MOLECULAR INSIGHTS INTO THE MODULATION OF GELATINASES BY THE GREEN TEA POLYPHENOL (-)-EPIGALLOCATECHIN-3-GALLATE (EGCG) IN HUMAN BREAST CANCER CELLS

A thesis submitted for the degree of

Doctor of Philosophy

To

INDIAN INSTITUTE OF TECHNOLOGY

GUWAHATI

By

GAURI DEB

Department of Biotechnology

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

GUWAHATI 781039, ASSAM, INDIA

April 2014

To my loving parents

(Maa and Baba)

For always being the wind beneath my wings

Acknowledgements

I am greatly indebted to many people in my life without whom this thesis would have remained a dream.

I owe my deepest gratitude to ***Dr. A. M. Limaye****, an amazing mentor who always believed in me and my abilities to do good research. I am fortunate to be the first graduate student of his lab and will always cherish some fond memories of working with him on the bench, during initial years of my PhD. I thank him for his valuable suggestions during lab meetings and patiently dealing with my scientific limitations and idiocracies. Without his support and guidance, it would have been impossible for me to go abroad and work on my thesis work.*

I would also like to thank...

My doctoral committee members – Dr. Aiyagari Ramesh, Dr. Biplab Bose and Dr. Gopal Das*, for their valuable suggestions during my work presentations.*

Dr. Sanjay Gupta *for giving me a wonderful opportunity to carry out a part of PhD work in his lab and providing excellent research facilities.*

Dr. Vijay S Thakur *for guiding me in my project and extending his help during lentivirus mediated gene knock down studies and ChIP assays.*

Past and present HODs of Dept. of Biotechnology *– Dr. Arun Goyal and Dr. V V Dasu for making available departmental facilities.*

The entire staffs of Dept. of Biotechnology, Academic affairs and External relations, IIT Guwahati *for efficient and timely help whenever required.*

Council for scientific and industrial research (CSIR) and Fulbright foundation, *for providing me the financial assistance during my graduate studies in India and the USA respectively.*

I acknowledge,

Dr. V. K. Dubey and Dr. V. Trivedi*: for providing access to fluorescence reader and microscope*

Dr. Hung-Ying Kao and Dr. Dipak Datta*: for providing us with breast cancer cell lines*

Dr. S. P. Kanaujia*: for molecular docking studies*

Contd....

I thank,

All the past and present members of my lab: *Ritesh, Anup, Sahil, Mohan, Abrar, Bhaskar, Abhay, Sheeba, Shalinee, Marine, Ajay and Raunak, for their help during experiments and also, making my life inside and outside the lab so enjoyable.*

Prajakta Ma'm, *who is one of the friendliest and loving person I have ever met. Thank you for always being the perfect host in all those high tea parties in your house and a great company during our lab outings.*

Dr. M. A. Laskar*, an amazing teacher and a mentor. Thank you for always being there for me and encouraging me to take up PhD studies.*

Dr. Vijay Thakur and Dr. Varsha Thakur *for all their love, care and for being my true guardians in the USA. Thank you for joining me in those 'house hunting' drives and always making sure that I don't feel homesick.*

Dr. Natarajan Bhaskaran, Mahalaxmi, Gautham and Amma*, who were like my extended family in the USA.*

Anup*, with whom I have always shared a special bonding. Thanks for being my best friend, companion and a source of happiness.*

Mithu, Ankana and Vinita *for being the awesome buddies, with whom I shared some of the best moments of my 'PhD Days'. Thank you guys for always being supportive and hearing me out in good and bad times.*

My friends, batchmates and seniors *for all the fun , friendship and care - Himangshu, Sonali, Arabinda, Shuchi, Shruthi, Kimjolly, Rohitas, Pranali, Debamitra, Nayanmoni, Deepti, Rekha, Anil, Mitun, Deeplina, Sushant, Prakash, Ayan, Nandkishore, Supriyo, Dr. Asim Das, Chokhalingam, Tushar, Ashok, Pojul, Dr. Manab Debadhikari and Dr. Abhay N Singh.*

My younger sister Sumita and my elder brother Gautam (Dada) *for their unconditional love, support and care.*

My parents and almighty god*, to whom I owe my very existence. Baba, you are always missed and I know you would have been proud of your 'Gauran'. Last but not least, I will be always indebted to the most wonderful person in my life, my mother, for her love and blessings*

Gauri Deb

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
DEPARTMENT OF BIOTECHNOLOGY
GUWAHATI-781039

DECLARATION

I hereby declare that the contents of the research work described in this thesis titled **"Molecular insights into the modulation of gelatinases by the green tea polyphenol (-)-Epigallocatechin-3-gallate (EGCG) in human breast cancer cells"**, is a presentation of my original research work carried out in the Department of Biotechnology, Indian Institute of Technology, Guwahati, India under the supervision of Dr. Anil Mukund Limaye and in the laboratory of Dr. Sanjay Gupta at Case Western Reserve University, Ohio, USA under the Fulbright –Nehru Doctoral and Professional research fellowship (2012-13).

Sincere efforts have been made to duly acknowledge the contributions from others for their ideas, technical help, references or any other help which may be involved in the completion of this thesis work.

April, 2014

Gauri Deb
Roll no: 09610613
Department of Biotechnology
IIT Guwahati

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
DEPARTMENT OF BIOTECHNOLOGY
GUWAHATI-781039

CERTIFICATE

This is to certify that the work described in the thesis titled **"Molecular insights into the modulation of gelatinases by the green tea polyphenol (-)-Epigallocatechin-3-gallate (EGCG) in human breast cancer cells"**, submitted by Gauri Deb (Roll no: 09610613) to Indian Institute of Technology Guwahati, India, for the award of the Degree of Doctor of Philosophy is an authentic record of the research work carried out under my supervision in the Department of Biotechnology, Indian Institute of Technology, Guwahati, India and under the guidance of Dr. Sanjay Gupta at Case Western Reserve University, Ohio, USA as part of the Fulbright-Nehru Doctoral and Professional research fellowship.

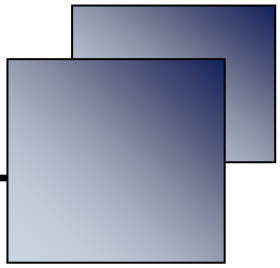
This thesis or any part thereof has not been submitted elsewhere for award of any other degree or diploma.

April, 2014

Dr. Anil Mukund Limaye
Thesis supervisor

CONTENTS

Chapter I



Introduction

Breast cancer represents a complex and diverse group of neoplasms originating in the tissues of the breast. Despite significant advances in diagnosis and treatment of breast cancer, several important issues related to its prevention, progression, recurrence and therapeutic resistance still remain unaddressed. Most of the newly diagnosed breast tumors express estrogen receptor α (ERα) and are initially responsive to endocrine therapy which targets the Estrogen–ERα signalling axis (Jemal *et al.*, 2009; Jordan and Brodie, 2007; Peng *et al.*, 2009). Over the course of treatment, majority of the estrogen responsive tumors eventually acquire hormone resistance and relapse as incurable metastatic tumors (Al Saleh *et al.*, 2011; Clarke *et al.*, 2001). Endocrine resistance (*de novo* or acquired) and tumor metastasis are the major barriers towards successful treatment of breast cancer.

A key step in the complex process of cancer progression and the establishment of metastasis is the remodelling of xtracellular matrix (ECM), which is brought about by diverse groups of proteases including the Matrix Metalloproteinases (MMPs), a family of 22 individual zinc-dependent endopeptidases in humans (Nagase and Woessner, 1999; Steffensen *et al.*, 2001; Sternlicht and Werb, 2001). A substantial body of data indicate the involvement of gelatinases/type IV collagenases (MMP-2 and MMP-9) in tumor invasion and metastasis (Bergers *et al.*, 2000; Fang *et al.*, 2000; Kleiner and Stetler-Stevenson, 1999; Sternlicht and Werb, 2001). The gelatinases are initially produced as zymogens in a latent form (pro-MMP), which are later activated by the proteolytic removal or disruption of pro-domain. Tissue inhibitors of matrix metalloproteinases (TIMPs) are endogenous MMP inhibitors and inhibit most of MMPs by binding to them in a 1:1 stoichiometry (Visse and Nagase, 2003). The balance between active MMPs and TIMPs determine tumor progression by regulating the net activity of MMPs (Bode *et al.*, 1999). TIMPs have been shown to possess tumor suppressive roles and upregulation of TIMPs have been shown to have an inhibitory effect on invasion and metastasis (DeClerck *et al.*, 1992; Imren *et al.*, 1996). Consistent to this low TIMP-3 protein expression in breast tumors has been shown to be associated with an aggressive tumor phenotype and poor disease-free survival (Mylona *et al.*, 2006)

Epidemiological studies have emphasized the cardinal role of *nutrition* in carcinogenesis. Dietary phytochemicals have been shown to possess anti-oxidant, anti-inflammatory and anti-cancer properties. (-)-Epigallocatechin-3-gallate (EGCG), the major active polyphenol present in green tea, has captured the interest of the scientific community for its purported health benefits and diverse effects demonstrated in various *in vitro* and *in vivo* models (Dreosti *et al.*, 1997; Schramm, 2013). It has been shown to target a wide range of cellular proteins thereby affecting diverse cellular functions. Evidences suggest that EGCG induces apoptosis and suppresses proliferation, angiogenesis and invasiveness in various breast cancer pre-clinical models (Bigelow and Cardelli, 2006; Braicu and Gherman, 2012; Stuart *et al.*, 2010). However mechanistic details are not completely understood.

Aims and scope of the present thesis work

One of the important aspects of the multifaceted role of EGCG in controlling tumor progression and metastasis is the inhibition of MMPs (Demeule *et al.*, 2000). Evidences indicate that the role of gelatinases (MMP-2 and MMP-9) in promoting breast carcinogenesis have been mostly studied in the context of matrix remodelling but it is still unclear if steroid hormones such as estrogens, which play critical role in breast tumor initiation and progression, affect gelatinolytic activity in breast cancer. About 70% newly diagnosed breast cancers are hormone dependent, express estrogen receptors, and require estrogen for growth (Jemal *et al.*, 2009; Tyson *et al.*, 2011). But none of the investigations reported so far have addressed the impact of EGCG treatment on estrogen regulated cellular phenotypes or molecular pathways. Hence, this study seeks to address the combinatorial effect of EGCG and β-estradiol (E2) on proliferation and invasion of human breast cancer cells.

The ability of EGCG to modulate the expression and activity of gelatinases, as demonstrated in other studies has emerged as an important lead, since they project EGCG (or green tea) as a potential anti-invasive or anti-metastatic agent (Farabegoli *et al.*, 2011; Sen *et al.*, 2009). However, the mechanistic details of EGCG mediated modulation of gelatinolytic activity have not been addressed

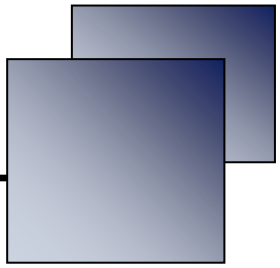
adequately. The present study aims to unravel the molecular mechanism of EGCG mediated inhibition of gelatinolytic activity.

TIMPs are important modulators of MMP activity and an imbalance in MMP-TIMP ratio drive tumorigenesis. *TIMP-3* over-expression in tumor cells is known to cause reduced primary growth, angiogenesis and induce apoptosis (Deng *et al.*, 2006; Zhang *et al.*, 2010). In breast cancer cells, *TIMP-3* repression has been linked to promoter DNA hypermethylation, which has been reported in 20–27% cases of primary breast tumors and invasive ductal carcinoma (Bachman *et al.*, 1999; Lui *et al.*, 2005). However, other epigenetic silencing mechanisms such as aberrant histone methylation and deacetylation patterns at the *TIMP-3* gene locus have not been characterized in breast cancer. The potential of green tea polyphenols (GTPs) to counteract cancer related epigenetic aberrations, by modulating the activity and expression of DNA methyltransferases (DNMTs) and histone modifying enzymes, is well known (Khan *et al.*, 2012; Thakur *et al.*, 2014). However, the effect of GTPs or EGCG on *TIMP-3* expression, in particular, has not yet been elucidated. This study seeks to explore and address the epigenetic regulation of *TIMP-3* by GTPs and EGCG.

The work presented in this thesis was carried out with the following specific objectives:

1. To study the combinatorial effect of EGCG and E2 on proliferation, apoptosis, cell cycle distribution, gelatinolytic activity, migration and invasion of human breast cancer cells.
2. To obtain mechanistic insights into EGCG driven modulation of gelatinolytic activity.
3. To address the epigenetic mechanisms underlying *TIMP-3* repression and study the effect of EGCG or GTP treatment on *TIMP-3* expression in human breast cancer cells.

Chapter II



Review of literature

Parts of this chapter have been published in Deb *et al.* (2013), *Epigenetics: official journal of the DNA Methylation society* and Deb *et al.* (2014), *Molecular Cancer Research*

BREAST CANCER – AN OVERVIEW

Cancer is a broad group of diseases characterized by uncontrolled cell growth and proliferation culminating in tumor formation and metastatic dissemination to other tissues. Conceptual progress made in cancer research over the past four decades have highlighted some essential hallmarks of cancer that provide a logical framework to understand the mechanistic underpinnings of the diverse and complex nature of neoplastic diseases. The acquired capabilities that dictate malignant transformation include growth signal autonomy, insensitivity to growth suppressors, resistance to cell death, replicative immortality, promotion of angiogenesis, modification or reprogramming of cellular metabolism, evading immune surveillance, tumor invasion and metastasis (Hanahan and Weinberg, 2011).

Based on the site of origin, there are more than 100 different types of cancers. Breast cancer is one of the most common malignancies and accounts for majority of cancer-related mortality among women worldwide. It represents a complex and heterogeneous group of pathologies that originate in the tissues (ducts or lobules) of the breast (Figure 2.1), with diverse histo-pathological features and clinical outcomes. Broadly breast cancer can be categorized into two types – ductal and lobular carcinoma. Ductal carcinoma is the most common type of breast cancer and originates in the lining of the milk ducts. Lobular carcinoma begins in the lobules (milk glands) of the breast. Both types of carcinoma have their benign and invasive forms namely ductal carcinoma *in situ* (DCIS), lobular carcinoma *in situ* (LCIS), invasive (infiltrating) ductal carcinoma (IDC) and invasive (infiltrating) lobular carcinoma (ILC). There are additional sub-types of invasive breast carcinoma, which are often classified by the features observed under the microscope. Examples include denoid cystic (or adenocystic) carcinoma, low-grade adenosquamous carcinoma (a type of metaplastic carcinoma), medullary carcinoma, mucinous (or colloid) carcinoma, papillary carcinoma and tubular carcinoma.

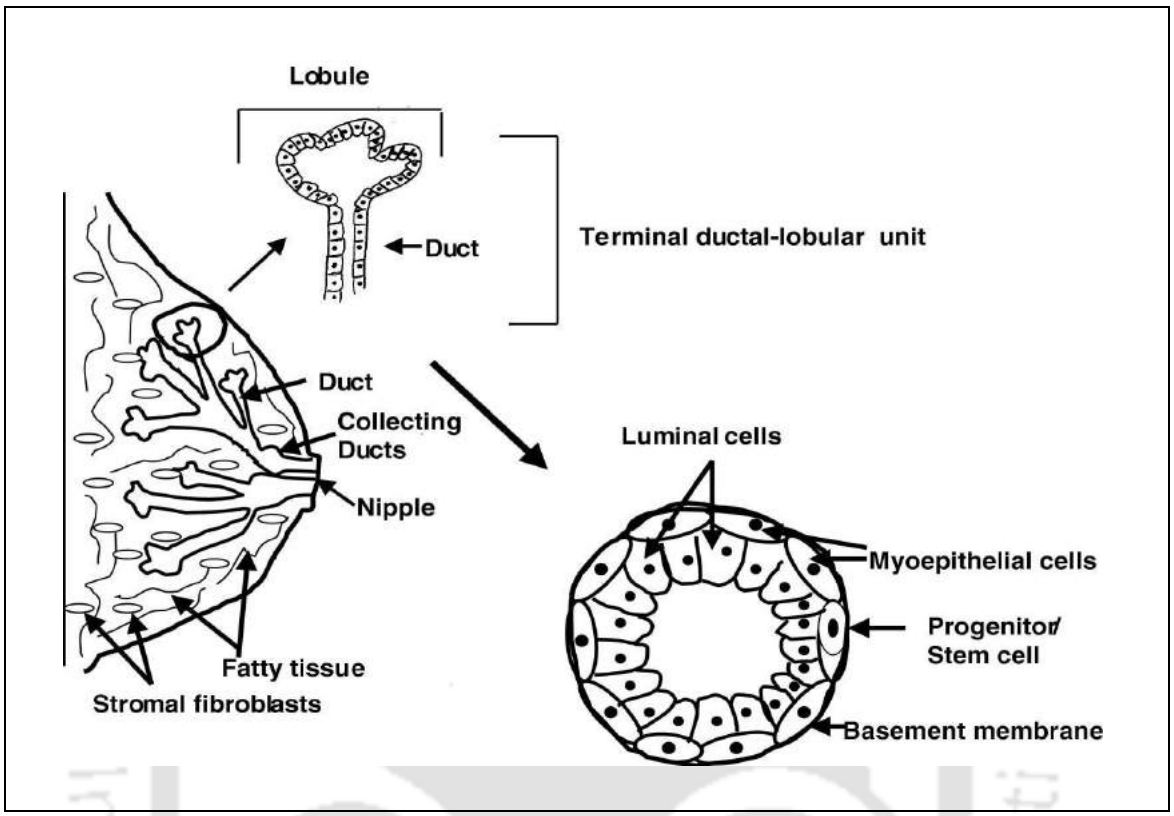


Figure 2.1. Schematic representation of terminal ductal–lobular unit of human breast in non-lactating women. Adapted from Dimri *et al.* (2005)

Breast Cancer Statistics

Although breast cancer occurs in both men and women, this malignant disease is more common among women and accounts for one tenth of all new cancers diagnosed worldwide (Kamangar *et al.*, 2006). According to the GLOBOCAN 2012 data (Ferlay *et al*; 2012), provided by International agency for Research on Cancer (IARC), WHO, the estimated number of breast cancer cases and the associated mortality rate in females across all ages in India was reported to be 27% and 21.5% respectively (Figure 2.2). When compared to world breast cancer statistics, the incidence and mortality rate of breast cancer is higher in India (Figure 2.2 and 2.3).

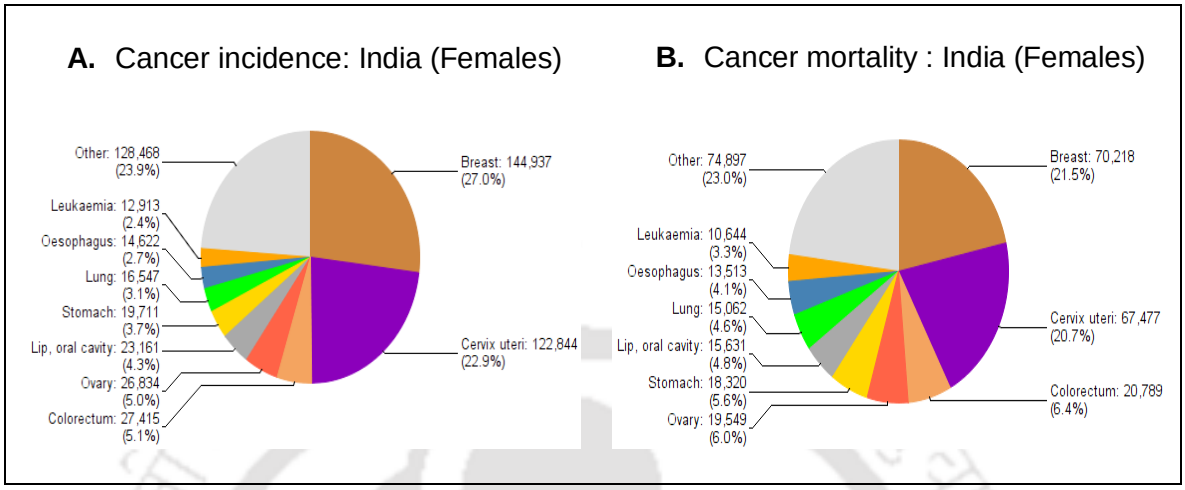


Figure 2.2. Cancer incidence and mortality rates among females (all ages) in India. *Panel* **A**: Estimated number of cancer cases (all ages, total: 537452) among females. *Panel* **B**: Estimated number of cancer related deaths (all ages; total: 326100) among females. Source: GLOBOCAN 2012 (IARC,WHO)

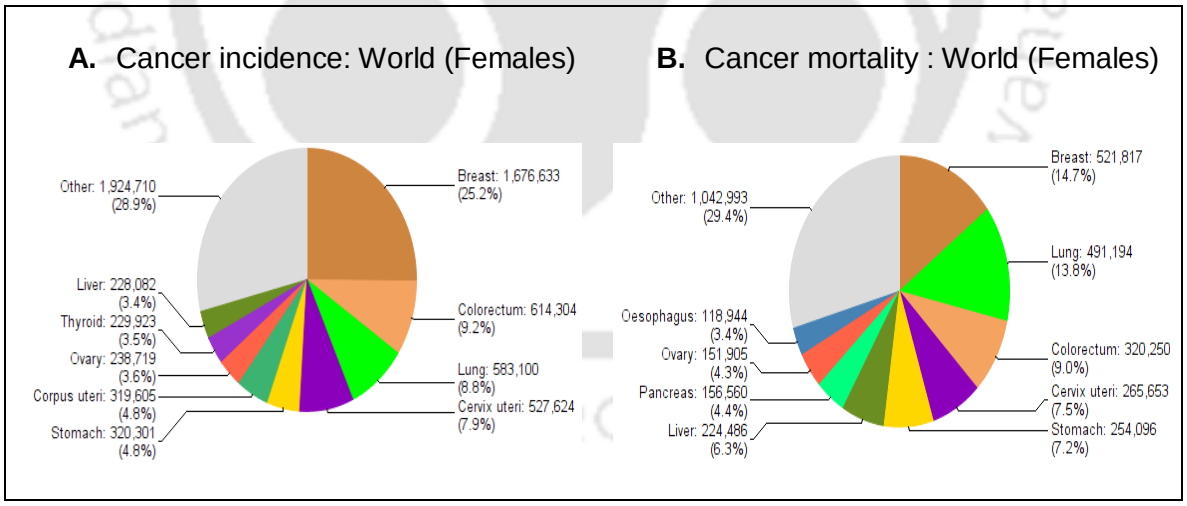


Figure 2.3. Cancer incidence and mortality rates among females (all ages) in the world. *Panel* **A**: Estimated number of cancer cases (all ages, total: 6663001) among females. *Panel* **B**: Estimated number of cancer related deaths (all ages; total: 3547898) among females. Source: GLOBOCAN 2012 (IARC,WHO)

Risk Factors

The strongest determinant of risk for developing breast cancer among females is age. However the risk of breast cancer occurrence is not same for all women in a given age group. Females with the following risk factors have increased chances of developing breast cancer (Source: National Cancer Institute, USA)-

- Genetic alterations in genes such as *BRCA-1* and *BRCA-2*
- Family history
- Reproductive or menstrual history
- Long term use of post-menopausal hormone therapy
- Radiation therapy
- Body weight
- Race - breast cancer diagnosed more often in white women than in African American/black, Hispanic/Latina, Asian/Pacific Islander, or American Indian/Alaska Native women.
- Alcohol consumption

Molecular profiling of breast cancer

Based on traditional immuno-histochemistry (IHC) techniques, breast cancer used to be broadly categorized into 3 major classes: Hormone-receptor-positive [Estrogen receptor (ER) and Progesterone receptor (PR)], Human Epidermal Receptor (HER) 2/*neu*-positive and triple negative (ER negative, PR negative, HER2/*neu* negative) breast cancers (Kittaneh *et al.*, 2013; Sorlie *et al.*, 2001; Sorlie *et al.*, 2003). However, in recent years seven different subtypes of breast cancer have been identified based on genome-wide expression profiling and hierarchical clustering (Hu *et al.*, 2006; Huang *et al.*, 2003; Perou *et al.*, 2000). The subtypes include luminal A, luminal B, luminal C, HER2-enriched, basal like, claudin low and normal breast like. All of these breast cancer subtypes display distinct features and natural histories (Kittaneh *et al.*, 2013). In general luminal A type tumors have the best, and basal like (triple negative) tumors the worst, prognosis. Each subtype varies in terms of response to therapy directed against them and overall survival, highlighting the need for a more specific and personalized treatment regimen.

Current treatment regimen

Treatment options available for breast cancer management include surgery, chemotherapy, radiation therapy, hormonal therapy and targeted therapy. The treatment for a particular patient depends on her risk profile, stage of the disease (depending on the tumor size, location, involvement of lymph nodes) and extent of metastasis. Surgery, radiation therapy and chemotherapy are general treatment options, which are routinely used in the treatment of any cancer type. But, hormone therapy is a specialized treatment option available for the management of hormone receptor (ER, PR, and Her2) positive breast cancers and involves the administration of aromatase inhibitors, selective estrogen receptor modulators (SERMs) and estrogen receptor down-regulators.

Endocrine therapies utilize drugs such as tamoxifen, herceptin and aromatase inhibitors (letrozole and exemestane) for ER-positive and Her2-overexpressing tumors (Curran and Wiseman, 2001; McDonnell *et al.*, 1995; Peng *et al.*, 2009). Chemotherapy is the standard treatment for tumors that are poorly differentiated and lack Her2, ER and PR. More effective therapeutic strategies with minimum side effects are therefore needed to improve the clinical outcome of patients with breast cancer (Hayes, 2000). Targeted therapies are designed to target only cancer cells as opposed to chemotherapy which destroys healthy, normal cells as well. This treatment strategy includes drugs such as avastin, herceptin and tykerb.

ROLE OF ESTROGEN AND ER IN BREAST CANCER

The ovarian steroid hormone E2 plays a pivotal role in controlling multiple physiological processes which include normal functioning and maintenance of reproductive, skeleton, cardiovascular and central nervous systems (Gruber *et al.*, 2002). Main target organs of estrogen in females include uterus, vagina, mammary glands and ovaries, while in males the target organs comprise the testes, prostate and epididymis. Although E2 has been shown to be an important regulator of normal breast morphogenesis and physiology, it has been also implicated in breast pathogenesis (Daniel *et al.*, 1987; Gruber *et al.*, 2002; Mueller *et al.*, 2002). Experimental and epidemiological studies have established that there exists a positive correlation between breast cancer risk and lifetime

high estrogen exposure due to early menarche, late menopause and estrogen replacement therapy (Ikeda and Inoue, 2004; Pike *et al.*, 1993). *In vitro* experiments have demonstrated that cells derived from breast and uterine tumors show enhanced cell proliferation in response to estrogenic stimulation (Foster *et al.*, 2001; Holinka *et al.*, 1986). Hence it is reasonable to assume that estrogen stimulated cell proliferation would result in increased spontaneous DNA replication errors, which may ultimately contribute to malignant transformation.

At the molecular level, majority of estrogen actions concerning the regulation of genes that orchestrate survival and proliferation are mediated through the ER (Osborne *et al.*, 2001). Majority of early human breast tumors show high ER expression levels as compared to normal breast tissue. ER status also correlates with histological grade of tumors. ER negative breast tumors are of high histological grade and display aggressive phenotype (Clarke *et al.*, 1994). Most ER positive tumors respond to anti-estrogen therapy utilizing ER antagonists such as tamoxifen. Since the tumors become resistant to tamoxifen over the course of treatment, a combined treatment regimen is preferred which involves the administration of both anti-estrogens and aromatase inhibitors such as letrozole and anastrazole. In summary, a substantial body of evidence suggests a key role of estrogens in breast tumorigenesis.

Estrogen Receptors (ERs)

ER has two subtypes – ERα and ERβ, which belong to the steroid/retinoid receptor gene superfamily and share structural similarity as well as mode of action (Ikeda and Inoue, 2004). Both these receptors have been reported to be expressed in bone, breast, urogenital tract cardiovascular tissue, gastrointestinal tract and central nervous system (Campbell-Thompson *et al.*, 2001; Gustafsson, 1999).

The structure and domain composition of these receptors are represented in Figure 2.4. There are four distinct functional domains present in these receptors – the N-terminal A/B region, the mid region also called the C- region or DNA binding domain (DBD), the D-domain or the hinge region and the C-terminal ligand binding domain (LBD) or E/F domain (Weihua *et al.*, 2003). A/B region modulates transcription through the activation function 1 (AF-1) sequence and

also interacts with p160 family of co-activators. AF-1 function is hormone or ligand independent. C domain participates in receptor binding to DNA through two zinc finger motifs. The hinge region plays an important role in regulating the conformation of the receptor by acting as a site of rotation and has been also implicated in the nuclear localization as well as DNA binding. LBD has multiple functions which include E2 (ligand) binding, receptor dimerization and interacting with heat shock proteins. This domain also harbors activation function 2 (AF-2), which requires hormone binding for its function. The function of F-domain is poorly understood. It may play a role in modulating estrogen and anti-estrogen responses and determine ERα/ERβ effects at the AP-1 sites (Weatherman and Scanlan, 2001).

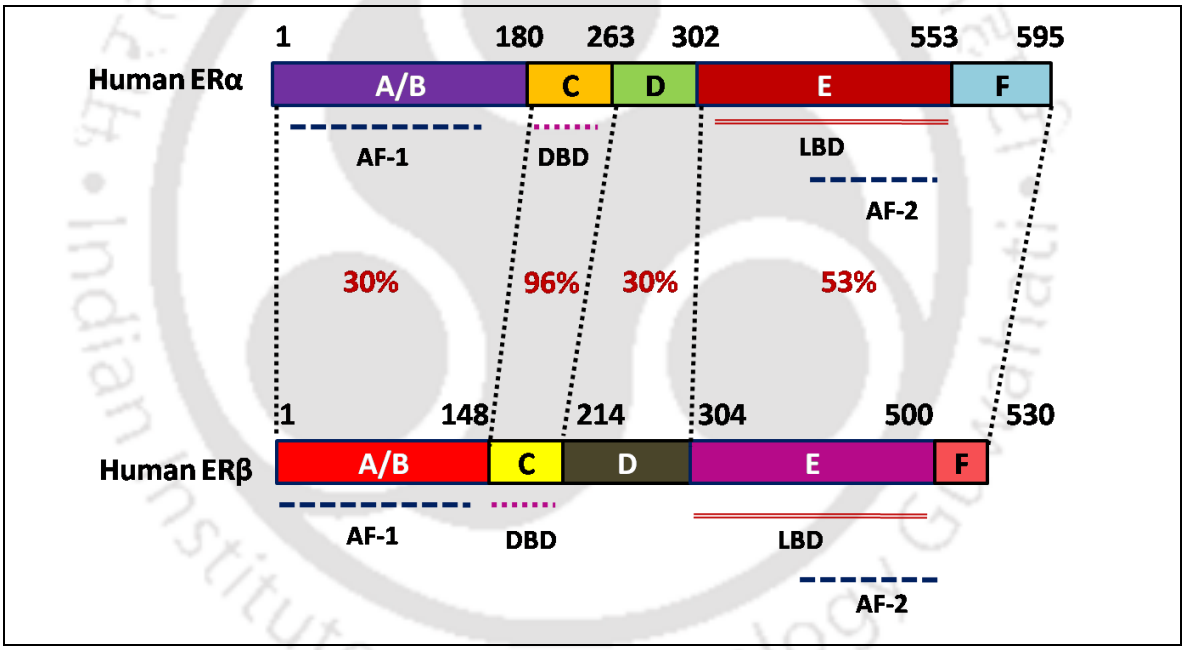


Figure 2.4. Schematic representations of human ERα and ERβ. ERs are composed of six regions (A-F). Percentage of amino acid identities between the corresponding regions is shown. DBD-DNA binding domain, LBD-Ligand binding domain, AF-1- activation function-1, AF-2- activation function-2

Mechanisms of ER mediated regulation of gene expression

Ligand dependent mode of action

The classical (ligand dependent) mode of action of ER involves regulation of genes harboring estrogen response elements (EREs) in their promoter region. Before binding to the ligand (E2), ERs are sequestered in the cytoplasm of target cells as

multiprotein inhibitory complexes in association with heat shock proteins (HSP 50, 70 and 90). Binding of E2 to ER leads to the dissociation of the HSPs and induces a conformational change in the receptor, which leads to its dimerization. The hormone-receptor complex then translocates into the nucleus and regulates the transcription of ERE containing target genes (Klinge, 2001). The consensus ERE sequence: 5'-GGTCAnnnTGACC-3' (where n denotes any random nucleotide) has been defined, based on the vitellogenin gene from *Xenopus laevis* (Klinge, 2001). The transcription of ERE regulated genes may be induced or repressed by the E2-ER complex, depending on the promoter context and cell type. Furthermore, promoter bound ER interacts with other cofactors (co-activators or co-repressors) to module target gene expression.

ERE independent genomic mechanism

Several non-ERE containing genes, have been also shown to be regulated by estrogen. Examples include genes encoding collagenase, ovalbumin, uteroglobin, Cyclin D and Cathepsin D (Duffy, 2006). In this mode of action, E2-ER complex interacts with DNA bound transcription factors at non-ERE sites such as AP-1, SP-1, NF-κB, upstream stimulatory factor (USF) and cAMP-like response element (CRE) sites (Jakacka *et al.*, 2001; Kushner *et al.*, 2000).

Estrogen independent mechanism

ER transcriptional activation has been demonstrated to be mediated by other cell surface signalling pathways involving EGF, HER2 and IGF, in addition to estrogen (Weigel and Zhang, 1998). EGF or IGF-1 mediated ER activation involves phosphorylation of ER at Serine-118 through mitogen-activated protein kinase (MAPK) signalling (Ali *et al.*, 1993; Kato *et al.*, 1995).

