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POST-TRANSLATIONAL MODIFICATIONS OF PROTEINS IN HUMAN BREAST CANCER: PROTEOMICS STUDIES OF PHOSPHORYLATION AND NITRATION AND IMPLICATION OF THESE PTMS IN TUMORIGENESIS

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"Histories make men wise; poems witty; the mathematics subtle; natural philosophy deep; moral grave; logic and rhetoric able to contend."

Francis Bacon

To my beloved and ever supportive family

Abstract

Breast cancer is the most common cancer in women. Even though improvements in diagnosis and treatment of breast cancer have been made, it is still the most common cause of cancer death in women. There is a great need to find biomarkers for early detection of the disease and novel drug targets to fight the cancer.

Cell immortalization is the prerequisite step in tumorigenesis, and identification of the biomarkers of immortalized cells may be helpful for early detection of cancer. In this thesis, we described identification of 71 immortalization-related proteins. We used proteomics and conditionally immortalized human breast epithelial cells. Identified proteins showed involvement in immortalization of such functional domains as cell proliferation and growth, death, cell assembly and organization, cellular movement, cell-to-cell signaling, and cell morphology. Kinase MAP2K3, was identified as down-regulated in immortalized cells. Overexpression of MAP2K3 in immortal human breast epithelial cells was sufficient to induce senescence. p38, p53 and pRB were modulated by MAP2K3. We also identified KSR2 as up-regulated in immortalized human breast epithelial cells and in human breast tumors.

Twenty-four proteins affected by hyperthermia of human primary breast epithelial cells were also identified. Among the proteins, TGF-β2 was found up-regulated. It induced HSP27 expression, and protected cells from cell death.

Aberrant protein tyrosine nitration has been associated with different diseases, including cancer. We explored changes in protein tyrosine nitration during the cell cycle, and observed that tyrosine nitration affected a number of cell cycle regulators.

Cross talk of different regulatory pathways may contribute to the resistance to the anti-cancer treatment. Targeting of multiple pathways has been regarded as a novel anti-cancer strategy. We showed that combined action of TGF-β1 and EGF involves changes in phosphorylation of 47 proteins. We observed that the convergence components of TGF-β1 and EGF, e.g., MEK1, CK1, can influence cell proliferation in the context of TGF-β1 and EGF signaling. Interestingly, we observed that the strongest inhibitory effect of Gefitinib (Iressa), EGFR kinase inhibitor, would be only when both EGF and TGF-β are highly active, and MEK1 and CK1 are inhibited. ZAK kinase was identified as a convergent target of TGF-β and EGF signaling, and was found contributing to the positive feedback regulation of cell migration upon combined TGF-β and EGF action.

We also studied effects of a long term exposure to EGF and estrogen on tumorigenesis of breast epithelial cells. We observed that the long-term exposure to EGF and 17β-estradiol may affect proliferation rate, colony formation, vessel formation, and stem cell features of human breast epithelial cells.

Thus, our findings provided insights into different mechanisms of tumorigenesis, and impact of cross-talk of signaling pathways on tumor development.

List of Publications

I. **<u>Min Jia</u>**, Nazariy Souchelnytskyi, Ulf Hellman, Michael O'Hare, Parmjit S. Jat, Serhiy Souchelnytskyi. Proteome profiling of immortalization-to-senescence transition of human breast epithelial cells identified MAP2K3 as a senescence promoting protein which is down regulated in human breast cancer. *Proteomics Clin Appl.* 2010 Nov; 4(10-11):816-28.

II. **<u>Min Jia</u>***, Serhiy Souchelnytskyi. Proteome profiling of heat-shock of human primary breast epithelial cells, a dataset report. *Cell Stress Chaperones.* 2011 Jul; 16(4):459-67.

III. **<u>Min Jia</u>**, Serhiy Souchelnytskyi. Kinase suppressor of ras 2 is involved in regulation of cell proliferation and is up-regulated in human invasive ductal carcinomas of breast. *Exp Oncol.* 2010 Sep; 32(3):209-12.

IV. **<u>Min Jia</u>***, Claudia Mateoiu, Serhiy Souchelnytskyi. Protein tyrosine nitration in the cell cycle. *Biochem. Biophys. Res. Commun.* 2011 Sep 23; 413(2): 270-6.

V. **<u>Min Jia</u>**, Andrey Alexeyenko, Claudia Mateoiu, Serhiy Souchelnytskyi. Network signaling by TGFβ1 and EGF in regulation of the cell proliferation as predictor of application of Iressa. *Manuscript*

VI. **<u>Min Jia</u>**, Serhiy Souchelnytskyi. Role of sterile alpha motif and leucine zipper containing kinase AZK (ZAK) in combined signaling by TGFβ1 and EGF. *Manuscript*

VII. **<u>Min Jia</u>**[#], Sara Cunha[#], Serhiy Souchelnytskyi. Transformation and stemness of human breast epithelial cells exposed to EGF and 17β-Estradiol. *Manuscript*

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# These authors contributed equally to the work

Papers not included in the thesis

I. **Min Jia***, Serhiy Souchelnytskyi. Comments on the cross-talk of TGF-β and EGF in cancer. *Exp Oncol*. 2011 33, 3, 1-4

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III. Kah Wai Lin, Ihor Yakymovych, **Min Jia**, Maria Yakymovych, Serhiy Souchelnytskyi. Phosphorylation of eukaryotic elongation factor eEF1A1 at Ser300 by type I transforming growth factor-β receptor results in inhibition of mRNA translation. *Curr Biol*. 2010 Sep 28; 20(18):1615-25

IV. Kah Wai Lin, **Min Jia**, Serhiy Souchelnytskyi. Book chapter: Application of bioinformatics tools in gel-based proteomics. *Proteomics/Book 2*, ISBN 979-953-307-693-4. Accepted

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Contents

List of abbreviations

AI	aromatase inhibitor
ALDH1	aldehyde dehydrogenase 1
BCRP1	breast cancer resistance protein 1
ALK	activin receptor-like kinase
CDKs	cyclin-dependent kinases
CSCs	cancer stem cells
DCIS	ductal carcinoma in situ
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
EPO	eosinophil peroxidase
ERK	extracellular signal-regulated kinase
ESR2	estrogen receptor 2
FASTKD2	FAST kinase domain 2
GC	gas chromatography
hTERT	catalytic subunit of human telomerase transcriptase
IDC	invasive (infiltrating) ductal carcinoma
IGF	insulin-like growth factor
ILC	invasive (infiltrating) lobular carcinoma
ILK	integrin-linked kinase
IMAC	immobilized metal affinity chromatography
JAK	janus family kinase
KLM	keyhole limpet hemocyanin
KSR2	kinase suppressor of ras 2
LAP	latency-associated protein
LC	liquid chromatography
LCIS	lobular carcinoma in situ
LTBP	latent TGF-β binding protein
MAPK	mitogen-activated protein kinase
MAP2K3	mitogen-activated protein kinase kinase 3
MALDI-TOF	matrix-assisted laser desorption ionization-time-of-flight
MoAbs	monoclonal antibodies
MS	mass spectrometry
mTOR	mammalian target of rapamycin
MPO	myeloperoxidase
NF-kB	nuclear factor kB
NOD/SCID	nonobese diabetes/severe combined immunodeficiency
NSCLC	non-small-cell lung cancer
PAI-1	plasminogen activator protein 1
PCR	polymerase chain reaction

PI3K	phosphatidylinositol 3-OH kinase
PDGF	platelet-derived growth factor
PD	population doublings
PTEN	phosphatase and tensin homolog deleted from chromosome 10
PTN	protein tyrosine nitration
SA-β-gal	senescence associated β-galactosidase
SCX	strong cation exchange chromatography
SP	side population
STAT	signal transducer and activator of transcription
SV40 LT	simian virus 40 large-tumor
TDLU	terminal ductal–lobular units
TGF-β	transforming growth factor-β
TiO_2	titanium dioxide
TK	tyrosine kinase
TKIs	tyrosine kinase inhibitors
TNF	tumor necrosis factor
TβRII	type II transforming growth factor-β receptor
VEGF	vascular endothelial growth factor
ZAK	sterile alpha motif and leucine zipper containing kinase AZK
2D-GE	two-dimensional polyacrylamide gel electrophoresis

1   Introduction

1.1   Breast Cancer

Cancer cells divide and grow uncontrollably, forming malignant tumors. It may invade surrounding tissues, and metastasize to distant organs. Worldwide, breast cancer is the most common invasive cancer in women. In 2008, breast cancer caused 458,503 deaths worldwide (13.7% of cancer deaths in women) [1].

1.1.1   Breast Structure

The breast lies between the second and sixth ribs, from the sternal edge to the edge of the axilla. It is composed of 15-20 lobes that radiate from the nipple. Each lobe is surrounded by fat and fibrous connective tissue and is divided into many lobules, also called ductal-lobular unit [2]. Lobules end in terminal ductal–lobular units (TDLUs) (Figure 1). It is believed that most breast cancers arise in the TDLU [3]. Histological studies of TDLU indicated two major types of cells: inner secretory luminal cells and outer contractile myoepithelial cells. The majority of breast carcinomas arise from luminal epithelial cells [4].
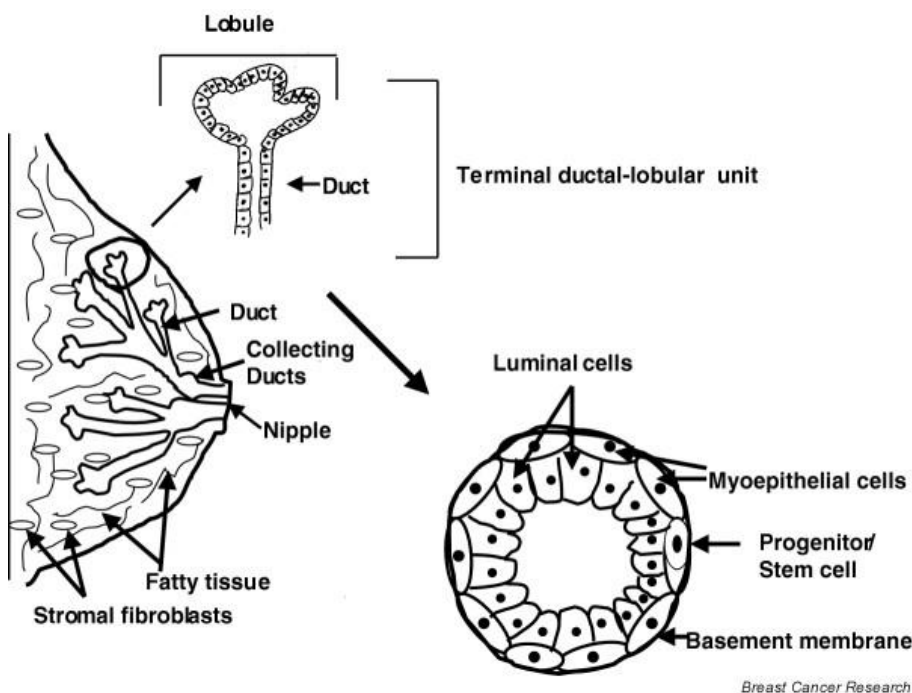

Figure 1: Structure of mammary gland. (From Dimri G, et al. [3]. Reproduced with permission).

1.1.2   Types of Breast Cancer

There are several different ways to classify human breast cancer, e.g., histopathology, grade, stage, receptor status and DNA arrays. Breast cancer is usually classified

primarily by its histological appearance, with division into non-invasive and invasive disease.

Non-invasive Breast Cancer

Ductal carcinoma in situ (DCIS) is the most common type of noninvasive breast cancer, accounting for about 15% of all new breast cancer cases in the U.S [157]. Ductal carcinoma in situ refers to an uncontrolled growth of cells that are confined to the breast duct. Lobular carcinoma in situ (LCIS) is characterized by abnormal changes in the cells that line the milk-producing lobules, or lobes, of the breast. LCIS is much less common and carries slightly less risk of invasive cancer than DCIS.

Invasive Breast Cancer

Invasive (infiltrating) ductal carcinoma (IDC) is the most common type of breast cancer. About 80% of invasive breast cancers are classified as invasive ductal carcinoma [157]. In infiltrating ductal carcinoma, cancer cells have penetrated the ductal wall and invaded surrounding breast tissue. The cells may then metastasize to other parts of the body through the bloodstream or lymphatic system.

Invasive (infiltrating) lobular carcinoma (ILC) begins in the milk-producing lobules where it extends into the adipose tissue of the breast. It is relatively uncommon, comprising about 10% of invasive breast cancers [157]. ILC can also be more difficult to detect by mammography [157].

Invasive papillary carcinoma is very rare, comprising less than 2% of invasive breast cancers [157]. Found predominantly in postmenopausal women, it is characterized by nodular densities that may be multiple and are frequently lobulated.

Phyllodes tumor is a rare fibroepithelial breast neoplasm, which resembles fibroadenoma; it accounts for 0.3% to 1% of all primary breast tumors and 2.5% of fibroepithelial breast lesions [157]. Phyllodes tumors can be either benign, borderline or malignant. Malignant tumors are very rare.

