studies discussed here. This discovery might have a profound impact on the future directions of CCN5 research as the PI3K-AKT pathway is one of the most frequently activated cell-proliferation pathways in human breast cancers (Manning & Cantley, 2007; Vivanco & Sawyers, 2002). The direct influences of CCN5 on the signaling cascade components, linking the integrin receptor-binding and the PI3K-AKT pathway inhibition, are yet to be experimentally elucidated. But, our experiments with the integrin blocking antibodies established that the CCN5-mediated PI3K-AKT inhibition is dependent on binding of CCN5 to the integrin receptors (Haque et al., 2015). Whether binding of CCN5 to the integrins, sequesters the integrin receptors from other activating binding partners or directly impose inhibitory conformational changes on the receptors, remains subject to investigation. The CCN proteins have been shown to directly bind to several growth factors and

also other ECM molecules in the extracellular space (Jun & Lau, 2011). Direct binding of CCN5 to these factors might also interfere with the PI3K-AKT pathway activation in the cancer cells.

The major impacts of CCN5 expression on the triple negative breast cancer cells which were discovered by our studies are - (i) promotion of cell-cycle arrest and (ii) induction of estrogen receptor-α (ER-α) expression. Both effects were dependent on inhibition of the PI3K-AKT pathway.

The first discovery which has been described in the second chapter (Chapter II), indicates that CCN5-mediated AKT inactivation results in stabilization of the FOXO3A transcription factor leading to expression of the Cyclin-Dependent Kinase Inhibitor (CDKN) $P27^{KIP1}$. This increase in expression leads to the growth arrest of the triple negative MDA-MB-231 cells both in-vitro and in the xenograft studies. The study also demonstrated that the phosphorylation status of both the $P27^{KIP1}$ and FOXO3A proteins, depended on activation level of the PI3K-AKT pathway, as both of these proteins are direct substrates of the AKT. The phosphorylation status of $P27^{KIP1}$ and FOXO3A regulates the nuclear-cytoplasmic localization and stability of both the transcription factors. However, it is well-established that AKT also exerts its kinase activity on a wide repertoire of other proteins. The substrates of AKT include apoptotic proteins such as BAD (Bcl2-associated death promoter), Bim (Bcl-2 interacting mediator of cell death), BCL-10 (B-cell CLL/Lymphoma 10); nuclear receptors such as ER (Estrogen Receptor), AR (Androgen Receptor); histone acetyltransferases such as CBP (CREB Binding Protein); cell cycle regulators such as CDK2 (Cyclin-Dependent Kinase 2); other Forkhead box transcription factors like FOXO1A, FOXO4 (Manning & Cantley, 2007; Vivanco & Sawyers, 2002). AKT- mediated phosphorylation of almost all these substrates upon activation of the PI3K-AKT pathway leads to cell survival and proliferation. So, we can argue that CCN5 mediated inhibition of the PI3K-

AKT pathway must have a multi-faceted effect on the cancer cells which also might contribute to the arrest of cell proliferation and induction of apoptosis. Further investigations into the phosphorylation status of the other AKT substrates will provide a broader understanding of other plausible mechanisms of CCN5 mediated action on cancer cell survival and proliferation.

The third chapter of this dissertation (Chapter III) describes that CCN5 expression in MDA-MB-231 cells and the mouse mammary epithelial cells influences ER-α expression status. We concluded from our observations that CCN5-induced AKT inhibition plays a key role in the induction of ER-α. It was already established that, the CCN5-mediated PI3K-AKT inhibition increases the FOXO3A protein level in breast cancer cells and that FOXO3A directly drives transcription of the ER-α gene (*ESR1*) (Guo & Sonenshein, 2004). We demonstrated that CCN5 induces ER-α expression at the transcriptional level (in the mouse mammary epithelial cells and the triple negative cancer cell line MDA-MB-231) by inhibiting the PI3K-AKT pathway and stabilizing FOXO3A. However, it can be argued that CCN5 also might play a role in relieving epigenetic repression of ER-α (*ESR1*) gene through mechanisms that are yet to be discovered. It has been indicated in a previous study that CCN5 might localize into the cell nucleus and directly interact with HDAC1 (Histone Deacetylase 1) (Sabbah et al., 2011). Recruitment of the transcriptional coactivators like p300/CBP by FOXO3A (F. Wang et al., 2009) can also lead to epigenetic activation of *ESR1* gene promoter. Investigations into these possibilities might provide further insights into CCN5 mediated induction of ER-α expression.

Loss of ER-α expression has been one of the critical reasons behind endocrine resistance of the breast cancer cells. Our studies indicate that ability to induce CCN5 expression in the tumor cells might restore ER-α expression in the resistant cancer cells and might provide the clinicians with a strategy to overcome the problem of endocrine resistance. Interestingly, it has been shown in

other studies that activated PI3K-AKT pathway can lead to endocrine resistance of ER-α expressing breast cancer cells. Phosphorylation of ER-α at the Ser167 residue, which is a direct substrate of AKT, has been shown to be critical for ligand-independent activation of ER-α leading to inhibition of tamoxifen-induced apoptosis (Campbell et al., 2001). Thus, the inhibitory effect of CCN5 on the PI3K-AKT pathway might also help overcome the endocrine resistance of ER-α expressing cells.

In the last section of the dissertation (Chapter IV), our observations from transgenic Neu (HER2) mouse model studies indicate that CCN5 expression in Neu overexpressing mammary epithelial cells lead to transcriptional upregulation of key Cyclin-Dependent Kinase inhibitors (P16INK4A and P21CIP1). We propose that this effect of CCN5 expression leads to a cell cycle arrest of the Neu-overexpressing cells resulting in slower tumor progression. Whether CCN5 induced inhibition of PI3K-AKT pathway should play any role in this model is still an unanswered question. Further studies into the phosphorylation levels of the FOXO3A and P27KIP1 proteins, (along with other AKT substrates) in the tumor tissues harvested from the mice, will help us to address this query.

Thus, after evaluating the results from all our studies collectively, we propose that – (a) therapy strategies leading to induction of CCN5 expression might have significant potential in breast cancer treatment and (b) CCN5 expression status in breast tumor tissues might have high prognostic value and help in prediction of resistance and recurrence of breast tumors. The next rational direction of CCN5 research would be the identification of ways, to induce overexpression of CCN5 protein or to deliver recombinant CCN5 protein specifically into the breast tumor tissue. CCN5 is a secreted protein, and most of the actions of CCN5 which have been elucidated are integrin receptor-mediated. We anticipate that ability to induce expression of

CCN5 in only a subset of tumor cells might also be beneficial, as the CCN5 protein secreted from the cells will be able to exert its role in an autocrine and paracrine fashion.

Our studies, as described in this dissertation discovers novel roles of CCN5 protein, which further establishes the capability of CCN5 to negatively regulate progression of breast tumors by exerting cell-cycle arrest in parallel with sensitizing the cancer cells to other therapeutic strategies.

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