Non genomic mechanisms

Cellular response of estrogen action through classical genomic pathway, like other steroid hormones, occurs slowly following exposure to the hormone. However, some effects of estrogen on target cells are rapid, which cannot be inhibited by transcription or translation inhibitors. These rapid changes include activation of adenylate and guanylate cyclases, nitric oxide synthase, MAPK pathway and changes in membrane transport of sodium and calcium ions (Hall *et al.*, 2001; Sak and Everaus, 2004). Some of these effects are believed to be mediated by cell surface receptors such as membrane bound ERs, G-protein

coupled receptor 30 (GPR30) and sex hormone binding globulin (SHBG) receptor (Fortunati *et al.*, 1999; Marquez and Pietras, 2001; Scaling *et al.*, 2014).

ER dependent mechanism of breast carcinogenesis

Estrogens through their receptor mediated actions induce proliferation of immature and mature breast tissue (Hofseth *et al.*, 1999), a factor causally related to tumorigenesis. The mechanisms by which increased cell proliferation facilitates cancer development has been described in detail by Preston-Martin *et al.* (1990). They pointed out that in rapidly dividing cells, the chances for errors in DNA replication are more. As the repair mechanisms operate less effectively due to short period of time in fast dividing cells, the DNA adducts or breaks may get converted into inheritable genetic mutations. ER mediated effects can also activate proto-oncogenes or oncogenes which synergize with other mutational events and promote oncogenesis.

ER independent mechanism of breast carcinogenesis

Experimental and clinical evidences suggest that ER independent mechanisms of estrogen action may also be involved in breast carcinogenesis (Eisen *et al.*, 2005; Foulkes *et al.*, 2004; King *et al.*, 2001; Lakhani *et al.*, 2002; Rebbeck *et al.*, 2009). Direct genotoxic effect of estrogen metabolites has been postulated to be a result of ER independent effects of estrogens (Cavalieri *et al.*, 2006; Fernandez *et al.*, 2006; Yager and Davidson, 2006). In the breast tissue, metabolism of E2 to form 2,3- and 3,4- catechols catalyzed by cytochrome P450 enzymes, which are further converted to 2,3- and 3,4- quinones by oxidative metabolism, can cause DNA damage (Cavalieri *et al.*, 2006). Furthermore, 3, 4-quinones form unstable adducts with adenine and guanine, which undergo depurination and form apurinic sites in the DNA backbone. These apurinic sites, through error prone DNA repair, results in the generation of point mutations. Interestingly, recent studies indicate that the levels of depurinating estrogen-DNA adducts are significantly higher in urine samples of women, who are at high risk or have breast cancer than those who are at normal risk for the disease (Gaikwad *et al.*, 2008; Gaikwad *et al.*, 2009). Cytochrome P450 reductase catalyzes the conversion of 2,3- and 3,4-quinones to semi-quinones. This establishes a redox

cycle to generate reactive oxygen species that cause oxidative damage to DNA (Cavalieri *et al.*, 2000).

Mechanisms of endocrine resistance in breast cancer

Most of the newly diagnosed breast tumors express ERs and are initially responsive to endocrine therapy. Endocrine therapy involves administration of anti-estrogens and aromatase inhibitors. Anti-estrogens such as fulvestrant and tamoxifen bind to ERα and neutralize ER mediated downstream signaling whereas aromatase inhibitors such as letrozole and exemestane block estrogen synthesis (Curran and Wiseman, 2001; McDonnell *et al.*, 1995). As shown in Figure 2.5, initially the tumors respond to hormone blockade and there is tumor regression. But over the course of treatment, majority of hormone responsive tumors acquire hormone resistance and relapse as incurable metastatic tumors.

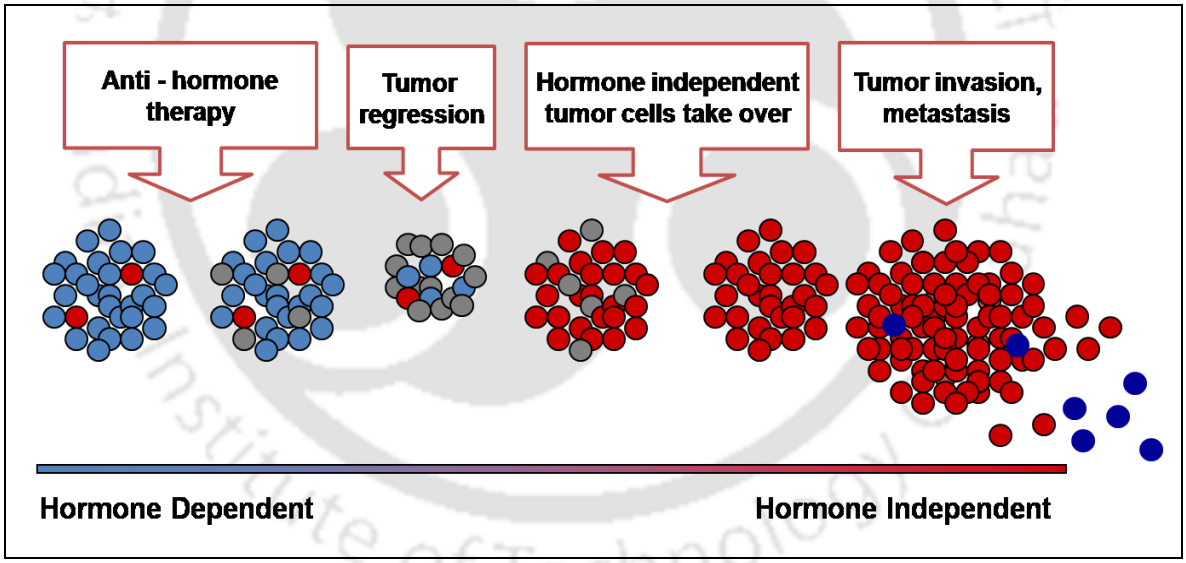


Figure 2.5. A schematic showing emergence of hormone independent state in breast cancer progression. Red circles represent hormone independent cells, blue circles represent hormone responsive cells and grey circles represent tumors cells targeted by endocrine therapy undergoing cell death.

Breast cancer cells which inherently lack ER expression become *de novo* resistant to endocrine therapy and therefore more than 90% of ER-negative tumors do not respond to anti-estrogen therapy. Mechanisms that have been proposed to explain the emergence of acquired hormone resistance in breast cancer may be broadly divided into three categories: (1) Loss or down-regulation

of ER expression, (2) aberrant ER signaling and (3) ligand independent ER activation. Clonal selection of ER-negative and estrogen insensitive cells during endocrine therapy has been reported to be a major cause of loss of ER expression (Sonoo *et al.*, 1999). Hypermethylation of ER promoter may also lead to its repression during tumorigenesis (Lapidus *et al.*, 1998). Furthermore, hypoxic microenvironment in the tumor due to tumor size and hormonal factors has been also demonstrated to repress ER expression by HIF-1α mediated proteasomal degradation of ER (Hockel and Vaupel, 2001; Stoner *et al.*, 2002).

Experimental and clinical evidences suggest that overexpression of HER2/EGFR family of growth factor receptors is closely associated with development of endocrine resistance, particularly to tamoxifen (Massarweh and Schiff, 2007). The cross talk among alternative (non-classical) signaling pathways has been also shown to activate ER in the absence of ligand mainly by the phosphorylation of ER at different sites (Al Saleh *et al.*, 2011; Osborne *et al.*, 2001). The discovery that estrogen independent activation of ER and tumor growth may occur via alternative signaling pathways involving epidermal growth factor receptor (EGFR), HER-2, MAPK, PI3K and vascular endothelial growth factor (VEGF) receptors, have prompted the development of novel therapeutic agents in breast cancer management.

In metastatic breast cancer, a missense mutation at tyrosine 537 residue in the ligand binding domain of ER protein has been reported (Weis *et al.*, 1996). This mutant ER was found to be constitutively active in the absence or presence of any ligand (E2 or tamoxifen) and has been associated with the development of hormone independent state in breast tumorigenesis. However, its clinical significance is questionable as other studies have reported this mutation only in 1% of primary breast cancer cases, most of which cause no change at the protein level (Roodi *et al.*, 1995). Furthermore, upregulated AKT activity in breast cancer cells causes phosphorylation at serine 167 residue in the ER protein, resulting in its activation (Campbell *et al.*, 2001). Interestingly, elevated AKT1 activity and AKT3 overexpression has been linked to estrogen independent growth and aggressive phenotype of breast tumors (Faridi *et al.*, 2003; Sun *et al.*, 2001). Other factors reported to cause endocrine resistance include drug pharmacokinetics, host endocrinology and immunological changes (Clarke *et al.*, 2001).

TUMOR INVASION AND METASTASIS

Metastasis is a complex, multistep process in which tumor cells escape from the primary site of tumor formation by invading through the basement membrane. They enter the vasculature, survive in the absence of adhesion, extravasate and finally establish a new tumor at a distant site in a favorable microenvironment. Among various molecular players involved in this process, matrix metalloproteinases (MMPs), a family of zinc dependent endopeptidases, have emerged as important regulators.

Matrix remodelling and MMPs in breast tumorigenesis

The ECM in association with various stromal cell types such as fibroblasts, adipocytes, endothelial and immune cells constitute the breast microenvironment. The tumor microenvironment is now recognized as a major regulator of breast carcinogenesis and response to treatment (Place *et al.*, 2011). Tumor progression is a complex process that involves multiple steps and one of the pivotal steps in this process of carcinogenesis and metastasis is the degradation of the ECM, carried out by a range of proteases including the MMPs (Hadler-Olsen *et al.*, 2013). MMPs normally function in tissue remodeling and wound healing processes and are predominantly synthesized by fibroblast cells. Aberrant MMP activity has been implicated in a number of pathological conditions especially in tumor progression and metastasis. In addition to degrading ECM components, MMPs further contribute to tumorigenesis by activating various chemokines, cytokines, growth factors and adhesion molecules, which promote tumor cell proliferation (for example MMP-3 and -7 release insulin like growth factor from ECM) and angiogenesis (for example MMP-1,-2,-9 and -14 mediated activation of angiogenic factors) (Schor and Schor, 2001). Beside MMPs, multiple other cellular proteins such as ECM proteins, integrins and other adhesion signaling receptors are also involved in cell migration (Schor and Schor, 2001).

MMP structure and composition

Although all MMPs share a common multi-domain structure consisting of an N-terminal signal peptide domain, a pro-domain, a Zinc ion containing catalytic

domain and a hemopexin-like domain; some deviations from this general structure have been reported (Visse and Nagase, 2003). For example gelatinases (MMP-2 and MMP-9) contain three fibronectin type II-like modules inserted within their catalytic domain (Figure 2.6), matrilysins (MMP-7 and MMP-26) are devoid of the hemopexin-like domain and the membrane-type MMPs contain a transmembrane domain at the C-terminal end of the hemopexin-like domain (Murphy *et al.*, 1994). These inherent unique structural variations present opportunities for designing novel therapeutic strategies selectively targeting a particular MMP class.

The signal peptide is hydrophobic in nature and acts as a secretory signal for the enzyme to be transported out of the cells.The Pro-domain shields the already formed active site in the zymogen (Pro-MMP) and blocks substrate access, thus keeping enzyme in an inactive or latent state. A mechanism called "cysteine switch" where the sulfhydryl group of the conserved cysteine residue coordinates with the zinc ion present in the catalytic centre, maintains the MMP in a latent state (Van Wart and Birkedal-Hansen, 1990). The basic architecture of the catalytic domain, known as the matrixin fold, consists of a five-stranded β sheet and three α helices. This structure is highly conserved in MMPs and is unaffected by insertion of the fibronectin domains. MMP-2 and MMP-9 have three repeats of fibronectin-type II domain inserted between the fifth β strand and helix 2 in the catalytic domain. The catalytic domain consists of a catalytic zinc ion and a zinc-binding (HEXXHXXGXXH) motif required for the Zn^{2+}-binding in the active site of the enzyme (Visse and Nagase, 2003). The C-terminal hemopexin-like domain determines the substrate specificity of the MMPs. The hemopexin domain is connected to the catalytic domain through a small proline-rich hinge region.

Regulation of MMP activity

MMPs are stringently regulated at multiple levels which include regulation at transcriptional, post-transcriptional and translation levels as well as their interaction with activators or inhibitors and cell surface localization. Most MMPs have overlapping substrate specificities. Hence the biological function of a particular MMP is largely dictated by their variable pattern of expression and is often indicative of their unique roles. In line with this, most MMPs are controlled at the transcriptional level, except MMP-2 which is regulated via a unique

activation mechanism and post-transcriptional mRNA stabilization (Overall *et al.*, 1991; Strongin *et al.*, 1995). Various inducible and suppressive stimuli affecting multiple signalling pathways regulate MMP gene expression. For example, cytokines and growth factors including interferons, interleukins, VEGF, tumor necrosis factor α (TNFα), platelet derived growth factor (PDGF) and the extracellular matrix metalloproteinase inducer EMMPRIN influence expression of MMPs by inducing the activation or expression of *c-fos* and *c-jun* proto-oncogenes. These gene products heterodimerize and bind to MMP gene promoters harboring activator protein-1 (AP-1) sites (Sternlicht and Werb, 2001). In some cases, same signal may have variable effects on different MMP promoters; for example, TGF-β represses MMP-1 and MMP-3 while it has an inducible effect on the transcription of MMP-13 (Uria *et al.*, 1998).

At the post-transcriptional level, several MMP transcripts are stabilized by mRNA stabilization; for example, phorbol esters and EGF stabilize MMP-1 and MMP-3 mRNA and PDGF and glucocorticoids stabilize MMP-13 transcripts (Delany *et al.*, 1995; Vincenti, 2001). The mRNA turnover in the case of MMP-1 was shown to be regulated by AU-rich sequence elements in the 3'-UTR (Vincenti, 2001). Most of the MMPs are constitutively secreted after translation but there are reports which suggest that secretory control does play a role in the regulation of some MMPs. In bone marrow, differentiating granulocytes synthesize MMP-8 and MMP-9 and store them in the granules of circulating neutrophils until its activation during inflammation (Hasty *et al.*, 1990). Most MMPs are first synthesized and secreted as pro-enzymes and maintained in a latent state, which are later activated by cysteine switch mechanism, when required for a cellular function. Unlike other MMPs, MMP-11, MMP-27 and MT-MMPs harbor an RXK/RR furin–like recognition motif are activated by intracellular subtilisin-type serine proteases, thus allowing them to be activated before they reach cell surface or secreted out of the cell (Pei and Weiss, 1995). Outside the cell, latent MMPs can be activated by already activated MMPs or by serine proteases. As an exception, MMP-2 is activated at the cell surface by a unique mechanism involving MT-MMPs and TIMP-2 and is thus refractory to activation by serine proteases (Strongin *et al.*, 1995).

TIMPs: Endogenous MMP inhibitors

TIMPs are natural inhibitors of MMPs and are involved in diverse biological processes including cell growth, tumor progression, apoptosis, invasion, metastasis and angiogenesis (Visse and Nagase, 2003). Four different TIMP (TIMP-1, -2, -3, -4) proteins are described in human and all four inhibit active form of most MMPs but with different affinities, by binding to the enzyme in a 1:1 stoichiometry through strong non-covalent interactions (Bode *et al.*, 1999) [Figure 2.6].

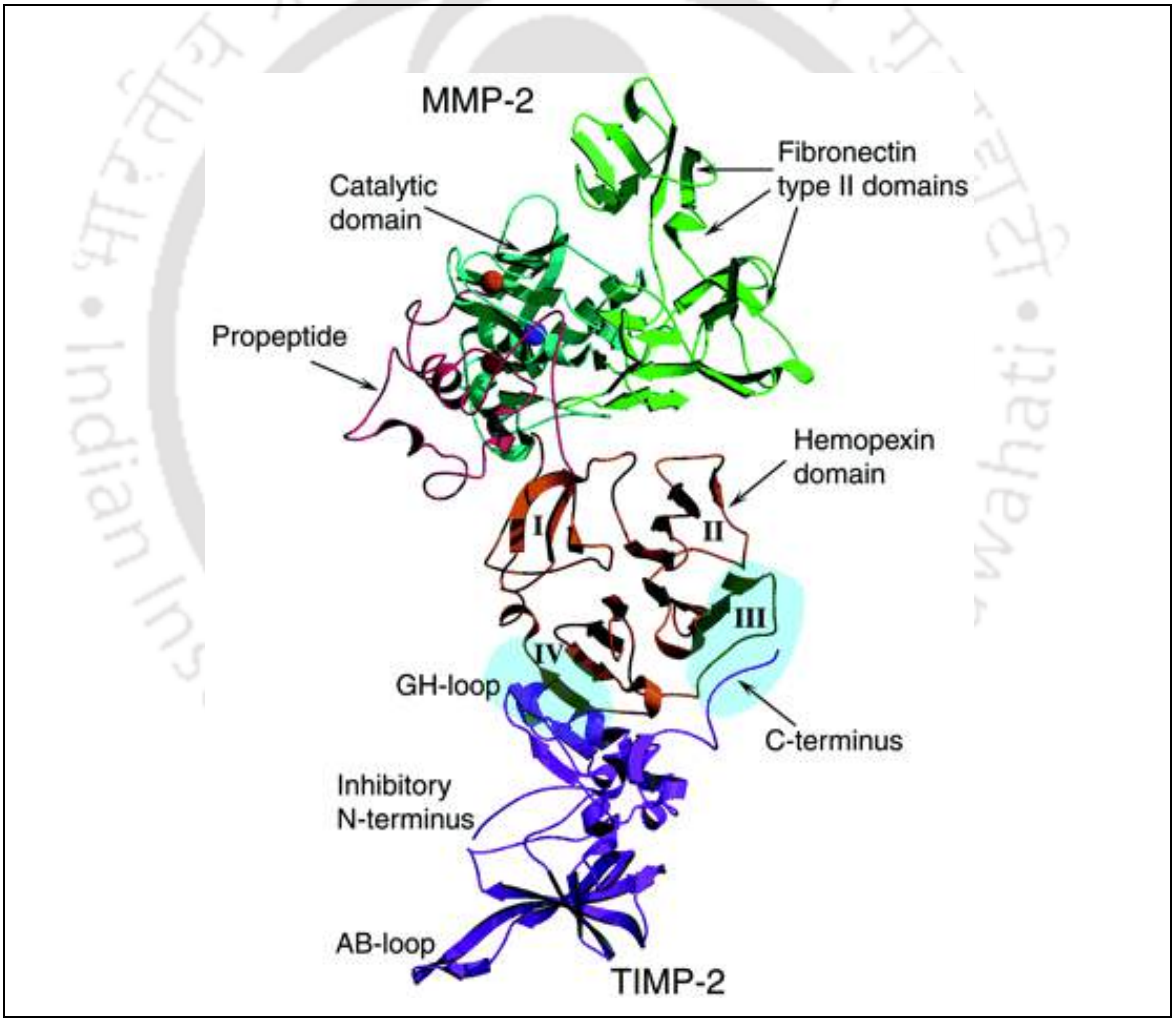


Figure 2.6. Structure of the pro MMP-2 /TIMP-2 complex. Adapted from Morgunova *et al*. (2002)

MMP inhibitors – development and failures

Till date, many different MMP inhibitors (MMPIs) have been designed to target MMPs in cancer (Hidalgo and Eckhardt, 2001). The first generations of MMPIs were peptidomimetics that mimic the structure of collagen at the site where MMPs bind to cleave it. They act as competitive inhibitors and chelate the zinc ion present at the MMP's active site (Hidalgo and Eckhardt, 2001). Most of these inhibitors in clinical development were hydroxamate derivatives such as Batimastat and Marimastat but they failed in clinical trials due to their low bioavailability and poor specificity for the MMPs thought to be involved in cancer progression (Day and Cohen, 2013). In order to combat these problems, non-peptidomimetic inhibitors were designed on the basis of the three-dimensional x-ray crystallographic conformation of the MMP active site. Some of the drugs tested in this category were tanomastat, prinomastat, BMS-275291 and CGS27123A. Results from these trials were disappointing and required early termination due to the poor survival of patients caused by dose-limiting toxicity. Some other classes of drugs which were tested for MMP inhibition include Tetracyclines and Biphosphonates (Fisher and Mobashery, 2006). However, all clinical trials of these MMPIs in advanced cancer patients have failed.

MECHANISMS OF BREAST TUMORIGENESIS

Breast tumorigenesis involves succession through defined clinical and pathological stages. It begins with hyper-proliferation of ductal epithelial cells, followed by progression into *in situ* and invasive carcinomas, and finally metastasis (Figure 2.7). At the molecular level, breast cancer initiates due to transforming events (genetic and epigenetic alterations) in a single cell. Accumulation of additional genetic changes combined with clonal expansion and selection drives tumor progression. Aberrant changes in breast tumor microenvironment (composed of extracellular matrix, fibroblasts, endothelial cells, myoepithelial cells, leukocytes and other cell types) also plays an important role in tumorigenesis.

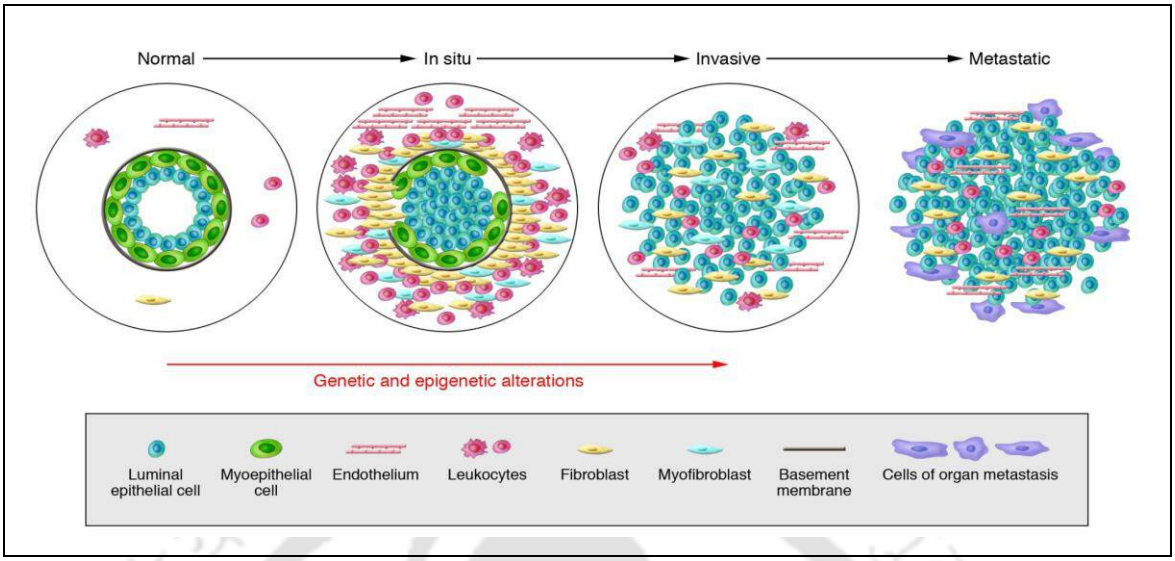


Figure 2.7. Hypothetical model of breast tumorigenesis. Reproduced with permission from Polyak (2007)

Genetic alterations and breast cancer

Inherited genetic mutations and acquired genomic aberrations are known to drive breast tumorigenesis. Germ line mutations in high-penetrance breast cancer susceptibility genes such as *BRCA1, BRCA2* and *TP53* have been reported to account for less than 25% of excess risk. However some recent studies have identified allelic variations and single nucleotide polymorphisms (SNPs) in moderate – and low- penetrance genes such as FGFR2, CASP9, LSP1, MAP3K1 and TNRC9 to be also associated with increased breast cancer risk (Cox *et al.*, 2007; Easton *et al.*, 2007; Hunter *et al.*, 2007; Stacey *et al.*, 2007).

High throughput next generation sequencing (NGS) of breast tumor genomes from all major molecular subtypes have discovered some key somatic alterations driving breast carcinogenesis. Some major studies in recent years have revealed the genetic landscape of breast cancer based on the sequencing results from some of the largest ever breast cancer datasets reported till now (Banerji *et al.*, 2012; Curtis *et al.*, 2012; Ellis *et al.*, 2012; Shah *et al.*, 2012; Stephens *et al.*, 2012). Stephens and co-workers studied somatic copy number changes and mutations in 100 breast tumor genomes, and identified mutations in new cancer genes such as *AKT2, ARID1B, CASP8, CDKN1B, MAP3K1, MAP3K13, NCOR1, SMARCD1* and *TBX3* that could emerge as key therapeutic targets

(Stephens *et al.*, 2012). Assessment of mutations, copy number and gene expression pattern in triple negative breast cancers highlighted that mutations may arise at several stages of tumorigenesis as the copy number aberrations and mutations vary significantly within the tumors (Shah *et al.*, 2012). Ellis and colleagues assessed the genomes of ER positive breast tumors and showed that tumors having large number of cells expressing Ki67, a protein associated with resistance to aromatase inhibitors, have high frequency of somatic mutations compared to tumors harboring low Ki67 positive cells (Ellis *et al.*, 2012). Some of the most frequently mutated genes in breast cancer have been summarized in Table 2.1.

Table 2.1. Top 21 most commonly mutated genes in breast cancer. Mutation frequencies (%) in all tumors or just within luminal (including $HER2^{+}$) and triple negative breast cancer (TNBC) subtypes

Gene	All (%)	Luminal	TNBC
TP53	35	26	54
PIK3CA	34	44	8
GATA3	9	13	0
MAP3K1	8	11	0
MLL3	6	8	3
CDH1	6	8	2
USH2A	5	4	8
PTEN	3	3	3
RUNX1	3	4	0
MAP2K4	3	4	1
NCOR1	3	3	1
RB1	3	2	5
TBX3	2	3	1
PIK3R1	2	3	2
CTCF	2	2	1
NF1	2	2	1
SF3B1	2	2	0
AKT1	2	2	0
CBFB	2	2	1
FOXA1	1	1	1
CDKN1B	1	1	0

Table reproduced with permission from Polyak and Metzger Filho (2012)

Epigenetic modifications and breast cancer

DNA methylation at CpG islands in the promoter region and covalent chemical modification of less structured, protruding N-terminal tails of core histones by methylation, acetylation, ubiquitination and phosphorylation, have emerged as two integral mechanisms of epigenetic regulation (Jones and Baylin, 2002). These epigenetic alterations in DNA which do not alter the nucleotide sequence are inheritable and potentially reversible unlike genetic changes. The transcription state of any gene may be predicted by deciphering the histone modification pattern at the promoter which is often referred to as the *Histone Code* (Sims and Reinberg, 2008).

DNA methyltransferases (DNMTs) catalyze addition of a methyl group at position 5 of a cytosine ring in the CpG dinucleotide sequences, which are densely enriched in gene promoters (Jones and Takai, 2001). DNMT1 is required for ensuring the maintenance of methylation patterns throughout each cell division whereas DNMT3A and 3B are *de novo* methyltransferases (Klose and Bird, 2006). In mammalian cells, DNA methylation is involved in controlling normal gene expression patterns; it has been shown to regulate processes such as X-chromosome inactivation and genomic imprinting, among several others (Csankovszki *et al.*, 2001; Jones and Takai, 2001; Kaneda *et al.*, 2004). During oncogenesis, silencing of critical tumor suppressor genes is often associated with aberrant DNA methylation patterns at their promoter regions (Jones and Baylin, 2002).

In addition to promoter DNA hypermethylation, two discrete histone modifications i.e. histone H3 lysine 27 trimethylation (H3K27me3) and histone H3 lysine 9 dimethylation (H3K9me2) catalyzed by histone lysine methyltransferases, contribute significantly to epigenetic silencing mechanism in mammals (Cao and Zhang, 2004; Jenuwein and Allis, 2001; Jenuwein *et al.*, 1998; Stewart *et al.*, 2005; Tachibana *et al.*, 2005). H3K27me3 mediated gene silencing involves evolutionarily conserved family of chromatin modulators called Polycomb group (PcG) proteins (Schuettengruber *et al.*, 2007). PcG proteins represent multi-protein complexes, which include the Polycomb repressive complexes: PRC1 and PRC2. H3K27me3 is a distinct histone modification catalyzed by histone methyltransferase enhancer of zeste homolog 2 (EZH2),

which is the catalytic component of PRC2, involved in the regulation of homeotic (Hox) gene expression and in the early steps of X-chromosome inactivation in women (Boyer *et al.*, 2006; Schuettengruber and Cavalli, 2009). PcG proteins play cardinal roles in establishing the repressed state of several genes during development and maintenance of embryonic stem cell (ESC) identity and pluripotency. Recent studies imply that PcG proteins are often deregulated in various cancer types and their over-expression is closely associated with carcinogenesis (Chase and Cross, 2011; Sauvageau and Sauvageau, 2010; Sparmann and van Lohuizen, 2006). EZH2 catalyzed H3K27me3 may serve as a docking site for PRC1, as well as DNA methyltransferases (DNMTs) (Figure 2.8). EZH2 mediated H3K27me3 may be an independent mechanism of epigenetic silencing of tumor suppressor genes in cancer (Kondo *et al.*, 2008; Rush *et al.*, 2009). Recent findings implicate that EZH2 is over-expressed in a wide range of cancer types especially in endocrine related cancers such as breast and prostate cancers (Kleer *et al.*, 2003; Varambally *et al.*, 2002).

Polycomb group (PcG) proteins – composition, recruitment and action

PcG proteins are a phylogenetically conserved group of multimeric protein complexes, initially characterized in Drosophila for their crucial role as negative regulators of homeotic genes required for segmentation (Lewis, 1978). PRC1 composition is variable and its core components in mammals include Bmi1 (also known as Pcgf4), Ring1b, Ring1a, and CBX proteins, PH1, PH2, NSPC1 (Pcgf1) and MEL18 (Pcgf2) (Levine *et al.*, 2002; Yu *et al.*, 2012). Ring 1a and Ring 1b ubiquitin ligases catalyzes mono-ubiquitination of Histone 2A at Lys 119 (H2AK119ub), which causes PRC1 dependent gene silencing (Wang *et al.*, 2004). ncRNA (non coding RNA) and H3K27me3 facilitate PRC1 recruitment to the target genes (Yap *et al.*, 2010). It blocks the ATP-dependent remodelling activity of SWI/SNF *in vitro* and thus maintains the repressed state of genes (Shao *et al.*, 1999) (Figure 2.8).

The mammalian PRC2 core complex consists of four components: EZH1/2, SUZ12, EED and RbAp46/48 (also called RBBP7/4) that catalyses the di-/tri-methylation of Histone 3 at lysine 27 (Margueron *et al.*, 2008; Schuettengruber *et al.*, 2007). Recent reports suggest that there are additional PRC2 components - AEBP2, PCLs and JARID2, which function as accessory units regulating the

function and enzymatic activity of PRC2 holoenzyme (Kim *et al.*, 2009; Nekrasov *et al.*, 2007; Walker *et al.*, 2010) (Figure 2.8). Despite the dynamic composition of PRC2, EZH1/2 remains its integral component for catalyzing H3K27 di/trimethylation (Abel *et al.*, 1996). EZH1 and EZH2 are two paralogs and PRC2-EZH1/PRC2 –EZH2 complexes function differently to maintain the repressed state of chromatin (Margueron *et al.*, 2008). EZH1 expression has been reported in dividing as well as differentiated cells whereas EZH2 is found in only proliferating tissues (Bracken *et al.*, 2003; Margueron *et al.*, 2008). Since both are part of PRC2 complexes, they regulate an overlapping array of target genes. Intriguingly, PRC2-EZH1 has low histone methyltransferase (HMTase) activity compared to EZH2 containing PRC2 complex, which suggests that the H3K27me3 repressive mark is established by PRC2-EZH2 (Margueron *et al.*, 2008). HMTase activity of PRC2-EZH1 may function in restoring the repressive methylation pattern (H3Kme2/-me3) of histones that could have resulted after demethylase activity or histone exchange.

EZH2 and its role in PRC2 complex

Human *EZH2* gene maps to the long arm of chromosome 7 at position 7q35 and encodes 746 amino acids protein, which belongs to histone-lysine methyltransferase family (Cardoso *et al.*, 2000). EZH2 contains several functional domains which are involved in mediating its interaction with other PRC2 and regulatory proteins besides histone methyltransferase activity. The major domains include CXC (cysteine-rich domain), SET (Su(var)3-9, enhancer of zeste, trithorax domain), ncRBD (non-coding RNA-binding domain) and a DNA binding domain (Margueron and Reinberg, 2011).

Although mutations in other PRC2 members have not been reported so far, recent studies in lymphoma and myeloid neoplasms have identified EZH2 mutations that may either cause gain-of-function or complete loss of histone methyltransferase activity (Chase and Cross, 2011). A heterozygous missense somatic mutation at Tyrosine (Y) 641 in the SET domain of EZH2 results in enhanced H3K27me3 levels in follicular lymphomas and diffused large B-cell lymphomas (Morin *et al.*, 2010). Interestingly in myeloid neoplasms, inactivating EZH2 mutations were found to be distributed throughout the gene, comprising of

missense, nonsense and premature stop codons, which lead to the loss of histone methyltransferase activity (Ernst *et al.*, 2010; Nikoloski *et al.*, 2010).