1.1.3 Breast Cancer Detection and Treatment

Improvement in early detection and treatment decrease the deaths from breast cancer over the past two decades [1; 5]. Nowadays, Clinical Breast Exam, Screening Mammogram, Breast Self-Examination and Magnetic Resonance Imaging are used for detection of breast cancer in clinical practice.

Five main types of therapies are available for the treatment of breast cancer: surgery, radiation therapy, chemotherapy, hormone therapy, and most recently, targeted therapy. Most patients are treated with surgery in combination with one or more additional therapies for optimal cancer management.

The primary treatment for breast cancer is surgery to remove the tumor. Radiation therapy uses high-energy X-rays or other types of radiation to destroy cancer cells. In contrast to radiation therapy and surgery, which are local therapies, chemotherapy is a systemic mode of treatment that affects tissues and organs throughout the entire body. It uses cytotoxic drugs to kill and prevent the growth of cancer cells. Hormonal

therapy has been extensively used in the treatment of patients with ER/PR-positive breast cancer. The mechanisms of action of hormonal agents include: 1) Blocking the interaction between estrogen and estrogen receptors with selective estrogen receptor modulators (SERM), e.g., tamoxifen (Nolvadex), raloxifene (Evista). In 1986, tamoxifen was the first drug of its kind approved as monotherapy for the treatment of early breast cancer in node-positive, postmenopausal women [6]. 2) Degrading the estrogen receptors with selective estrogen receptor down-regulators (SERD). Currently, fulvestrant is the only FDA-approved SERD for treatment of breast cancer. 3)Inhibiting estrogen production with an aromatase inhibitor (AI), e.g., letrozole (Femara), anastrazole (Arimidex), and exemestane (Aromasin). These drugs inhibit the action of the aromatase enzyme which converts androgens into estrogens by a process called aromatization [7].

Targeted therapy is directed at specific targets in the body (cellular receptors, proteins and enzyme systems) to treat the cancer. Such therapies include monoclonal antibodies and small molecules. Trastuzumab (Herceptin), a monoclonal antibody, has been used since 1998 to treat certain cases of advanced breast cancer by targeting cancer cells overexpressing HER-2/neu, which account approximately 20% to 30% of breast cancers [8].

1.2 Mechanisms of Tumorigenesis

There are two current ideas that attempt to explain the carcinogenesis. They are the clonal evolution model and the cancer stem cell hypothesis (Figure 2).
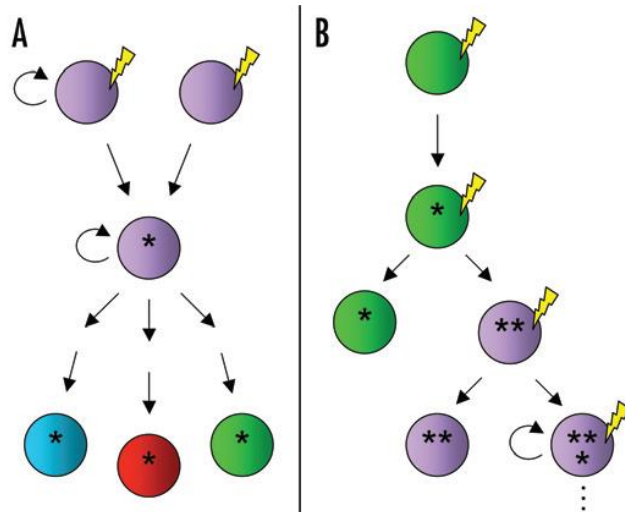
Figure 2: Two proposed explanations of cancer initiation and progression. (A) Cancer stem cells model, and (B) cloncal evolution model. In both (A) and (B), circles represent cells (with purple indicating stem or progenitor properties), lightning bolts represent mutagenesis, and stars represent mutations. The first star in each circle stands for the multiple mutations needed to convert a normal cell to a cancer cell. (From Campbell LL., Polyak K. [9]. Reproduced with permission)

1.2.1   Clonal Evolution

Nowell first proposed the clonal evolution model in 1976 [10]. The clonal evolution model of carcinogenesis proposes that cancer cells over time acquire various combinations of mutations within a tumor and that genetic drift and stepwise natural selection for the fittest, most aggressive cells drive tumor progression. According to this idea, tumor initiation takes place once multiple mutations occur in a random single cell, providing it with a selective growth advantage over adjacent normal cells. As the tumor progresses, genetic instability and uncontrolled proliferation allow the production of cells with additional mutations and hence new characteristics. These cells may leave a large number of offspring by chance, or the new mutations may provide a growth advantage over other tumor cells such as resistance to apoptosis.

Based on this model, in the process of tumorigenesis, abnormal cells display several hallmarks that can be distinguished from those of normal counterparts. These include immortalization or bypass of senescence, evasion of apoptosis and anti-growth signals, growth factor independence, enhanced glycolysis, anchorage-independence, resistance to contact inhibition, angiogenesis, degradation of matrix components, invasion, migration, etc (Figure 3).
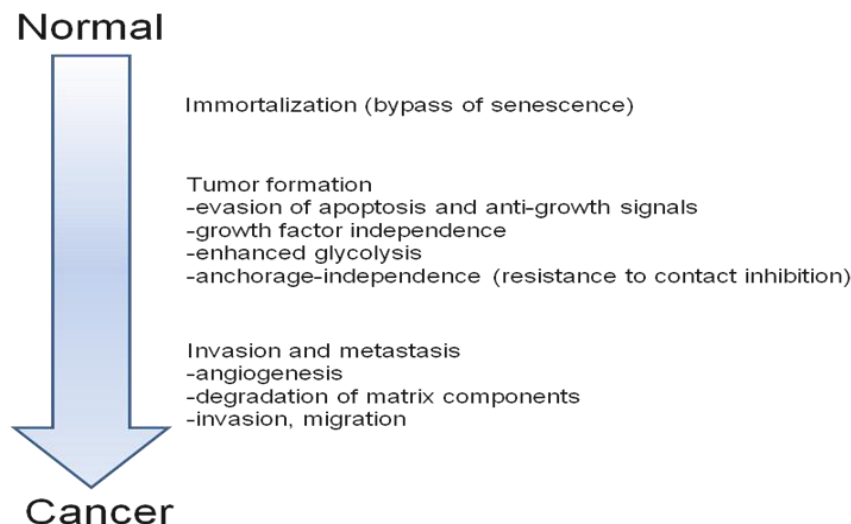
Figure 3: Simplified model of tumorigenesis.

1.2.1.1 Cell Senescence

Normal somatic cells grown in culture cease to proliferate, senesce, after a finite number of divisions. This phenomenon, first described by Hayflick in 1965, is referred to as replicative senescence [11]. Epithelial cells have two stages of senescence. The first stage, termed M0, is a transient growth plateau that occurs after only a few cell divisions [12; 13; 14] . Arrest of epithelial cells in M0 is associated with an increase in p16INK4a protein expression, but it is not a consequence of telomere shortening [12; 13; 14]. Following inactivation of p16INK4a, and in some cell types the inactivation of p53, epithelial cells from M0 may continue to proliferate for approximately another 20–70 population doublings before the second stage of growth arrest, termed M1 or agonoescence [13]. Epithelial cell M1 growth arrest is associated with telomere shortening [13; 14]. M1-arrested epithelial cells do not express p16INK4a. There are a

higher percentage of M1 epithelial cells stained for Annexin-V, a marker of apoptosis, than in M0-arrested epithelial cells [12].Senescent cells arrested in G1 phase of the cell cycle, remain viable and metabolically active and possess a characteristic transcriptional prolife that distinguishes them from quiescent cells. They are characterized by expression of β-galactosidase (SA-β-gal) at pH 6 [11], plasminogen activator protein 1(PAI-1) overexpression and altered cell morphology characterized by giant cell size, increased cytoplasmic granularity and a single large nucleus [15].

1.2.1.2 Cell Immortalization

In order to form tumors, potential cancer cells must overcome this senescence barrier that limits their proliferative potential (Figure 4). This process is called cellular immortalization, in which cells escape senescence and acquire an infinite lifespan. Cell immortalization is the prerequisite step in human carcinogenesis. It provides the opportunity for immortal cells to accumulate sufficient number of genetic mutations, and eventually results in promotion of carcinogenesis. It has long been known that loss of a key tumor suppressor gene, such as p53, or activation of telomerase contribute to spontaneous cellular immortalization [14; 16; 17].
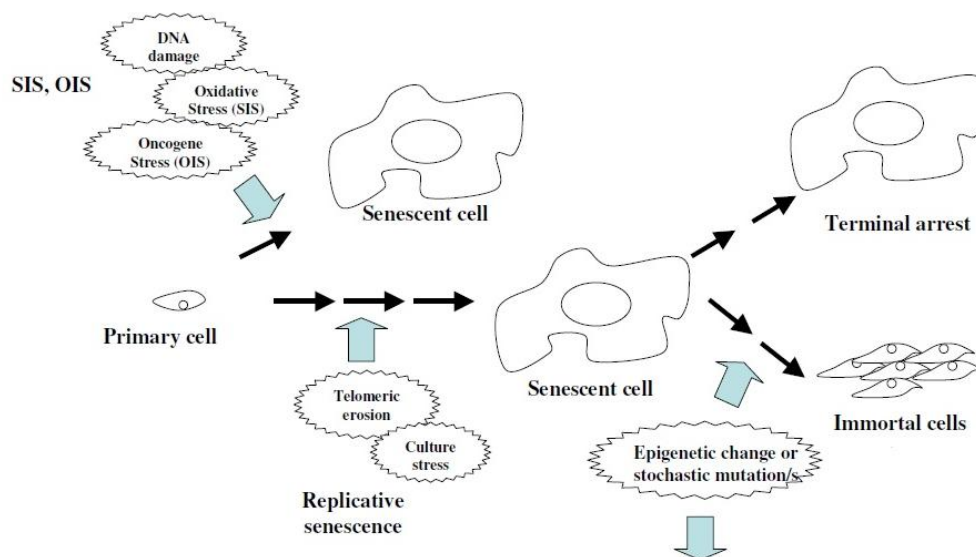
Figure 4: Primary cells reach immortalization after overcoming the senescence barrier in culture. Epigenetic changes and/or stochastic mutation/s can lead it to escape from senescence. (From Lleonart E M., et al. [18]. Reproduced with permission)

In vitro immortalization models have been developed using cells immortalized chemically, virally or with a biological agent. Mortal cells can be immortalized artificially by stabilizing the telomeres by overexpression of hTERT [19], through the addition of a chemical mutagen such as aflatoxin B1 [20], or by transduction with viral oncogenes such as SV40 T-antigen, adenovirus E1a/E1B and HPV16-E6 or -E7 [21; 22; 23; 24]. These viral oncogenes act by inhibiting key genes such as the tumor suppressor gene proteins p53 and pRB, thus allowing the cell to bypass senescence and become immortal [22; 24].

1.2.2 Cancer Stem Cells

The cancer stem cell hypothesis states that a particular subset of tumor cells with stem cell-like properties, called "cancer stem cells" drive tumor initiation, progression, and recurrence. By definition, these cells have the abilities to self-renew indefinitely and to differentiate, and have characteristics of normal adult stem cells [9]. Cancer stem cells are thought to persist as a small fraction of the cells in a tumor. According to the cancer stem cell hypothesis, tumor progression is a result of the metastatic spread of these cells, and cancer recurrence is caused by their resistance to therapy.

Breast CSCs have been identified by Al-Hajj et al. in specimens from patients with advanced stages of metastatic breast cancer [25]. They demonstrated that cells with a specific cell-surface antigen profile (CD44+/CD24−) could successfully establish themselves as tumor xenografts. Recent studies have provided definitive evidence for the existence of CSC populations within breast cancer cell lines.

Several techniques have been employed for the characterization of cancer stem cells. They include cell surface markers, anchorage-independent cell culture, ALDEFLUOR assay, and Side population (SP) technique.

Expression of cell surface markers

Expression of cell surface markers has been widely used to isolate stem cells, but the choice of marker can greatly vary depending on tissues or species. CD44+/CD24-/low/lin- is the most common used surface marker in the study of breast stem cells. The pioneering study by Al-Hajj and colleagues used breast cancer xenografts to isolate a population of cells able to initiate tumors in NOD/SCID mice by the combined expression of cell surface markers CD44+/CD24-/low/lin- [25]. As few as 200 of these cells generated tumors in NOD/SCID mice whereas 20,000 cells that did not display this phenotype failed to do so. The NOD/SCID tumors recapitulated the entire heterogeneity of the initial tumor. Furthermore, the CD44+/CD24-/low/lin- cell population was able to reinitiate tumors in NOD/SCID mice, and retained this ability after serial passages [25]. Thus, these cells, which were able to self-renew, to differentiate, and displayed tumorigenic capacity, had CSC features. Flow cytometry using cell surface markers have been successfully applied to mice and human samples to detect and isolate stem cell populations.

Anchorage-independent cell culture

Human mammary stem and progenitor cells were able to survive in suspension and produce spherical colonies (mammospheres) composed of both stem and progenitor cells. These non-adherent mammospheres were enriched in early progenitor/stem cells and able to differentiate along the three mammary epithelial lineages and to clonally generate complex functional structures in reconstituted three dimensional culture systems as well as reconstitute human normal mammary gland in mice [26]. The mammosphere assay, based on the unique property of stem/progenitor cells to survive and grow in serum-free suspension, was also successfully used to establish long-term cultures enriched in stem/progenitor cells from tumor samples. The mammospheres formed in breast tumor showed an increase in side population (SP) fraction and in CD44+/CD24-/Lin/low- cells, overexpressed neoangiogenic and cytoprotective factors,

expressed the putative stem cell marker OCT4, and displayed high tumorigenic potential in NOD/SCID mice [27].

ALDEFLUOR assay

The ALDEFLUOR assay is based on the enzymatic activity of aldehyde dehydrogenase 1 (ALDH1), a detoxifying enzyme responsible for the oxidation of retinol to retinoic acid. High ALDH1 activity is associated with several types of murine and human stem hematopoietic and neural stem and progenitor cells [28; 29; 30]. This method has been recently used with success to isolate stem and progenitors cells from mammary tissues.

Side population (SP) technique

The SP technique has been used for many years to isolate both normal and tumor stem cells from different organs and species [31; 32]. It is based on the abilities of stem cells to exclude vital dyes. Normal and cancer stem cells express transmembrane transporters, such as the ATP-binding cassette protein, ABC transporter ABCG2/BCRP1 (breast cancer resistance protein 1). These molecules exclude dyes such as Hoechst 33342 or Rhodamin 123 from the cells, a property not found in differentiated cells that remain positive for the dye. The SP fraction from uncultured mammary cells represented ~1% of cells [33]. In contrast, in mammospheres, the SP fraction represented 27% of the cells and could generate bi-lineage colonies when cultured under differentiating conditions [33].

1.3   TGF-β Signaling

1.3.1   TGF-β Superfamily

Almost all cells secrete TGF-β and have TGF-β receptors. The TGF-β superfamily consists of 33 different pleiotropic cytokines that influence cell proliferation, migration, and differentiation [34; 35; 36]. It is composed of the TGF-βs (TGF-β1, -β2, and β3, which are highly homologous), the bone morphogenic proteins, growth and differentiation factors, and activins. Five type II receptors and seven type I receptors (also termed activin-receptor like kinases (ALKs) have been identified. Auxilliary co-receptors have also been identified that regulate access of TGF-β superfamily members to signaling receptors [37].

TGF-β is synthesized as a latent complex consisting of the mature TGF-β homodimer noncovalently linked to the amino-terminal remnant termed latency-associated protein (LAP). This small latent complex can be covalently bound to the latent TGF-β binding protein (LTBP) and stored in the extracellular matrix (ECM) [38; 39]. In this large latent complex, TGF-β is in an inactive conformation and requires processing for the activation, release, and to be capable of binding to receptors. Proteolytic cleavage of the LTBP and integrin mediated conformational change lead to release mature TGF-β from the LAP, generates active TGF-β [40; 41]. Activation of the latent TGF-β complex is crucial for regulating TGF-β activity in tissues [42; 43].

1.3.2 TGF-β Signaling

1.3.2.1 Smad Dependent TGF-β Signaling

TGF-β family member binds to the Type II receptor and recruits Type I, whereby Type II receptor phosphorylates and activates Type I. The Type I receptor, in turn, phosphorylates receptor-activated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5, and Smad8). Once phosphorylated, R-Smads associate with the co-mediator Smad, Smad4, and the heteromeric complex then translocates into the nucleus. In the nucleus, Smad complexes activate specific genes through cooperative interactions with other DNA-binding and coactivator (or co-repressor) proteins [38; 44; 45] (Figure.5).
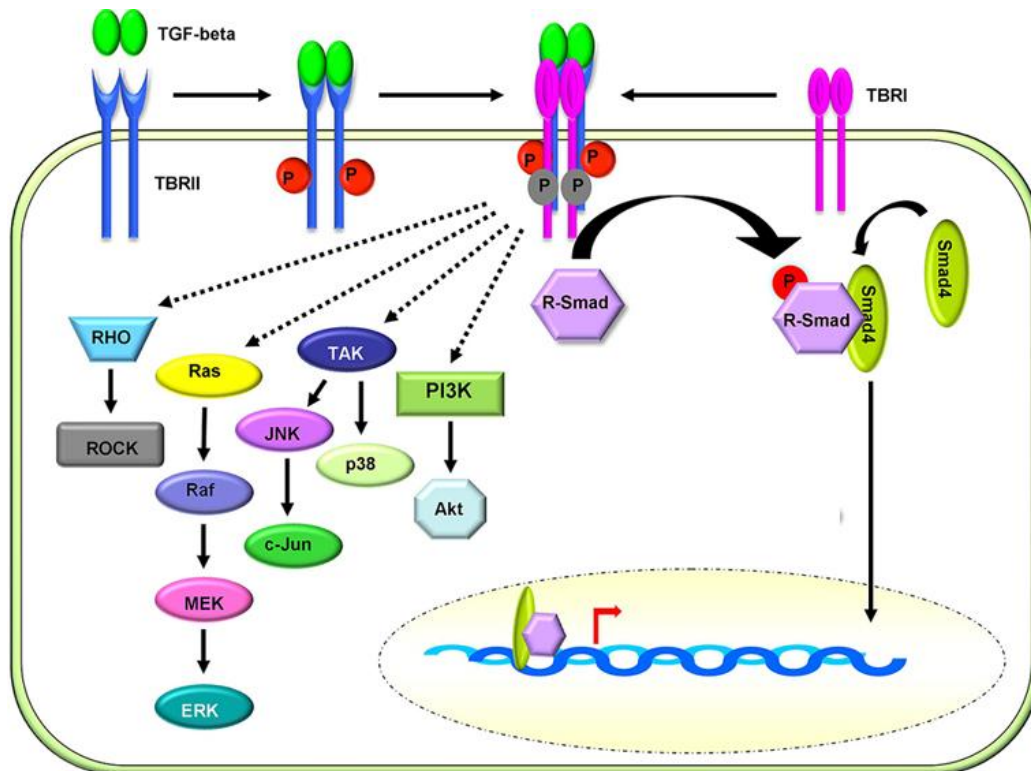
Figure 5. Schematic depicting the Smad and Smad-independent TGF-β signaling systems (Modified from Reiner JE., et al. [46]. Reproduced with permission).

1.3.2.2 Smad-Independent TGF-β Signaling

In addition to its ability to activate Smad2/3, TGF-β also can stimulate Smad2/3-independent pathways (Figure 5), whose activation and biological activities span all cellular compartments, e.g., integrin and Focal Adhesion Signaling, PI3K (phosphatidylinositol 3-kinase pathway), AKT, and mTOR, Rho-family GTPases, MAP kinase family of dual-specificity protein kinases, which includes ERK1/2 (extracellular signal-related kinase 1 and 2), JNK (c-Jun N-terminal kinase), eEF1A1, p38 MAPK, Nuclear Factor-κB (NF-κB) and Lysyl Oxidase (LOX) [47].

1.3.3   TGF-β and Cancer

In cancer, TGF-β plays a dual role. In premalignant stages, TGF-β acts as a tumor suppressor by inhibition of proliferation of epithelial cells and the induction of apoptosis. However, in later stages the epithelial cells become refractory to the growth inhibitory properties of TGF-β by mutations in TGF-β receptors or intracellular signaling components such as Smads [48]. In addition, tumor cells frequently start to secrete high levels of TGF-β [49]. Moreover, TGF-β becomes tumor promoting in a paracrine way by stimulating a favorable microenvironment by promoting angiogenesis [50], suppression of the immune response [51; 52], and hyperactivation of fibroblasts into cancer-associated myofibroblasts [34; 53]. Furthermore, TGF-β also acts on tumor cells directly by stimulating an epithelial to mesenchymal transition (EMT). In this process, epithelial cells lose their epithelial characteristics and acquire a mesenchymal phenotype, which increases their migratory and invasive capacity [36; 54].

Spontaneous epithelial tumors are significantly increased in mice with mammary epithelium-specific expression of dominant negative TβRII [55]. In human neoplastic breast lesions, TβRII down-regulation is correlated with progression and aggression of both in situ and invasive breast carcinomas [56]. Reduced nuclear levels of phosphorylated Smad2/3 are associated with high tumor grades and larger tumor size [57]. These data suggest the suppressive role of TβRII in breast tumorigenesis. TβRI and TβRIII also play a suppressive role in mammary gland tumorigenesis [37]. In breast cancer patients with lymph node and distant metastasis, TGF-β levels in plasma are increased and may be predictive of local and distant metastases [46]. TGF-β signaling also contributes to the resistance of breast cancer cells to DNA-damaging chemotherapeutic agents through modulating the MutS homolog 2 (MSH2) expression [58]. TGF-β may induce immunosuppression and lead to resistance and relapses of breast cancer [59].

1.3.4   Therapeutical Agents Targeting TGF-β Pathway

The therapeutic potential of the TGF-β signaling pathway is ascribed to its supportive function in late stage tumors [60]. A number of approaches to inhibit TGF-β or its signaling effectors have shown promise in anticancer treatment. They are generally classified into four categories: (1) TGF-β expression inhibition at the translational level with antisense oligonucleotides (ASO); (2) blockade of TGF-β interaction with its receptors using monoclonal antibodies; (3) small molecule inhibitors; and (4) peptide aptamers to Smad proteins [61].

1.4   EGFR Signaling

1.4.1   EGFR/erbB Family and Signaling

Epidermal growth factor (EGF) was first discovered in 1962 from new-born mice by Stanley Cohen, as one of the first growth factors that were isolated [62]. The receptor for human EGF, EGFR, was purified two decades after EGF was first discovered [63; 64]. The human EGFR family comprises four closely related receptors: EGFR (HER-1, and erbB1), EGFR-2 (HER-2 and erbB2), HER-3/ erbB3, and HER-4/ erbB4, which are transmembrane glycoproteins containing an extracellular ligand binding domain

and an intracellular receptor tyrosine kinase (RTK) domain [65; 66]. Ligands binding to EGFR family include EGF, transforming growth factor (TGF)-α , amphiregulin (AR), epiregulin (ER), neuroregulin (NR), heparin-binding EGF, and betacellulin [67; 68].

The physiologic downstream signal-transduction pathways modulated by EGFR have been well-documented [69; 70]. Following binding of a specific ligand to its extracellular domain, EGFR undergoes homodimerisation or heterodimerization (with other ErbB family members, including HER2 and HER3) to induce activation of the tyrosine kinase (TK) domain. This leads to auto-phosphorylation of critical tyrosine residues, which serve as attachment sites for various cellular-docking proteins to activate signaling cascades and affect gene transcription (Figure 6) [71; 72].
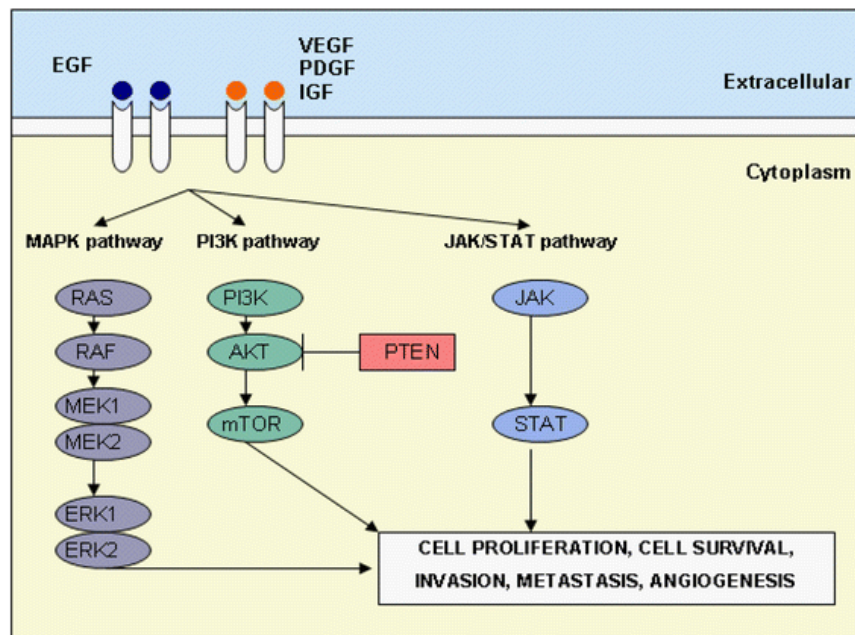

Fig. 6 EGF binding to EGFR and subsequent downstream signaling pathways (From Zahorowska B., et al. [73]. Reproduced with permission).

These cascades include the Ras-Raf-MAPK pathway, whereby the activation of Ras initiates a multistep phosphorylation cascade and activation of ERK-1/2, which regulate cell growth and proliferation [72; 74]. EGFR also relays signals through the PI3K to AKT, which directly activates multiple anti-apoptotic factors and also affects cell proliferation through the mammalian target of rapamycin (mTOR) pathway [72]. The third major pathway involves the signal transducers and activators of transcription (STAT), which exerts action on gene transcription and protein translation [75; 76].

1.4.2   EGFR/erbB Signaling and Cancer

Dysregulation of EGFR/erbB pathways can promote tumor processes including angiogenesis and metastasis and is associated with poor prognosis in many human malignancies [77; 78]. There are several mechanisms that may lead to aberrant receptor activation; some of them include receptor overexpression, e.g., 20% to 30% of breast cancers are noted to overexpress the Her2 [8; 79], mutations, as well as ligand-dependent and -independent mechanisms, which in turn lead to

phosphorylation of key tyrosine residues and the recruitment of cytoplasmatic substrates. This results in initiating downstream mitogenic events.