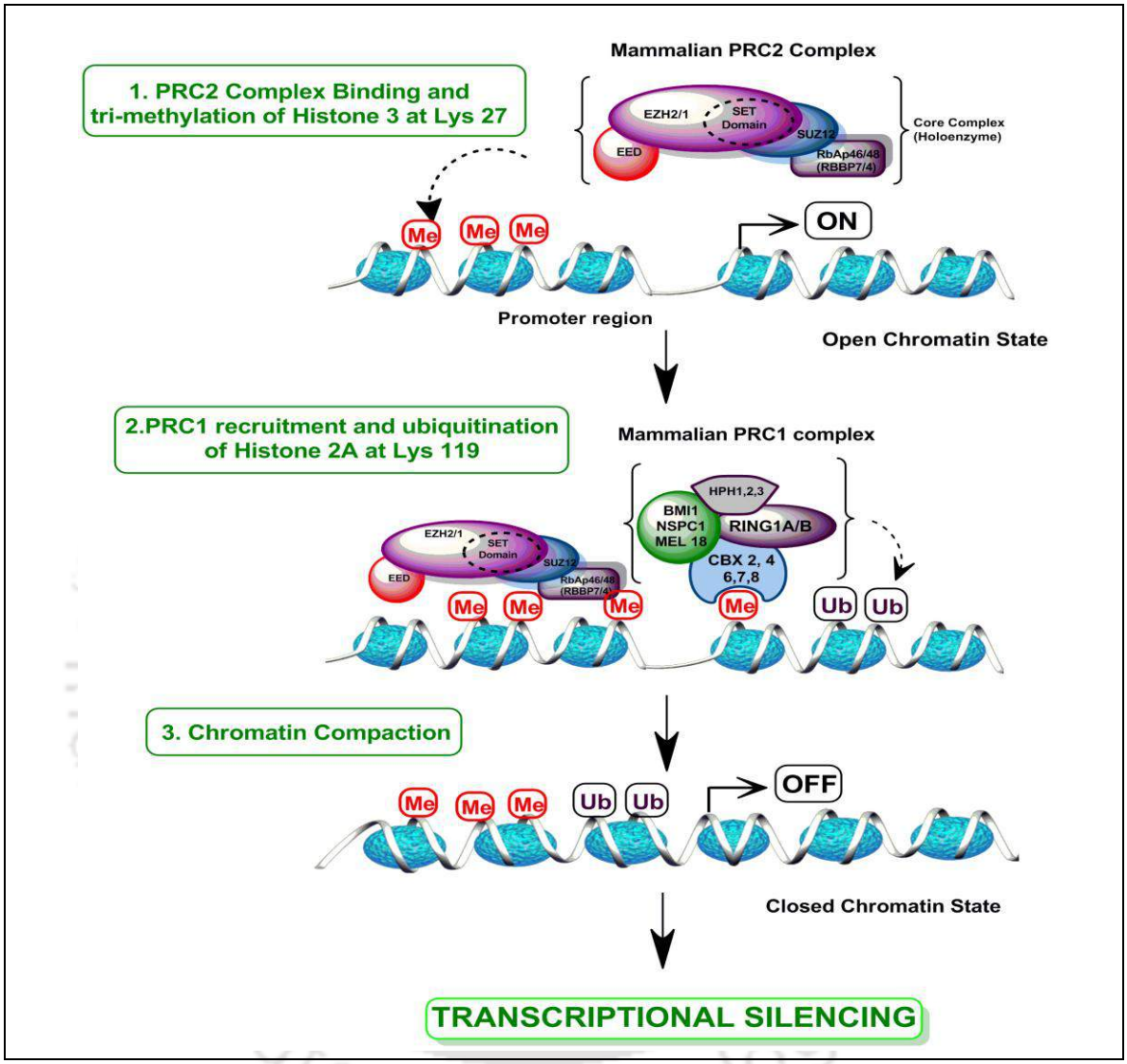


Figure 2.8. Polycomb dependent mechanism of EZH2 action in transcriptional repression. Reproduced from Deb *et al.* (2013)

EZH2 protein is subjected to various post-translational modifications. Phosphorylation of EZH2 by AKT1 at Serine-21 reduces its H3K27me3 activity whereas phosphorylation at Thr-345 by CDK1 and CDK2 is required for maintenance of H3K27me3 repressive marks at target gene promoters (Cha *et al.*, 2005; Chen *et al.*, 2010). Furthermore, a recent study demonstrated that EZH2 and SUZ12 are potential targets for sumoylation in both *in vivo* and *in vitro* conditions (Riising *et al.*, 2008). In Drosophila PcG proteins are recruited to

polycomb response elements (PREs) containing target genes with the help of several other DNA binding proteins such as GAF, Pipsqueak, Zeste, or PHO . In mammals, PRC2 is targeted to CpG islands which are GC rich sequences but surprisingly no consensus sequence elements for PcG recruitment have been identified to date in mammals (Margueron and Reinberg, 2011). Recent reports suggest that YY1, the mammalian orthologue of the Drosophila PRE DNA-binding protein PHO and RYBP which interacts with both PcG proteins are involved in the PRC2 recruitment process (Ku *et al.*, 2008; Wilkinson *et al.*, 2006; Xi *et al.*, 2007). Long ncRNAs have also emerged as potential elements involved in PRC2 recruitment. HOTAIR ncRNA promotes PRC2 recruitment *in trans* and is associated with the transcriptional repression of HOXD locus (Rinn *et al.*, 2007; Tsai *et al.*, 2010). Similarly XIST and KCNQ1OT1 ncRNAs have also been implicated in PRC2 gene targeting (Kohlmaier *et al.*, 2004; Maenner *et al.*, 2010; Pandey *et al.*, 2008; Plath *et al.*, 2003).

There are two possible molecular mechanisms for EZH2 action based on its role as a transcriptional activator or repressor. As an integral component of PRC2, the canonical role of EZH2 is of a histone methyltransferase (Figure 2.8). SET domain of EZH2 catalyze methylation of lysine in succession such that each methylation event serve as a substrate for the next (H3K27me2 is mono-methylated to form H3K27me3) and each methylation mark represent functionally distinct chromatin state. H3K27me3 has been implicated in the recruitment of PRC1 complex, suggesting that both PcG protein complexes function in gene silencing. However there are PRC2 target genes that lack H2AK119ub and genes targeted by PRC1 in the absence of PRC2, highlighting the discrepancy in the exact functional relationship between both protein complexes (Eskeland *et al.*, 2010; Ku *et al.*, 2008; Schoeftner *et al.*, 2006). Trimethylation of H3 may pose steric hindrance for RNA Pol II and other proteins binding to target gene promoters and repress transcription.

The second mechanism of EZH2 action highlights its lesser known role as a transcriptional inducer. Xu *et al.* (2012) reported that in castration resistant prostate cancer (CRPC), EZH2 functions independent of other PRC2 members and Akt-1 mediated phosphorylation of EZH2 at serine 21 allows it to interact with androgen receptor (AR) at many solo genes. They also demonstrated that depletion in EZH2 does not alter AR levels but there is reduction in AR-

associated lysine methylation (Lys-630 and 632). AR methylation catalyzed by Set 9 enhances its transcriptional activity (Gaughan *et al.*, 2011; Ko *et al.*, 2011). Therefore, methylation of AR or AR-associated proteins is a potential mechanism for EZH2 driven transcriptional activation.

In breast cancer cells, EZH2 has been reported to induce gene transcription in two different ways depending on the ER status (Figure 2.9). In luminal-like MCF-7 breast cancer cells, EZH2 acts as a bridge to physically link ERα and Wnt signaling components β-catenin and TCF, on the cyclin B1 and c-Myc promoters (Shi *et al.*, 2007). EZH2 also interacts with Mediator complex through its domain II independent of the SET domain involved in HMTase activity and induces transcription by its interaction with RNA polymerase II. In ER-negative, basal like MDA-MB-231 cells, EZH2 forms a ternary complex with NF-κB components RelA and RelB and activates transcription of NF-κB target genes such as TNF and IL6 (Lee *et al.*, 2011). In both types of cancer, although EZH2 functions independent of PRC2 as a transcriptional activator, the mechanisms are different.

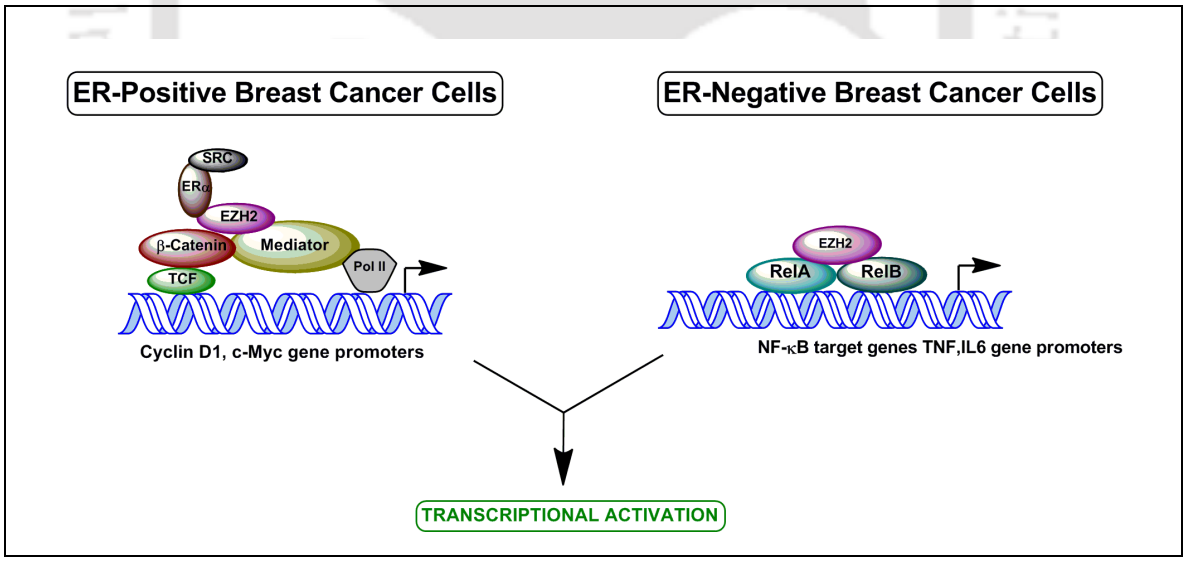


Figure 2.9. Polycomb independent mechanism of EZH2 action in human breast cancer cells. When over-expressed in ER-positive luminal like MCF-7 breast cancer cells, EZH2 functions as a transcriptional activator by acting as a bridge to physically link ERα and Wnt signalling components β-catenin and TCF, on the Cyclin B1 and c-Myc promoters. It was also demonstrated that EZH2 also associates with Mediator complex through its domain II independent of the SET domain involved in HMTase activity and might enhance transcription by its interaction with RNA polymerase II. In ER-negative, basal like MDA-MB-231 cells, EZH2 forms a ternary complex with NF-κB components RelA and RelB and activates transcription of NF- κB target genes such as TNF, IL6. Reproduced from Deb *et al.* (2013)

Crosstalk between H3K27me3 and DNA methylation

Epigenetic modulations such as aberrant promoter DNA hypermethylation and histone modifications, which govern chromatin remodelling, have emerged as important players in tumor onset and progression. Interesting experimental studies addresses the functional relationship between PcG proteins and DNA methylation that leads to epigenetic inactivation of tumor suppressor genes in various cancer types. Previous studies showed that the crosstalk between DNA methylation and histone modifications was mediated by a group of proteins possessing methyl DNA binding characteristics such as methyl CpG binding protein 2 (MeCP2), methyl CpG binding domain protein 1 (MBD1) and Kaiso/ ZBTB 33 (Zinc finger and BTB domain containing protein 33)(Jones *et al.*, 1998; Nan *et al.*, 1998). These proteins bind to methylated CpG islands on target gene promoters and recruit histone deacetylases (HDACs) and histone methyltransferases containing protein complexes, adding further to target gene silencing. But the evidences linking DNA methylation and PRC-mediated silencing are partial and sometimes conflicting. Recent technological developments have led to multiple mechanistic hypotheses on the underlying process.

Early studies in Drosophila and *C. elegans* suggest the involvement of PRC2-mediated repressive machinery in gene silencing but no detectable DNA methylation in the chromatin (Simon and Lange, 2008). Vire *et al.* (2006) first reported the role of EZH2 and its H3K27me3 repressive mark in the recruitment of DNMTs to EZH2 target promoter in human osteosarcoma cells. They described that EZH2 is essential for the binding of DNMT but DNMT is not required for EZH2 or H3K27me3 binding to the MYT1gene promoter. Bisulfite genomic sequencing data of MYT1 and WNT1 promoters in EZH2 over-expressing and knockdown cells showed that EZH2 is required for CpG methylation of EZH2 target promoters. McGarvey *et al.* (2007) demonstrated that EZH2 might act as an epigenetic modulator when promoter is not densely methylated. They showed that EZH2 knockdown leads to increased expression of unmethylated or basally expressing genes but not hyper-methylated completely silenced genes such as $p16^{INK4a}$ in U2OS cells. Contrary to previous reports, they found that EZH2 depletion does not reduce promoter DNA methylation of the target genes and

proposed a dominant role of dense CpG island DNA methylation in maintaining the heritable repressive state of target genes as compared to various other repressive chromatin marks.

To further address the issue of PcG proteins recruiting DNMTs and establish a functional link between them, Kondo *et al.* (2008) analyzed DNA methylation at several H3K27me3 modified CpG islands in human prostate cancer cell line PC3 and breast cancer cell line MCF-7. Using bisulfite pyro-sequencing in 2-6 adjacent CpG sites in PC3 cells, they confirmed that methylation was very low in the candidate genes ranging from 6.3% to 39.3% and only three out of eight genes having methylation above 15%. Such low levels of DNA methylation compared to what has been generally observed, is insufficient in causing silencing of tumor suppressor genes. Global assessment of DNA methylation and H3K27me3 in PC3 and MCF-7 cells focusing on genes detectable on both ChIP-chip and DNA methylation arrays, using methylated CpG island microarrays, revealed that H3K27 based silencing and DNA methylation are independent events. However there was some overlap between H3K27me3 and DNA methylation which suggest that there may be genes targeted by both silencing mechanisms, although it seems relatively rare.

Absence of DNA hypermethylation at PRC occupied regions in embryonic carcinoma cells and lack of DNA hypomethylation after EZH2 knockdown in cancer cells hint at different gene sets being targeted for repression by these two silencing machineries. However some gene profile studies comparing chromatin states in tumor cells versus cancer cells reveal that H3K27me3 enriched EZH2 target genes in normal cells subsequently become abnormally hyper-methylated in tumor cells during cellular transformation (Ohm *et al.*, 2007; Schlesinger *et al.*, 2007; Widschwendter *et al.*, 2007). In conclusion, the current view supported by comprehensive genome-wide and functional analysis suggest that DNA methylation and polycomb-mediated silencing collaborate in epigenetic silencing of certain genes depending on cellular context but is probably not as directly related as previously suggested by some reports.

Crosstalk between H3K27me3 and other histone modifications

Histone modification is a well conserved and important mechanism of transcriptional regulation. Emerging evidences suggest that the modification patterns of histone can be linked to their biological function and act as a 'histone code', to predict the transcriptional states of genes. Histone modifications at positions H3-K4 (trimethylation), H3-K9/18 (Acetylation) at promoter/transcription start site regions and H3-K36 (dimethylation) at 5-coding regions, are hallmarks of actively transcribed genes (Barrand *et al.*, 2010; Barski *et al.*, 2007; Bell *et al.*, 2007). Many studies comprehensively describe H3 K4me3 and H3K36me2 antagonizes polycomb mediated silencing at H3K27 (Schmitges *et al.*, 2011; Yuan *et al.*, 2011). This inhibition requires the presence of these methylation marks on the same histone tail subject to EZH2 mediated K27 methylation. Similarly, H3K27 acetylation and H3K27me3 are mutually exclusive. Hence, acetylation of H3 at K27 functionally antagonizes PRC2 mediated silencing in both mammalian and fly systems (Pasini *et al.*, 2010; Schwartz *et al.*, 2010; Tie *et al.*, 2009). This highlights the role of HDACs in EZH2 mediated silencing although they are not core subunits of PRC2. HDACs could deacetylate K27 side chains to make available ε-amino groups for methylation by PRC2 complex. Also, two recent studies establish that phosphorylation of serine-28 (Ser28) located immediately to H3K27 by kinases MSK1 and MSK2, also disrupt the functioning of PcG silencing pathway (Gehani *et al.*, 2010; Lau and Cheung, 2011).

In summary, recent literature advocates the fact the PRC2-mediated gene silencing mechanism(s) has evolved to sense the surrounding epigenetic landscape and subject to cues from other chromatin marks based on the transcriptional state of the chromatin (O'Meara and Simon, 2012). But further studies addressing the structural organization of PRC2 active site and its regulatory domains are needed to completely unravel its mechanism of inhibition.

Molecular insights into EZH2 mediated Breast tumorigenesis

Several experimental studies have established the fact that elevated EZH2 levels in human breast carcinomas is associated with the aggressive ER-negative basal-like phenotype characterized by lack of ER expression, nuclear polymorphism

and lack of BRCA1 protein (Bachmann *et al.*, 2006; Collett *et al.*, 2006; Gonzalez *et al.*, 2009; Kleer *et al.*, 2003). It represses several essential tumor suppressor genes ultimately leading to metastasis in breast carcinomas (Table 2.2) (Du *et al.*, 2012; Fujii *et al.*, 2008; Lee *et al.*, 2011; Ren *et al.*, 2012; Taniguchi *et al.*, 2012; Truax *et al.*, 2012; Yang *et al.*, 2009; Zeidler *et al.*, 2005). Deregulation of EZH2 contributes to ER-negative breast cancer progression. EZH2 knockdown in breast cancer cells causes decreased proliferation and delayed the G2/M cell-cycle transition and *in vivo* studies demonstrate that EZH2 down regulation significantly decreased breast xenograft growth and improved survival (Gonzalez *et al.*, 2009). EZH2 knockdown upregulated BRCA1, which is a nuclear protein that plays a major role in DNA repair and estrogen receptor modulation (Gonzalez *et al.*, 2011).

The proposed mechanisms underlying EZH2 overexpression which ultimately causes uncontrolled cell proliferation leading to tumorigenesis in breast cancer cells are summarized in Figure 2.10. Bracken *et al.* (2003) reported that the expression of two PRC2 complex components i.e. EZH2 and EED is regulated by E2F transcription factors. Upon pRB phosphorylation, E2F dissociates from pRB-E2F complex and the activated E2F directly binds to the promoters of EZH2 or EED containing putative E2F binding sites and transactivate their transcription. The study further confirmed that although the abrogation of EZH2 or EED expression could significantly decrease positive regulators of cell proliferation such as cyclinD1 (CCND1), cyclinE1 (CCNE1), cyclinA2 (CCNA2) and cyclinB1 (CCNB1), there was no increase in the expression of negative regulators of cell cycle e.g. p14ARF. This study provides a direct link between pRB-E2F pathway mediated growth control and PcG regulated essential chromatin modifications.

In another study, Fujii *et al.* (2011) reported that the MEK-ERK-Elk-1 pathway, which is commonly upregulated in various cancer types, is linked to EZH2 overexpression in triple-negative and ERBB2-overexpressing breast cancer cell lines. Computational analysis revealed that EZH2 promoter harbor three Elk-1 binding motifs and other sequence elements for NF-κB, c-Myb, STAT1 and SRF (serum response factor) recruitment. Treatment with MEK inhibitor (U0126) decreased EZH2 levels in triple negative breast cancer type MDA-MB-231 cells and ERBB2-overexpressing SKBr3 cells. Also siRNA mediated knockdown of Elk-1, caused a significant decrease in EZH2 mRNA expression that was similar to

the decrease caused by U0126 treatment. Further they demonstrated that MEK inhibitor treatment significantly decreased association of phospho- Elk-1 with the EZH2 promoter in breast cancer cells. In conclusion the study suggests that MEK/ERK pathway activated via KRAS mutation, EGFR amplification and ERBB2 amplification in triple-negative and ERBB2-overexpressing cells causes EZH2 overexpression.

Under cancer-predisposed hypoxic conditions in breast tumor initiating cells (BTICs), EZH2 expression was reported to be regulated by HIF transcription factor (HIF1α). Chang *et al.* (2011) identified a consensus sequence for HIF response element (HRE) by promoter analysis of EZH2 promoter. They further demonstrated that a hypoxic microenvironment induces HIF1α binding to the HRE and transactivate EZH2 expression. Increased EZH2 expression down-regulates double-strand break repair protein RAD51 expression, which ultimately results in impaired DNA repair and accumulation of genomic abnormalities. Further they demonstrated that EZH2 mediated down-regulation of RAD51 causes expansion of self-renewing BTIC population and RAF1 amplification which further activate downstream p-ERK-β-catenin signaling. EZH2 mediated RAF1-ERK signaling promotes BTIC expansion and aggravates breast cancer condition.

Studies by Cha *et al.* (2005) have shown that Akt-mediated phosphorylation of EZH2 at a highly conserved serine 21 residue, inhibited its H3K27 methyltransferase activity. Although EZH2 phosphorylation had no impact on the integrity of PRC2 complex, there was marked decline in the affinity of EZH2 for Histone H3, which ultimately results in decreased H3K27me3. Interestingly, another study reported that elevated EZH2 in breast cancer cells induces phosphoinositide-3-kinase/Akt (PI3K/Akt) pathway by specifically activating Akt isoform 1(Gonzalez *et al.*, 2011). Increased EZH2 levels positively correlated with elevated Phospho-Akt1 (Ser473) and decreased nuclear localization of phospho-BRCA1 (Ser1423). Accumulation of BRCA-1 protein in the nucleus promotes tumorigenesis by causing aberrant mitosis, aneuploidy and genomic instability.

Table 2.2. Some metastasis associated target genes epigenetically repressed by EZH2 in Breast Cancer.

EZH2 target genes	Possible functional role in metastasis	Mechanism	Model system
FOXC1	Increased *in vitro* and *in vivo* migration and metastasis	Enhanced H3K27me3 and reduced H3 and H4 acetylation at the promoter	MCF-7 MDA-MB-231
$P57^{KIP2}$	Aberrant cell proliferation and tumor growth	Increased H3K27me3 Reduced H3K9/14 acetylation and H3K4me3 at the promoter	SK-BR-3 BT-474 MDA-MB-231 MCF10A
RUNX3	Increased cell proliferation	H3K27me3 and HDAC1 mediated repression	MCF-7 LNCaP
RKIP	Accelerates cell invasion and metastasis	H3K27me3 and H3K9me3 mediated repression and involvement of Snail in the recruitment of EZH2 repressive complex	T47D MCF-7 MDAMB231
CIITA	Decreased expression of cell surface MHC II which enables tumor cells to escape immune system	Enhanced H3K27me3 repressive mark at the promoter	MDA-MB-435 variants
Kruppel like Factor 2	Suppression of cell growth and anti-angiogenesis	Increase in H3K27me3 and depletion of phosphorylated serine 2 of RNA polymerase at the promoter	MDA-MB-231 MCF-7
NF-κB targets such as *IL6 ,TNF, BIRC3 and IL8*	Inflammation , oncogenesis and tumor dissemination	Binding of ternary complex containing EZH2 , RelA and RelB to target promoters positively modulate their expression and released cytokines induces a positive feedback loop resulting in constitutive expression of NF-κB	MDA-MB-231
IL6 , IL8		In ER positive cells PRC2 complex is recruited by ER and EZH2 functions as an epigenetic repressor of target genes by catalyzing H3K27me3	MCF-7 T47D

Table reproduced from Deb *et al*. (2013)

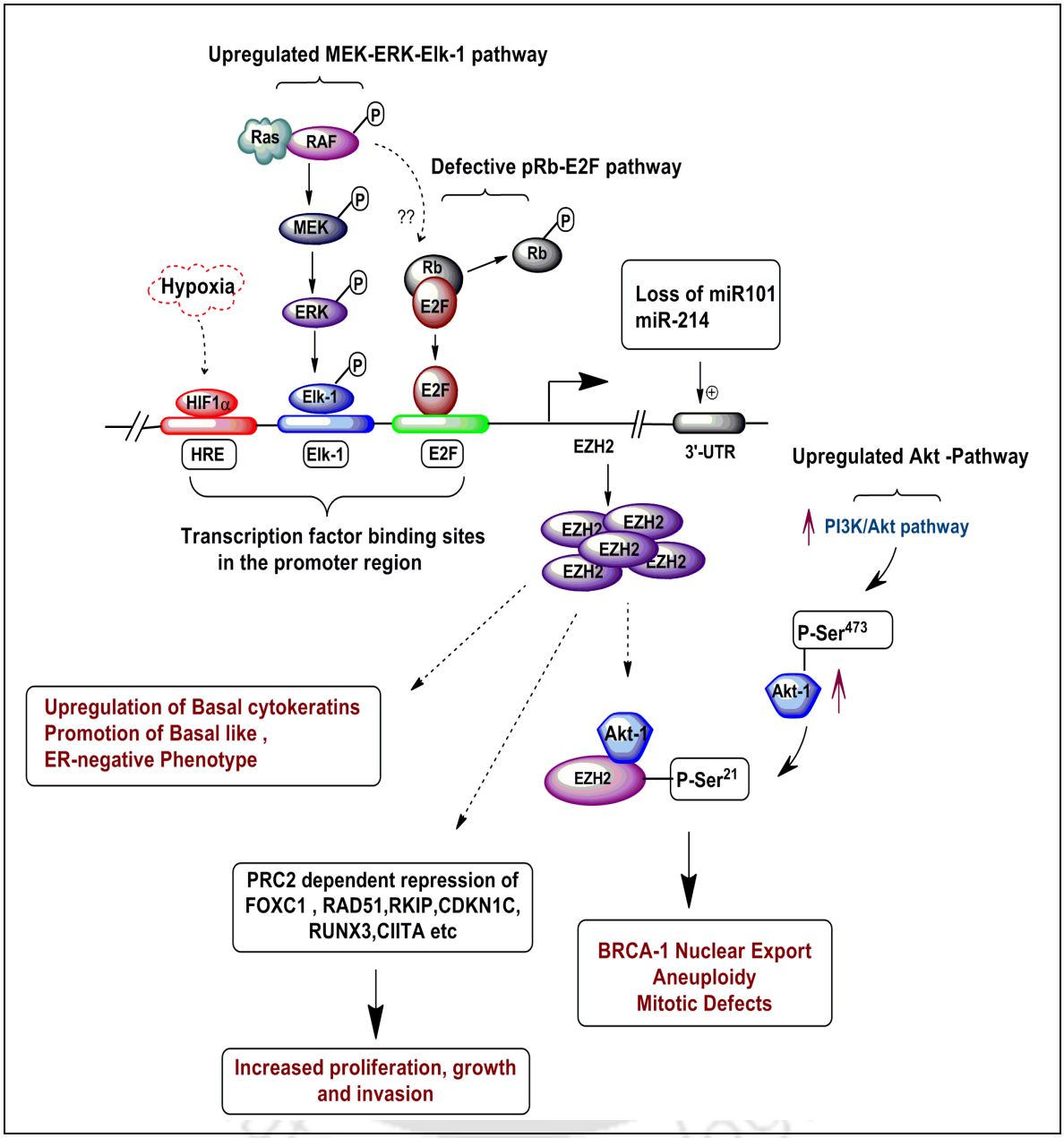


Figure 2.10. Molecular insights into EZH2 driven Breast tumorigenesis. In Breast cancer cell, EZH2 expression is regulated by several factors such as hypoxia induced HIFα, pRB-E2F and MEK-ERK-Elk1 pathways. Besides these, genomic loss of miR101 and miR214 also up-regulates EZH2 expression. Elevated EZH2 levels leads to the transcriptional repression of several tumor suppressor genes such as FOXC1, RAD51, RKIP, CDKIC, RUNX3, CIITA etc, by PRC2 mediated H3K27 trimethylation. High EZH2 protein levels are associated with increased expression of phospho-Akt-1 (Ser473) and decreased nuclear localization of phospho-BRCA1 (Ser1423).EZH2 mediated nuclear shuttling of BRCA-1 protein in ER negative basal like breast cancer cells is one of its PRC2 independent functions. Nuclear retention of BRCA-1 protein leads to aneuploidy, aberrant mitosis and genomic instability, which ultimately promotes tumorigenesis. Reproduced from Deb *et al*. (2013)

GREEN TEA AND CANCER CHEMOPREVENTION

Tea

Tea (*Camellia sinensis*) is one of the most widely consumed beverages in the world, which is now cultivated in more than 30 countries. Annual tea production has been estimated to be about 2.5 million metric tons (Butt and Sultan, 2009; Katiyar and Mukhtar, 1996). It is consumed in a variety of forms, such as green, black and oolong tea, which vary in their processing methods. Moist humid climate is suitable for tea cultivation, which is available in north-eastern part of India, Sri Lanka, Tibet, Korea and southern China (Butt and Sultan, 2009).

Botanical classification

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Dilleniidae
Order	Theales
Family	Theaceae – Tea family
Genus	*Camellia*
Species	*Camellia sinensis*

***Camellia sinensis* (Tea)**

Source : Butt and Sultan (2009)

Tea- processing and manufacturing

'Green tea' refers to the product manufactured by steaming or initial heating of fresh tea leaves at an elevated temperature, which inactivates the enzyme polyphenol oxidase (PPO) (Cabrera *et al.*, 2006). PPO catalyzes the oxidation of tea catechins and conversion of flavanols into dark polyphenolic compounds produces the color in black tea (Balentine *et al.*, 1997). Black tea is a fully

fermented product while oolong tea is in semi- fermented or partially oxidized form. White tea is produced from steaming of new buds and young leaves, which are shielded from sunlight to avoid chlorophyll production. Of the total worldwide tea production, 78% is black tea, 20% is green tea and 2% is oolong tea (Balentine *et al.*, 1997; Cabrera *et al.*, 2006; Katiyar and Mukhtar, 1996).

Green tea – chemical composition

The chemical composition of green tea is complex, which varies with the geographical origin, climate, season, time of harvest, horticultural practices and position of leaf on the harvested shoot. The main components of dried green tea leaves include: polyphenols (10-25%) and plant alkaloids (~4%) which include caffeine, theophylline and theobromine. Other constituents include proteins (15–20% dry weight), carbohydrates (5–7% dry weight), vitamins (B, C, E), minerals and fiber (Butt and Sultan, 2009; Cabrera *et al.*, 2006; Mukhtar and Ahmad, 1999; Mukhtar *et al.*, 1992).

Majority of health related benefits associated with green tea consumption has been attributed to polyphenols particularly flavonoids (phenol derivatives). Green tea does not undergo fermentation and therefore contains greater amounts of catechins than in black or oolong tea. The major flavonoids present in green tea include catechins (flavon-3-ols) such as (−)-epicatechin (EC), (−) - epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigalocatechin-3-gallate (EGCG) [Figure 2.11].

EGCG is the most abundant catechin present in green tea and accounts for 59% of total catechin content, followed by EGC (~19%), ECG (~13.6%) and EC (~6.4%) (McKay and Blumberg, 2002) (Schramm, 2013). EGCG has been demonstrated to be the strongest anti-oxidant among all other green tea catechin, with free radical scavenging properties (Butt and Sultan, 2009; Mukhtar *et al.*, 1992). A cup of green tea (2.5g of green tea leaves/200 mL of water) may contain 90mg of EGCG (Hakim *et al.*, 2000; Wu and Wei, 2002). The plasma bioavailability of EGCG, when administered as pure compound or tea in humans, has been found to be in the range of 0.1-7μmol/L and concentrations over 100μmol/L observed in saliva. No significant excretion of EGCG was reported in urine (<0.1% of dose) (Howells *et al.*, 2007).

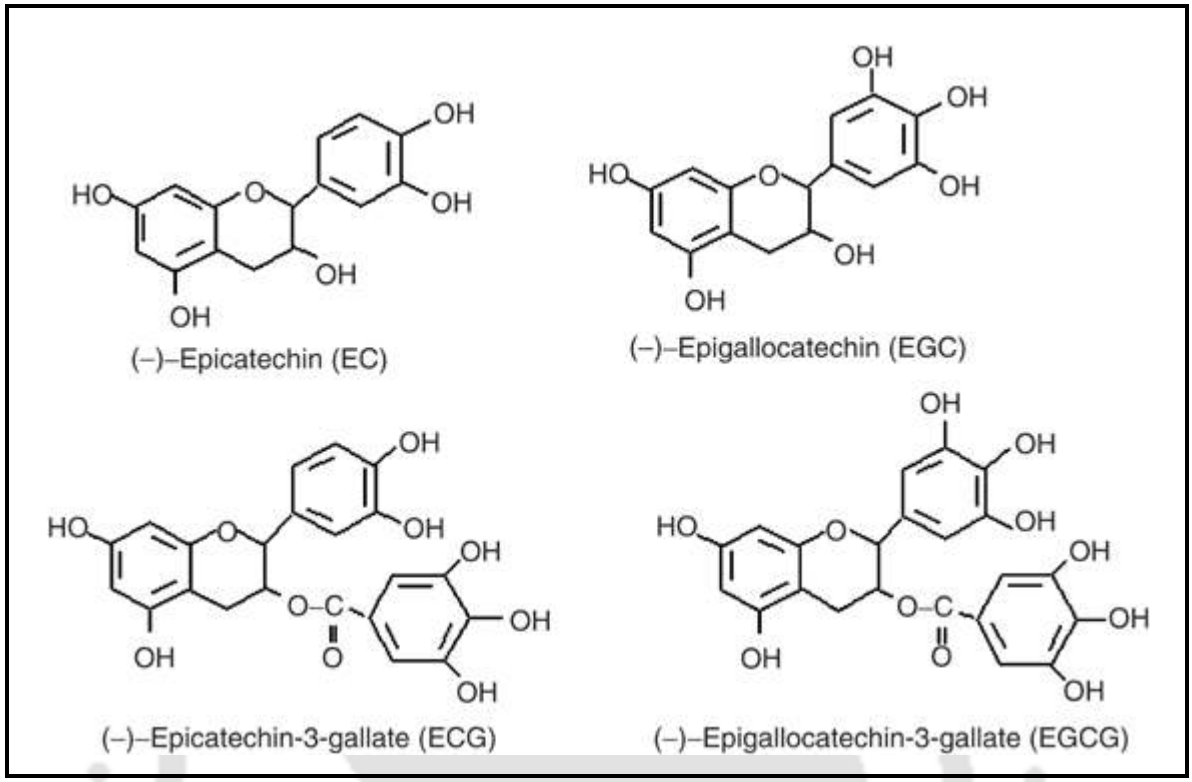


Figure 2.11. The structure of green tea catechins. Reproduced with permission from Bigelow and Cardelli (2006)

Green tea and cancer chemoprevention

Tea polyphenols or the catechins have been shown to reduce the risk of various diseases, including cancer (Dreosti *et al.*, 1997; Isemura and Timmermann, 2013). Green tea polyphenols particularly EGCG has been a subject of global interest due to its purported health benefits. It constitutes more than 50% of the total catechin isolated from green tea and has been widely studied for its anticancer effects in tissue culture, animal models and more recently in clinical trials (Schramm, 2013; Suzuki *et al.*, 2012). Extensive experimental studies using *in vitro* and *in vivo* model systems have demonstrated that EGCG possess anti-proliferative, anti-mutagenic, anti-oxidant, anti-bacterial and anti-tumorigenic properties (Cheng *et al.*, 1991; Cooper *et al.*, 2005a; Cooper *et al.*, 2005b; Crespy and Williamson, 2004; Katiyar *et al.*, 1992; Lambert and Elias, 2010).