The roles that EGFR/erbB and its ligands play in breast cancer have been a subject of intensive study and controversy [80; 81; 82]. Some retrospective immunohistochemical studies have indicated that EGFR overexpression in primary tumors is an indicator of poor prognosis [82; 83], whereas other similar studies have failed to establish such a link [80]. EGFR/erbB is expressed in 18–35% of breast cancers but is not overexpressed relative to the normal breast epithelia [81]. Overexpression of Her2 has been associated with a more aggressive phenotype with decreased survival [84; 85].

1.4.3   Anti-EGFR/erbB Targeted Therapies

Two major classes of EGFR-targeted therapies have been developed: the anti-ErbB monoclonal antibodies (MoAbs) and ErbB-specific TK inhibitors (TKIs). Although both classes target the EGFR and HER-2 retrospectively, they differ mechanistically and appear to differ in the clinical profile, too [86]. Anti-EGFR MoAbs such as cetuximab (ImClone) and panitumumab (Amgen) bind to the extracellular domain of EGFR on the surface of tumor cells, thus preventing EGFR ligands from interacting and activating the receptor, as well as receptor-ligand internalization. By contrast, TKIs such as gefitinib, trade name called Iressa (Astra Zeneca) and erlotinib (Roche, Nutley, N.J., USA) block the binding of adenosine triphosphate to the intracellular TK domain of EGFR, thereby blocking TK activity and subsequent intracellular signaling [87; 88]. In this regard, anti-EGFR MoAbs have now been approved for the treatment of advanced colorectal cancer and head and neck carcinomas, and EGFR TKIs have been approved for the treatment of advanced non-small-cell lung cancer (NSCLC) and pancreatic carcinoma. Further, lapatinib (GlaxoSmithKline) – a dual TKI – has been designed for dual suppression of the EGFR (ErbB1) and HER-2 signaling network and was approved by the US FDA in 2007 for the therapy of breast cancer patients treated with first line capecitabine (Roche) [89].

1.5   Protein Phosphorylation

Reversible protein phosphorylation is one of the most important and well explored post-translational modifications. It is estimated that 30- 50% of the proteins are phosphorylated at some time point [90]. Phosphorylation is a major regulatory mechanism that controls many basic cellular processes. It may mediate a signal from the plasma membrane to the nucleus using a cascade of proteins, by which to regulate physiological and pathological processes such as cell growth, proliferation, differentiation and apoptosis [91; 92]. Protein phosphorylation may result in alteration in protein- protein interactions, protein intracellular localization, and its activity [90; 91]. Approximately 30% of drug discovery programs and R&D investment by the pharmaceutical industry target protein kinases. Knowledge of exactly when and where phosphorylation occurs and the consequences of this modification for the protein of interest can lead to an understanding of the detailed mechanism of the protein action, and ultimately to the discovery of new drug targets.

1.5.1 Detection of Phosphoproteins

1.5.1.1 Radioactive Labeling of Proteins with ^{32}P Isotope

Radioactive labeling of proteins with ^{32}P or ^{33}P is the oldest, but still one of the most sensitive approaches for detection of phosphorylation. Under the appropriate condition, the phosphoryl groups of ^{32}P or ^{33}P are enzymatically added to the proteins. The phosphorylated proteins are then detected by autoradiography. Therefore, radioactive labeling detects all types of phosphorylation, and is not specific to only one type of phosphorylation. The proteins can be labeled with ^{32}P/^{33}P isotopes in vitro and in vivo.

1.5.1.2 Phospho-Specific Antibodies

In 1981, the first documented phospho-antibody was produced in rabbits immunized with benzonyl phosphonate conjugated to keyhole limpet hemocyanin (KLH) [93]. This antibody broadly recognized proteins containing phosphotyrosine. After that, there has been a rapid development in production of the phospho-antibodies. Nowadays, a large amount of phospho specific antibodies targeted to different amino acids (Ser, Thr, Tyr) at distinct sites in proteins have been produced, and widely used in the basic and clinic research [93; 94]. The major decisive factor for selection of antibodies is their specificity in detection of a phosphoprotein. Therefore the quality of antibodies becomes the key concern on their applications.

1.5.1.3 Phosphoprotein Staining

Currently, Pro-Q Diamond has increasingly become the first choice of phosphoprotein dye [95]. It is a fluorescent dye, and is suitable for the detection of phosphoserine-, phosphothreonine-, and phosphotyrosine-containing proteins directly in polyacrylamide gels. The sensitivity of ProQ- Diamond staining has been improved significantly, and is down to 1- 16 ng. However, it is still considerably less sensitive than radioactive methods.

1.5.1.4 Mass Spectrometry (MS)

Mass spectrometry is one of the most modern techniques for detection of phosphorylation. Introduction of MS has significantly advanced the research in protein phosphorylation [96]. It may be applied not only for detection of phosphorylation, but also identification of phosphorylation sites. Detection of phosphorylation by MS has been based on mass spectrum generated by trypsin-digested peptides. The mass shift of m/z 79.9 or neutral loss m/z 80 or 98 compared to its theoretical peptide mass has normally been considered as occurrence of phosphorylation. MS provides also a high speed and high sensitivity means for detection of phosphorylation. However, there are several inherent difficulties for the analysis of phospho-proteins. Firstly, signals from phosphopeptides are generally weaker as compared to non-phosphorylated peptides, as they are negatively charged and poorly ionized by MS performed in the positive mode. Secondly, it can be difficult to observe the signals from low-abundance phospho-proteins of interest in the high-background of abundant non-phosphorylated proteins. To overcome these

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drawbacks, enrichment of phophoproteins or phosphopeptides before MS is necessary to apply.

1.5.2 Isolation and Enrichment of Phosphorylated Proteins and Peptides

1.5.2.1 Immunoprecipitation

Phosphospecific antibodies are an efficient tool for enrichment of phosphorylated proteins [97]. Antibodies specific to phosphorylated residues are used to immunoprecipitate full-length proteins and phosphopeptides. Nowadays, a variety of commercial phospho-specifc antibodies with high quality are available, especially antibodies to phosphotyrosine.

1.5.2.2 Immobilized Metal Affinity Chromatography (IMAC)

IMAC [98] is the most frequently used technique for phosphopeptide and phosphoprotein enrichment, although it was originally introduced for purification of His-tagged proteins. It employs metal chelating compounds which are covalently bound to a chromatographic support for the coordination of metal ions. Phosphorylated peptides or proteins are bound to the IMAC stationary phase by electrostatic interactions of its negatively charged phosphate group with positively charged metal ions bound to the column material via nitriloacetic acid (NTA), iminodiacetic acid (IDA), and Tris (carboxymethyl) ethylenediamine (TED) linkers. Immobilized metal ions such as Ni^{2+}, Co^{2+}, or Mn^{2+} were initially shown to bind strongly to proteins with a high density of histidines. However, immobilized metal ions of Fe^{3+}, Ga^{3+}, and Al^{3+} have been demonstrated to show better binding with phosphopeptides.

1.5.2.3 Strong Cation Exchange Chromatography (SCX)

Strong cation exchange chromatography has been used in the enrichment of phosphorylated peptides [99]. This procedure is based on the fact that under acidic conditions (pH 2.7) phosphorylated peptides are single positively charged and amenable to further separation from nonphosphorylated peptides that usually have a net charge of 2+ at low pH. One of the main advantages of this method is that complex peptide mixtures can be analyzed directly, since it can be connected directly to LC-MS/MS for identification or sequencing [100]. However, this strategy does not have high specificity and the fractions enriched in phosphopeptides also contain a high percentage of contaminants. Therefore, it's very common to combine SCX with other enrichment methods, i.e. IMAC and TiO_2.

1.5.2.4 Titanium Dioxide (TiO$_2$)

A promising alternative to the use of IMAC for the enrichment of phosphorylated peptides was first described by Pinkse et al [101]. The approach is based on the

selective interaction of water-soluble phosphates with porous titanium dioxide microspheres via binding at the TiO_2 surface. Phosphopeptides are trapped in a TiO_2 precolumn under acidic conditions and desorbed under alkaline conditions. An increased specificity for phosphopeptides has been reported. Another advantage of this approach is that it can be easily coupled with a LC-ESI-MS/MS or LC-MALDI MS/MS workflow [102]. Nevertherless, TiO_2-based columns may retain nonphosphorylated acidic peptides. Peptide loading in 2, 5-dihydroxybenzoic acid (DHB) has been described to efficiently reduce the binding of nonphosphorylated peptides to TiO_2 while retaining high binding affinity for phosphorylated peptides. This improved TiO_2 procedure was found to be more selective than IMAC.
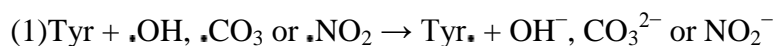
1.5.2.5 Chemical Modification

A number of chemical modification strategies were developed in which the phosphate group has been replaced with a moiety that is chemically more stable than phosphate. One such method employs β-elimination of the phosphate from phosphothreonine or phosphoserine and results in the formation of dehydroaminobutyric acid or dehydroalanine, respectively. This product can be detected directly using tandem MS [103]. Alternatively, Michael addition is used to add a reactive thiol to dehydroaminobutyric acid or dehydroalanine to allow attachment of an affinity tag. Biotin is a widely used affinity tag and it permits purification of the chemically modified (previously phosphorylated) peptides [104]. This chemical modification is not applicable to phosphotyrosine residues and suffers from side reactions in which nonphosphorylated serine can be tagged.

1.6   Protein Tyrosine nitration (PTN)

1.6.1   Definition, Characteristics and Sources of Tyrosine Nitration

Tyrosine nitration is a covalent post-translational protein modification that occurs in vivo as a consequence of oxidative stress [105]. Tyrosine is modified in the 3-position of the phenolic ring through the addition of a nitro group (NO_2) (Figure 7). Mitochondria is believed to be the primary locus for protein tyrosine nitration (PTN). To nitrate Tyr, nitrogen dioxide ($\cdot NO_2$) reacts with a tyrosyl radical (Tyr$\cdot$) at diffusion-limited rates ($3 \times 10^9 \, M^{-1} \, s^{-1}$) [106] . Once a Tyr$\cdot$ is generated on free or protein-bound Tyr by reaction with one electron oxidants (reaction 1), $\cdot NO_2$ can then react with the Tyr$\cdot$ to give NO_2Tyr (reaction 2) [107].

(1) Tyr + $\cdot$OH, $\cdot CO_3$ or $\cdot NO_2$ → Tyr$\cdot$ + OH$^-$, CO_3^{2-} or NO_2^-
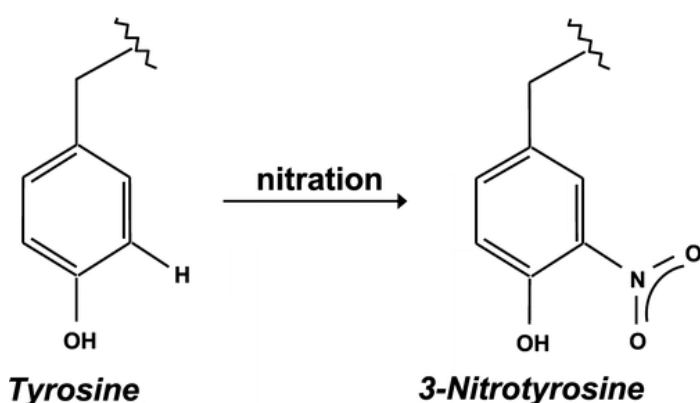
(2) Tyr$\cdot$ + $\cdot NO_2$ → NO_2Tyr.

Figure 7: Formation of 3-nitrotyrosine under the action of a nitrating agent.

An essential contribution to reaction 2 is $\cdot NO_2$, which can be formed both enzymatically and non-enzymatically by three primary pathways [107]. Generation of peroxynitrite (ONOO−), via the radical–radical reaction of nitric oxide ($\cdot NO$) with superoxide ($O_2 \cdot -$), can lead to the formation of $\cdot NO_2$. Peroxynitrite is in fast, dynamic equilibrium with its conjugated acid, peroxynitrous acid (ONOOH, pKa 6.8), which can rapidly undergo homolytic cleavage to yield $\cdot NO_2$ and $\cdot OH$ [106]. Formation of $\cdot NO_2$ can also occur via homolytic cleavage of nitrosoperoxocarbonate (ONOOCO2−) [108], formed by the reaction of ONOO− and carbon dioxide (CO_2) [109]. The intermediate ONOOCO2− decays by homolysis of the peroxidic bond to form $\cdot NO2$ and carbonate anion radical ($CO_3 \cdot -$). Heme peroxidases, such as myeloperoxidase (MPO) and eosinophil peroxidase (EPO), contribute to Tyr nitration in the presence of hydrogen peroxide (H_2O_2) by converting NO2−, the primary metabolic end product of $\cdot NO$, to $\cdot NO_2$ [110]. PTN has been considered to be a stable post-translational modification, and there is increasing evidence of an in vivo denitration process [111].

1.6.2 Biological Functions of PTN and PTN-Related Diseases

There is increasing number of studies focused on PTN on protein activity. It can result in a loss, an increase, or no effect on protein function [112; 113]. Oxidative modifications have been shown to increase the susceptibility of modified proteins to proteolysis. The removal of oxidized proteins can be seen as a defense mechanism against the consequences of oxidative stress [114]. An increase in proteolytic degradation as a result of exposure of isolated proteins or cells in culture to peroxynitrite was observed [114; 115]. PTN might trigger mitochondrial proteolytic enzymes [114; 115]. In addition, PTN was also observed to affect cellular function by interfering with tyrosine phosphorylation [114; 116]. The shift in pKa of the phenolic hydroxyl group due to nitration may be the major cause for this interference (tyrosine kinases phosphorylate the neutral phenolic hydroxyl group rather than the negatively charged phenolate), but steric hindrance and a distortion of the local protein structure may also contribute. The reverse situation has also been observed, in that phosphorylation of tyrosine prevents subsequent PTN. Therefore, nitration and phosphorylation are two competing chemical reactions at a given tyrosine residue. Furethermore, PTN also has been reported contribution to increase the cell immunogenity [117]. Endogenous proteins are normally not immunogenic due to immunological tolerance. Studies of the recognition of tyrosine nitrated proteins by T-cells showed that PTN of a tyrosine in a T-cell receptor (TCR) contact position may

result in the formation of an immunogenic neoepitope [118; 119]. Moreover, PTN also found to be involved in regulation of signaling transduction, e.g. NF-kB, IkB. For example, tyrosine nitration triggers dissociation of IkB from NF-kB, this results in the activation of a pathway that plays an important role in cancer and inflammation [111; 120].

Currently, accumulating data show a strong link between protein PTN and the mechanisms involved in formation of many pathological conditions and diseases: post-radiation response, acute and chronic inflammation, acute and chronic rejection of the allograft, chronic hypoxia, tumor vascularization and microenvironment, atherosclerosis, myocardial infarction, chronic obstructive pulmonary disease, diabetes, Parkinson's disease, Alzheimer's disease, and others [121; 122; 123; 124; 125; 126; 127; 128; 129; 130; 131; 132; 133; 134; 135].

1.6.3   Detection of Nitrotyrosine

Several methods have been developed for the detection of nitrotyrosine [136; 137; 138; 139]. They include antibody- based, mass spectrometry, and fluorogenic tagging.

Antibody based detection was the first used method for the detection of PTN. Currently, nitrotyrosine-directed antibodies have been used to detect protein containing nitrotyrosine in cell/tissue homogenate, blood plasma, and tissue section [111; 127].

Mass spectrometric, proteomics-based strategies allow for the identification of individual proteins that are nitrated. Two-dimensional (2D) gel electrophoresis based proteomics allows to separate a cell/tissue homogenate on the 2D gels, detect the nitrated proteins using an anti-NO_2Tyr antibody, and then identifying the peptides generated by in-gel proteolytic digestion using matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) MS. In the case of NO_2Tyr containing proteins, intact molecules can be measured directly by detecting the corresponding covalent modification with a mass difference of $+ 45$. When performing MALDI-TOF MS analysis, the decomposition products of NO_2 involving the loss of one and two oxygens ($- 16$ and $- 32$ Da, respectively) can also be observed [111]. Shotgun based proteomics also has been widely established to identify and quantify the PTN [138; 139].

The method of fluorogenic tagging involves two reaction steps to convert the 2-nitrophenol functionality of protein-bound nitrotyrosine chemoselectively into a fluorophore [140]. In the first reaction step, the 2-nitrophenol functionality is reduced to a 2-aminophenol functionality by sodium dithionite. In the second step, the 2-aminophenol functionality is converted into a fluorophore by reaction with salicylaldehyde and Al3+. This novel histochemical staining selectively detects 2-nitrophenol, as well as 2-nitrosophenol and 2-aminophenol functionalities. This method can detect nitrotyrosine in tissue sections by fluorescence microscopy [107]. This method is also applicable for staining protein-bound 2-nitrophenol functionalities on western blot membranes in complex biological samples such as blood plasma [107].

1.7 Proteomics in Cancer Studies

1.7.1 Definition and Application in Cancer Research

The word "proteome" was coined by Wilkins in 1994 [141]. The proteome is the entire complement of proteins, including the modifications made to a particular set of proteins, produced by an organism or system. This will vary with time and distinct surroundings, that a cell or organism undergoes.

Proteomics is the large-scale study of proteins, particularly their structures and functions. In general, proteomics can be classified into three types based on distinct applications. (1) Protein expression proteomics, this approach hold promise for identifying disease markers (tumor markers) that could be important in early detection, diagnosis and in monitoring effectiveness of treatment and eventually leads to the design of novel treatments. (2) Functional proteomics, it includes analysis of protein-protein or protein-DNA/RNA interactions and activitites of proteins. (3) Structural proteomics, the main goal of this approach is to map out the structure of protein complexes or the proteins present in sub-cellular localization or organelles. Proteomics therefore, provide a powerful approach for the analysis of normal and transformed cell functions from complex mixtures for the identification of disease specific markers, uncovering novel endpoints for the evaluation of chemoprevention agents, and protein based drug targets for better treatments [142].

1.7.2 Concepts in Proteomics

Based on different approaches, proteomics includes gel-based and shotgun proteomics (Figure 8). Liquid chromatography of proteins and capillary electrophoresis are other two techniques for analysis of full-length proteins, which are under strong development.

1.7.2.1 Two-Dimensional Gel (2-DG) -based Proteomics

Two-dimensional polyacrylamide gel electrophoresis (2D-GE) is a technique that has been described for the first time already in 1975 by O'Farrell [143] . Proteins are separated according to their isoelectric point by isoelectric focusing in the first dimension, and according to their molecular weight in the second dimension. Since these two parameters are unrelated, it is possible to obtain an almost uniform distribution of protein spots across a two-dimensional gel. The resulting map of protein spots can be considered as the protein fingerprint of that sample. Since polyacrylamide has the same UV absorbance as proteins, the proteins have to be stained in order to make the spots visible on the gel. Coomassie blue staining is the most popular protein staining method. It is inexpensive, easy to use and has a wide linear range that makes relative quantification easy. However, Coomassie blue staining is rather insensitive and a large number of proteins remain undetected using this method. In contrast, silver staining has a 20 to 50 times higher detection limit but a limited dynamic range [144]. The use of radioactive or fluorescent labels solves the problem of lack of linearity in staining intensity. To circumvent the problem of inter gel variability, the method of differently labeling the different protein samples and separate them on the same gel rather than following the paradigm of one sample −

one gel has been developed, two dimensional difference gel electrophoresis (DIGE) [145]. An image is obtained by scanning the gel, and image analysis software enables spots of different intensity to be identified by superimposing the images. After excising the spots of interest, in situ tryptic digestion and subsequent analysis by MALDI-TOF (time-of flight) mass spectrometry, differentially occurring proteins can be identified based on the masses of their tryptic fragments.
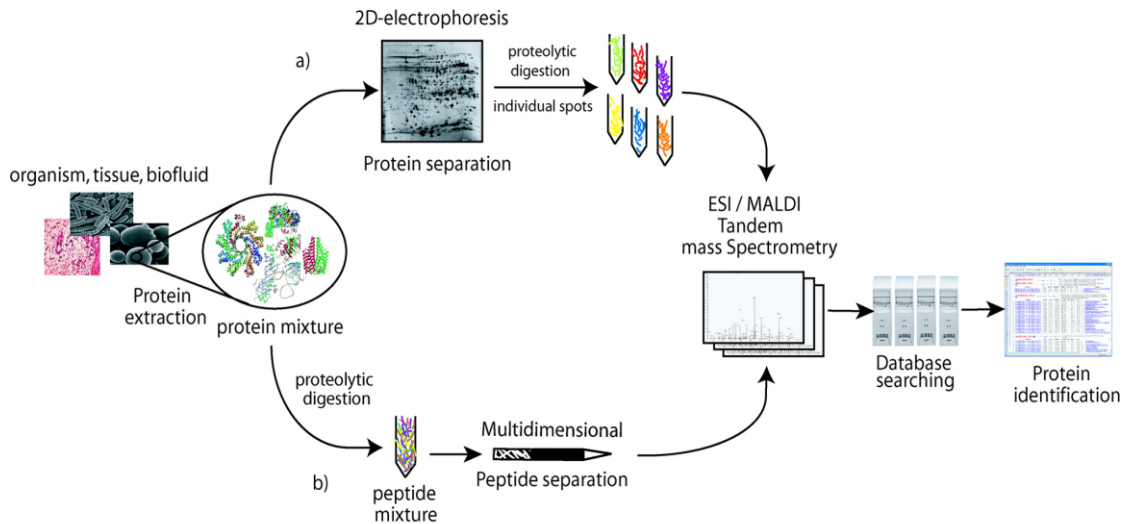

Figure 8. Schematic description of 2D-GE based and gel-free (shotgun) proteomics.

1.7.2.2 Gel- Free Shotgun Proteomics

By means of recently developed bottom-up (shotgun) proteomics techniques, hydrophobic proteins as well as peptides can be analyzed. Instead of using 2D-PAGE, these approaches use multi-dimensional liquid chromatography coupled to tandem mass spectrometry to separate and identify the peptides obtained from the enzymatic digest of an entire protein extract [149]. It is important to realize that in this approach it is not the protein but peptides are separated and subjected to tandem mass spectrometric analysis. Mass spectrometric identification of these peptides allows the determination of the protein content of the initial sample. Since peptides can be more easily separated by liquid chromatography than proteins, a peptide based proteomic analysis can be performed much faster and cheaper than a complete gel-based analysis.

2 Present study

2.1 Aims

General aims of the thesis are to uncover the mechanism of tumorigenesis of breast cancer, and investigate the crosstalk of signaling pathways during cancer development.

The specific aims for each paper are described as below:

Ⅰ To explore the mechanisms of immortalization of breast epithelial cells, by proteome profiling of conditionally immortalized primary human breast epithelial cells.

Ⅱ To better understand the response to hyperthermia of immortalized primary human breast epithelial cells, we performed a proteomics study of these cells cultured at 37°C or 39°C.

Ⅲ To study the expression of Kinase Suppressor of Ras 2 (KSR2) in human breast tumors and its effect on proliferation of breast epithelial cells.

Ⅳ To investigate protein tyrosine nitration in the cell cycle.

Ⅴ To identify the convergent phosphoproteins of TGF-β1 and EGF signaling pathways, systemic analyze their functions, and evaluate their involvement in cell proliferation in the context of TGF-β1 and EGF.

Ⅵ To explore ZAK, the convergent target of TGF-β1 and EGF, impact on TGF-β1- and EGF-dependent regulation of the cell proliferation, migration and apoptosis.

Ⅶ To explore mechanisms of tumorigenesis of breast cancer activated upon prolonged exposure of cells to EGF and estrogen. Specifically, stemness features of human breast epithelial cells.

2.2 Materials and methods

2.2.1 Materials

Conditionally immortalized human primary epithelial cells were generated using constructs and methods described by O´Hare et al. [143]. Notably, the catalytic subunit of human telomerase (hTERT) and a temperature-sensitive mutant of simian virus 40 large-tumor antigen were used (non-DNA binding non-Bub1 binding thermolabile T antigen; [143]). The luminal 226Lts4 and 226LU19 cells were used. Expression of LT antigen was confirmed by immunoblotting, and hTERT was monitored by TRAP assay (O'Hare and Jat; unpublished data). 184A1, MCF10A and COS7 cells were obtained from ATCC (Manassas, USA), and were cultured in media recommended by ATCC.

2.2.2   Two-Dimensional Gel Electrophoresis

Samples were dissolved in 2-D GE buffer (8 M urea, 4 % CHAPS, 0.5 % DTT, IPG buffer, pH 3-10). 100 μg of protein were subjected to isoelectrofocusing (IEF) using 18 cm linear IPGDry strips with a pH range of 3 to 10 (GE Healthcare, Uppsala, Sweden). IEF was performed in an IPGphor (GE Healthcare, Uppsala, Sweden) using the following protocol: rehydration, 10 hr; 50 V, 3 hr; 1 000 V, 1 hr; 8 000 V, 10 hr. After IEF, strips were equilibrated in 50 mM Tris-HCl, pH 8.8, 6 M urea, 2.0 % SDS, 30 % glycerol with 1 % DTT for 10 min, and then for 10 min in the same buffer containing 4 % iodoacetamide instead of DTT. Equilibrated strips were placed on top of 10 % polyacrylamide gels and were fixed with 0.5 % agarose in 62.5 mM Tris-HCl, pH 6.8, 0.1 % SDS. SDS-PAGE was performed in a Dalt-Six following the manufacturer's recommendations (constant power 50 W, for 6-8 hr; GE Healthcare, Uppsala, Sweden). Gels were fixed in 10 % acetic acid and 20 % methanol for 10-12 hrs. Proteins were detected by silver staining. For each condition we generated at least 3 gels using samples from 2 separate experiments.

2.2.3   Gel Image Analysis

Silver-stained gels were scanned in an ImageScanner with the MagicScan32 software and analyzed with calculation of volumes of spots by the ImageMaster 2-D Platinum software (GE Healthcare, Uppsala, Sweden). The statistical significance of changes was evaluated using the ImageMaster 2-D Platinum Version 6.0 software; embedded in the software Student's t-test was used.