Green tea and breast cancer – Epidemiological studies

Epidemiological data suggests that there exists an inverse correlation between green consumption and breast cancer. In a case control study conducted among Chinese-, Japanese-, and Filipino-American women, Wu *et al.* (2003) reported that green tea consumption was associated with a reduced breast cancer risk in women having a low-activity catechol-O-methyltransferase (COMT) allele. COMTs catalyze rapid methylation of tea catechins and differential methylation potential between individuals may modulate the chemopreventive property of green tea polyphenols by altering the bioavailability of the catechins. In another study, Yuan *et al.* (2005) demonstrated that green tea consumption in women possessing the high-activity angiotensin-converting enzyme, have reduced breast cancer risk. A hospital based study conducted by Zhang *et al.* (2007) in Zhejiang province, China, also showed significant reduction in breast cancer risk with regular green tea consumption. However, data obtained from some prospective cohort studies conducted in Japan, Singapore and China showed no association between breast cancer risk and green tea consumption (Dai *et al.*, 2010; Inoue *et al.*, 2008; Iwasaki *et al.*, 2010; Nagano *et al.*, 2001; Suzuki *et al.*, 2004).

In summary, case control studies provide convincing evidence between green tea consumption and reduced breast cancer risk, however supporting data from cohort studies are lacking. Predominance of post-menopausal women in cohort studies and the menopausal status of women have been suggested to be the possible reasons for the differences in results (Wu and Butler, 2011).

***In vitro* effects of EGCG in breast cancer**

Green tea polyphenols have been shown to exert anticancer effects in breast cancer by modulating multiple mechanisms which include inhibition of cellular proliferation by induction of cell cycle arrest, apoptosis, and the suppression of oxidative stress, angiogenesis, invasion, and metastasis (Braicu and Gherman, 2012; Chisholm *et al.*, 2004; Farabegoli *et al.*, 2011; Kavanagh *et al.*, 2001; Schramm, 2013; Tachibana, 2009; Yiannakopoulou, 2014). Recent reports highlight the lesser known role of green tea polyphenols particularly EGCG as 'epigenetic modulators' targeting epigenetic alterations that occur during carcinogenesis, by modulating the activity or expression of DNMTs, histone

modifying enzymes and miRNAs (Khan *et al.*, 2012; Thakur *et al.*, 2014). EGCG has been demonstrated to inhibit DNMTs, Class I HDACs, histone methyltransferase EZH2 and various miRNAs involved in epigenetic repression of critical suppressor genes in various cellular systems and restore their tumor suppressive activity in delaying cancer progression (Choudhury *et al.*, 2011).

Although a plethora of literature has detailed the *in vitro* effects of EGCG in breast cancer cells, very few studies have provided the insights into the molecular mechanisms. Studies by Tachibana *et al.* (2004) demonstrated that a cell surface 67kDa laminin receptor (67LR) confers EGCG sensitivity to cancer cells. In breast cancer, 67LR overexpression has been shown to be associated with poor prognosis and metastasis (Martignone *et al.*, 1993). Umeda *et al.* (2008) demonstrated that EGCG binds to 67LR at the cell surface and induces dephosphorylation of myosin phosphatase targeting subunit 1 (MYPT1) at Thr-696 through eukaryotic translation elongation factor 1A (eEF1A) . This leads to the activation of myosin phosphatase which induces alterations in actin cytoskeleton and might ultimately lead to growth inhibition.

EGCG has been shown to decrease the proliferation of ER positive breast cancer cells such as MCF-7 and BT474, as well as ER negative breast cancer cells such as BT-20, MDA-MB-231, MDA-MB-468 and Hs78t (Chisholm *et al.*, 2004; Kavanagh *et al.*, 2001; Liang *et al.*, 1999; Roy *et al.*, 2005). Liang *et al.* (1999) showed EGCG treatment causes cell cycle arrest by inducing p21 and p27 protein levels in MCF-7 cells which corroborates with other studies showing similar effects in MDA-MB-231 cells (Masuda *et al.*, 2002). Furthermore, Masuda *et al.* (2002) showed that following treatment with EGCG, basal as well as TGF-α induced auto-phosphorylation of EGFR increases in MDA-MB-231 cells. In addition, there was inhibition of constitutive and TGF-α induced AKT, STAT3 and NF-κB activity in these cells, however the phosphorylated ERK remained unchanged. HER-2/neu and EGFR related downstream signalling pathways were also shown to be inhibited following treatment with EGCG (Masuda *et al.*, 2003; Pianetti *et al.*, 2002). This was demonstrated to be associated with the inhibition c-fos and cyclin D1 promoter activity and STAT activity. Finally, hepatocyte growth factor (HGF)/met signaling pathway, which is known to regulate cell proliferation , survival and invasion , has also been shown to be a target of EGCG

action (Bigelow and Cardelli, 2006). In summary, evidences suggest that EGCG modulates multiple signalling pathways regulating cell proliferation and survival in breast cancer cells.

In vivo effects of EGCG in breast cancer

In vivo studies using a mixture of green tea polyphenols or purified EGCG have demonstrated a negative effect of green tea catechins on tumor growth and progression. Kavanagh *et al.* (2001) demonstrated that Sprague Dawley rats with DMBA induced mammary tumors administered with green tea as the sole drinking fluid exhibit reduced tumor burden and invasiveness. Similar studies in rats with chemical induced mammary tumors, have also shown a protective effect of green tea extracts on tumor load and overall survival (Bhide *et al.*, 1994; Hirose *et al.*, 1994; Hirose *et al.*, 1997; Hirose *et al.*, 2002).

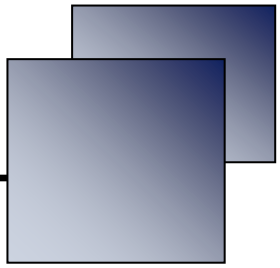
Studies conducted using breast cancer xenografts in mice corroborate with the findings in animal models, which demonstrated that following green tea consumption, there was delay in tumor onset as well as decreased tumor growth, weight and endothelial vessel density (Sartippour *et al.*, 2001; Thangapazham *et al.*, 2007). Baliga *et al.* (2005) demonstrated that there was decreased tumor growth in BALB/c immuno-competent mice inoculated with 4T1 mouse mammary carcinoma cells, which consumed EGCG rich GTP supplemented drinking water. This was accompanied with an elevation Bax/Bcl-2 ratio, caspase 3 activation and reduction in proliferating cell nuclear antigen (PCNA). Studies conducted in female athymic mice inoculated with MCF-7 or MDA-MB-231 cells, receiving EGCG in their drinking fluid , also demonstrated reduced tumor burden (Liao *et al.*, 1995; Thangapazham *et al.*, 2007).

Clinical trials of EGCG in breast cancer

At present, there are five ongoing clinical trials with green tea in breast cancer patients (clinicaltrials.gov identifiers – NCT00917735, NCT00516243, NCT00949923, NCT00676793 and NCT01060345). A phase II trial aims to study the effects of green tea extract on breast cancer compared to placebo in post menopausal women (NCT00917735) and include biomarkers endpoints such as mammographic density, plasma insulin-like growth factor 1 (IGF-1), IGF binding protein 3, estrone, estradiol, androstenedione, sex hormone binding globulin,

urinary estrogen metabolites and plasma F2-isoprostanes. Another randomized phase I trial aims to identify the best dose and side effects of green tea extract in treating hormone receptor negative (stage I-III) breast cancer patients (NCT00516243). A pilot study including 50 women with newly diagnosed DCIS has been initiated to identify biomarkers for better understanding the role of green tea as chemopreventive and chemotherapeutic agent (NCT01060345).

Chapter III



Materials and Methods

CELL CULTURE

Cell lines

MCF-7 (ERα positive), human breast cancer cells were obtained as gifts from Dr. Dipak Datta, CSIR-CDRI, Lucknow, India and Dr. Hung-Ying Kao, Department of Biochemistry, School of Medicine, Case Western Reserve University, Ohio, USA. MDA-MB-231(ERα negative) was either from National Centre for Cell sciences (NCCS), Pune, India or obtained as gift from Dr. Hung-Ying Kao. Original source of all breast cancer cell lines used for the studies conducted in this thesis was American Type Culture Collection (ATCC), Manassas, VA. 293T kidney cancer cell line was procured from ATCC. The human fibrosarcoma cell line HT1080 was obtained from NCCS.

Culture conditions

MCF-7 cells were routinely maintained in phenol red containing Dulbecco's Modified Eagle medium (DMEM) F12 and MDA-MB-231 cells were cultured in phenol red containing RPMI-1640 media. HT1080 cells were maintained in phenol red containing DMEM medium. For routine culture DMEM-F12 or RPMI-1640 were supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin and 100µg/mL streptomycin (M1). Cells were maintained in 25 cm^2 tissue culture flasks under standard culture conditions of 37^0C in a humidified atmosphere and 5% CO_2. However, treatment of cells with EGCG and E2 was carried out in phenol red-free DMEM-F12 or RPMI-1640, 10% charcoal-stripped and heat inactivated FBS, 100U/mL penicillin and 100µg/mL streptomycin (M2).

Sub-culturing

Confluent cells were washed with Dulbecco's Phosphate buffered Saline (DPBS) and treated with 0.5% Trypsin–EDTA for a brief period, depending on the cell line. After the cells detached, they were resuspended in 1mL M1 or M2 to neutralize enzyme action. Cell suspension was centrifuged at 1200rpm for 5 min and the supernatant was removed. The cell pellet was resuspended in 1mL fresh medium, counted and seeded in an appropriate density depending on the experiment.

Freezing cells

Cells were trypsinized, resuspended in medium M1 and pelleted to remove trypsin. The cell pellet was resuspended in cell freezing medium containing 10% DMSO, 40% FBS and 50% basal growth medium (DMEM or RPMI-1640, depending on the cell line). The cells were equilibrated at 4˚C, -20˚C and -80˚C before storage in liquid nitrogen.

Thawing frozen cells

Cryovials containing frozen cells were thawed in a water bath maintained at 37°C and transferred into a 15mL centrifuge tube. 5mL pre-warmed M1 medium was added slowly to the thawed cells. The cell suspension was mixed and transferred into a 25cm^2 tissue culture flask which was incubated under standard culture conditions of 37^{0}C in a humidified atmosphere and 5% CO_2. After 6h, the culture medium was gently aspirated from the flask without disturbing the cells which adhered to the substratum. Appropriate medium M1 was replenished in the tissue culture flask and adherent cells were allowed to grow under standard culture conditions.

MATERIALS AND REAGENTS

Cell culture materials

Tissue culture flasks (25cm^2), culture dishes (35mm and 90mm) and 96-well plates were purchased from Greiner Bio-One, Germany. DMEM-F12 (with and without phenol red) was from Invitrogen Corporation, USA. Fetal Bovine Serum (FBS) was purchased from PAA Laboratories, GmbH, Austria. Charcoal/dextran treated FBS was purchased from Hyclone (Thermo Scientific™ HyClone™, USA). Antibiotic Solution (100X) and trypsin-EDTA were from HiMedia, Mumbai, India.

Chemicals, hormones and enzymes

EGCG, E2, 5-aza-2'-deoxycytidine (5-Aza-CdR), trichostatin A (TSA) and Propidium Iodide (PI) were purchased from Sigma-Aldrich, USA. 3-Deazaneplanocin A (DZNep) was purchased from Cayman Chemical Company, USA. Polyphenon E, a high quality green tea extract, was obtained from Mitsui

Norin Co. Ltd, Japan and hereafter referred to as green tea polyphenols [GTP(s)]. All the chemicals were dissolved as concentrated stock solutions in DMSO, ethanol or PBS. They were stored at -80˚C and diluted at the time of use in culture media. Human MMP-2, active (E.C Number 3.4.24.24) was purchased from Calbiochem, EMD Millipore, USA (Cat. No. PF023). MMP-2 specific quenched fluorescent substrate MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2 [MCA= (7-metoxycoumarin-4-yl)acetyl, Dpa = 3-(2, 4-dinitrophenyl)-L-2, 3-diaminopropionyl; Nva = L-norvaline] was purchased from Merck Millipore, Germany (Product No. 444212), dissolved in 100% DMSO to prepare a 10mM stock and stored in small aliquots at -80˚C until use. Collagen IV (SC-29010) was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). Other salts, solvents and buffer components used in this study were from Merck, SRL or Fischer scientific.

Antibodies

Anti-TIMP-3 (SC-373839), anti-HDAC-1 (SC-7872), anti-HDAC-2 (SC-6296), anti-HDAC-3 (SC-11417), anti-HDAC-8 (SC-11405), anti-β-actin (SC-47778), goat anti-mouse IgG-HRP (SC-2005), bovine anti-goat IgG-HRP (SC-2350) and goat anti-rabbit IgG-HRP (SC-2004) antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). Anti-H3K27me3 monoclonal antibody (mAbCam6002) was purchased from AbCam (Cambridge, MA); anti-acetyl H3 K9/18 antibody (07-593) was purchased from Upstate (Lake Placid NY), anti-Histone H3 (Clone A3S) antibody and anti-EZH2 (07-689) antibody were purchased from Millipore (Billerica, MA).

TREATMENT PROTOCOLS

Experiments to study the effect of E2 treatment on breast cancer cell viability and proliferation

E2 was dissolved in ethanol and a stock solution of 10mM was prepared that was stored at -20°C until use. MCF-7 and MDA-MB-231 cells (1×10^5) were seeded in 35mm culture dishes and allowed to grow in M1 medium. After the cells were 50-60% confluent, they were washed with DPBS (×1) and M2 medium (×2) to remove traces of phenol red containing medium. The cells were maintained in M2

medium for 6-8h and then treated with various doses of E2 (10nM, 1nM and 0.1nM) or vehicle (ethanol) alone in M2 medium. The plates were maintained at 37°C and 5% CO_2 in a humid environment. After 72h, viable cell count assay was performed using trypan blue dye exclusion method. For time dependent experiments, similar procedure was used except that only one E2 dosage of 10nM was chosen and cell counts were performed after every 24/48/72h.

Experiments to study the effect of E2, EGCG or both on breast cancer cell viability, proliferation and gelatinolytic activity

EGCG was dissolved in ethanol (20mg/mL stock) and stored at -20°C until use. For studying the dose dependent effect of EGCG on breast cancer cell viability, MCF-7 and MDA-MB-231 cells (8×10^3 cells per well) were seeded with M1 medium in a 96-well plate and incubated for 36h in a CO_2 incubator maintained at 37˚C and 5% CO_2. The experiment was then started by replacing the spent M1 medium in groups of wells with fresh M1 medium containing increasing concentrations of EGCG, from 2 to 200µM. Wells containing M1 medium treated with ethanol alone served as controls. The cells were treated for 72h before proceeding with MTT assay.

In experiments to study the combinatorial effect of E2 and EGCG on breast cancer cell viability and proliferation, the same protocol was followed except that the M1 medium was replaced with fresh M2 medium and further incubated for 3-4h. The spent M2 medium was replaced with fresh M2 medium containing E2, EGCG or both. Cells treated with vehicle (ethanol) alone served as controls. Before proceeding with MTT assay, the culture supernatants (conditioned medium) from each experiment was collected, centrifuged at 8000rpm to remove cell debris and stored at -80˚C in small aliquots for gelatin zymography assay.

Experiments to study the effect of EGCG on extracellular or *in vitro* MMP-2 activity by gelatin zymography

Incubation of HT1080 conditioned medium or purified MMP-2 with EGCG

Equal volumes (30µL) of HT1080 supernatant were incubated with various doses of EGCG (final concentration 0 to 200µM) at 37°C for 45 min. For assays using human MMP-2 (active), 10ng of enzyme was incubated with various doses of

EGCG (0-5µM) in phosphate buffered saline (pH 7.2, with $CaCl_2$ and $MgCl_2$) at 37°C for 45 min. The samples were then loaded in gelatin-PAGE for analysis.

Pretreatment of HT1080 conditioned medium or purified MMP-2 with EDTA followed by incubation with EGCG

Equal volumes of HT1080 supernatants or 10ng of human MMP-2 in PBS were incubated with 10mM EDTA for 15 min at 37°C, followed by the addition of EGCG stock to get the desired concentration in the solution. The concoctions were incubated for 45 min at 37°C and then loaded in gelatin –PAGE gel.

Pretreatment of EGCG with divalent cations followed by incubation with HT1080 conditioned medium for gelatin zymography

EGCG (2mM), $CaCl_2$ (1M, 100mM, and 10mM) and $MgCl_2$ (1M, 100mM and 10mM) stocks were prepared. EGCG (2mM) was incubated with desired concentration of salts (for example if the concentration of salt required is 100mM, 1M stock was used in the cocktail mix) at 37°C for 15 min. The concoction was diluted 10 times with HT1080 supernatant to get the desired final concentration of divalent salt (1-100mM) and 200µM EGCG. The mixture was incubated at 37°C for 45 min. The samples were then analyzed by gelatin zymography.

Treatment of breast cancer cells with green tea polyphenols and other epigenetic related drugs

MCF-7 and MDA-MB-231 were plated at 20% seeding density and allowed to attach for 24h in medium M1. Medium was replaced and cells were treated with 10µM 5-Aza-CdR, 40ng/mL TSA (0.13228µM), 5µM DZNep, 10µg/mL GTP and 20µM EGCG for indicated period of time. Concentration of 10µg/mL GTP corresponds to 14.0µM of EGCG. For combined treatment with TSA and DZNep, cells were cultured with 40ng/mL TSA for 48h followed by the addition of 5µM DZNep for 24h. In these experiments, the media were replaced every alternate day with the fresh media containing the appropriate treatment. At the end of the experiments, the cells were harvested and used for the extraction of RNA or protein. Same treatment protocol was used in experiments involving ChIP assay, wound healing assay and gelatin zymography as described in chapter VI.

CELL VIABILITY ASSAYS

Trypan blue dye exclusion method

10µL of 0.4% trypan blue was mixed with equal volume of cell suspension and incubated at room temperature for 1-2 min. 10µL of this mixture was applied to a hemocytometer and live cells which remain unstained due to dye exclusion were counted under light microscope.

MTT Assay

8×10^3 cells per well were plated in a 96-well culture plate. After treatment of cells for the stipulated period of time, the media from control and treated cells were aspirated out. The cells were washed with DPBS. To measure the viability of cells at different experimental conditions, MTT assay was used. 100µL of MTT reagent (0.5 mg/mL MTT in DMEM-F12 or RPMI-1640 depending on the cell line) was added to each well and incubated at 37˚C and 5% CO_2. After 3h, the MTT reagent was removed and the formazan crystals were dissolved in 100µL of DMSO. The reaction product was quantified spectro-photometrically by measuring absorbance at 570nm (A_{570nm}) and background correction absorbance was also recorded at 690nm (A_{690nm}). The difference between the two absorbance values (A_{570nm}–A_{690nm}) was determined as a measure of cell viability.

APOPTOSIS ASSAY

Extent of apoptosis was estimated by flow cytometry using Annexin V-FITC Apoptosis Detection Kit (Cat. No PF032, Calbiochem, EMD Biosciences, USA) as per the manufacturer's protocol. In this assay, fluorescein isothiocyanate (FITC) conjugate of Annexin V (Annexin V-FITC) is used to detect apoptosis by flow cytometry. Propidium iodide is used to distinguish between viable, early apoptotic and necrotic or late apoptotic cells. Necrotic cells bind Annexin V-FITC and get stained with propidium iodide while propidium iodide will be excluded from viable (FITC negative) and early apoptotic (FITC positive) cells. Final stages of apoptosis involve necrotic-like disintegration of the total cell, thus cells in late apoptosis will be labeled with both FITC and propidium iodide.

Cells (1×10^5 per well) were seeded in a 6 well plate and allowed to grow. After 24-36h (depending on cell line), the cells were treated with vehicle (ethanol), 10nM E2, 40µM EGCG and 10nM E2 + 40µM EGCG, for 72h. At specified time point, the spent media from each well was transferred to a 15mL conical tube and placed on ice (this media may contain cells that have detached from the plate during the cell death process). The adherent cells were washed with DPBS, trypsinized and resuspended in spent media collected in the previous step. The cells were harvested by centrifugation at 400g for 5 min at 4˚C. Cells were gently resuspended in 0.5mL cold DPBS and centrifuged at 1000g for 5 min at room temperature. DPBS was removed and cells were resuspended in 0.5mL cold 1X binding buffer (provided by the manufacturer). Cells were then stained with 4µL Annexin V-FITC at room temperature in the dark. After 15 min, the cells were centrifuged (1000g, 5 min), supernatant was removed and gently resuspended in 0.5mL cold 1X binding buffer. 10µL propidium iodide (PI) was added and samples were placed on ice away from light.

The samples were then analyzed by flow cytometry with a FACSCalibur instrument (Becton-Dickinson, USA). Fluorescence intensity of FITC and PI stained cells was measured in FL1 and FL3 channel in log mode respectively.

CELL CYCLE ANALYSIS

Flow cytometry based analysis of cells stained with propidium iodide (PI) was used for determining cell cycle distribution pattern.

Serum starvation, treatment and fixation

Cells (1×10^5 per well) were seeded in 6 well plate and after 24-36h of seeding, cells were allowed to grow in 1% FBS containing M2 medium (serum starvation) overnight (12-15h). After 12h the cells were released from serum starvation by replacing the media with fresh 5% FBS containing M2 medium. After 45-60 min, the cells were appropriately treated for stipulated time periods in 10% FBS containing M2 medium. At desired time points, the cells were washed with DPBS, trypsinized and harvested in 1.5mL micro-centrifuge tubes. The cell pellet was again washed with cold DPBS and centrifuged at 1500rpm for 5 min at 4˚C. The cell pellet was resuspended in 50µL cold DPBS and 450µL cold methanol was

added. The samples were first kept on ice for 30 min and then stored at -20˚C until required for analysis.

RNase digestion, PI staining and analysis by flow cytometry

The cells were washed with cold DPBS twice with centrifugation at 2500rpm for 7 min each time to remove methanol. 50µL RNase A (from 100µg/mL stock) was added to the cells suspended in 450µL DPBS and allowed to incubate at 37˚C for 30 min. The cells were chilled at 4˚C for 10 min and then 200µL PI (from 50µg/mL stock) was added and kept on ice, away from light for 30 min. The cells were then analyzed by flow cytometry (FACSCalibur, Becton, Dickinson and Company, USA). PI stained nuclei were analyzed in FL2 channel (585/42 band pass filter), and a dot plot of FL2-area *vs.* FL2-width was used to discriminate doublets. Histogram of FL2-area for gated population was plotted for visual representation of cells in different stages of cell cycle. All cell cycle data analysis was performed using ModFit LT software (Verity software house).

GELATIN ZYMOGRAPHY

Samples were subjected to zymography on 7.5% SDS-PAGE gel co-polymerized with 0.1% gelatin. Gel was washed in 2.5% Triton-X-100 for 30 min (3 times) to remove SDS and was then incubated overnight (14-16h) in activation buffer (2.5% Triton-X-100, 10mM Tris–HCl pH 7, 5mM $CaCl_2$, 1µM $ZnCl_2$). After incubation, the gels were stained with 0.5% coomassie brilliant blue for 1h. This was followed by destaining the gel with methanol: glacial acetic acid: water (30:10:60) until clear bands were visible against blue background.

INVASION ASSAY

A quantitative cell migration and invasion assay was performed with ThinCert™ cell culture inserts (Greiner bio-one, Germany) in a 24 well format.

Coating of the cell culture inserts with collagen type IV

24-well ThinCert™ cell culture inserts with translucent polyethylene terephthalate (PET) membrane (Pore size 8µm) were placed in the wells of a 24 well plate. Collagen IV (1mg/mL) was thawed overnight at 4°C and diluted 1:10

with 0.05N HCl to yield a protein concentration of 100ng/µL. 60µL of the collagen solution (33.6µL of the collagen IV solution was mixed with 26.4µL 0.05N HCl) was added to each ThinCert™ cell culture insert (10µg collagen IV per cm^2 culture surface area) and dried for 3-4h at 37˚C in 5% humidified CO_2 incubator . Non-coated ThinCert™ cell culture inserts were used as controls in both migration and invasion assays.

Preparation and treatment of cells

MDA-MB-231 cells were seeded 35mm dishes (1.5×10^5 cells per dish) in M1 medium and appropriate treatment of cells with EGCG or E2 alone or in combination was carried out in M2 medium. After 48h, 5×10^4 cells from each treated group (i.e. vehicle, 10nM E2, 20µM EGCG and 20µM EGCG ± 10nM E2) were seeded in the upper chamber of the insert in serum free RPMI-1640 culture medium. The lower chamber was supplied with complete medium which contained 10% FBS. Appropriate treatment conditions were present in both the chambers of the apparatus. The cells were allowed to migrate through collagen IV coated / uncoated membranes for 18h.

Detachment and quantification of the cells

The non migrated cells present on the upper side of the membrane were removed by swabbing with cotton buds and those migrated to the other (reverse) side of the membrane were fixed, permeabilized and stained with 0.05% crystal violet dye. The stained cells were first photographed in bright field microscope under 20X and then dissolved in 1% SDS solution at 37˚C for 1h and absorbance was read at 595nm.

Calculation of invasion index (I %)

To determine the invasive properties of the studied cell lines the **invasion index (I %)** was calculated according to the following formula:

$$\mathbf{I}\,\% = \frac{[(\mathbf{ABS})\mathbf{1} - (\mathbf{ABS})\mathbf{0}]}{[(\mathbf{ABS})\mathbf{2} - (\mathbf{ABS})\mathbf{0}]} * \mathbf{100}$$

(ABS) 1= absorbance units obtained from cells that migrated through collagen IV coated membrane towards 10% FBS

(ABS) 2 = absorbance units obtained from cells that migrated through an uncoated membrane towards 10% FBS

(ABS) 0 = absorbance units obtained from cells that passively passed through an uncoated membrane in the absence of FBS

MMP-2 ACTIVITY ASSAYS USING FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) BASED PEPTIDES

End point assay

MMP-2 specific FRET peptide substrate MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2 contains the fluorophore MCA and the quencher Dpa. The substrate stock solution was prepared in 100% DMSO as a 10mM stock. To determine the effect of EGCG on MMP-2 inhibition, endpoint assays were conducted in 150µL reactions by incubating 10ng active MMP-2 (Final molar concentration = 1.01 nM) with various doses of EGCG (0-40µM) in an assay buffer (10 mM Tris–HCl pH 7, 5mM $CaCl_2$, 1µM $ZnCl_2$) at 37˚C for 30 min. To measure the residual enzyme activity, peptide substrate was added (final concentration = 20µM) and the reaction mixture was incubated in dark at 37˚C for 1h.

Substrate control (containing only 20µM substrate in assay buffer without MMP-2), Vehicle control (containing 20µM substrate + 10ng MMP-2 + 3µL DMSO) and assay buffer control (containing 150µL assay buffer only) were included in each assay. Fluorescence was measured on Synergy HT Multimode Microplate reader (BioTeK, USA) using λ_{max} excitation 324nm and λ_{max} emission 393nm. To address the possibility of EGCG interfering with the FRET donor- quencher pair itself, a separate experiment was performed where 20µM EGCG was added to reaction mixture (10ng MMP-2 and 40µM peptide substrate in assay buffer) pre-incubated at 37˚C for 2h. At 2h time point, MMP-2 mediated peptide hydrolysis yields maximum fluorescence values (saturation) representing complete hydrolysis. Fluorescence was measured before and after EGCG addition every 15 min for 30 min.

To study the effect of EDTA on MMP-2 mediated hydrolysis of FRET based fluorogenic peptide, 10ng active MMP-2 in assay buffer was incubated with various doses of EDTA (0.1mM to 100mM) for 15 min at 37˚C , followed by the

addition of fluorogenic substrate (final concentration 20µM). The mixture was incubated for 1h at 37˚C, away from light and the residual enzyme activity was monitored by measuring fluorescence as described previously. To study the effect of EDTA pretreatment followed by EGCG treatment on MMP-2 mediated fluorogenic substrate hydrolysis, 10ng of human MMP-2 in assay buffer was incubated individually with 10mM EDTA for 15 min at 37°C, followed by the addition of EGCG stock to get the desired concentration (5µM and 10µM) in the reaction mixture. The concoctions were incubated for 45 min at 37°C, followed by the addition of peptide substrate (20µM) and fluorescence was measured after 1h incubation at 37˚C. To study the effect of divalent cations on EGCG mediated decrease in MMP-2 catalytic activity, EGCG was pre-incubated in excess cation salts containing assay buffer (10 mM Tris–HCl pH 7, 15 mM $CaCl_2$, 3µM $ZnCl_2$) at 37˚C for 15 min. The cation concentrations ($CaCl_2$ and $ZnCl_2$) were three times (3X) in excess as compared to assay buffer used for other routine fluorogenic substrate assays. Purified MMP-2 (10ng) was then added to the mixture, followed by incubation at 37˚C. After 30 min, peptide substrate (20µM) was added and fluorescence was recorded after 1h as described previously.

MMP-2 Inhibition Kinetic studies

Preliminary studies were performed to determine if the MMP-2 mediated hydrolysis of FRET peptide followed 1st order exponential increase for product formation with time. 10ng MMP-2 was incubated with a fixed FRET peptide substrate concentration of 40µM at 37°C in the assay buffer and fluorescence was measured every 5 min. The data was plotted as RFUs *versus* time and fitted using the 1st order exponential rate equation in GraphPad prism software.

For kinetics studies, 10ng MMP-2 enzyme (1.01nM) was incubated with a range of MMP-2 specific FRET peptide substrate concentrations (0-80µM) in the presence and absence of fixed inhibitor (i.e. EGCG) concentration of 5µM at 37˚C in the assay buffer and fluorescence was measured every 15 min. Using data points from only the linear portion of the hydrolysis curve, rates of hydrolysis were obtained from plots of fluorescence *versus* time for each substrate concentration in the presence and absence of EGCG. The slope obtained from these plots was divided by the fluorescence change corresponding to complete hydrolysis to obtain rates of hydrolysis in units of µM/min as described by

Lauer-Fields *et al.* (2008). The kinetic analysis were performed by fitting the initial velocity (µM/min) as a function of substrate concentration (µM) in the presence and absence of inhibitor, using non-linear regression analysis (global fitting) program in GraphPad Prism version 5.00 for Windows and kinetic parameters were computed.

RNA ISOLATION

Total RNA was extracted from cell lines after appropriate treatment at indicated time points for cDNA preparation using Trizol reagent (Ambion, Life technologies) as per manufacturer's protocol. Briefly, 0.5mL Trizol was added to the cells in the $35mm^2$ culture dish after removing spent medium. After addition of the reagent on the monolayer, the cells were lysed by repeated passing through a syringe to form a homogenous lysate. The samples were allowed to stand for 5 min at room temperature to permit complete dissociation of nucleoprotein complexes. 0.2mL chloroform per 1mL Trizol reagent was added. After 15-30s of vigorous shaking, samples were allowed to stand for 5-10 min at room temperature. The samples were centrifuged at 12000g for 15 min at 4˚C. The aqueous phase containing RNA was transferred to a fresh tube and 0.5mL 100% isopropanol per 1mL Trizol was added to precipitate the RNA. After 10 min incubation at room temperature, samples were centrifuged at 12000g for 10 min at 4˚C. The supernatant was removed and RNA pellet was washed with 1mL of 75% chilled ethanol and centrifuged again at 12000g for 10 min at 4˚C. RNA pellet was air dried, dissolved in suitable amount of sterile water and stored at -80˚C for long term use.

Removal of genomic DNA contamination from RNA

Contaminating DNA in the total RNA was digested by DNase I (New England Biolabs Inc, USA) treatment for 20 min at 37˚C in DNase I digestion buffer supplied by the manufacturer. The total RNA was then cleaned using RNeasy mini kit (Qiagen Sciences Inc, USA). Quality and quantity of extracted RNA was determined using Nanodrop spectrophotometer (Thermo Scientific, USA).

SEMI QUANTITATIVE RT-PCR

2µg of total RNA from each sample was subjected to reverse transcription reaction using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The resultant cDNAs were diluted five times and 2 µl was used as template for PCR reaction with gene specific primers for TIMP-3, MMP-2, MMP-9 and GAPDH expression levels using semi-quantitative PCR. For each gene, the number of cycles of amplification required for product formation in the linear range of detection by agarose gel electrophoresis was determined separately. The primer sequences along with their annealing temperatures, number of cycles used and amplicon length have been listed in Table 3.1. Each reaction was carried out in a reaction volume of 20µL containing 2µL cDNA, 10µL 2X PCR master mix (Promega, Madison, WI) and forward and reverse primers (100ng each in final reaction). PCR products were run in 2% ethidium bromide-agarose gel at 110V in TBE buffer and visualized in an UV transilluminator. Images of ethidium bromide stained bands were captured using Kodak gel imaging system. For each gene, the band intensities were normalized against GAPDH. The normalized band intensities obtained for controls have been assigned the value of 1 or 100 and those obtained for various treatments were plotted as fold increase or percent with respect to control, respectively.