2.2.4   Mass Spectrometry

Protein spots were excised from the gels, destained and subjected to in-gel digestion with trypsin (modified, sequence grade porcine, Promega, USA), as described earlier [150]. Tryptic peptides were concentrated and desalted using microC18 ZipTip's (Millipore, USA). Peptides were eluted with 65 % acetonitrile, containing the matrix α-cyano-4-hydroxycinnamic acid, and applied directly onto the metal target and analyzed by MALDI TOF MS on a Bruker Ultraflex III (Bruker Daltonics, Bremen, Germany). Embedded Bruker software (FlexAnalysis) was used to process the mass spectra. Peptide spectra were internally calibrated using autolytic peptides from the trypsin (MH+ 842.51, 1045.56, 2211.10 and 3337, 76). To identify proteins, we performed searches in the NCBInr sequence database using the ProFound search engine (http://65.219.84.5/service/prowl/profound.html). One missed cleavage, alkylation with iodoacetamide and partial oxidation of methionine were allowed. Search parameters were set on mass tolerance less than 0.1 Da, no limitations of pI, limits of Mr of + 20 kDa and - 20kDa, as compared to the migration position of a spot in the 2D gel, and "mammalian" was selected for species search. Significance of the identification was evaluated according to the probability value, ''Z'' value, mass precision of the matched peptides and sequence coverage.

2.2.5 Systemic Analysis

Systemic analysis of obtained data was performed using GoMiner (http://discover.nci.nih.gov/gominer/), Ingenuity Pathway Analysis (IPA; www.ingenuity.com), and Cytoscape tools.

GoMiner allows classification of identified proteins into biologically coherent categories and assesses these categories.

Functional and pathway analysis was performed using Ingenuity Pathway Analysis (IPA), a tool for description of networks and signaling pathways. See www.ingenuity.com for detailed description of IPA; IPA can also be evaluated via free access trials offered on the www.ingenuity.com. IPA operates with a proprietary database which is based on a thorough analysis of reported experimental data. IPA considers only those experimental data which have been evaluated by independent researchers. This ensures that only confirmed results are taken into consideration for building a network. Experimental results which have not been reported by multiple laboratories or may have controversial interpretations are not considered by IPA. Such stringent selection of experimental data is required to exclude building of incorrect dependencies and exclude false-positive relations. Settings for the network analysis were taken as recommended by IPA, e.g. the number of connections and components between two dataset-defined components. A dataset containing identified proteins was uploaded into the Ingenuity Pathway Analysis application, and networks were generated. Fischer's exact test was used to calculate a p-value determining the network connectivity.

For the analysis with Cytoscape, we generated a network using identified by us proteins, MiMi tool and KEGG database. The network was viewed in Cytoscape, betweenness was computed by Centiscape tool, and subnetworks were extracted by MCODE tool.

2.2.6 Immunoblotting

Cell lysates were resolved on SDS polyacrylamide gels and transferred onto Hybond P membranes (GE Healthcare, Piscataway, NJ). Membranes were blocked with 5 % (w/v) BSA and then incubated with a primary antibody against target proteins with dilutions, as recommended by the manufacturer, and followed by an HRP-conjugated secondary antibody (GE Healthcare, Uppsala, Sweden). The proteins were visualized using Luminol Reagents (Santa Cruz Biotechnology Inc.).

2.2.7 Immunohistochemistry

AccuMax breast cancer arrays (ISU ABXIS Co., Ltd, Seoul, South Korea) were used to immunohistochemistry staining. Each array slide contains 45 cases of cancer tissues and 4 non-neoplastic tissues. In addition to AccuMax, we used BRC961 human breast cancer tissue microarrays from US Biomax (US Biomax Inc., Rockville, USA). Arrays were stained with primary antibody. Antigen retrieval was performed using DakoCytomation target retrieval solution high pH (DAKO, Carpinteria, USA). The slides were stained with VECTASTAIN Elite ABC kits (Vector Laboratories Inc.,

Burlingame, USA) following the manufacturer's instruction, counterstained with hematoxylin and mounted with Fluoromount G (Southern Biotechnology, Birmingham, USA). The stained tissues were photographed using a Leica DFC camera and images were acquired with Leica QWin Standard software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). Intensity of staining was evaluated as absent (-), weak (+) and strong (++), when no staining ("absent" or (-)), less than 30 % of cells staining and a weak signal ("weak" or (+)), and more than 90 % of staining and a strong signal ("strong" or (++)) were observed, respectively. We evaluated staining in malignant (epithelial) cells of tumors, and epithelial cells of normal tissues.

2.2.8   Immunoprecipitation

The protein concentration was adjusted to 3 mg/mL and the solution was subjected to IP with 40 μL of protein agarose-bound primary antibody overnight at 4 °C. The protein-bound beads were collected by centrifugation at 12 000 rpm for 1 min and washed thoroughly (3 times) with the lysis buffer. The bounded proteins were eluted with a buffer containing 100mM DTT, 1% SDS, 50mM Tris/HCl, pH 6.8 for 5min at 95 °C.

2.2.9   Construct and Transfection

The construct was made by a polymerase chain reaction (PCR)-directed approach, and subcloned in pcDNA3(-) vector (Invitrogen). Site-directed mutagenesis was performed with a QuikChange Site-Directed Mutagenesis Kit (Stratagene), and all constructs were verified by DNA sequencing. For the generation of stably transfected MCF-7 cells, MCF-7 cells were transfected in 6-wells plates using LipofectAMINE 2000 reagent, as recommended by the supplier (Invitrogen). After 48 hours transfection, cells were transferred to 10-cm petri-dish, and were under selection of 400 μg/ml of G418 for 3 weeks. Single colonies were picked up, and were under expansion culture for 3 weeks. Validation of protein expression in the stable transfected cells was examined by immunoblotting with specific antibodies.

2.2.10   Cell Culture and Synchronization

We used the cell lines as followed: 184A1, MCF10A, MCF7, COS7. All cell lines were obtained from ATCC (Manassas, VA). Cells were cultured in media recommended by ATCC.

MCF-7 cells were synchronized at G0/G1 phase by starving the cultured cells for 48 hrs in DMEM medium supplemented with 0.1% FBS. Synchronization of the MCF-7 cells at the S phase was followed by methods [151]. In brief, media was replaced from proliferating MCF-7 cells with 10% FBS-supplemented medium containing 2 mM hydroxyurea (H8627, Sigma-Aldrich) for 16 h. The media was removed and the cells were washed three times with PBS solution and incubated in 10% FBS-supplemented DMEM for 13 h. Subsequently, hydroxyurea was added from a 500 mM stock solution to yield a final concentration of 2 mM hydroxyurea, and the cells were incubated for another 13 h. Replacement of the media with one without hydroxyurea permitted the cells to progress along the cell cycle in a synchronized fashion. Cell synchronization in

G2/M phase were obtained by incubating exponentially growing cells in complete DME medium containing 0.4 μg/ml nocodazole (M1404, Sigma-Aldrich) for 16 h. Synchronous progression along the cell cycle was obtained by removing the medium, washing with PBS solution and incubating the cells with 10% FBS-supplemented medium.

2.2.11 Cell Immortalization, Proliferation, Apoptosis and Migration Analysis

Cell immortalization assay

Population doubling (PD) rate of cells: 10^5 cells were plated into each well in 12-well plates in triplicates. Every 3 days, cells were trypsinized from plates and cell numbers were counted. At each split, 10^5 cells were reseeded to each well in fresh plates and allowed to grow until the next split. Population doublings were calculated with the formula PD= log(n2/n1)/log2, where n1 is the number of cells seeded and n2 is the number of cells recovered.

Senescence-associated (SA) β-galactosidase activity assay: SA β-galactosidase activity is a marker of senescent cells [11]. After transfection, cells were washed with PBS, fixed for 15 min in 3% formaldehyde at room temperature, washed, and incubated at 37°C overnight with fresh senescence-associated (SA)-β-Gal stain solution: 1 mg/ml X-Gal in dimethylformamide, 40 mmol/l citric acid and phosphate buffer pH 6.0, 5 mmol/l potassium ferrocyanide, 5 mmol/l potassium ferricyanide, 150 mmol/l sodium chloride, and 2 mmol/l magnesium chloride. After incubation, at least 200 cells in several fields were examined and SA β-gal positive cells were counted. Statistical significance of observed differences was evaluated with Student's t-test.

Cell proliferation assay

[^{3}H]thymidine incorporation assay

Cells were seeded in 48-well plates for proliferation assays. Cells were incubated with 1 μCi/ml of [^{3}H]thymidine for the last 2 h of the 24 h incubation time period. Radioactivity incorporated in DNA was measured as described earlier [150]. Statistical significance of observed differences was evaluated using Student's t-test.

MTT assay

Cell proliferation was measured by using CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, Promega Biotech AB, Stockholm, Sweden). MTT assay was performed according to the manufacturer's recommendations. Statistical significance of observed differences was evaluated using Student's t-test.

Cell apoptosis assays

Cell apoptosis assays were determined by using Cell Death Detection ELISAPLUS (Roche, Germany). Briefly, cell lysates were placed in a streptavidin-coated micro-plate. A mixture of anti-histone-biotin and anti-DNA-POD was added and incubated for 2 h at 25°C. After removal of unbound antibodies by a washing step, POD was

determined photometrically at 405 nm with ABTS as substrate. Statistical significance of observed differences was evaluated using Student's t-test.

Cell migration assays

A disposable 96 well chemotaxis chamber (CHEMO-TX 101-8, Chemo TX, USA) was utilized to quantify cellular migration. These assays were performed following the manufacture's recommendation. After the overnight incubation, non migrated cells and excess media were gently removed using tissue. The filters were then washed twice in PBS (no Ca^{+2} or Mg^{+2}) and fixed with 1% formaldehyde in PBS buffer. The cells migrated through filter were stained with 0.5 % crystal violet. The filters were scanned and quantization was performed by using ImageJ software.

2.2.12   Mammosphere Culture

Cells were trypsinized and mechanically separated and, when necessary, passed through 40-μm filters to obtain single cell suspensions that were plated at less than 10,000 cells per mL in super–low-attachment plates. Number of mammosphere was counted every 3 days for 2 weeks.

2.2.13   FACS

Cell cycle

Cells were collected by trypsinization and fixed for at least 30 min in 70% ethanol at −20 °C. Then, ethanol was removed, and cells were stained with staining solution (PBS supplemented with 0.1% (v/v) sodium citrate, pH 7.4, 0.2 mg/mL RNase A, 20 μg/mL propidium iodide). Cell cycle was analyzed in a FACscan flow cytometer (Becton Dickinson)

Surface markers

Approximated 1×10^6 cells were trypsinized to obtain single cell suspension. The cells were washed twice in cold PBS + 1% BSA, and subsequently incubated at 4°C with either 1:25 diluted CD44-FITC antibody (clone ML5, BD Pharmingen), CD24-APC antibody (clone G44-26, BD Pharmingen) in PBS + 1% BSA for 30 minutes in the dark. After incubation, the cells were washed twice in cold PBS + 1% BSA and resuspended in 400 μl cold PBS + 1% BSA for flow cytometry analysis within 1 hour.

2.2.14   Isolation and Enrichment of Phosphoproteins

Fe-IMAC technology, reported by Dubrovska and Souchelnytskyi, was used to enrich for phosphorylated proteins. Phosphorylation status of proteins was confirmed by counter-staining of gels with phosphor-specific fluorescence dye ProQ-Diamond.

2.2.15 Metabolic Labeling of Cells with ^{32}P Orthophosphate

Subconfluent cells were labeled in phosphate-free medium containing 0.5% FBS, 20 mM HEPES, pH 7.2, and [^{32}P]orthophosphate (1.0 mCi/ml). After stimulation with 10 ng/ml of TGFβ1 or 5 ng/ml EGF alone and in combination for 60 min, the cells were washed and lysed in a lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 100 units/ml aprotinin). The cell lysates were subjected to immunoprecipitation using the anti-ZAK antibody (301-993A, Bethyl); immunoprecipitates were then subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The radioactivity in the gel was quantified by exposure in a Phosphorimager (Molecular Dynamics).

2.3 Results and Discussion

2.3.1 Paper 1

Proteome profiling of immortalization-to-senescence transition of human breast epithelial cells identified MAP2K3 as a senescence promoting protein which is down regulated in human breast cancer

Up to 70 % of neoplasia in human breast do not give rise to fully malignant tumors. Discrimination between benign and malignant neoplasia is of importance for selection of treatment. Immortalization is one of the first changes in cells undergoing carcinogenic transformation. Identification of biomarkers of immortal cells is considered as an efficient way for early detection and prevention of carcinogenesis.

In paper 1, the goals were to perform proteome profiling of the immortalized cells, provide insights into molecular mechanisms of immortalization, and evaluate the selected identified proteins as markers of early steps of tumorigenicity.

In this paper, we used conditionally immortalized primary human breast epithelial cells as a model. These cells were generated by expression of hTERT and temperature-sensitive mutant (U19tsA58) of simian virus 40 large-tumor (LT) antigen. The cellular status of immortal and non-immortal were controlled by temperature. At 34°C the cells are immortal but when cultured at 39°C, they rapidly cease proliferating, and undergo senescence.