Table 3.1. Primer sequences used for semi-quantitative RT-PCR analysis

Gene	Primer Sequences	Ref.	Amplicon (bp)	No. of cycles	PCR conditions
Human TIMP-3	F: 5'- CTACCTGCCTTGCTTTGTGAC-3' R: 5'-ATCCTCGGTACCAGCTGCAG-3'	Shin and Kim (2012)	140	25	95˚C (30s) 58˚C (30s) 72˚C (30s)
Human MMP-2	F: 5'-AGATCTTCTTCTTCAAGGACCGGTT-3' R-5'-GGCTGGTCAGTGGCTTGGGGTA-3'	Munaut *et al.* (2003)	225	30	95˚C (30s) 66˚C (30s) 72˚C (30s)
Human MMP-9	F-5'- GCGGAGATTGGGAACCAGCTGTA-3' R- 5' – GACGCGCCTGTGTACACCCACA-3'	Munaut *et al.* (2003)	209	30	95˚C (30s) 66˚C (30s) 72˚C (30s)
GAPDH	F-5'- CAACGGATTTGGTCGTATTGG-3' R-5'- GCAACAATATCCACTTTACCAGAGTTAA -3 '	Thakur *et al.* (2012)	72	25	95˚C (30s) 55˚C (30s) 72˚C (30s)

WESTERN BLOTTING

Whole cell lysate preparation

Cells grown in culture dishes were washed with cold DPBS and culture plates were placed on ice. 200µL (per 90 mm culture plate) ice cold whole cell lysis buffer [Tris-HCl-50mM, NaCl-150mM, Triton X-100-1%, EGTA-1mM, Sodium pyrophosphate-20mM, pH-7.4, protease inhibitors cocktail (10ul/mL) , NaF (10 mM), DTT (1 mM), PMSF (0.1 mM) and sodium orthovanadate (1 mM)] was added and the plate was incubated on ice. After 30- 45 min, the cells were scraped off, collected in cold micro-centrifuge tube and centrifuged at 13000rpm for 25 min. The supernatant was transferred into a fresh micro-centrifuge tube and used for western blotting after protein estimation.

Protein estimation

Total protein concentration was determined using protein estimation kit based on BCA method (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. In brief, 750µL of reagent A was mixed with 15µL of reagent S. 25µL of this mixture was added to each well of a 96 well plate according to the number of protein samples to be quantitated. 5µL of whole cell lysate was then added to the wells. 200µL of reagent B was added per well and the plate was covered with plastic top. The plate was placed on a shaker for 15 min to ensure proper mixing of the reagents and then absorbance was recorded at 650nm in a microplate multimode reader.

Electrophoresis, blotting and detection

40µg protein was resolved by SDS-PAGE (4-15% or 4-20% precast polyacrlamide gels; Bio-Rad) and transferred to nitrocellulose membrane (Millipore, USA). Non specific binding sites on membrane were blocked using 5% non-fat skimmed milk for 1h and then incubated with the primary antibody (1:500 dilution in 5% skimmed milk or BSA) overnight at 4˚C. The membrane was washed three times with tris buffered saline containing 0.05% tween 20 (TBST), followed by the incubation with suitable HRP conjugated secondary antibody for 1h at room temperature. The secondary antibodies were detected using Clarity Western ECL substrate (Bio-Rad, USA), followed by exposure to X-ray films for visualization.

BISULFITE MODIFICATION AND METHYLATION-SPECIFIC PCR

Genomic DNA from control (vehicle treated), 5-Aza-CdR, GTP and EGCG-treated cells was isolated using the Quick-gDNA MiniPrep Kit (Zymo Research, USA) as per the manufacturer's instructions. Bisulfite modification of genomic DNA (2µg) was using EZ DNA Methylation –Gold Kit (Zymo, Orange County, CA) followed by additional desalting and purification using the DNA Clean and Concentrator-5 Kit (Zymo). DNA was suspended in 10µl of water and stored at -20˚C. 100ng of bisulfite treated DNA was used as a template for Methylation specific PCR (MSP) reaction, which was performed using primer sequences described in Table 3.2 that specifically recognized -91 to +25 region in TIMP-3 promoter. Universal methylated human DNA standard (Zymo Research, USA) and water were used as positive and negative controls respectively. PCR products were resolved in 2% ethidium bromide-agarose gel along with low DNA mass ladder (Invitrogen, Carlsbad, CA) and visualized under UV light.

Table 3.2. Primer sequences used for MSP analysis of TIMP-3 promoter

Gene	Primer sequence	Reference	PCR conditions
Unmethylated TIMP-3 (UM)	F: 5'-TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT-3' R: 5'-CCCCCAAAAACCCCACCTCA-3'	Bachman *et al.* (1999)	95˚C 59˚C 72˚C } 30 cycles
Methylated TIMP-3 (M)	F: 5'-CGTTTCGTTATTTTTTGTTTTCGGTTTC-3' R: 5'-CCGAAAACCCCGCCTCG-3'		95˚C 59˚C 72˚C } 30 cycles

GENERATION OF KNOCKDOWN CELL LINES USING LENTIVIRUS MEDIATED TRANSFECTION

Packaging cells 293T were plated in 90mm plates with 20% cell density one day prior to transfection in DMEM medium containing 10% heat inactivated FBS without penicillin-streptomycin. Cells were transfected with 6µg of pLKO1 (control vector only), shEZH2 40075, shHDAC-1 4814, shHDAC-2 4819, shHDAC-3 4826 or shHDAC-8 4849 vector (Open Biosystems, Thermo Fisher,

Pittsburgh PA) along with second generation packaging construct (pCMV-dR8.74) and pMD2G using Lipofectamine/Plus reagent (Invitrogen Corp., Carlsbad, CA) as per manufacturer's protocol. Media was collected for two subsequent days and layered onto breast cancer cells MCF-7 and MDA-MB-231 to infect them with virus after adding 10µl of 4mg/mL polybrene per 10mL medium and further sterilization by passing through 0.45µM micro filters. 48h after infection, virus-containing media was replaced with DMEM +10% FBS. Twenty four hour later cells were put under selection using 1µg/mL puromycin for 2 weeks. Selected cells were tested for gene knockdown using Western blot analysis.

ACID EXTRACTION OF HISTONES

Control and treated cells were harvested and washed twice with ice cold DPBS, which was supplemented with 5mM Sodium Butyrate to maintain the levels of histone acetylation. The cells were resuspended in Triton Extraction Buffer (TEB: PBS containing 0.5% Triton X-100, 2mM PMSF, 0.02% NaN_3) at a cell density of 10^7 cells per mL. The cells were lysed on ice for 10 min with gentle stirring. The samples were then centrifuged at 2000rpm for 10 min at 4˚C and the supernatant was discarded. The cells were washed in half the volume of TEB and centrifuged as before. The pellet was resuspended in 0.2N HCl at a cell density of 4×10^7 cells per mL and the histones were acid extracted overnight at 4˚C. The samples were centrifuged at 2000rpm for 10 min at 4˚C. The supernatant was collected and protein content was determined as described previously. The aliquots were stored at -20˚C until use.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

This protocol was adapted from Strahl-Bolsinger *et al.* (1997) with some minor modifications.

Crosslinking

Control and treated cells in culture dishes were fixed with 1% formaldehyde in the growth medium for 15 min at room temperature. The reaction was stopped by adding glycine, to a final concentration of 0.125M and further incubation for 5 min at room temperature. The cells were then washed with ice-cold DPBS, scraped off in 5mL ice-cold DPBS and pelleted by centrifugation at 5000g for 1

min. The cell pellets were washed extensively in ice-cold DPBS using repeated cycles of resupension and centrifugation and stored in -80˚C until use.

Lysis and Micrococcal Nuclease (MNase) Digestion

500µL of ChIP lysis buffer (50mM HEPES pH 7.5, 140mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate) was added to the each cell pellet, vortexed for 15s and incubated on ice for 10 min. The lysates were centrifuged at 9000g for 3 min to harvest the nuclei and supernatants were discarded. Nuclei were resuspended in 500µL MNase digestion buffer [50mM Tris-HCl pH 8.0, 5mM $CaCl_2$, 200µL Protease inhibitors cocktail (PIC)] containing 1.5µL MNase (ChIP grade, 10 Units /µL), vortexed and incubated at 37˚C water bath for 15 min. The reaction was stopped by addition of 100µL of MNase stop solution (200mM EGTA, pH 8.0), vortexed briefly and incubated on ice for 5 min. The samples were centrifuged at 9000g for 5 min to recover the nuclei. The nuclei were resuspended in high salt ChIP lysis buffer (50mM HEPES pH 7.5, 140mM NaCl, 1% Triton X -100, 0.1% sodium deoxycholate) and incubated on ice for 15 min with vortexing for 15s every 5 min. The samples were centrifuged at 9000g for 5 min and supernatants which contain the fragmented chromatin were transferred to fresh 1.5mL tubes. The samples were either stored at -80˚C or used immediately for immunoprecipitation.

Immunoprecipitation (IP)

Aliquots of fragmented chromatin equivalent to 50µg protein were incubated with 2µL of primary antibody specific to EZH2, H3K27me3, and H3K9/18Ac along with 30µL agarose protein A/G beads in 1mL ChIP buffer (high salt) overnight at 4˚C in rotator. The immuno-precipitates were washed extensively in a series of buffers, pelleting the beads after each wash. The washing protocol was - 2×1mL ChIP lysis buffer, 2×1mL ChIP lysis buffer (high salt), 2×1mL ChIP wash buffer(10mM Tris pH 8.0, 250mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1mM EDTA), 2×1mL TE.

Reverse Crosslinking, DNA purification and PCR amplification

To the beads obtained after IP, 75µL elution buffer (50mM Tris pH 8.0, 1% SDS, 10mM EDTA) was added followed by overnight incubation at 65˚C overnight for

reversing the protein DNA crosslinking. Loading control (Input) containing 150µL elution buffer and 50µg protein taken for the IP was incubated for 6h or overnight at 65˚C. DNA was purified using phenol-chloroform-isoamyl reagent followed by ethanol precipitation. DNA was then dissolved in nuclease-free water.

Immunoprecipitated DNAs, beads only or input DNA controls were subjected to PCR amplification for 30 cycles of the following cycling conditions: stage 1, 95˚C for 5 min (1 cycle); stage 2, 95˚C for 30s and 60˚C for 30s and 72˚C for 1 min (30 cycles); and stage 3, 72˚C for 7 min (1 cycle). Primers used for detection of the TIMP-3 promoter region containing the YY1 binding were from Shin and Kim (2012). PCR products were subjected to electrophoresis on a 2% ethidium bromide-agarose gel and visualized under UV light using Kodak gel imaging system.

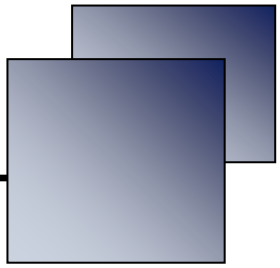
WOUND HEALING ASSAY

Cells were seeded in 6-well culture dishes and grown until 90-95% confluence. Using a sterile pipette tip, at least three scratch wounds per plate were made followed by a brief wash with 1X DPBS to remove any floating cells. Then, the cells received following treatments: 10µM 5-Aza-CdR, 40ng/mL TSA, 5µM DZNeP, 20µM EGCG and 10µg/mL GTP. The cells were incubated at 37˚C in a 5% CO_2 humidified incubator and photographed at indicated time points using a digital camera attached to an inverted microscope. Cell migration areas were calculated using Image J version 1.47 (NIH, USA).

STATISTICAL ANALYSIS

All quantitative data has been represented as mean ± standard deviation. The number of replicates for each experiment has been indicated in respective figure legends. The digital images were quantified using Image J software (NIH, USA). Statistical analysis between control and treated groups was performed using two-tailed Student's t-test. Multiple comparisons were performed using one way ANOVA followed by Tukey's test. The level of significance designated statistically significant are as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Chapter IV



Combinatorial effect of EGCG and β-estradiol on the proliferative and invasive characteristics of human breast cancer cells

INTRODUCTION

Estrogens are known to cause breast cancer proliferation through two modes i.e. ER dependent (genomic) or ER independent (non-genomic) pathways (Heldring *et al.*, 2007; Ikeda and Inoue, 2004). In ER mediated actions, upon ligand (E2) binding to nuclear receptor (ERα), E2- ERα complex translocates to the nucleus and activates the transcription of genes involved in proliferation, differentiation and survival. Examples include genes involved in cell cycle such as *C-MYC*, *CYCLINS D, A* and *E* (Preston-Martin *et al.*, 1990; Yue *et al.*, 2013). The ER independent oncogenic effects of estrogens have been demonstrated to occur through the actions of estrogen metabolites such as 2,3- or 3,4-catechols, 2,3- or 3,4- quinones and semi-quinones (Cavalieri *et al.*, 2006; Yager and Davidson, 2006). All these metabolites cause DNA damage and are associated with high degree of oxidative damage and genotoxicity in breast tissue (Cavalieri and Rogan, 2011; Yue *et al.*, 2013). In addition, non genomic effects of E2 in breast cancer cells has been attributed to alternative signalling pathways via membrane bound ER (mbER) and G-protein coupled receptor (GPCR-30). Binding of E2 to mbER complex cause de-palmitoylation, release of ERα from caveolin-1, activation of downstream signalling proteins such as AKT, Protein Kinase C, tyrosine kinase Src and facilitates the movement of ERα to membrane microdomains (Bjornstrom and Sjoberg, 2004; Sotgia *et al.*, 2006). mbER-E2 signalling has been shown to affect cell proliferation, survival and apoptosis (Marino *et al.*, 2002). Furthermore, E2 has been reported to act as an agonist in GPCR-30 signalling (Filardo and Thomas, 2012). Upon ligand binding, E2-GPCR-30 complex stimulates adenylate cyclase and induces cAMP mediated modulation of EGF signalling pathway (Filardo *et al.*, 2002). Taken together, existing reports strongly advocate the central role of estrogen in breast tumorigenesis.

Till date, molecular mechanisms of how EGCG affects cell proliferation, apoptosis, migration and cell cycle by modulating the function of a wide range of cellular targets have been extensively studied in breast cancer models (Bigelow and Cardelli, 2006; Demeule *et al.*, 2000; Schramm, 2013; Stuart *et al.*, 2010). However the question as to how EGCG impacts estrogen regulated cellular phenotypes has not been addressed. This question is of relevance to our notion of EGCG as a potential chemopreventive or chemotherapeutic agent against breast

cancer, which is an estrogen dependent malignancy in majority of the newly diagnosed cases. Therefore the first aim of this work was to address the combinatorial effect of EGCG and E2 on proliferation, apoptosis, cell cycle distribution, gelatinolytic activity, migration and invasion of breast cancer cells differing in their ER status. Here, we have used MCF-7 and MDA-MB-231 breast adenocarcinoma cells, representing two different phenotypes of breast cancer conditions and ER status, as the *in vitro* cellular models. MCF-7 represents less invasive, ER positive and hormone responsive breast cancer condition while MDA-MB-231 shows more aggressive, highly migratory, ER negative and estrogen insensitive *in vitro* behavior. In this chapter, we have demonstrated that EGCG decreases viability, proliferation, gelatinolytic activity (MMP-2 and MMP-9) and induces apoptosis in the breast cancer cells irrespective of the ER status or estrogenic stimulation. In addition, EGCG was also found to inhibit the migration and invasion of MDA-MB-231 breast cancer cells.

RESULTS

Dose and time dependent effect of E2 on MCF-7 and MDA-MB-231 viability and proliferation

MCF-7 cells were treated with varying concentrations of E2 for a period of 72h and the numbers of viable cells were counted on the basis of Trypan Blue dye exclusion. 1nM and 10nM concentrations of E2 were found to significantly increase the number of viable counts by 1.3 and 1.8 fold respectively (Figure 4.1A). A time course experiment showed that 10nM E2 significantly increased the number of viable MCF-7 cells as early as 48h (Figure 4.1C). By 72h there was 1.7 fold increase in viable cell count, which is similar to the observation in the previous experiment. In a dose dependent experiment with MDA-MB-231 cell line carried out for 72h, none of the concentrations of E2 increased the number of viable cells (Figure 4.1B). 10nM E2 treatment for a period of 72h was used as an optimum dosage for observing estrogen induced effects in subsequent studies.

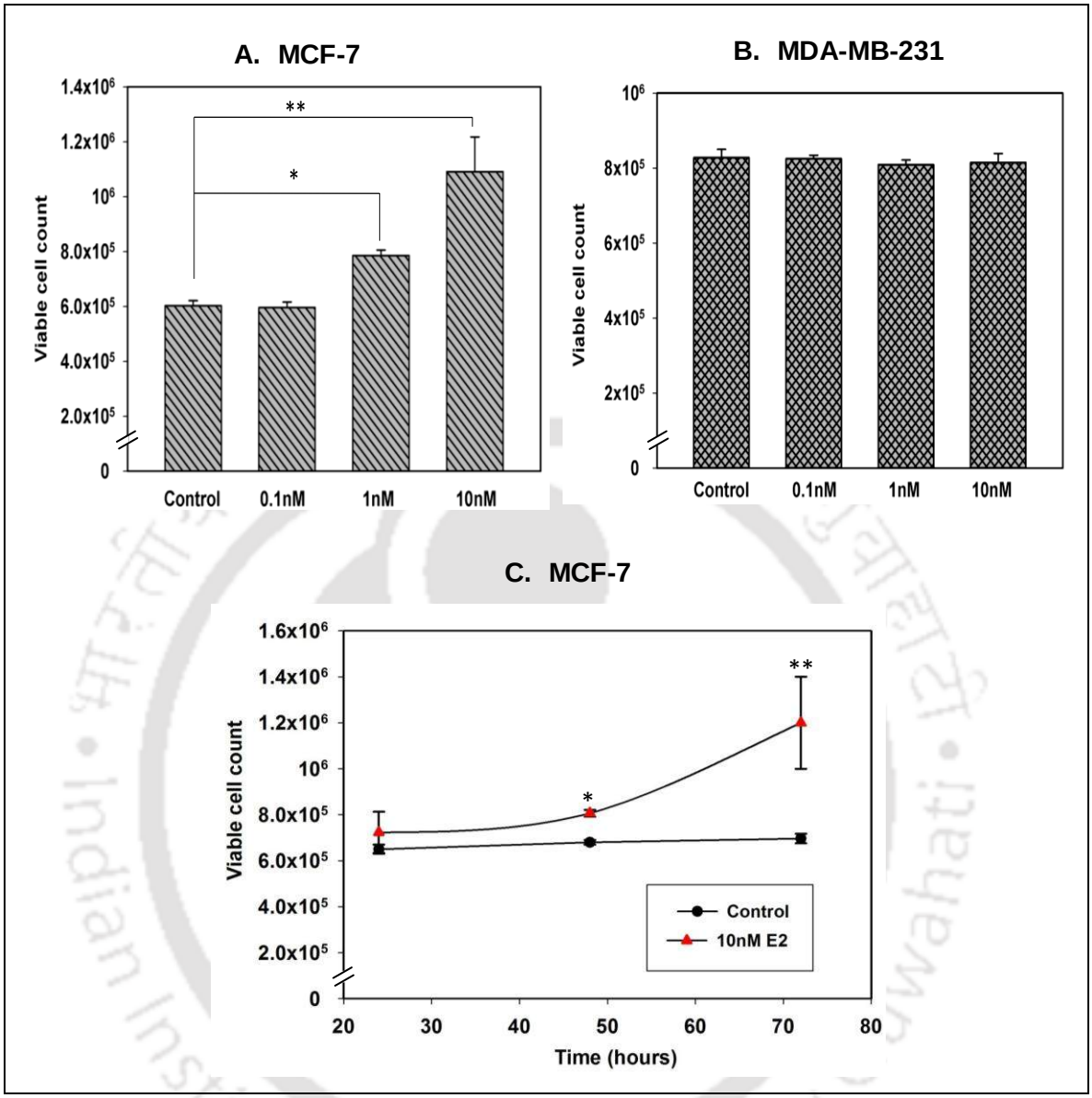


Figure 4.1. Dose and time dependent effect of E2 on breast cancer cell viability and proliferation. 1×10^5 cells were seeded in 35mm culture dishes and allowed to grow in M1 medium. After 24-36h, the cells were treated with various doses of E2 (0-10nM) for a period of 24-72h in M2 medium. Viable cell count was determined by trypan blue dye exclusion method. *Panel* **A**: Viable cell count of MCF-7 cells treated with E2 for 72h. *Panel* **B**: Viable cell count of MDA-MB-231 cells treated with E2 for 72h. *Panel* **C**: Time dependent effect of 10nM E2 on MCF-7 viable cell count. Each bar represents Mean +/- SD (n=3). *p < 0.05, **p < 0.01, ***p< 0.001.

Dose dependent effect of EGCG on MCF-7 and MDA-MB-231 cell viability

MTT assays performed with varying doses of EGCG (2-200µM) showed that there was a significant dose dependent decrease in cell viability with increasing concentration of EGCG in both MCF-7 and MDA-MB-231 breast cancer cells (Figure 4.2). In MCF-7 cells, IC_{50} value of EGCG (EGCG concentration at which 50% reduction in cell proliferation was observed) was found to be ~80µM whereas in MDA-MB-231 breast cancer cells it was found to be much lower (~45µM) as compared to MCF-7.

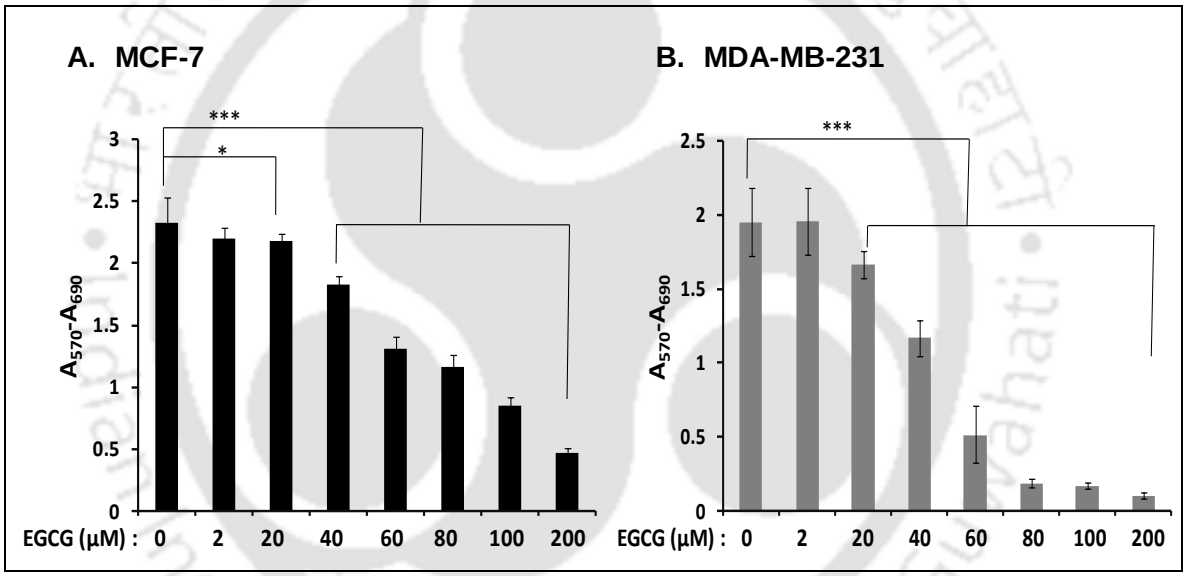


Figure 4.2. Dose dependent effect of EGCG (2-200µM) on the viability of breast cancer cells. 8×10^3 cells were seeded per well in a 96 well plate with medium M1 and allowed to grow. After 36h, the spent medium was replaced with fresh M1 medium containing only vehicle (ethanol) or various doses of EGCG (as indicated). After 72h, MTT assay was performed to determine cell viability. Each bar represents mean +/- SD (n=10). *p< 0.05, **p< 0.01, ***p < 0.001.

Combined effect of E2 and EGCG on the viability and proliferation of MCF-7 and MDA-MB-231

We explored the combined effect of EGCG and E2 on the viability and proliferation of both breast cell lines using MTT assay. In these experiments, 20-60µM doses were chosen so that there was no severe cytotoxic effect of EGCG on the cells while evaluating the combined effect. In MCF-7, 10nM E2 treatment for

72h significantly induced cell proliferation with respect to control (vehicle treated) cells (Figure 4.3A). These data confirm the estrogen responsiveness of MCF-7 cells and corroborate with our previous observation (Figure 4.1A and 4.1C). Since MDA-MB-231 is an estrogen insensitive cell line, no significant increase in cell proliferation was observed for 10nM E2 treated groups with respect to control (Figure 4.3B). 20-60µM EGCG significantly reduced cell viability with respect to control and 10nM E2 treated groups in both cell lines (Figure 4.3). However, no significant difference was observed between combined EGCG + E2 treated and only EGCG treated groups in both MCF-7 and MDA-MB-231 cells (Figure 4.3).

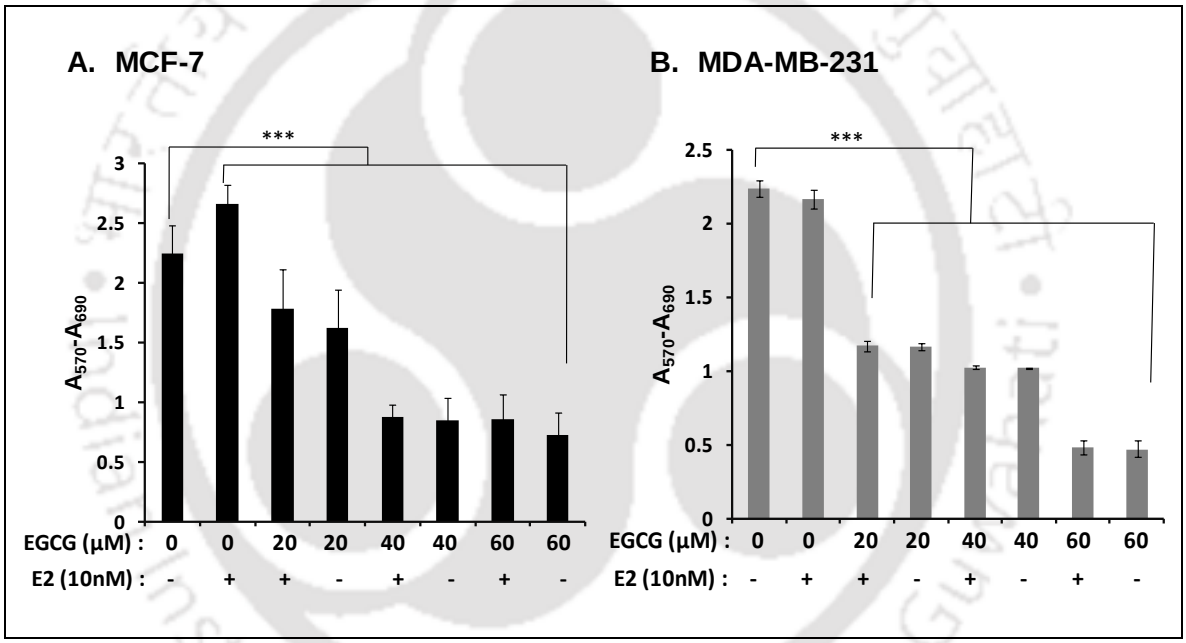


Figure 4.3. Effect of E2, EGCG or both on the viability and proliferation of breast cancer cells. 8×10 ^{3} cells were seeded per well in a 96-well plate with M1 medium and allowed to grow. After 36h, the spent medium was replaced and cells were incubated with fresh M2 medium for 3-4h followed by treatment with only vehicle (ethanol), E2 (10nM), EGCG (20-60µM) or E2 (10nM) + EGCG (20-60µM). After 72h, MTT assay was performed to determine cell viability and proliferation. Each bar represents Mean +/- SD (n=10). *p< 0.05, **p<0.01, ***p< 0.001

Hence, we may draw two inferences from these experiments-

(a) E2 is unable to induce breast cancer cell proliferation in the presence of EGCG.

(b) The effectuality of EGCG remains unhindered even in the presence of high exogenous E2 levels.

Combined effect of E2 and EGCG on MCF-7 and MDA-MB-231 cell morphology and nuclear integrity

The morphology and nuclear integrity of E2 and EGCG treated cells were studied by bright field microscopy and PI staining. Cells treated with 40µM EGCG alone or combinations with E2 were fewer in number and irregular in appearance as compared to control and 10nM E2 treated cells in both MCF-7 and MDA-MB-231 (Figure 4.4). Propidium iodide (PI) staining of MDA-MB-231 cells treated with EGCG showed no major changes in nuclear staining pattern as compared to only vehicle treated cells (Figure 4.4B). However, PI stained EGCG treated MCF-7 cells were discrete, round and brightly stained (Figure 4.4A).

Effect of E2, EGCG and combined treatment on the cell cycle distribution of MCF-7 and MDA-MB-231 cells

We studied the cell cycle distribution in MCF-7 and MDA-MB-231 cells treated with E2, EGCG or both by flow cytometry. As shown in Figure 4.5, 40µM EGCG induced a significant increase (7.52%) in G0/G1 phase in 10nM E2 stimulated MCF-7 cells while there was a concomitant decrease (7.43%) in S-phase cells as compared to control (vehicle treated) cells. MCF-7 cells in G2/M phase remained unaffected by EGCG treatment alone or in combination with E2. In MDA-MB-231 cells, no significant changes in cell cycle distribution pattern were observed after EGCG or E2 treatment (Figure 4.6).

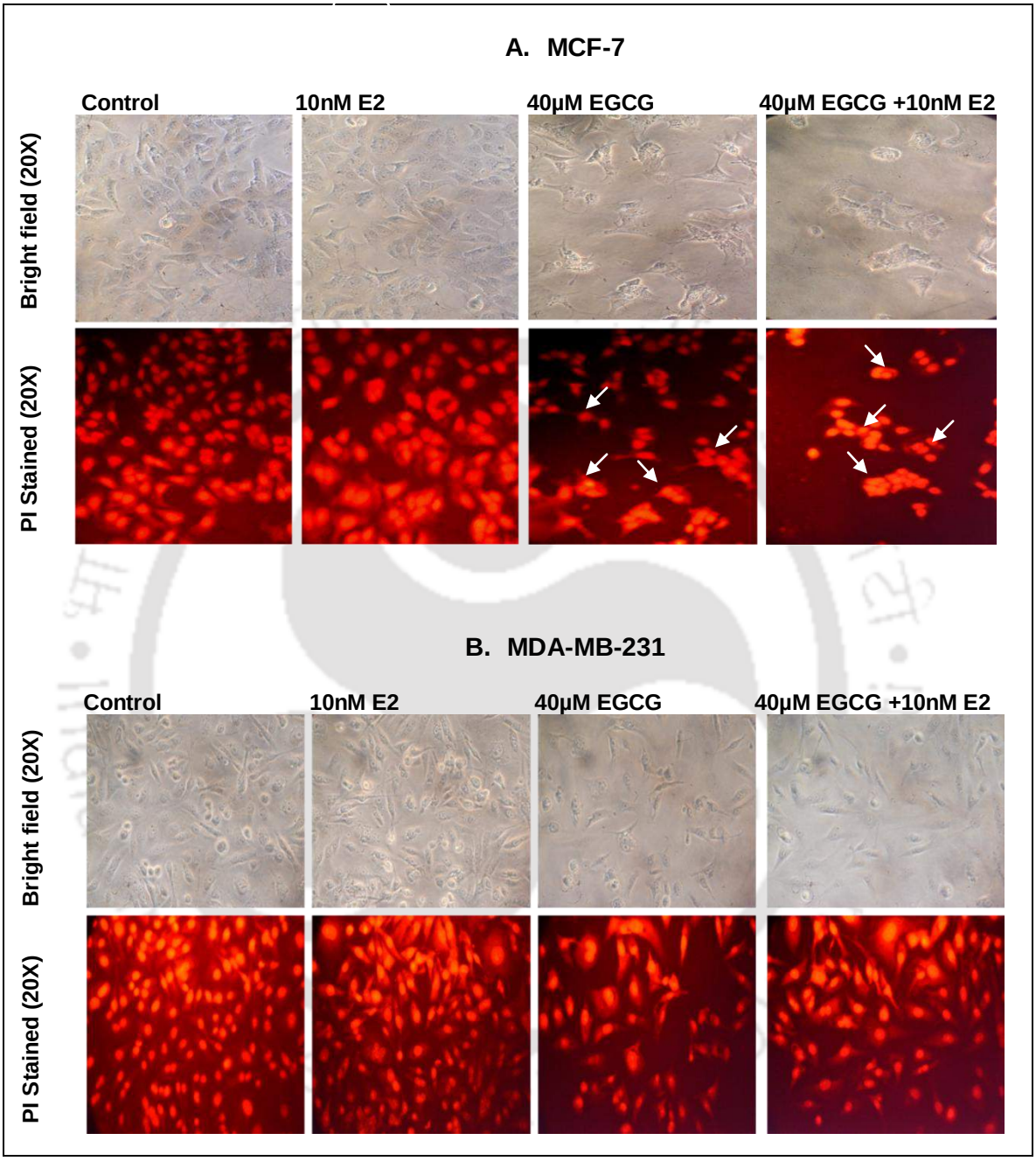


Figure 4.4. Effect of E2, EGCG or both on the breast cancer cell morphology and nuclear integrity. 1×10^5 cells were seeded in 35mm culture dishes and allowed to grow in M1 medium. After 24-36h, the cells were treated with vehicle (ethanol), E2 (10nM), 40µM EGCG or both E2 (10nM) and 40µM EGCG in M2 medium. After 72h, the spent media were removed and cells were washed with DPBS. The cells were fixed and stained with PI for 10 min at 4˚C in dark. The cells were then photographed under 20X magnification using white light (bright field) and green laser beam (PI stained) in an inverted microscope.

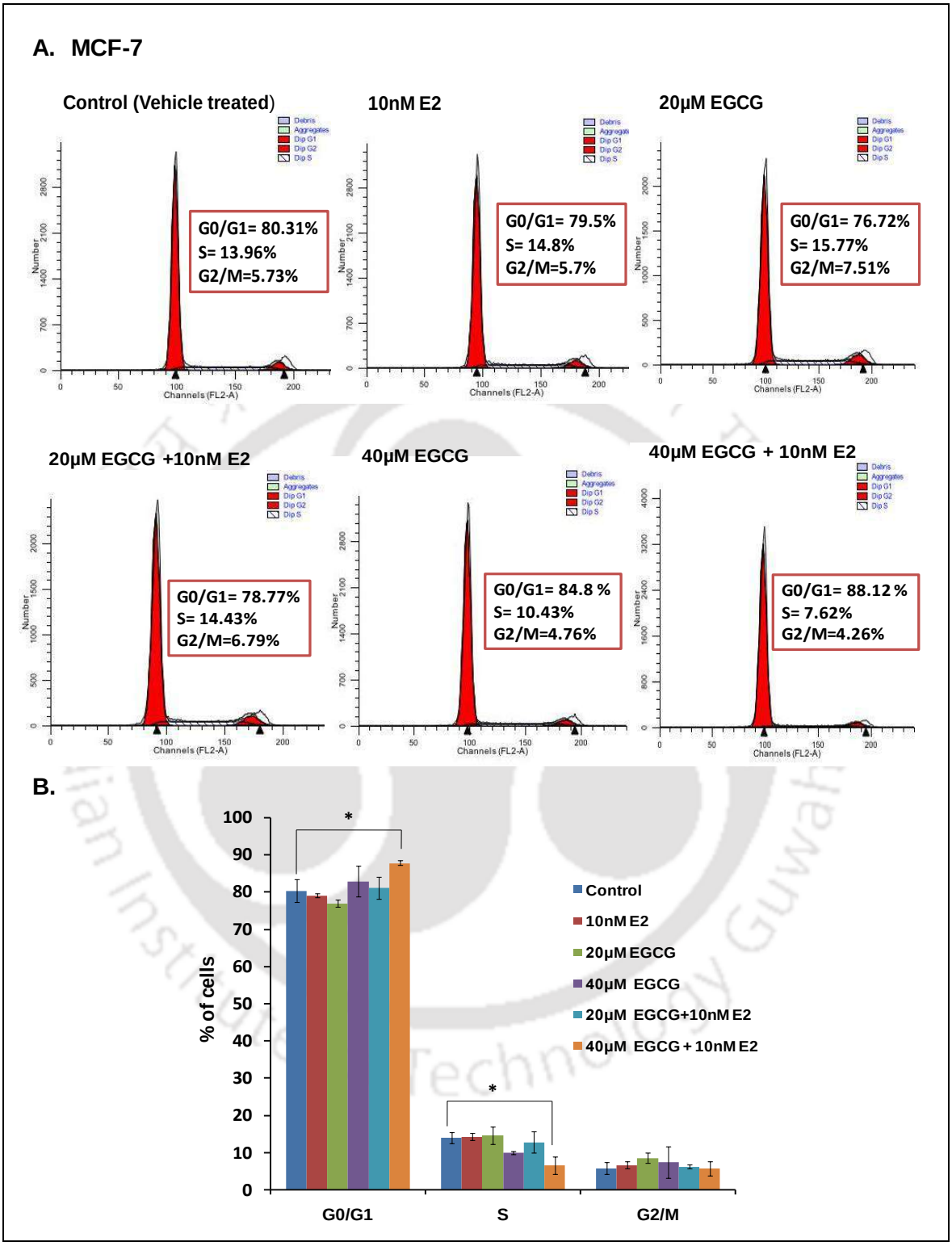


Figure 4.5. Effect of E2, EGCG or both on the cell cycle distribution of MCF-7 cells. Overnight (12-15h) serum starved cells were treated with only vehicle (Control), 10nM E2, 40μM EGCG or 10nM E2+ 40μM EGCG for 24h. The cells were fixed, PI stained and then analyzed by flow cytometry. *Panel* **A**: Histogram profiles generated using ModFit LT software. *Panel* **B**: Bar graph showing percentage of cells in each phase of the cell cycle after treatment. Each bar represents mean +/- SD (n=3). *p< 0.05, **p< 0.01, ***p< 0.001.

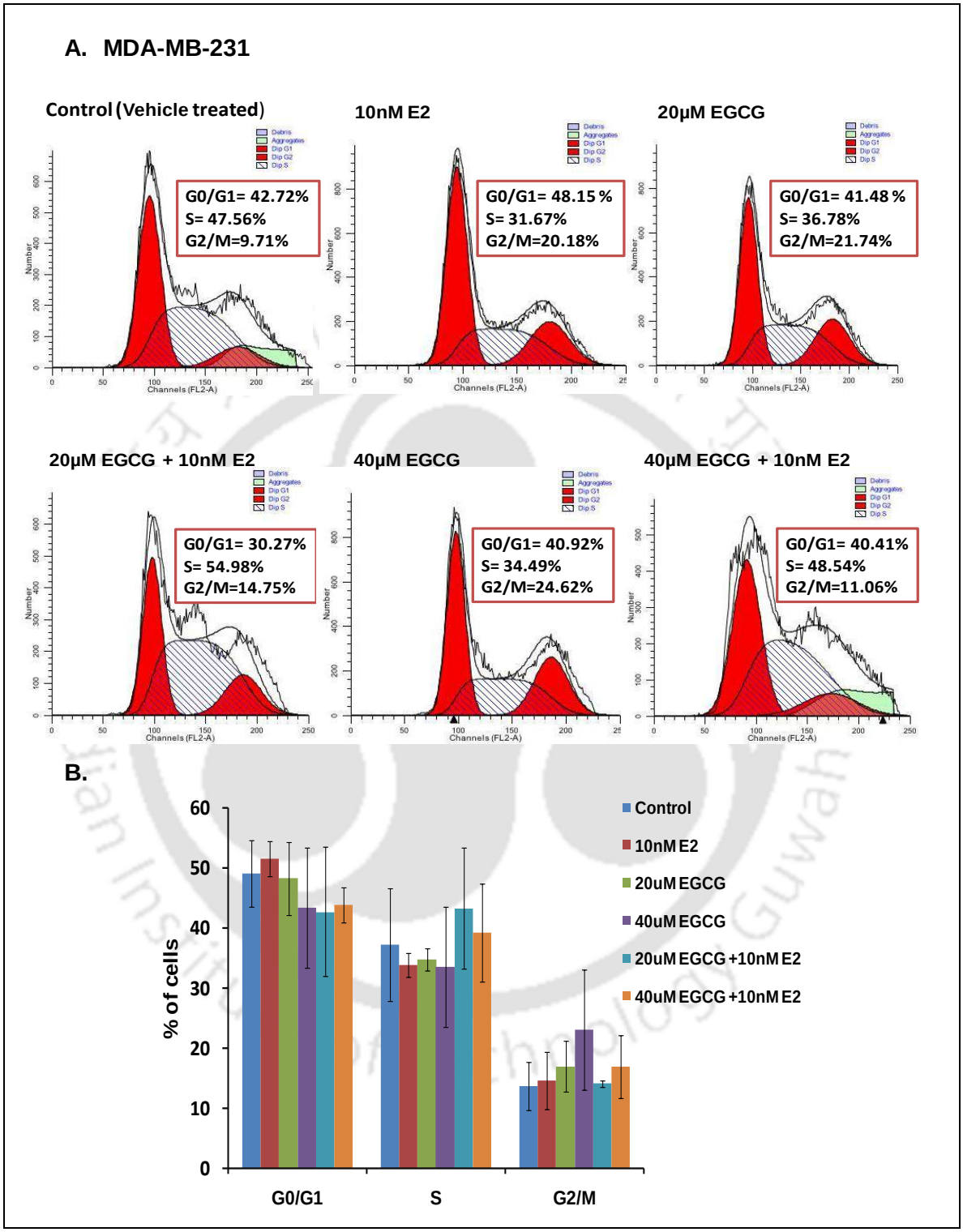


Figure 4.6. Effect of E2, EGCG or both on the cell cycle distribution of MDA-MB-231 cells. Overnight (12-15h) serum starved cells were treated with only vehicle (Control), 10nM E2, 40µM EGCG or 10nM E2+ 40µM EGCG for 24h. The cells were fixed, PI stained and then analyzed by flow cytometry. *Panel* **A**: Histogram profiles generated using ModFit LT software. *Panel* **B**: Bar graph showing percentage of cells in each phase of the cell cycle after treatment. Each bar represents mean +/- SD (n=3). *p< 0.05, **p< 0.01, ***p< 0.001.

Detection of apoptosis in E2, EGCG or combined treated MCF-7 and MDA-MB-231 cells

In our earlier experiments, we have demonstrated that EGCG alone or in combination with E2 caused a significant decrease in breast cancer cell viability and proliferation, regardless of the ER status or estrogenic stimulation. To evaluate whether the EGCG mediated anti-proliferative effect on breast cancer cells was due to the induction of apoptosis, we investigated the effect of E2, EGCG or both on apoptosis in MCF-7 and MDA-MB-231 cells using flow cytometry based Annexin V-FITC PI staining.

The total percentage of apoptotic cells represents the sum of late apoptotic cells shown in upper right (UR) quadrant and early apoptotic cells shown in lower right quadrant (LR) of the scatter plot. As shown in Figure 4.7, the total number of apoptotic cells in 40µM EGCG treated MCF-7 cell line were found to be 28.73%, which represents a marked increase of 17.15% as compared to control (vehicle treated) cells (11.58%). In combined treated (EGCG+E2) group, the total number of apoptotic cells increased by 21.3% as compared to control. Notably, 10nM E2 treatment also caused 5.56% increase in apoptosis as compared to control. Therefore, the increase in apoptotic index observed in combined treated MCF-7 cells may be attributed to the additive effect of both EGCG and E2 treatment.

In MDA-MB-231 cells, 40µM EGCG treatment caused 17.25% increase in the total percentage of apoptotic cells as compared to control whereas in combined treated (E2 + EGCG) cells, the total increase was found to be 13.99% (Figure 4.8). Compared to MCF-7, 10nM E2 mediated induction of apoptosis was found to marginal (1.96%) in MDA-MB-231.

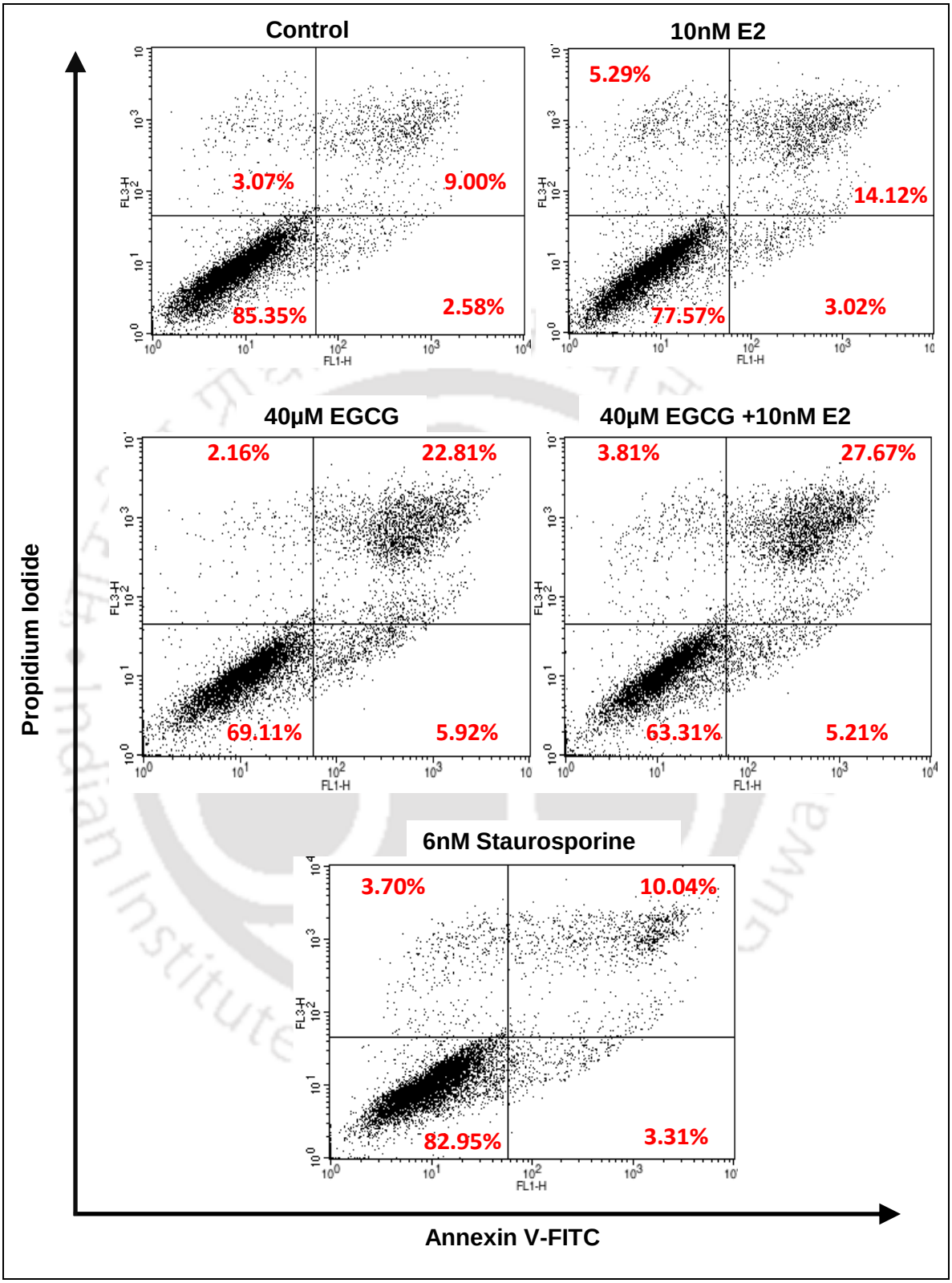


Figure 4.7. Determination of apoptosis (%) after treatment with E2, EGCG or both in MCF-7. Cells were treated with only vehicle (Control), 10nM E2, 40µM EGCG and 10nM E2 + 40µM EGCG for 72h and apoptotic cell population was determined by flow cytometry based analysis of Annexin-V-FITC PI stained cells. MCF-7 cells treated with 6nM Staurosporine for 1h served as positive control.

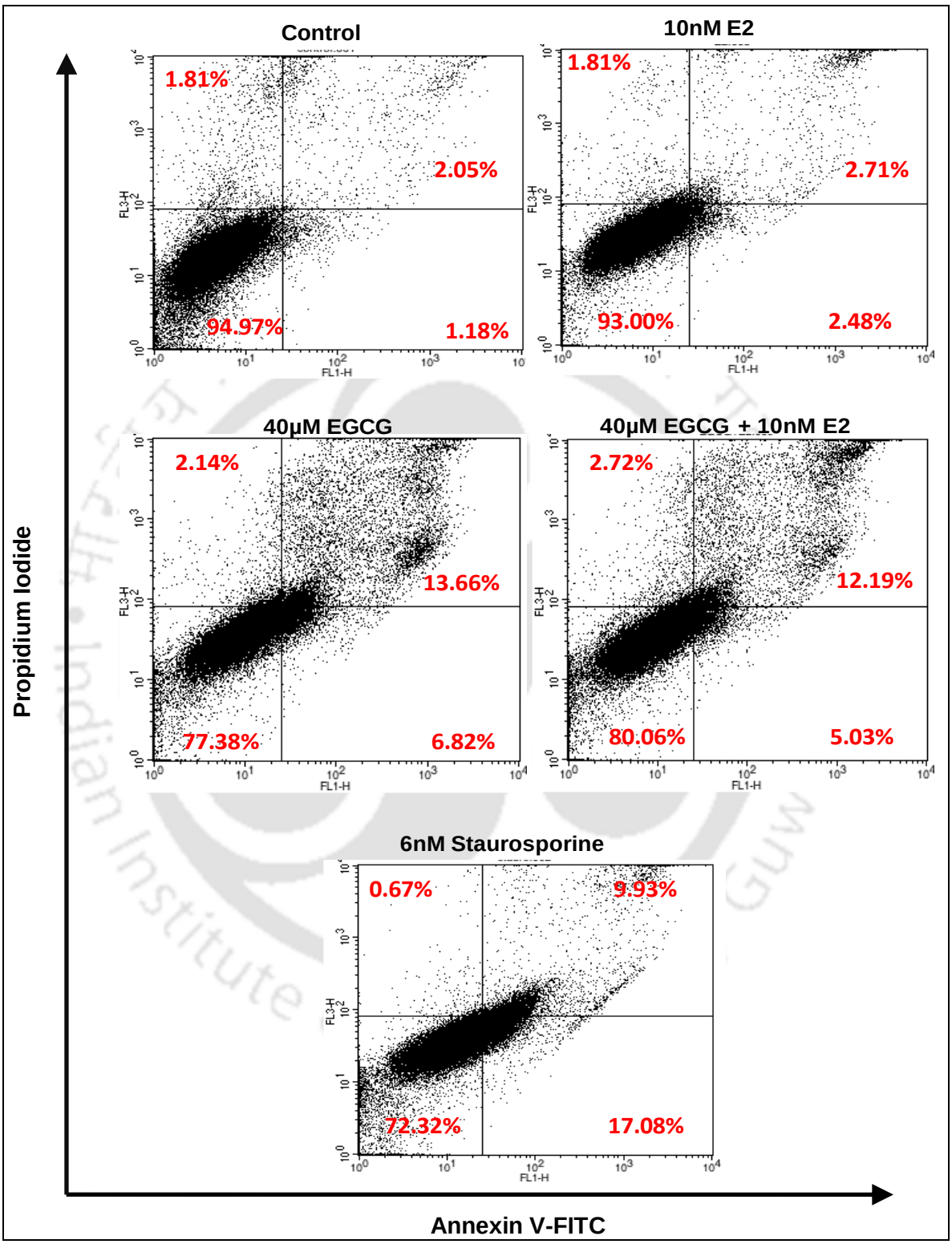


Figure 4.8. Determination of apoptosis (%) after treatment with E2, EGCG or both in MDA-MB-231. Cells were treated with only vehicle (Control), 10nM E2, 40µM EGCG and 10nM E2 + 40µM EGCG for 72h and apoptotic cell population was determined by flow cytometry based analysis of Annexin-V-FITC PI stained cells. MDA-MB-231 cells treated with 6nM Staurosporine for 1h served as positive control.

Effect of E2, EGCG or both on the gelatinolytic activity (MMP-2 and MMP-9) in MCF-7 and MDA-MB-231

Breast cancer cells were treated with different doses of EGCG (2µM, 20µM and 200µM) and conditioned media were collected after 72h. The conditioned medium from each treated group was analyzed by gelatin zymography for determining MMP-2 and MMP-9 activity. In this assay, the gelatin substrate present in the polyacrlamide gel gets digested by gelatinases and appears as white clear bands. As shown in Figure 4.9, 200µM EGCG treatment in breast cancer cells caused a marked decrease in MMP-2 and MMP-9 activity.

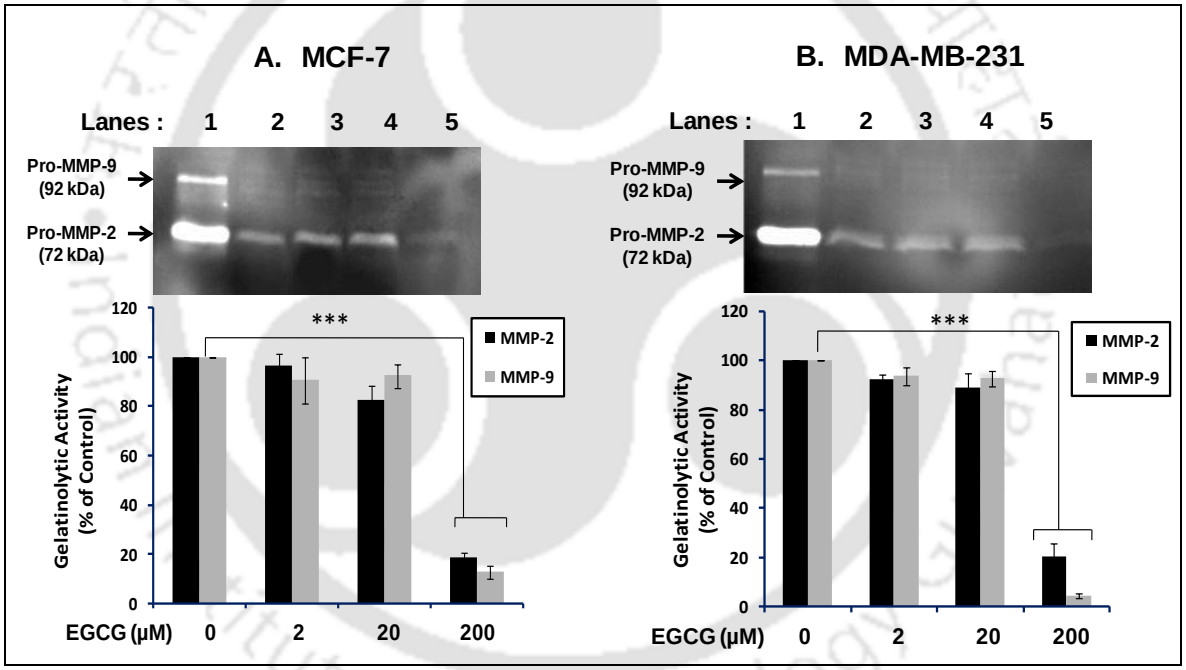


Figure 4.9. Effect of different doses of EGCG on the gelatinolytic activity in breast cancer cell lines. After 72h of treatment period with different doses of EGCG, the culture supernatants were collected and equal volumes of samples were analyzed for gelatinolytic activity by gelatin zymography. Lanes: **1**. HT1080 conditioned medium; **2.**Control (vehicle treated); **3.** 2µM EGCG treated; **4.** 20µM EGCG treated; **5.** 200µM EGCG treated. Densitometry based quantitation of pro-MMP-2 and pro-MMP-9 bands were done using Image J. The normalized band intensities for control were assigned the value of 100% and those for EGCG treated samples were plotted as percentage with respect to control. Bars represent mean +/- SD (n=3). *p< 0.05, **p< 0.01, ***p< 0.001.

In our previous experiments, we have shown that 200µM EGCG caused more than 80% decrease in cell viability. Hence, we choose 40µM EGCG dosage for exploring the effect of E2, EGCG or both on the gelatinolytic activity in MCF-7 and MDA-MB-231 cells. As shown in Figure 4.10, 40µM EGCG significantly decreases gelatinolytic activity in both breast cancer cells irrespective of the presence of E2 in the medium or their ER status.

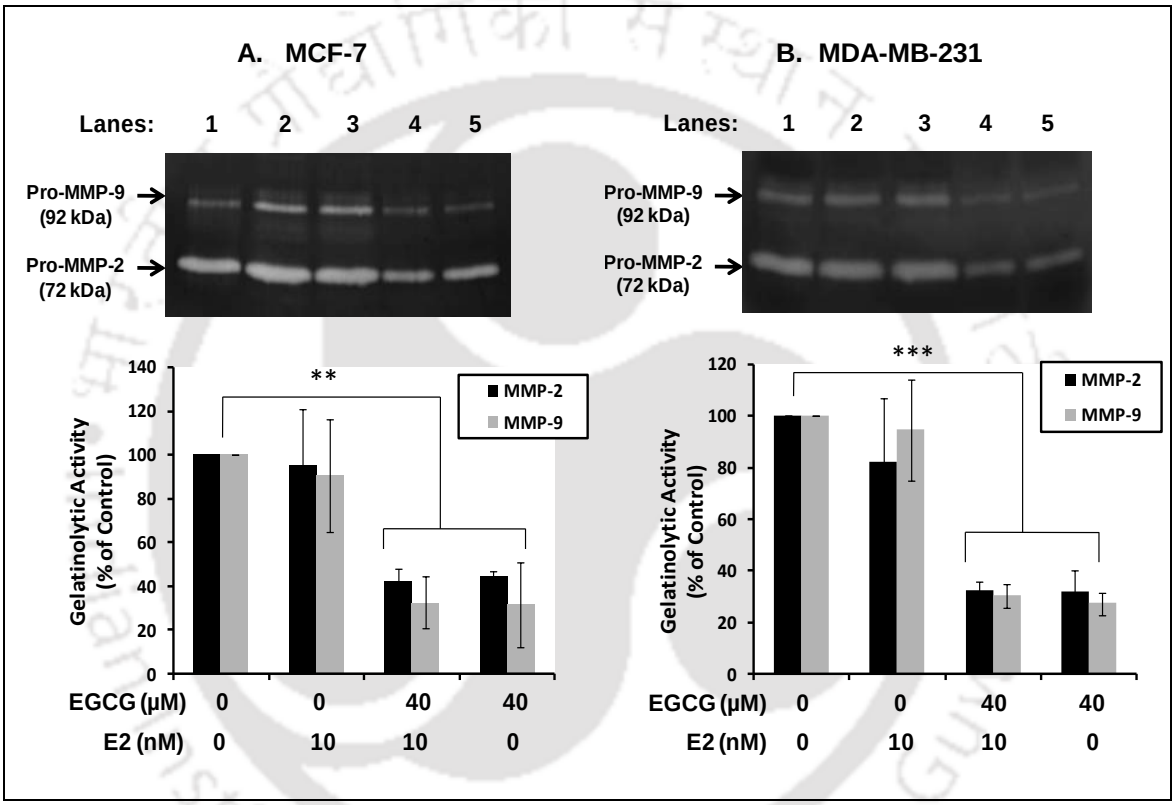


Figure 4.10. Effect of E2, EGCG or both on the gelatinolytic activity in breast cancer cells. Cells were treated with vehicle (ethanol), 10nM E2, 40µM EGCG and 40µM EGCG + 10nM E2 for 72h and conditioned media obtained from various treated groups were analyzed by gelatin zymography. Lanes: **1**. HT1080 spent medium; **2.**Control (vehicle treated); **3.** 10nM E2 treated; **4.** 40µM EGCG treated; **5.** 40µM EGCG+10nM E2 treated. Densitometry based quantitation of pro-MMP-2 and pro-MMP-9 bands were done using Image J. The normalized band intensities for control were assigned the value of 100% and those for treated samples were plotted as percentage with respect to control. Bars represent mean +/- SD (n=3). *p< 0.05, **p< 0.01, ***p< 0.001.

Effect of E2, EGCG or both on breast cancer cell migration and invasion

To gain an insight into the functional effect of EGCG mediated down-regulation of gelatinolytic activity in the breast cancer cells, *in vitro* migration assays were performed. Cells were treated with vehicle, 10nM E2, 20µM EGCG or both and allowed to migrate along a chemoattractant (10% FBS) gradient from an upper compartment through collagen IV coated/ uncoated PET membranes (8µm) into a lower compartment. MDA-MB-231 cells which are highly invasive breast cancer cells exhibited significantly high invasive index (90.46%) in control (vehicle treated) group. 20µM EGCG alone or in combination with 10nM E2 caused a significant (45-48%) reduction in their invasive capability in a 18h time period (Figure 4.11). The cells which migrated or invaded through the collagen IV matrix were stained with crystal violet as shown in Figure 4.12. There was no statistically significant difference observed in invasive index between EGCG alone or combined treated groups. Since MCF-7 cells are inherently non-migratory in nature, they did not display significant migration across the membrane even at 24h time period (data not shown). These data provide evidence that EGCG causes significant decrease in the invasive capacity of ER negative MDA-MB-231 breast cancer cells.

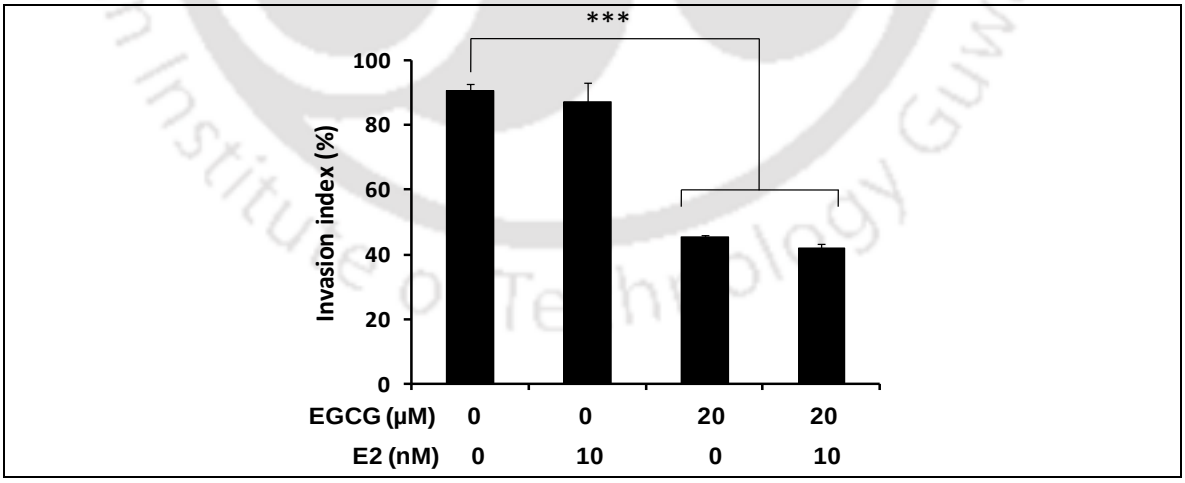


Figure 4.11. Effect of E2, EGCG or both on the invasive index of MDA-MB-231 cells. Cells migrated to the other side of the PET membrane were stained using crystal violet and then dissolved in 1% SDS solution at 37˚C for 1h. Absorbance reading was taken at 595nm and the invasive index for each treatment condition was calculated as described in chapter III (Materials and Methods). Passive migration of cells through collagen IV coated or uncoated membrane under serum free condition in lower chamber served as negative control. Bars represent average invasive index ± SD (n=3). *p< 0.05, **p< 0.01, ***p< 0.001.

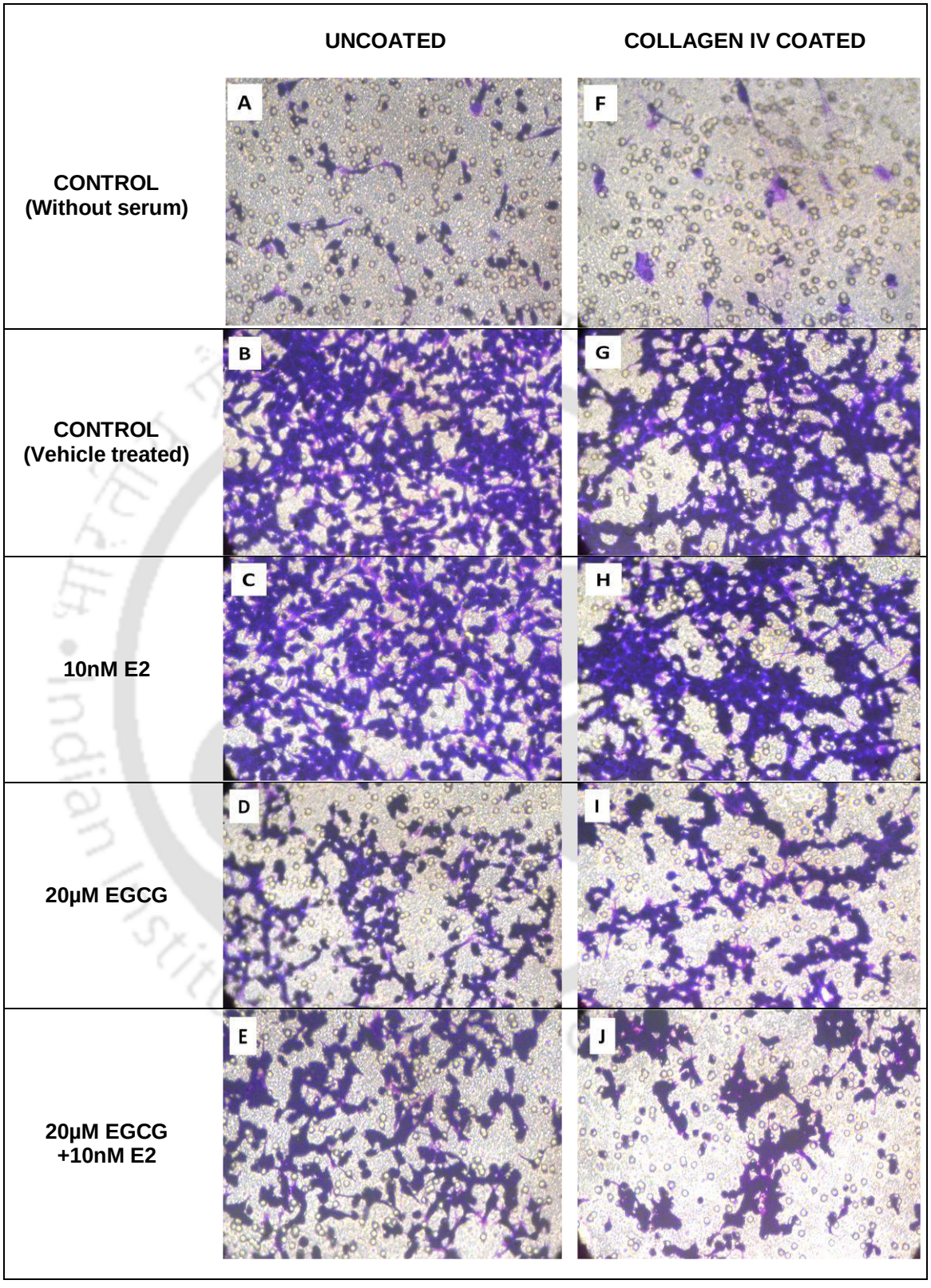


Figure 4.12. Effect of E2, EGCG or both on the invasion and migration of MDA-MB-231 cells. 20X bright field images of 0.05% crystal violet stained cells that migrated through uncoated PET membranes **(A-E)** and collagen IV coated PET membrane **(F-J)**.Passive migration of cells through collagen IV coated or uncoated membrane under serum free condition in lower chamber served as negative control.

DISCUSSION

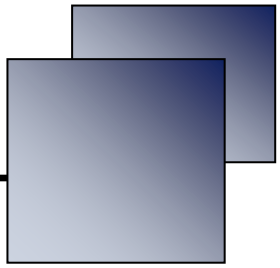
A plethora of literature supports the anti-tumor characteristics of EGCG against a variety of cancer types including endocrine related cancer such as breast cancer (Baliga *et al.*, 2005; Kushima *et al.*, 2009; Ogunleye *et al.*, 2010; Seely *et al.*, 2005; Thangapazham *et al.*, 2007). Since breast cancer is an estrogen dependent malignancy, it becomes essential to evaluate the effects of EGCG on various cellular phenotypes known to be regulated directly or indirectly by estrogen in breast cancer cells. Previous work from our own laboratory aimed at understanding the impact of EGCG treatment on estrogen responsive genes showed that EGCG significantly induces the steady state mRNA expression levels of two classical estrogen target genes, namely Trefoil Factor 1 (pS2) and Progesterone Receptor (PR) in ER positive MCF-7 cells (Mohan *et al.*, 2013). Similarly other studies have demonstrated that EGCG can generate ER mediated responses *in vitro*. For example, studies by Goodin *et al.* (2002) demonstrated specific binding of EGCG to both ERα and ERβ and showed that EGCG can evoke ER mediated transcriptional induction of reporter genes. In an independent study, Kuruto-Niwa *et al.* (2000) have shown that at sub-micromolar concentrations EGCG synergizes with estrogen to induce the estrogen response element (ERE)-driven reporter gene activity indicating that the EGCG can evoke estrogenic responses at the level of gene expression. However there are some studies which advocate that EGCG may act an antagonist of ER mediated signalling by demonstrating down-regulation of pS2 levels at high EGCG doses [>200µM] (Farabegoli *et al.*, 2011). In summary, EGCG has been shown to interact directly or indirectly with ER or ER mediated signalling, however none of the investigations so far have addressed the functional effect of EGCG in combination with E2 on breast cancer cells.