To explore the proteome changes upon immortalization of human breast epithelial cells, we generated 2D gels of conditionally immortalized cells cultured at permissive 34°C and at non-permissive 39°C. 95 proteins were identified as differentially expressed. Among them, 71 proteins were immortalization-dependent, while changes of 24 of the proteins were observed also in the control temperature-insensitive SV40 LT cells, i.e. in cells which would remain immortal at the higher temperature.

We validated changes in expression of 3 proteins selected from the proteins identified by proteomics (MAP2K3, CK2α, and KSR2). The immunoblotting assays confirmed changes in the protein expression observed in the proteomics study.

Clustering of identified proteins into functional groups showed that immortalization affected proteins involved in cell proliferation and growth, death, cell assembly and organization, cellular movement, cell-to-cell signaling and interaction, and cell

morphology, as main functional domains Analysis of the network generated by our identified proteins and proteins they closely related to suggested possible involvement of signalling pathways related to activities of epidermal growth factor (EGF), platelet-derived growth factor (PDGF), progesterone, tumor necrosis factor (TNF), interferon-γ, and interleukins (IL12, IL29). These regulators have not been reported as changed at the early steps of induction of senescence, but as affected later upon immortalization-senescence transition of cells. Among intracellular regulators, p53, AKT, Erk, HIF1A, H-Ras, NFkB, JNK, p38, PKC, PLCγ, RET and Myc were present as highly-connected hubs in the network formed by the identified proteins.

We selected MAP2K3 for further study, as this kinase has been reported to contribute to the oncogenic Ras-induced crisis in cell proliferation and senescence. In our proteomics screen, 49% down-regulation of MAP2K3 was observed upon reversal of the non-immortal phenotype. Primary human epithelial cells are known to be difficult to transfect transiently. These limitations prompted us to use established cultured cells for functional studies, as a more efficiently transfectable and controlled model. We selected two human breast epithelial cells, 184A1 and MCF10A. These cells are immortal, but non-tumorigenic, e.g. do not form tumors. Therefore, 184A1 and MCF10A cells may be used as a model for studying early steps of transformation of human breast epithelial cells. We calculated the population doubling rate and measured senescence-associated (SA) β-galactosidase, to investigate the impacts of MAP2K3 on cell senescence. Population doubling rate of MAP2K3-expressing cells was decreased already after 3 days, and was halted by day 6, while the control cells continued to proliferate. When senescence-associated (SA) β-galactosidase was measured, we observed that expression of MAP2K3 strongly increased its activity. Both approaches suggested that overexpression of MAP2K3 in 184A1 and MCF10A cells resulted in pronounced senescence of cells. Measurement of cell proliferation showed that expression of MAP2K3 had an inhibitory effect, which is in agreement the increase in the proportion of senescent cells. The network analysis indicated the potential involvement of p53, Erk1/2 and p38 in the immortalization-senescence transition and regulation of cell proliferation. Therefore, we measured the levels of p53, p38, pRB and Erk1/2 in cells transfected with MAP2K3 expression vector. We observed that enhanced expression of MAP2K3 led to increase of p53, pRB and p38 expression, but not Erk1/2. This suggests that p38, p53 and pRB may be involved in MAP2K3 activity in human breast epithelial cells, and provide support for further mechanistic signalling studies.

The observations with cultured human breast epithelial cells confirmed the proteomics data, and suggested that MAP2K3 may be an important regulator of senescence. To explore whether MAP2K3 expression is changed in clinical samples, we performed an immunohistochemistry study of human breast cancers using a tissue microarray consisting of 30 cases of infiltrating ductal carcinomas (IDC), 8 cases of papillary carcinomas, 4 cases of infiltrating lobular carcinomas (ILC), 3 cases of phyllodes tumors and of 8 normal breast tissues. Immunohistochemistry staining showed significant decrease of MAP2K3 staining in malignant tumor cells, as compared to epithelial cells in normal ducts. The decrease in MAP2K3 staining was observed independently of TNM gradation, ER, PR and ErbB2 status, indicating that MAP2K3 down-regulation may be an early event in carcinogenic transformation.

In this work, we observed that MAP2K3 was down-regulated in immortalized cells, and expression of MAP2K3 in immortal human breast epithelial cells was sufficient to induce senescence. This indicates a potential role of MAP2K3 in immortalization.

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This was supported by our finding that MAP2K3 is down-regulated in cancer, as compared to normal human breast epithelial cells. The lack of correlation of MAP2K3 down-regulation with TNM gradation, ER, PR, and ErbB2 status indicated that the decrease of MAP2K3 expression is an early event in tumorigenesis. Thus, our proteomics data provide an insight into the complexity of mechanisms involved in the early response to immortalization-senescence transition, and identify additional regulatory pathways, as compared to the genomic and transcriptomic profiling. MAP2K3 expression analysis confirmed that this kinase may be considered for further evaluation as a marker of immortalization of human breast epithelial cells to discriminate potentially carcinogenic cells from non-transformed cells of a benign neoplasia.

2.3.2   Paper 2

Proteome profiling of heat-shock of human primary breast epithelial cells, a dataset report

The heat-shock response is a reaction in cells and organisms to elevated temperatures (heat shock or heat stress). Cells subjected to heat have to be able to adjust to such a shock if they are to survive. The classical responses to heat shock involve the induction of heat shock proteins, which include chaperones and proteases, required for protein refolding and degradation. Besides mentioned forehead, it's believed that many other proteins also involved in heat shock response. Relatively little is known about systemic features of the response to heat shock in human somatic cells.

The goals of paper 2 were to perform proteome profiling of heat-shock cells, identify the heat shock associated proteins, and explore the possible functions of the identified proteins involved in.

The primary immortalized human breast epithelial cells cultured under 37°C and 39°C was used (describe in paper 1). 2-D gels of proteins extracted from immortal cells cultured at 37°C and 39°C were generated. On average, 900 protein spots were reproducibly observed in each gel. Twenty-four proteins were unambiguously identified by MALDI-TOF MS as heat shock related proteins. Among them, fifteen proteins were found to be up-regulated and nine proteins were down-regulated in response to elevated temperatures.

To explore biological processes and functions that could be affected by the identified proteins, we performed a systemic study using GoMiner, Ingenuity Pathway Analysis and Cytoscape. The GoMiner tool clustered the identified proteins based on various cellular processes. Involvement of the identified proteins in regulation of cell adhesion, cell communication, cell cycle and cell development was predicted. An analysis of affected intracellular regulatory mechanisms showed that most of the identified proteins were involved in cellular metabolism (TGFB2, ZFP91, HSPB1, PANK1, GLP1R, AGPS, ACHE, FTSJ1, PTPRE, CUL5, PRDX2). The other functional domains included cell development (ACHE, TGFB2, PRDX2, CUL5, PCOLCE, HSPB1), cell communication (PTPRE, ANPP5A, ACHE, ITGA6, GLP1R, TGFb2), cellular component organization (ITGA6, TGFB2, ACHE), cell cycle (CUL5, TGFB2), cell proliferation (TGFB2, ACHE, CUL5), cell adhesion (ACHE, ITGA6, TGFB2) and in cell motility (HSPB1, TGFB2, ACHE).To explore systemic features of the signaling mechanisms represented by the identified proteins, we generated networks of

interactions between the identified proteins and proteins and genes that may be affected by the identified proteins. The network generated by MiMi tool showed 170 nodes, including 24 proteins identified by us, and 146 proteins they interacted with. The nodes with highest betweenness were TAF1, HSPB1(HSP27), HNF4A, ITGA6, HNF4A, GFAP, VIM, MAX and TGFB.

We validated the changes of expression of HSP27 and TGF-β2 from the heat shock response proteins identified by proteomics. For validation, we used breast epithelial cells at different stages of transformation, including primary breast epithelial cells, tumorigenic breast cells MCF-7 and metastatic breast cancer cells MDA-MB-231. The immunoblotting assays showed that HSP27 and TGF-β2 were up-regulated upon 39°C hyperthermia in all three breast epithelial cells mentioned above, which confirmed the protein expression changes observed in the proteomics study.

To investigate whether HSP27 expression would be dependent on TGF-β2, we treated MCF-7 and MDA-MB-231 cells with TGF-β2 ligand or TGF-β neutralizing pan-specific antibody. Our data showed that the TGF-β2 induced HSP27 expression, and the neutralizing TGF-β antibodies decreased HSP27 expression, as compared to control non-treated cells, both at 37°C and at 39°C. A stronger difference was observed with cells incubated at 37°C. Thus, our results validated the proteomics data related to expression of HSP27 and TGF- β2, and showed that TGF- β2 may be a stimulator of HSP27 expression. To explore the roles of TGF-β in the heat shock response, we treated cells with TGF-β2 ligand to enhance the effects of TGF-β, or used neutralizing pan-specific TGF-β antibody to decrease the effects of endogenous TGF-β. In Cell Death Detection ELISAPLUS assay, we observed that TGF-β2 had no significant effects on cell apoptosis at 37°C in both MCF-7 and MDA-MB-231 cell. MDA-MB-231 cells were more sensitive to 39°C heat shock, as compared to MCF-7 cells. MDA-MB-231 cells had a significantly higher number of apoptotic cells in all three groups (control, TGF-β2, Ab-TGF-β), as compared to MCF-7 cells. In agreement with the notion that TGF-β2 protects against the cell death-inducing effect of hyperthermia, we observed that the addition of TGF-β2 to the cells protected them from cell death by 31 % and 42 % in TGF-β2-treated MCF-7 and MDA-MB-231 cells at 39°C, as compared to the control group and the TGF-β neutralizing antibody-treated group.

To investigate the role of TGF-β2 on cell proliferation upon heat shock, we performed a [³H]thymidine incorporation assay. We observed that TGF-β2 inhibited MCF-7 and MDA-MB-231 cell proliferation at 37°C. Hyperthermia decreased cell proliferation rate in both cell lines to the levels observed for the TGF-β2-treated cells. There was no significant difference in the rate of proliferation between the 37°C and the 39°C TGF-β2-treated groups.

Thus, systemic analysis predicted a number of functions that may be affected by hyperthermia. Our functional studies indicated that TGF-β2 protected cells from cell death, and that TGF-β2 stimulated expression of HSP27 in the studied cells. Results reported here also provide a list of proteins which can be further explored in the context of heat shock response.

2.3.3 Paper 3

Kinase suppressor of ras 2 is involved in regulation of cell proliferation and is up-regulated in human invasive ductal carcinomas of breast

In paper 1, we identified KSR2 was up-regulated in immortalized human breast epithelial cells. In paper 3, we aimed to study the expression of KSR2 in human breast tumors and its effect on proliferation of breast epithelial cells.

KSR2 is a scaffold protein in mitogen-activated protein kinase (MAPK) pathways. To explore potential impact of KSR2 on cell proliferation and generic MAPK pathways in unbiased way, we used FunCoup tool (http://funcoup.sbc.su.se). The generated network showed potential involvement of Raf-1, mitogen-activated kinases 1 and 3 (MAPK1 and MAPK3), mitogen-activated kinase kinase 1 (MAP2K1) and MAPK scaffold protein 1 (MAPKSP1). These molecules provide further links to regulators of the cell cycle, p53 and hTERT. A number of other KSR2 interactors involved in regulation of the cell cycle were reported, although they have to be validated [152]. Thus, analysis of the KSR2-centered network indicated that KSR2 may have an impact on cell proliferation.

To manipulate KSR2 level in cells subjected to cell proliferation assays, we enhanced expression of KSR2 by transfecting cells with a specific vector or down-regulated KSR2 with a specific siRNA. We studied two human breast epithelial cell lines, MCF10A and 184A1, which are both non-tumorigenic and considered to have normal phenotype of human breast epithelial cells. These cells can be cultured in vitro, and therefore are immortalized. Both cell lines also express KSR2. However, 184A1 cells may undergo immortalization crisis, and have lower proliferation potential, as compared to MCF10A cells. We observed that down-regulation of KSR2 had a strong inhibitory effect on proliferation of both cell lines. KSR2 overexpression had rather weak effect on cell proliferation, which was more pronounced for 184A1 cells, but not significant for MCF10A cells. This could be explained by the presence of endogenous KSR2 in a quantity already sufficient for its normal functions.

To explore whether KSR2 expression could be altered in breast tumors, we performed immunohistochemistry study of human breast tissue microarray. All samples of normal breast tissue did not show KSR2 staining. KSR2 staining in tumor samples was detected mainly in cytoplasm of cells. No correlations of KSR2 expression with TNM gradation of tumors were observed.

KSR2 plays an important role in activation of various signaling events, with reported role in signaling by p38 MAPK and MAP2K3. We found that KSR2 is involved in regulation of cell proliferation, and is upregulated in tumor epithelial cells in human invasive ductal, invasive lobular and papillary carcinomas of breast. In conclusion, our findings provide evidence of a potential role of KSR2 in tumorigenesis, indicating that expression of KSR2 may be changed already upon acquisition by cells the ability to non-limited proliferation.

2.3.4   Paper 4

Protein tyrosine nitration in the cell cycle

Protein tyrosine nitration is a low abundant post-translational modification. It is a potential marker of oxidative/nitrosative stress [153; 154], and associated with many physiological and pathological processes such as neurodegenerative diseases, cancer and inflammatory diseases [128; 129; 132]. Cell cycle is an important mechanism involved in different biological functions. It has been reported that Redox and Reactive

nitrogen species (RNS) (nitric oxide (NO) and peroxynitrite (ONOO_)) may affect the cell cycle, which are sources of protein tyrosine nitration. However, relatively little is known how 3-tyrosine nitration changes in the cell cycle.

The goals of paper 4 were to identify the 3-tyrosine nitrated proteins at different phases of the cell cycle, and explore their possible functions during cell cycle.

 To enrich for tyrosine nitrated proteins, we used immunoprecipitation with anti-3-NT antibodies. We optimized binding, washes, and elution of nitrated proteins from antibody beads. Enriched proteins were then analyzed using 1DE, followed by Coomassie brilliant blue staining or immunoblotting with antibodies specific for nitrotyrosine. Coomassie brilliant blue-stained gels showed that the protein fraction eluted with buffer containing 1% SDS has high abundance of proteins. Our trials with elution with nitrotyrosine showed that significant quantities of nitrated proteins were not released from the beads. The low efficiency of nitrotyrosine elution may be attributed to differences between pure nitrated tyrosine and nitrated tyrosine in proteins used for generation of the antibody. We estimated that at least 90% of tyrosine nitrated proteins could be enriched and recovered using described here protocol.

We synchronized cells in G0/G1, S and G2/M phases. To monitor cell synchronization, we analyzed cells by flow cytometry. MCF-7 cells were synchronized in G0/G1 phase by a serum starvation for 48 hours, with 84% of cells in G0/G1 phase. Hydroxyurea double block was used to enrich for cells in the S phase, with 77% of cells in S phase, and 16 hours nocodazole treatment was used to synchronize mitotic cells, with 88 % of cells in G2/M phase.

To monitor tyrosine nitration of proteins during cell cycle progression, we performed immunoblotting of total cell extracts from cells synchronized in different phases of the cells cycle. The lowest level of protein tyrosine nitration was observed in cells in the G0/G1 phase, as compared to S and G2/M phase. The majority of nitroproteins in G0/G1 phase were distributed in molecular range 50 to 75 kDa. The most abundant presence of tyrosine nitrated proteins was observed in cells in S phase. Quantification of immunoblots indicated that the distribution of nitrated proteins may be as 1: 10: 7 for cells in G0/G1, S and G2/M phases, respectively. This indicates that the total tyrosine nitration of proteins changes during the cell cycle, with the highest nitration level in S phase.

To identify tyrosine nitrated proteins, we used described before immuno-enrichment protocol, and separated tyrosine nitrated proteins by 2DE. Peptide mass fingerprinting with MALDI TOF mass spectrometry was used. We identified 27, 37 and 12 proteins in 2D gels representing tyrosine nitration enriched proteins in G0/G1, S and G2/M phases, respectively.

Validation of nitrated proteins was performed by immunoblotting with protein-specific antibodies of IP- enriched nitrotyrosine containing proteins from different cell cycle phases. Here we examined the nitration status of 3 identified proteins, intergrin-linked kinase (ILK), FAST kinase domain 2 (FASKD2) and estrogen receptor 2 (ESR2). We observed that ILK was significantly nitrated in G0/G1 phase as compared to S and G2/M phase, and the ratio of band intensity was approximately 7: 1: 2 for cells in G0/G1: S: G2/M phases. The level of nitration of FASTKD2 was low at G0/G1 phase, increased in S phase, and back to the similar level as G0/G1 phase in G2/M phase. The ratio of FASTKD2 nitration in three phases was approximately 1: 3: 1 (G0/G1: S: G2/M). The nitration of ESR2 gradually increased during progression from G0/G1 =>

S => G2/M, with the ratio of intensity 1: 1.5: 4. Thus, observed tyrosine nitration status of ILK, FASKD2 and ESR2 confirmed results obtained by 2DE.

To gain an insight into functions of the identified proteins, we used Gominer and Funcoup analysis tools. We found that the identified proteins may be involved in various biological functions. The most abundant functional domains they affected included cell growth and proliferation, cell death, metabolism, localization and signaling. As we were interested in potential impact of nitrated proteins on the cell cycle, we focused on proteins that may regulate cell cycle and cell proliferation. We observed that 7 proteins identified in G0/G1 phase synchronized cells (KHDRBS2, ILK, CDKN1A, ZBTB33, STAB1, PRKACG) were earlier described as potential regulators of the cell cycle. Among cell proliferation-regulating proteins, 8 proteins (RAP1GAP, RIOK3, AHSA1, NSF, CTSS, SCFD1, PCK1, FRMD7) were identified in S phase, and 4 nitrated proteins (ZNF777, ZPHP3, VAV3, ITIH4) were indentified in synchronized cells in G2/M phase.

In paper 4, we report identification of proteins enriched for tyrosine nitration in cells synchronized in G0/G1, S or G2/M phases of the cell cycle. We identified 27 proteins in cells synchronized in G0/G1 phase, 37 proteins in S phase synchronized cells, and 12 proteins related to G2/M phase. Nineteen of the identified proteins were previously described as regulators of cell proliferation. Thus, our data indicate which tyrosine nitrated proteins may affect regulation of the cell cycle.

2.3.5   Paper 5

Network signaling by TGFβ1 and EGF in regulation of the cell proliferation as predictor of application of Iressa

Cell proliferation is controlled by many different positive and negative signals (growth factors, cytokines et al). In breast cancer, TGF-β plays dual roles during cancer development. In early stage cancer, TGF-β inhibits cell proliferation, and can induce cell apoptosis. However, at later stage cancer, it acts as tumor promoter. It promotes cell mobility, invasion and metastasis. To understand how the TGF-β and EGF signaling interact may provide insights on mechanisms of cancer development, and further improve the anti-cancer treatment.

The goals of paper 5 were to identify convergent phosphoproteins of TGF-β1 and EGF signaling. Systemic analyze the identified proteins, with focus on the ones involved in regulation of cell proliferation. Interrogate the involvement of the selected proteins in regulation of cell proliferation.

First, we performed cell proliferation assay to test how TGF-β1 and EGF would act on MCF7 human breast carcinoma cells. We observed that proliferation of MCF7 human breast carcinoma cells was stimulated by EGF, inhibited by TGF-β1, and EGF partially reverted inhibitory action of TGF-β1.

To identify phosphoproteins co-regulated by TGF-β1 and EGF, we performed enrichment of phosphoproteins from MCF7 cells using developed by us Fe-IMAC method. To confirm phosphorylation status of the separated proteins, the gels were counterstained with phosphor-specific ProQ Diamond stain. Image analysis, followed

by MALDI-TOF mass spectrometry identified 47 proteins affected by combined action of TGF-β1 and EGF, as compared to single treatments and control cells.

We performed systemic analysis aiming to identify these regulatory mechanisms and proteins of key importance for these regulatory mechanisms. Analysis of the networks for functional domains affected by the identified proteins showed that cell cycle, DNA replication, drug metabolism and regulation of post-translational modifications were the top affected molecular processes. Systemic analysis identified CK1 (CSNK1A1) and MEK1 (MAP2K1) as two kinases that may have an impact on the TGF-β1 and EGF cross-talk. CK1 and MEK1 are kinases. Therefore, to evaluate their impact on cells, their kinase activities would have to be targeted primarily. We used U0126 inhibitor of MEK1 and D4476 inhibitor of CK1 at 10 μM concentrations suggested as specific by numerous studies [155; 156]. To explore whether TGF-β1/EGF cross-talk would affect impact of anti-cancer drugs, we treated cells with Iressa. We observed that the U0126 and D4476 inhibitors alone had no effect on cell proliferation, but when cells were treated with TGF-β1, EGF or/and Iressa, the inhibitors modulated cell proliferation significantly. We observed the strongest inhibitory effect of Iressa would be only when both EGF and TGF-β are highly active, and MEK1 and CK1 are inhibited.

In this study, we investigated the crosstalk of TGF-β1 and EGF signaling, and examined the effects of the common targets in anti-cancer drug, Iressa. Our interrogation data indicate also that combination of anti-EGF and anti-TGF-β therapies have to include evaluation of the convergence targets, and not only levels of TGF-β, EGF and their receptors.

2.3.6 Paper 6

Role of sterile alpha motif and leucine zipper containing kinase AZK (ZAK) in combined signaling by TGF-β1 and EGF

In paper 5, we identified ZAK kinase as a convergence target in TGF-β1 and EGF signaling. ZAK has been reported to regulate cell cycle and apoptosis. There has been no exploration of ZAK kinase in the context of TGF-β1 and EGF signaling. Here we explored ZAK impact on TGF-β1- and EGF-dependent regulation of the cell proliferation, migration and apoptosis.

ZAK was identified as a convergent target of TGF-β1 and EGF signaling. ZAK phosphorylation was found to be enhanced by a single treatment of cells with TGF-β1 or with EGF. To validate this observation, we performed ^{32}P labelling of the treated cells. In agreement with phosphoproteomics data, TGF-β1 or EGF-treated cells showed higher ^{32}P incorporation in ZAK protein, as compared to the control and double treated cells. This indicated that TGF-β1 or EGF promoted ZAK phosphorylation, while the combinatorial treatment showed similar phosphorylation level as in the control cells.

We generated MCF-7 cells stably transfected with wild-type ZAK kinase, and cells stably transfected with kinase-dead ZAK. We observed that the wild-type ZAK over-expressing cells showed enhanced cell migration upon TGF-β1 or EGF treatment. Combined treatment with TGF-β1 and EGF induced maximal increase of the cell migration, among all tested conditions. However, the ZAK kinase-inactive mutant abrogated the enhancement, which indicated that ZAK kinase activity may be a positive regulator of cell migration by the network signaling by TGF-β1 and EGF. Assays of

cell proliferation and apoptosis analysis did not show significant differences between different treatments in ZAK overexpressing cells. Therefore, ZAK kinase contributes to the positive feedback regulation of cell migration upon combined TGF-β1 and EGF action.

2.3.7   Paper 7

Transformation and stemness of human breast epithelial cells exposed to EGF and 17β-Estradiol

Exposure of breast epithelial cells to EGF and estrogen may have a strong impact on breast tumorigenesis, leading to enhanced incidence of cancer. Anti-EGF and anti-estrogen therapies are used in treatment of breast cancer. However, knowledge of molecular mechanisms governing cell growth upon exposure to EGF and estrogen is not fully understood.

In paper 7, the goals were to explore mechanisms of tumorigenesis activated upon prolonged exposure of cells to EGF and estrogen. Specifically, we were interested in an impact of the exposure on stemness features of human breast epithelial cells.

We generated clones of MCF-7 cells exposed to a long-term treatment with EGF and/or 17β-estradiol, alone or in combination. Clones were selected for their enhanced proliferation. We explore cell proliferation, death, migration and invasiveness of the clones. Stem cell-like markers were also studied. We observed that the exposure to EGF and 17β-estradiol enhanced proliferation rates of MCF-7 cells, as compared to the clones of control non-exposed cells. EGF exposure led to enhanced colony formation by the cells, while exposure to 17β-estradiol reduced it. Mice tumorigenesis study showed no significant differences in tumor take for each clone. However, 17β-estradiol-exposed clones had a lower tumor volume, as compared to other groups. MIB1 staining indicated that EGF and EGF&17β-estradiol clones had higher rate of cell proliferation, while estrogen clones had significantly reduced cell proliferation rate, when compared to the control group. Vessel score calculated by vWF staining of tumor tissue, showed similar response pattern as in proliferation assay. Notably, EGF and EGF&17β-estradiol clones had enhanced angiogenesis, while estrogen clones showed inhibited vessel formation, as compared to the control group. EGF and EGF&17β-estradiol clones also showed enhanced breast cancer stem cell features.

Proliferation rate, colony formation, vessel formation, and stem cell features of human breast epithelial cells after a long-term exposure to EGF and 17β-estradiol were affected.

2.4 Conclusions

Based on the findings in the thesis, we can conclude that

1   MAP2K3 was down-regulated in immortalized cells, and expression of MAP2K3 in immortal human breast epithelial cells was sufficient to induce senescence. This indicates a potential role of MAP2K3 in immortalization. This was supported by our finding that MAP2K3 was down-regulated in

cancer, as compared to normal human breast epithelial cells. The lack of correlation of MAP2K3 down-regulation with TNM gradation, ER, PR, and ErbB2 status indicated that the decrease of MAP2K3 expression is an early event in tumorigenesis.

2   TGF-β2 and HSP27 were up-regulated in response to hyperthermia. TGF-β2 stimulated expression of HSP27 in the cells, and protected cells from cell death.

3   KSR2 expression was up-regulated in immortalized human breast epithelial cells and in human breast tumors. It may contribute to un-limited cell proliferation.

4   Tyrosine nitrated proteins may affect regulation of the cell cycle.

5   Combination of anti-EGF and anti-TGF-β therapies has to include evaluation of the convergence targets, and not only levels of TGF-β, EGF and their receptors.

6   ZAK kinase contributed to the positive feedback regulation of cell migration upon combined TGF-β1 and EGF action.

7   Proliferation rate, colony formation, vessel formation, and stem cell features of human breast epithelial cells were affected after long-term exposure to EGF and 17β-estradiol .

3 Acknowledgements

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