In the present study, we have addressed the functional effect of combined EGCG and E2 treatment on cellular phenotypes such as proliferation, apoptosis, cell cycle distribution, gelatinolytic activity, migration and invasion of breast cancer cells differing in their ER status. Our investigations demonstrated that EGCG (20-60µM) cause significant decrease in proliferation and induce apoptosis in breast cancer cells regardless of the ER status or the high exogenous E2 levels in the surrounding medium. This ER independent anti-proliferative effect of EGCG

on breast cancer cells observed in our studies is consistent with some other studies reported in literature (Chisholm *et al.*, 2004; Kavanagh *et al.*, 2001; Kushima *et al.*, 2009; Liang *et al.*, 1999; Thangapazham *et al.*, 2007). Previous studies have also demonstrated that EGCG treatment significantly induce apoptosis in various breast cancer cell lines including MCF-7 and MDA-MB-231 which is in line with our observations in the present study (Chisholm *et al.*, 2004; Roy *et al.*, 2005). Furthermore, we showed that the efficacy of EGCG in inducing apoptosis in breast cancer cells remain unhindered in the presence of estrogen. In fact, 10nM E2 in combination with 40µM EGCG demonstrated an additive effect in causing apoptosis in ER positive MCF-7 cells. The mechanisms by which EGCG induce apoptosis in breast cancer cells include cell cycle arrest and modulation of cyclin dependent kinase inhibitors (CDKIs) such p21 and p27 has been reported by others (Liang *et al.*, 1999) and was not the focus of the present study. However we demonstrated that 40µM EGCG in combination with 10nM E2 for 24h time period caused a significant G0/G1 arrest of MCF-7 cells while the cell cycle distribution of control (vehicle treated), 40µM EGCG or 10nM E2 alone treated cells remained unaffected. In MDA-MB-231 cells, no significant changes in the cell cycle distribution pattern could be observed in control or treated groups. We also showed that EGCG treatment significantly reduced gelatinolytic activity (MMP-2 and MMP-9) and invasive potential of the breast cancer cells irrespective of the ER status or the presence of estrogen in the surrounding medium.

Our observation that the efficacy of EGCG remained unhindered even in the presence of elevated estrogen levels in the surrounding environment, which in part mimics the *in vivo* breast cancer condition, lead us to conclude that EGCG may emerge as a potential natural chemotherapeutic agent in breast cancer treatment. To our knowledge, this is the first study to evaluate the anti-tumor properties of EGCG on breast cancer cells in the context of estrogen action. We believe that further investigations into the efficacy of EGCG in suppressing tumor growth in breast cancer pre-clinical models would hopefully provide more comprehensive picture.

Chapter V



Modulation of extracellular secreted matrix metalloproteinase -2 activity by EGCG

INTRODUCTION

Aberrant proteolytic activity of MMPs has been implicated in various pathologies including chronic inflammation such as arthritis, periodontal disease as well as in different stages of neoplastic growth and dissemination (Hadler-Olsen *et al.*, 2013; Sternlicht and Werb, 2001). Gelatinases (MMP-2 and MMP-9) have been demonstrated to degrade different components of extracellular matrix particularly collagen IV rich basement membrane and thus facilitate the escape of tumor cells from the primary site of tumor initiation to establish secondary metastases (Curran and Murray, 1999; Liotta and Stetler-Stevenson, 1990). They are over-expressed in a variety of malignant tumors such as breast, brain, ovarian, pancreas, colorectal, bladder, prostate and lung cancer as well as in hematological malignancies (Chaudhary *et al.*, 2013; Hadler-Olsen *et al.*, 2013). Therefore, among all MMPs known to be involved in tumorigenesis, gelatinases have attracted considerable scientific interest as potential anti-cancer targets (Stetler-Stevenson, 1990; Stetler-Stevenson *et al.*, 1992). Elevated gelatinolytic expression and activity are often associated with tumor aggressiveness and poor prognosis.

The failure of synthetic MMP inhibitors such as Batimastat, Marimastat, Prinomastat etc, in the clinical trials, has prompted a global search for natural, non-toxic and selective inhibitors (Belotti *et al.*, 1999; Hidalgo and Eckhardt, 2001; Zucker *et al.*, 2000). Several epidemiological, *in vitro* and *in vivo* studies have highlighted the health benefits associated with green tea consumption. In the past two decades, EGCG has attracted the attention of scientific community as a potential chemopreventive agent. The ability of EGCG to modulate the expression and activity of gelatinases, as demonstrated in several studies has emerged as an important lead, since it projects EGCG (or green tea) as a potential anti-invasive or anti-metastatic agent (Farabegoli *et al.*, 2011; Garbisa *et al.*, 2001; Sen *et al.*, 2010; Sen *et al.*, 2009). EGCG was shown to down-regulate MMP-2 mRNA and protein levels as well as act as an inhibitor of MMP-2 enzyme (Garbisa *et al.*, 2001). However, the mechanistic details were not addressed adequately.

In the present study, we have demonstrated that EGCG inhibits MMP-2 activity in a cell free environment via a non - Zn^{+2} chelation mechanism. Furthermore, divalent cations were shown to have a negative effect on EGCG mediated MMP-2 inhibition. Enzyme kinetic studies revealed that EGCG acts as an uncompetitive inhibitor of MMP-2 enzyme.

RESULTS

Dose dependent inhibition of human MMP-2 by EGCG under cell-free environment

MCF-7 conditioned medium treated with various doses of EGCG (2-200µM) showed a marked decline in gelatinolytic activity in a cell free environment (Figure 5.1A). For further confirmation, culture supernatants were obtained from confluent cultures of HT1080 cells, a fibrosarcoma cell line with over-expression of gelatinases and used as source of MMP-2 for our studies. To investigate the direct effect of EGCG on Pro-MMP-2 in a cell–free environment, aliquots of HT1080 conditioned media were incubated with various doses of EGCG (0-200µM) at 37˚C for 45 min. Incubation with only high dose of EGCG (200µM) caused a significant decrease ($p<0.05$) in Pro-MMP-2 gelatinolytic activity as demonstrated by gelatin zymography (Figure 5.1B and 5.1C). However, when purified and active human MMP-2 was incubated with various doses of EGCG (0-5µM) followed by gelatin zymography, inhibition was achieved at a much lower EGCG concentration, with an IC_{50} value (concentration that inhibited 50% of the catalytic activity of the enzyme) of 3.05µM (Figure 5.2A and 5.2B).

Since gelatin zymography is primarily a qualitative technique for detection of MMPs, we used MMP-2 specific fluorogenic substrate assay for further confirmation of our preliminary observations. The assay utilizes triple helical peptide substrate that is highly selective for MMP-2. As shown in Figure 5.2C, EGCG (2-40µM) significantly inhibited hydrolysis of triple helical FRET peptide substrate MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2 by MMP-2 (active) in a dose dependent manner. The IC_{50} value for this EGCG mediated MMP-2 inhibition was computed to be 4.23µM. Taken together, our results suggest that EGCG significantly inhibits *in vitro* MMP-2 activity in a dose dependent manner.

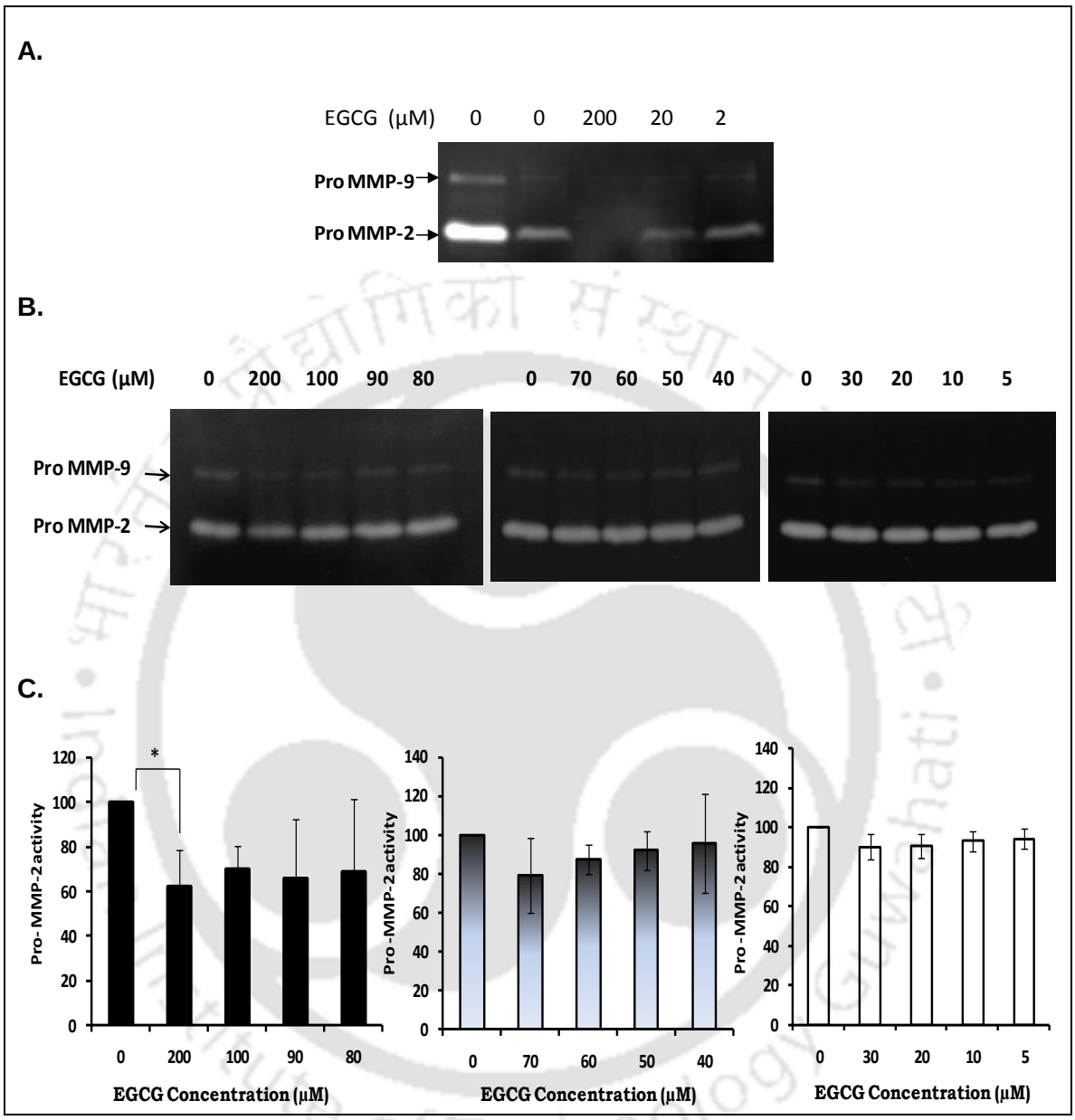


Figure 5.1. EGCG mediated decrease in MMP-2 gelatinolytic activity under cell free environment. *Panel* **A**: Gelatin zymograph gel showing dose dependent decrease in gelatinolytic activity in MCF-7 conditioned medium. Equal volume of conditioned medium was incubated with various doses of EGCG (2-200µM) at 37˚C for 45 min and loaded into a gelatin-PAGE gel. HT1080 conditioned medium was used as a positive control and loaded into the first lane (extreme left) of the gel. *Panel* **B**: Equal volume of HT1080 conditioned medium was incubated with various doses of EGCG (0-200µM) at 37˚C for 45 min and loaded into a gelatin-PAGE gel. Gelatin zymographs showing dose dependent effect of EGCG on MMP-2 and MMP-9 activities. *Panel* **C**: Graphical representation of data shown in panel **B**. Densitometry based quantitation of pro-MMP-2 bands using Image J. The normalized band intensities for control were assigned the value of 100% and those for EGCG treated samples were plotted as percentage with respect to control. Bars represent mean +/- SD (n=3). *p< 0.05, **p< 0.01, ***p< 0.001.

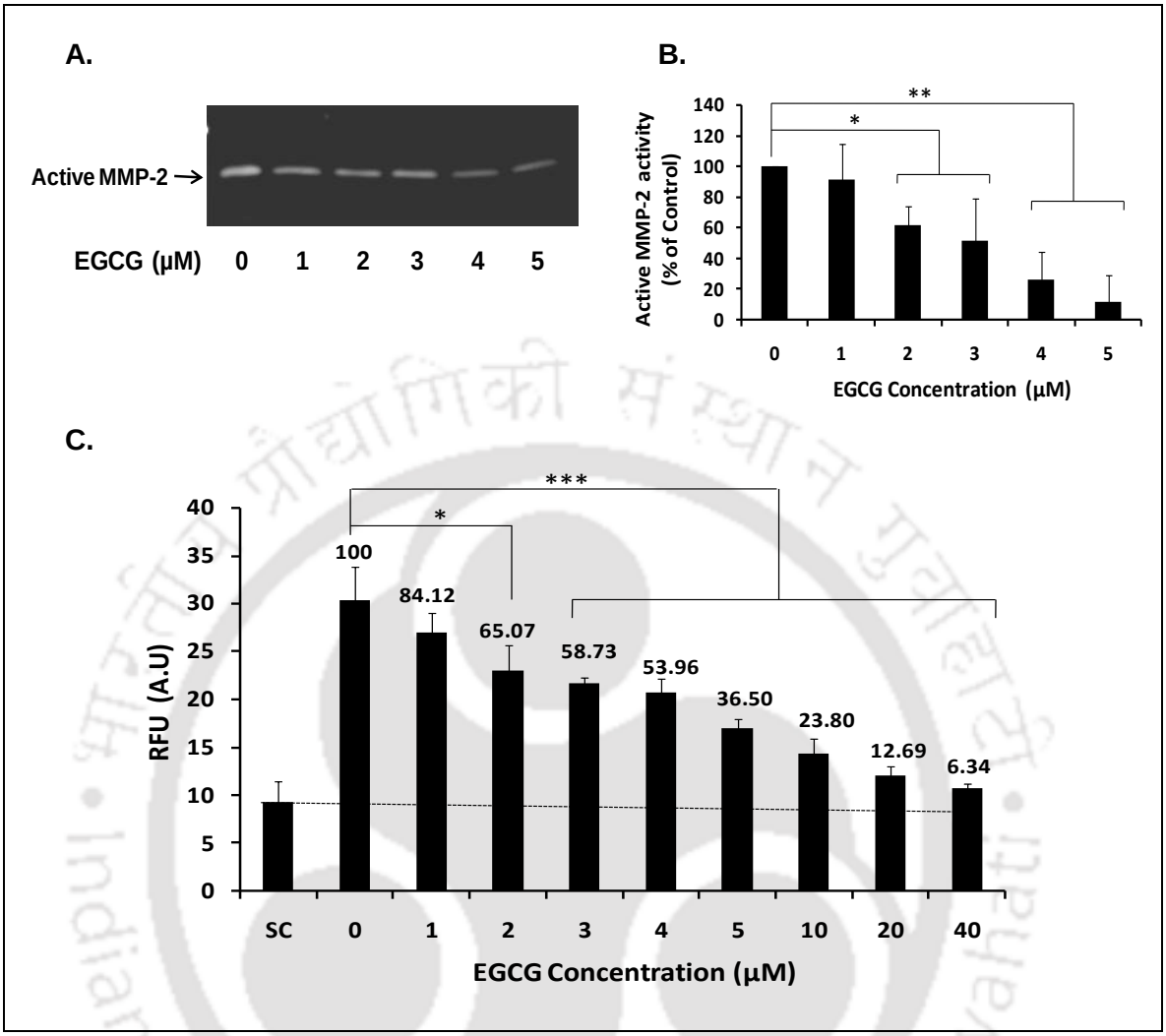


Figure 5.2. EGCG mediated decrease in active MMP-2 activity. Purified, active MMP-2 (10ng) was incubated with various doses of EGCG (0-5µM) at 37˚C for 45 min and analyzed for gelatinolytic activity using gelatin-PAGE gel and MMP-2 specific FRET based assay. *Panel* **A:** Gelatin zymography gel image showing dose dependent decrease in MMP-2 activity following incubation with different doses of EGCG. *Panel* **B**: Densitometry based quantitation of MMP-2 bands using Image J. The normalized band intensities for control were assigned the value of 100 % and those for EGCG treated samples were plotted as percentage with respect to control. Bars represent mean +/- SD (n=3). *Panel* **C**: FRET assay based analysis of active MMP-2 incubated with various doses of EGCG (1-40µM) for 45 min at 37˚C. SC is the substrate control and represents background fluorescence value obtained by auto-hydrolysis of fluorogenic substrate in the absence of MMP-2 in assay buffer. To measure the residual enzyme activity, peptide substrate was added after appropriate treatment with EGCG and the reaction mixture was incubated in dark at 37˚C for 1h. Substrate control (containing only 20µM substrate in assay buffer without MMP-2), Vehicle control (containing 20µM substrate + 10ng MMP-2 + 3µL DMSO) and assay buffer control (containing 150µL assay buffer only) were included in each assay. Fluorescence was measured at λ_{max} excitation 324nm and λ_{max} emission 393nm. Bars represent average RFU ± SD (n=3). Numbers above each bar represents relative RFU values normalized with respect to control (set to 100%). *p< 0.05, **p< 0.01, ***p< 0.001.

Divalent cations have negative effect on EGCG mediated inhibition of MMP-2

EGCG with tri-hydroxyl group (pyrogallol structure) on the B ring and the gallate moiety at the C ring is a potent natural anti-oxidant molecule with free radical scavenging and iron chelating properties. One could argue that EGCG being a potent metal chelator, may be chelating the Zn^{+2} metal ion present at the enzyme active site or removing essential cofactors such as Zn^{+2} and Ca^{+2} from surrounding environment, which are required for gelatinolysis and thus causing decreased gelatinolytic activity.

To test this, we pretreated HT1080 conditioned media or purified active MMP-2 with 10mM EDTA followed by incubation with EGCG and analyzed the residual gelatinolytic activity by gelatin zymography and MMP-2 specific fluorogenic substrate assay respectively. Interestingly, the decrease in MMP-2 activity was found to be more pronounced and significant in EDTA pretreated samples compared to only EGCG treated HT1080 conditioned media as demonstrated by gelatin zymography (Figure 5.3A). It is intriguing to note that EDTA pretreatment followed by 200µM EGCG treatment completely abolishes Pro-MMP-2 gelatinolytic activity, which can be detected in 200µM EGCG alone treated sample. Similarly, 10mM EDTA pretreatment of active MMP-2 containing assay buffer, followed by incubation with 10µM EGCG caused a significant decrease in gelatinolytic activity compared to 10µM EGCG, 10mM EDTA and vehicle treated (Control) groups, as demonstrated by gelatin zymography and fluorogenic substrate assays (Figure 5.3B and 5.3C).

If EGCG being a potent metal chelator may be removing the essential cofactors, culminating in decreased activity of MMP-2, it is arguable that synthetic metal chelators like EDTA, glycine or citrate which can also chelate these metal ions should also show the same effect. To investigate this aspect, HT1080 culture supernatant was incubated with varying doses of various known chelating agents such as EDTA or citrate at 37°C for 45 min. It is clear from the gelatin zymography that EDTA and citrate have no significant effect on the activity of either of the gelatinases (Pro-MMP-9 and Pro-MMP-2) as the bands are of equal intensity (Figure 5.4A and 5.4B). In addition, incubation of active MMP-2 with 0.1

to 10mM EDTA had no significant effect on MMP-2 catalyzed hydrolysis of triple helical FRET peptide substrate (Figure 5.4C).

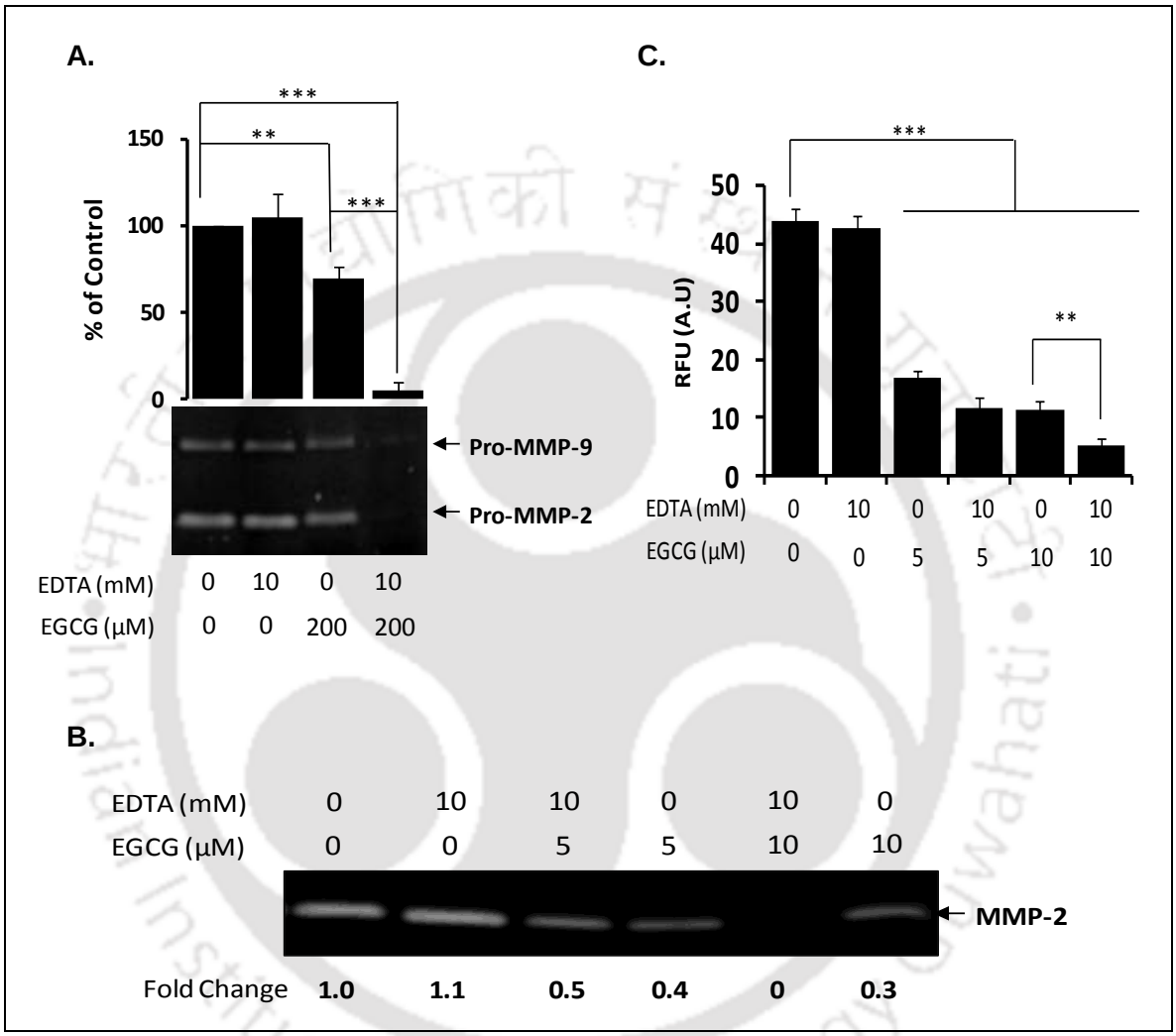


Figure 5.3. Pretreatment with EGCG with EDTA potentiates its efficacy to inhibit MMP-2 activity. *Panel* **A**: HT1080 conditioned medium was incubated with 10mM EDTA at 37˚C for 15 min followed by the addition of 10mM EGCG stock to get the desired concentration (200µM) in the supernatant and the concoction was incubated at 37˚ C for 45 min and then analyzed for gelatinolytic activity by gelatin zymography. Graphical representation of densitometric analysis of MMP-2 bands by Image J (bottom panel). Bars represent mean MMP-2 band intensity ± SD. *Panel* **B**: 10ng active MMP-2 was incubated with 10mM EDTA in assay buffer at 37˚C for 15 min followed by the addition of 10mM EGCG stock to get the desired concentration (as indicated) in the supernatant. The concoction was analyzed by gelatin zymography after 45min. incubation at 37˚C. *Panel* **C**: 10ng human MMP-2 was incubated with 10mM EDTA for 15 min at 37°C, followed by the addition of EGCG stock to get the desired concentration (5µM and 10µM) in the reaction mixture. The concoctions were incubated for 45 min at 37°C, followed by the addition of peptide substrate (20µM) and fluorescence was measured after 1 hour incubation at 37˚C, as described previously. Bars represent average RFU ± SD (n=3). *p< 0.05, **p< 0.01, ***p< 0.001.

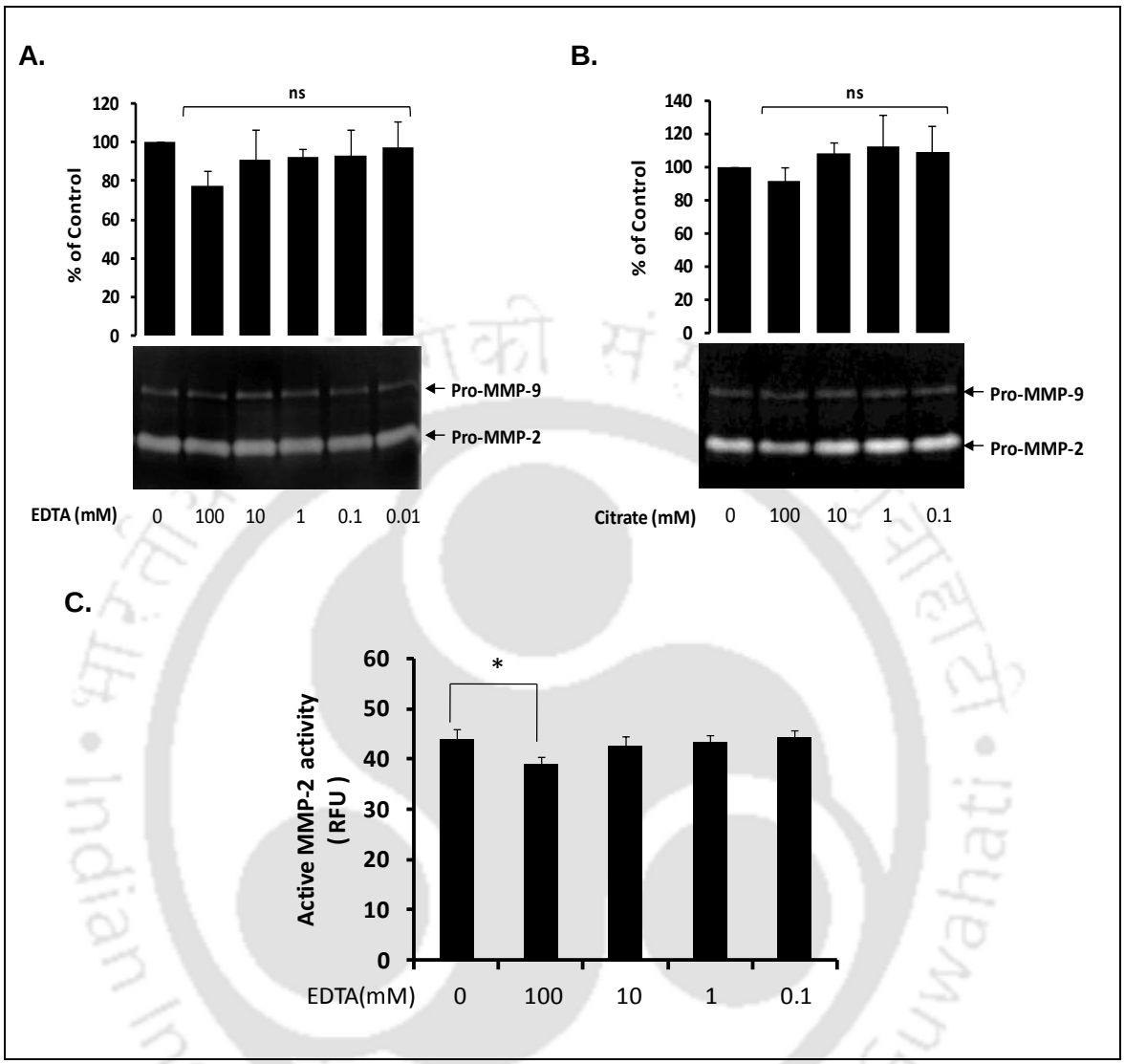


Figure 5.4. Effect of chelating agents on MMP-2 activity. *Panel* **A** and **B**: Equal volumes of HT1080 conditioned medium was incubated with various doses of EDTA (0-100mM) and citrate (0-100mM) for 45 min at 37˚C, followed by gelatin zymography analysis. Densitometry based quantitation of pro-MMP-2 bands using Image J. The normalized band intensities for control were assigned the value of 100% and those for treated samples were plotted as percentage with respect to control. Bars represent mean +/- SD (n=3). *Panel* **C**: 10ng active MMP-2 in assay buffer was incubated with various doses of EDTA (0.1mM to 100mM) for 15 min at 37˚C , followed by the addition of fluorogenic substrate (final concentration 20µM). The mixture was incubated for 1h at 37˚C, away from light and the residual enzyme activity was monitored by measuring fluorescence as described earlier. Bars represent average RFU ± SD (n=3). *p < 0.05, **p< 0.01, ***p< 0.001, ns: non-significant with respect to control.

Partial reversal of EGCG mediated inhibition of MMP-2 activity by divalent cations

To further confirm the negative effect of divalent cations on EGCG mediated inhibition of MMP-2 *in vitro*; EGCG was incubated with excess divalent cations prior to its interaction with MMP-2 enzyme and then examined for its inhibitory activity. Pre-incubation of EGCG with divalent cations, followed by addition of HT1080 conditioned media resulted in partial reversal of EGCG mediated decrease in gelatinolytic activity (Figure 5.5A). Similarly, there was a significant reversal of active MMP-2 inhibition in excess divalent salts containing 10µM EGCG samples as compared to only 10µM EGCG treated sample (Figure 5.5B). These data demonstrated that the efficacy of EGCG in MMP-2 inhibition is sensitive to the cations present in the surrounding environment and removal of these cations prior to interaction between EGCG and MMP-2 enzyme may potentiate its efficacy.

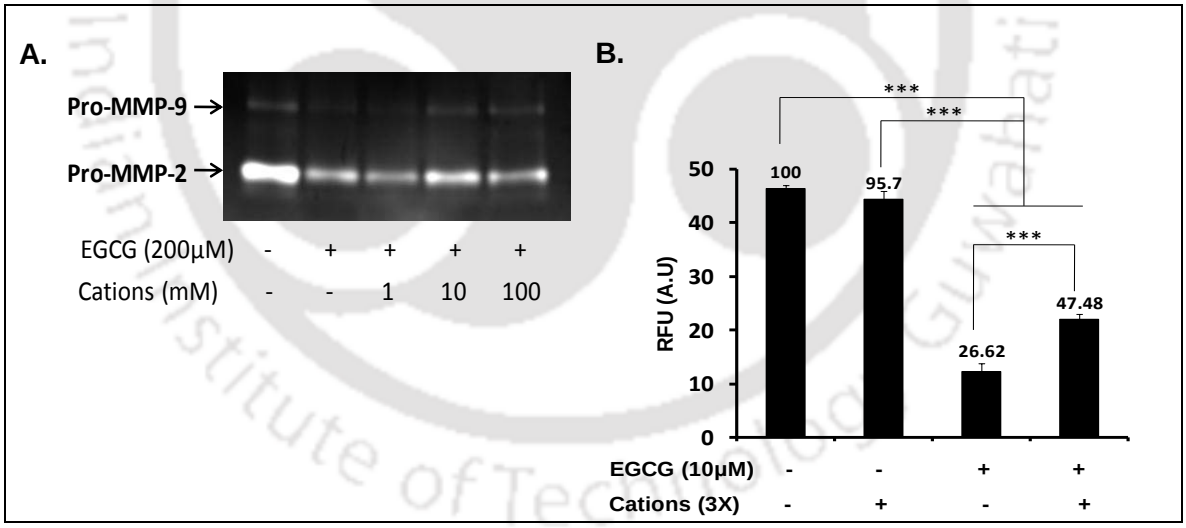


Figure 5.5. Effect of divalent cations on EGCG mediated inhibition of MMP-2 activity. *Panel* **A**: Gelatin zymography gel showing effect of cations on EGCG mediated reduction in pro-MMP-2 activity. EGCG (200µM) was pretreated with divalent cations (0-100mM) and then incubated with HT1080. The concentration of cations indicated here refers to the individual concentration of each divalent salt ($CaCl_2$ and $MgCl_2$) in the final mixture. *Panel* **B**: EGCG was pre-incubated in assay buffer (10 mM Tris–HCl pH 7, 15mM $CaCl_2$, 3µM $ZnCl_2$) containing excess of divalent cations at 37˚C for 15 min. The concentration of cations ($CaCl_2$ and $ZnCl_2$) was three times (3X) more in this buffer as compared to assay buffer used in routine MMP-2 fluorogenic assay. Purified MMP-2 (10ng) was then added to the mixture, followed by incubation at 37˚C. After 30 min, peptide substrate (20µM) was added and fluorescence was recorded after 1h as described previously.

EGCG acts as an uncompetitive inhibitor of MMP-2

Preliminary data confirmed that MMP-2 mediated hydrolysis of FRET peptide substrate showed 1st order exponential increase in the RFU values with time (R^2 = 0.955) at fixed concentration of enzyme (10ng) and substrate (40µM) [Figure 5.6].

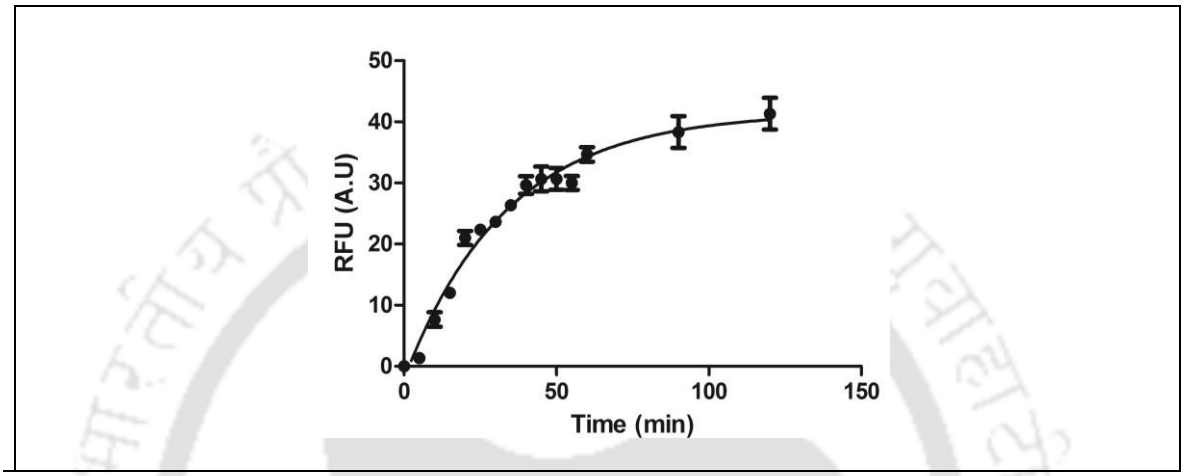


Figure 5.6. MMP-2 mediated hydrolysis of FRET peptide substrate. 10ng MMP-2 was incubated with a fixed FRET peptide substrate concentration of 40µM at 37°C in the assay buffer and fluorescence was measured every 5 min as described earlier. The data was plotted as RFUs *versus* time and fitted using the first order exponential rate equation in GraphPad prism software. Each data point represents mean RFU ± SD (n=3).

In separate experiments, initial velocities (V_0) were obtained for each substrate concentration (0-80µM) in the presence and absence of EGCG (5µM), by determining the slope of linear portion of the hydrolysis curve from the initial range of time points, during which the reaction is linear. The mechanism of MMP-2 inhibition by EGCG was determined using non-linear global fitting of initial velocity values (µM/min) versus various MMP-2 specific fluorogenic substrate concentration (µM), in the presence and absence of EGCG (Figure 5.7). Analysis of statistical and fitting parameters output revealed EGCG to be an uncompetitive inhibitor of MMP-2 (R^2=0.8263). In the presence of 5µM EGCG, K_m value for MMP-2 mediated catalysis of FRET peptide was found to decrease from 21.09µM to 15.14µM and there was a reduction in V_{max} from 1.257µM Min^{-1} to 0.6320µM Min^{-1}. The constant of inhibition (Ki) for the binding of EGCG to enzyme was found to be 11.69µM.

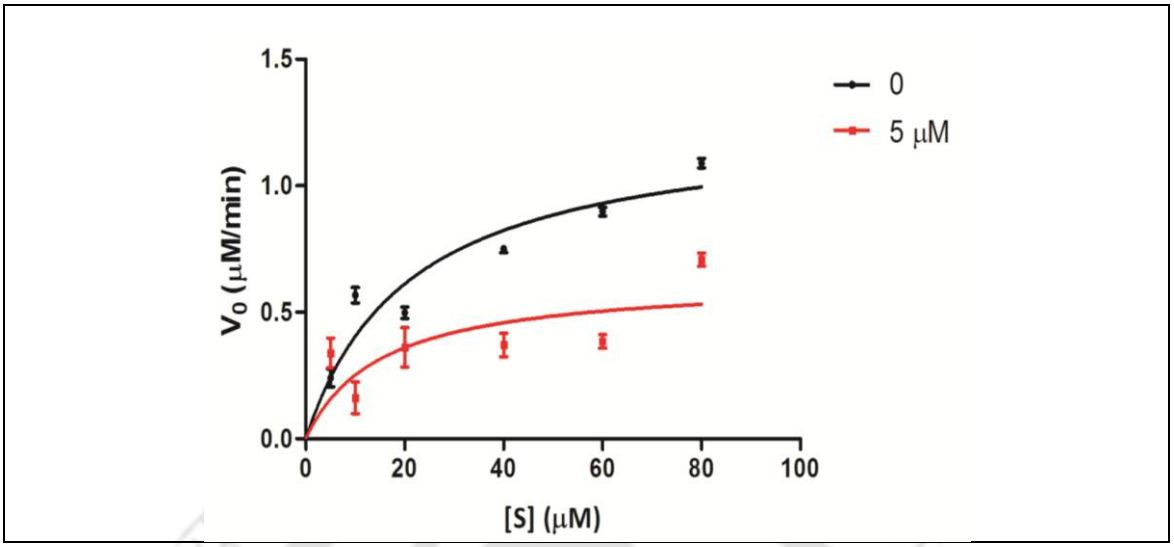


Figure 5.7. Michaelis-Menten plot showing the effect of EGCG (5μM) on MMP-2 catalyzed hydrolysis of FRET peptide substrate. 10ng MMP-2 enzyme (1.01 nM) was incubated with a range of MMP-2 specific FRET peptide substrate concentrations (0-80μM) in the presence and absence of fixed inhibitor (i.e. EGCG) concentration of 5μM at 37˚C in the assay buffer and fluorescence was measured every 15 min as described earlier. The initial velocity (μM/Min) was plotted as a function of substrate concentration (μM) in the presence and absence of inhibitor and fitted using non-linear regression analysis (global fitting) program in Graph Pad Prism software Each point in the graph represents mean ± SD (n=3).

DISCUSSION

As MMPs are complex, multifunctional group of enzymes with overlapping substrate specificities, they are tightly regulated at multiple levels including transcription, secretion, cell surface localization, activation and interaction with TIMPs. Green tea catechins have been shown to exert their chemopreventive and chemotherapeutic effects by modulating multiple cellular pathways and molecular players, directly or indirectly regulating MMPs. EGCG was reported to exert its anti-invasive and anti-angiogenic effects mostly by inhibiting type IV collagenases i.e. MMP-2 and MMP-9, which are frequently over-expressed in several cancer types (Deng and Lin, 2011; Farabegoli *et al.*, 2011; Garbisa *et al.*, 2001; Khoi *et al.*, 2013; Park *et al.*, 2010; Qin *et al.*, 2012; Sen *et al.*, 2010; Sen *et al.*, 2009). Evidences suggest that the molecular mechanism of EGCG mediated inhibition of gelatinases is complex and varies in different cancer types. In general, EGCG was shown to suppress the steady state mRNA levels, protein levels, secretion and activation of MMP-2.

In the present study, our objective was to understand the molecular mechanism of direct inhibition of MMP-2 by EGCG under cell free environment and investigate the effect of divalent cations on EGCG mediated inhibition of MMP-2, which in part mimics *in vivo* extracellular environment. Although previous studies have reported EGCG to be a direct inhibitor of MMP-2, the mechanistic insights into the EGCG mediated MMP-2 inhibition still remain to be elucidated (Demeule *et al.*, 2000; Garbisa *et al.*, 2001). Here, we demonstrated that EGCG inhibits extracellular or secreted MMP-2 (Pro/active) in a dose dependent manner in a cell free environment which may be attributed to the direct interaction of this phytochemical with the enzyme. This observation is supported by some previously published literature. Garbisa *et al.* (2001) demonstrated a dose dependent inhibition of MMP-2 in HT1080 conditioned media by EGCG via a direct, precipitation independent and non-zinc chelation mechanism under cell free conditions. Another study reported EGCG mediated direct inhibition of MT1-MMP could potentially lead to the accumulation of non-activated MMP-2 at the cell surface, indirectly restraining MMP-2 activity (Dell'Aica *et al.*, 2002). Studies by Cheng *et al.* (2003) demonstrated that EGCG may form a reversible complex with MMP-2 without affecting its binding with denatured or native type I collagen by enhancing MMP-2-TIMP-2 interaction and ultimately leading to decreased gelatinolytic activity.

Polyphenols, which are present in abundance in green tea, contain multiple hydroxyl groups and therefore possess strong acid –base buffering capacity and ability to form chelate rings by coordinating with ions such as Zn^{+2}, Cu^{+2} and Mn^{+2} (Kumamoto *et al.*, 2001; Tay *et al.*, 2013). EGCG like other catechins is a potent anti-oxidant with free radical scavenging and metal chelation properties (Frei and Higdon, 2003; Higdon and Frei, 2003). Here we showed that in cation free environment, which we achieved experimentally by pre-treating the conditioned media or purified MMP-2 in assay buffer containing $ZnCl_2$ and $CaCl_2$ with EDTA, there is better EGCG mediated MMP-2 inhibition. This may be due to the chelation of divalent cations present in the surrounding medium by EDTA, which were previously coordinating with EGCG. We also showed that treatment of HT1080 conditioned medium or purified MMP-2 with synthetic chelating agents like EDTA and glycine had no significant effect on MMP-2 activity. Furthermore, we showed that incubation of EGCG with divalent cations prior to

its interaction with MMP-2 partially reverses its MMP-2 inhibitory activity. Hence, our studies demonstrate that divalent cations have negative effect on EGCG mediated MMP-2 inhibition. In conclusion, our data suggests that the divalent cations present in the surrounding environment may mask the ability of EGCG to inhibit MMP-2, by making it less available to interact with the enzyme. To our knowledge, this is the first study to demonstrate the negative effect of divalent cations on EGCG's efficacy to inhibit MMP-2 in a cell free environment. This finding has important pharmacological implications and may explain why *in vivo* effects of EGCG are observed at concentrations much higher than that measured in regular green tea consumers.

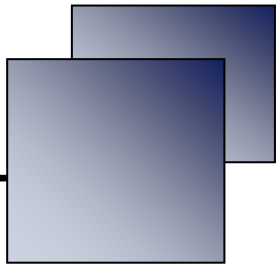
Ongoing *in silico* molecular docking work by other colleagues in our laboratory revealed that EGCG binds to fibronectin type II repeat 3 region of MMP-2 enzyme, away from the Zn^{+2} ion containing catalytic site. MMP-2 and MMP-9 mediated collagenolysis have been shown to utilize three fibronectin-like type II modules inserted into the catalytic domain (collagen binding domain) for proper substrate (collagen and elastin) positioning. This finding is line with our study where we demonstrated that EGCG may act as an uncompetitive inhibitor of MMP-2. EGCG was found to make interactions with Asn-245, Ser-338, Pro-348, Cys-349, Ser-362 and Pro-391 residues located in the fibronectin type II repeat 3 of MMP-2 (Manuscript under preparation).

Recent evidences suggest that specific binding sites (exosites), located away from the catalytic site of MMPs help in the proper positioning of the substrates during hydrolysis and are indispensable for catalysis (Lauer-Fields *et al.*, 2008; Mikhailova *et al.*, 2012; Overall, 2002; Xu *et al.*, 2004). Therefore, compounds which may block or interfere with exosite mediated substrate interactions may emerge as a highly selective MMP inhibitor. Studies by Mikhailova *et al.* (2012) identified three exosite residues (R^{368}, F^{297}, R^{252}) located in collagen binding domain of human MMP-2 essential for gelatinolysis. These molecular docking studies demonstrated that EGCG binds to amino acid residues in fibronectin type II repeat 3, which are in close vicinity of exosites critical for gelatinolysis. This finding corroborate with earlier findings where we and other groups have demonstrated that EGCG mediated MMP-2 inhibition is independent of zinc chelation at the catalytic site. To our knowledge this is the first study to provide

detailed molecular insight into MMP-2 inhibition in which EGCG targets the exosites instead of the active site.

This feature of EGCG mediated MMP-2 inhibition sets it apart from the synthetic small molecule inhibitors. Our findings implicate that EGCG may emerge as a natural, non toxic, selective MMP-2 inhibitor targeting unique sites which are structurally and functionally non-redundant among other MMP classes.

Chapter VI



Epigenetic induction of tissue inhibitor of matrix metalloproteinase-3 (TIMP-3) by green tea polyphenols in breast cancer cells

The results discussed in this chapter are published in Deb *et al.* (2014), *Molecular Carcinogenesis*

INTRODUCTION

Cancer has long been perceived as a diverse group of disorders that are induced by genomic instability caused by clonally selected genetic changes such as mutation, deletion, insertion, inversion, translocation in critical tumor suppressor genes and oncogenes. However recent advances in the field of epigenetics have now broadened this concept of carcinogenesis. Epigenetic abnormalities caused by genome-wide hypomethylation, promoter DNA hypermethylation mediated silencing of tumor suppressor genes, and modulation in the expression profiles of histone modifying enzymes are closely associated with tumorigenesis (Hatziapostolou and Iliopoulos, 2011; Lechner *et al.*, 2010). The cross-talk between genetic and epigenetic regulatory mechanisms is prevalent and widely reported in literature (Choi and Lee, 2013). Mutations in several epigenetic modifiers involved in DNA and histone modifications lead to aberrant gene expression patterns ultimately resulting in cancer (Plass *et al.*, 2013).

Breast cancer like several other human malignancies represents a heterogeneous group of neoplasms driven by multiple genetic and epigenetic aberrations (Polyak, 2007). Mutations which lead to either oncogenic activation or silencing of critical tumor suppressor genes have been implicated in breast cancer etiology and linked to breast cancer risk. For example, specific mutations in *BRCA1* and *BRCA2* genes are frequently observed in 20-25% of hereditary breast cancers and 5-10% of all breast cancers (Polyak, 2007; Polyak and Metzger Filho, 2012). However, in the past two decades, epigenetic mechanisms underlying deregulation of genes resulting in breast cancer initiation and progression have gained considerable attention. For example, epigenetic mechanisms have been shown to be associated with loss of *ERα* expression in breast cancer. Nearly one-third of primary breast cancer cases are ER negative and genetic changes alone proved insufficient to account for the loss of ER in all such cases. Recent studies have revealed that the loss of *ERα* expression in breast cancer etiology could be due to epigenetic silencing caused by promoter DNA hypermethylation in 41% of breast cancer cases and *ERα* promoter hypermethylation was found to be highly increased in triple negative breast cancers (Dworkin *et al.*, 2009; Hervouet *et al.*, 2013).

Interestingly, several reports suggest that treatment of ERα negative breast cancer cell lines with 5-Aza-CdR (DNA methyltranferase inhibitor) and TSA (histone deacetylases inhibitor) could restore ERα expression and tamoxifen sensitivity (Fan *et al.*, 2008; Jang *et al.*, 2004). Epigenetic mechanisms have also been implicated in the development of acquired resistance to endocrine therapy in breast cancer treatment (Trimarchi *et al.*, 2011). Besides epigenetic deregulation of ER expression, several other genes such as *CCND2* (cyclin-D2), *SFN* (stratifin/14-3-3 protein σ), *RUNX3*, *RIZ* (retinoblastoma protein-interacting zinc finger protein), *BRCA1* have also been shown to be the targets of epigenetic aberrations during breast carcinogenesis (Jovanovic *et al.*, 2010). In a recent study, Brglez *et al.* (2014) demonstrated that differential mRNA expression levels of secreted phospholipases A_2 (hGIIA, hGIII and hGX) in aggressive triple negative breast cancer cells is a consequence of epigenetic modulation via DNA methylation and histone acetylation. Other epigenetic aberrations such as altered expression of DNA and histone modifying enzymes have also been implicated in breast cancer pathogenesis. For example histone methyltranferase EZH2 has emerged as a promising biomarker indicating breast cancer progression and disease aggressiveness (Yoo and Hennighausen, 2012). EZH2 and repressive chromatin H3K27me3 levels have been found to increase steadily as the disease progresses from a benign tumor to clinically evident metastasis causing epigenetic silencing of a cohort of genes critical for normal cellular functions. Highest EZH2 levels are associated with the ER-negative basal like phenotype of breast cancer cells that represents the most aggressive form of breast carcinoma characterized by nuclear polymorphism, lack of ERα and BRCA1 protein (Ding and Kleer, 2006; Kleer *et al.*, 2003). All these evidences underscore the cardinal role of epigenetic aberrations in breast carcinogenesis. Therapeutic targeting of these epigenetic aberrations for better disease management is increasingly being considered as a viable option.

In recent years *Nutri-epigenetics* which focuses on the influence of naturally occurring dietary phytochemicals on epigenetic mechanisms has emerged as an attractive area of research with the potential of providing novel chemopreventive agents (Khan *et al.*, 2012; Thakur *et al.*, 2014). For several decades the chemopreventive and chemotherapeutic benefits of green tea catechins, EGCG in particular, were largely attributed to their multifaceted effects on various

signalling pathways involved in cell proliferation, apoptosis, invasion and angiogenesis. However it is now widely accepted that GTPs are potent natural 'epigenetic modifiers' which could modulate the activity of DNMTs, HDACs and HMTs, depending on the cellular context and cancer type (Khan *et al.*, 2012). Pandey *et al.* (2010) demonstrated that green tea polyphenol mediated promoter demethylation and chromatin remodelling significantly induces Glutathione-S-transferase pi (*GSTP1*) expression in human prostate cancer cells. Studies by Kato *et al.* (2008) showed that the treatment of oral cancer cells with EGCG partially reversed the hypermethylation status of the *RECK* (reversion-inducing cysteine-rich protein with Kazal motifs) gene, a negative regulator of MMPs. Li *et al.* (2010) reported that EGCG in combination with TSA synergistically reactivated ERα expression in ER negative MDA-MB-231 breast cancer cells, by modulating histone acetylation and methylation pattern as well as decreasing the binding of transcriptional repressor complex Rb/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 at the *ERα* promoter.

In breast cancer low levels of TIMP-3 protein expression has been shown to be associated with an aggressive tumor phenotype and poor disease-free survival (Mylona *et al.*, 2006). TIMP-3 is a secreted protein which binds strongly to ECM through glycosaminoglycans, unlike other members of TIMP family (Chambers and Matrisian, 1997; Visse and Nagase, 2003). Studies have demonstrated that *TIMP-3* over-expression in tumor cells causes reduced primary growth and angiogenesis and induces apoptosis (Deng *et al.*, 2006; Zhang *et al.*, 2010). *TIMP-3* repression has been linked to promoter DNA hypermethylation in approximately 20-27% cases of primary breast tumors and invasive ductal carcinoma (Bachman *et al.*, 1999; Lui *et al.*, 2005). However, other epigenetic silencing mechanisms such as aberrant histone methylation and deacetylation at the *TIMP-3* gene locus are not completely understood in the context of breast cancer.

In this chapter, we have addressed the impact of green tea catechins on TIMP-3 expression in two *in vitro* models of breast cancer, namely MCF-7 and MDA-MB-231, and the epigenetic mechanisms involved therein. Here we provide evidence that class I HDACs and EZH2 catalyzed histone modifications play key roles in *TIMP-3* gene silencing. We further demonstrated GTP and EGCG mediated

induction of *TIMP-3* expression and the associated epigenetic mechanism in breast cancer cells. A detailed study into the molecular mechanism revealed that GTP and EGCG treatment decreased EZH2 catalyzed H3K27me3 with a corresponding increase in the deposition of transcriptionally active acetylated Histone H3 at Lysine 9/18 (H3K9/18Ac) specifically at the *TIMP-3* gene promoter.

RESULTS

EZH2 and class I HDACs mediate epigenetic silencing of *TIMP-3* gene in human breast cancer cells

We investigated the effect of known pharmacologic agents that modulate epigenetic pathways such as 5-Aza-CdR, TSA and DZNep on *TIMP-3* expression in MCF-7 and MDA-MB-231 cells. Treatment with TSA or DZNep alone or in combination significantly induced *TIMP-3* steady state mRNA and protein levels in MCF-7 and MDA-MB-231 (Figure 6.1). This provided a proof of concept that alterations of histone modifications may lead to the modulation of *TIMP-3* expression. Treatment with 10µM 5-Aza-CdR had no significant effect on *TIMP-3* steady state mRNA and protein levels in both MCF-7 and MDA-MB-231 cells (Figure 6.1).

For further investigation, we used methylation specific PCR (MSP) to analyze the methylation status of the *TIMP-3* gene promoter with and without 5-Aza-CdR treatment. Our data show that *TIMP-3* promoter was unmethylated in both MCF-7 and MDA-MB-231 cells as no methylation specific band could be detected (Figure 6.2). However DNA bands associated with unmethylated *TIMP-3* promoter locus were observed in both cell lines with or without 5-Aza-CdR treatment. The MSP results suggested that *TIMP-3* promoter methylation may not be the mechanism responsible for its repression in MCF-7 and MDA-MB-231 cells.

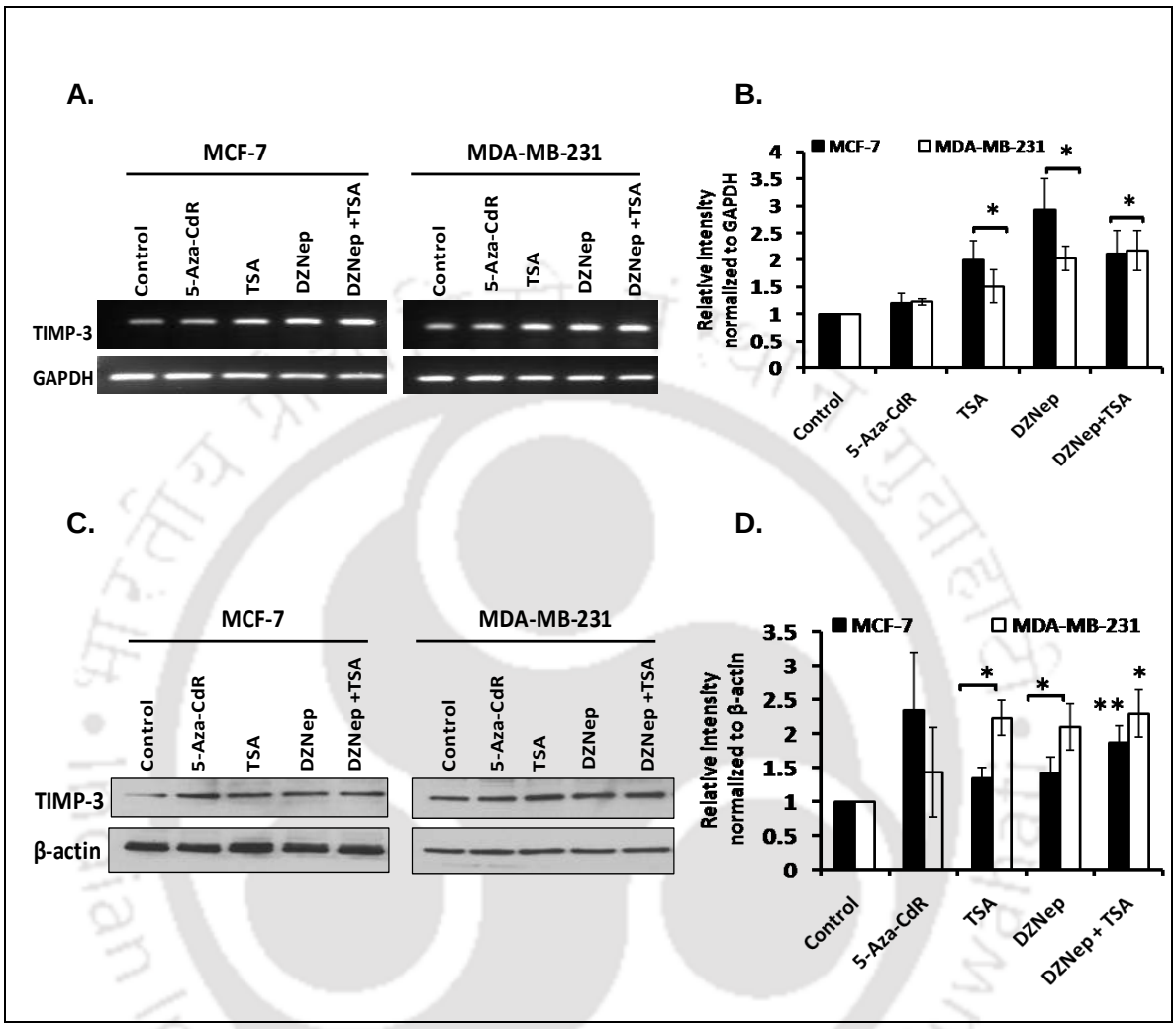


Figure 6.1. Induction of *TIMP-3* mRNA and protein levels following treatment with various epigenetic drugs. *Panel* **A**: A semi-quantitative RT-PCR analysis of the effect of 5-Aza-CdR (10μM), TSA (40ng/mL), DZNep (5μM) and combined DZNep (5μM) + TSA (40ng/mL) for a period of 72 h on *TIMP-3* mRNA levels in MCF-7 and MDA-MB-231 cells; GAPDH was used as an internal control. *Panel* **B**: Graphical representation of data in **A** showing relative intensities of *TIMP-3* bands normalized to GAPDH. The normalized band intensities obtained for controls were assigned the value of 1 and those obtained for various treatments were plotted relative to control. Bars represent the mean relative *TIMP-3* levels ± SD (n=3). *Panel* **C**: Western blot analysis of TIMP-3 expression in MCF-7 and MDA-MB-231 treated with 5-Aza-CdR (10μM), TSA (40ng/mL), DZNep (5μM) and DZNep + TSA for 72h. 40μg total protein was loaded in each lane and β- actin was used as loading control. *Panel* **D**: Graphical representation of data in **C** showing relative intensities of TIMP-3 bands normalized to β-actin. TIMP-3 basal expression levels in control were taken as 1 and those for other treatment groups were expressed relative to control. Bars represent the mean TIMP-3 levels ± SD (n=3). Two-tailed Student's t-test was used to compare TIMP-3 expression levels between individual treatment groups and control, *p< 0.05, **p< 0.01, ***p< 0.001.

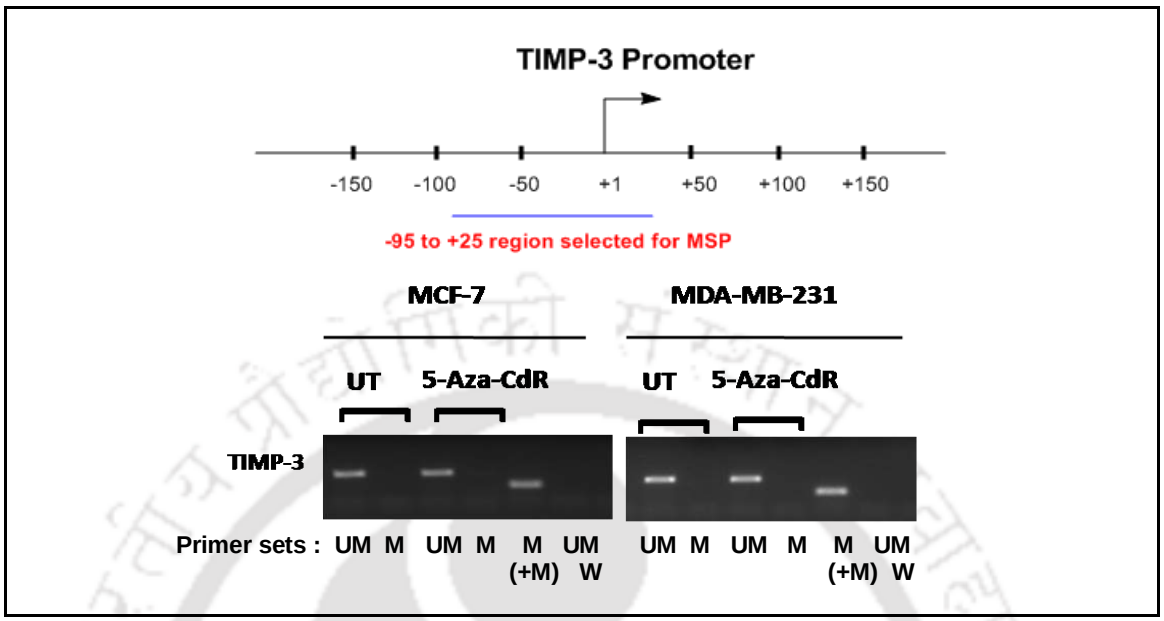


Figure 6.2. Analysis of CpG promoter methylation status of *TIMP-3* in MCF-7 and MDA-MB-231 using methylation-specific PCR. Genomic DNA from only vehicle treated (UT) and 10μM 5-Aza-CdR treated cells for 72h was isolated and bisulfite modification of genomic DNA (2μg) was performed followed by additional desalting and purification. DNA was suspended in 10μl of water and stored at -20˚C. PCR reaction was performed using MSP primer sequences that specifically recognize *TIMP-3* promoter sequence in the region from -91 bp to 125 bp.The presence of a visible PCR product in lanes UM or M indicates the presence of unmethylated or methylated *TIMP-3* respectively. Universal methylated human DNA standard (+M) and water (W) were used as template for positive and negative controls. The data shown is a representative of three independent experiments.

To address the role of histone methylation and deacetylation in the modulation of *TIMP-3* expression, we knocked down class I HDACs (HDAC-1, -2, -3, and -8) and histone methyltransferase EZH2 in MCF-7 and MDA-MB-231 cells using specific shRNAs for each target. Knockdown of HDACs and EZH2 was confirmed by western blotting (Figure 6.3A). A very high percentage of knockdowns of HDAC-2, HDAC-8 and EZH2 proteins were achieved in both the cell lines. The focus of our study was to investigate TIMP-3 levels in Class-I HDACs and EZH2 knockdown breast cancer cells. Interestingly, TIMP-3 protein levels were induced upon class I HDACs or EZH2 knockdown in both cell lines (Figure 6.3B). Knockdown of EZH2 also resulted in 2.5 and 7.2 fold increase in TIMP-3 protein levels in MCF-7 and MDA-MB-231 respectively. Taken together, the results discussed so far strongly suggest that *TIMP-3* repression in breast cancer is mediated by these histone modifying enzymes.

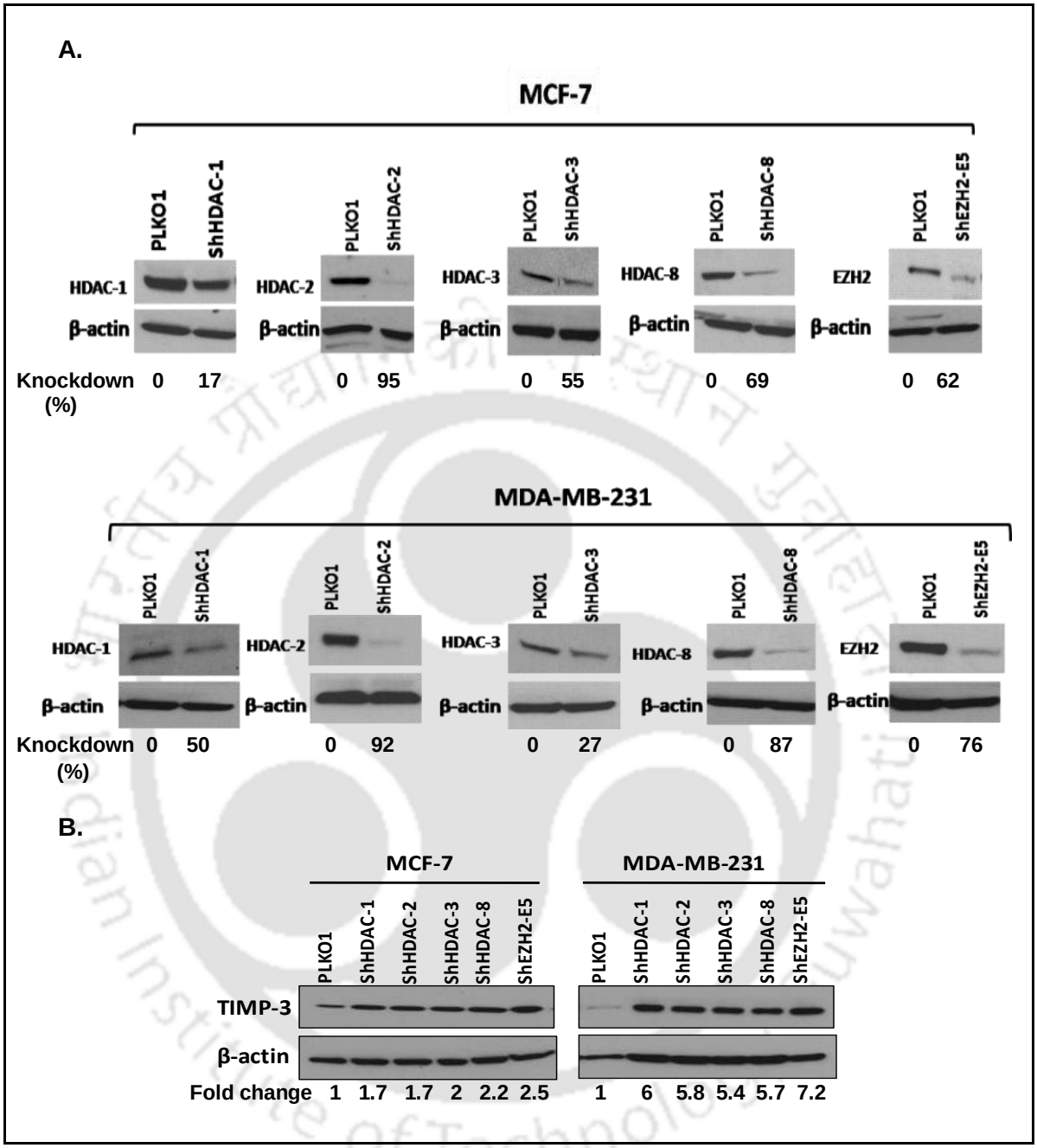


Figure 6.3. Induction of TIMP-3 protein levels upon Class I HDACs and EZH2 knockdown in breast cancer cells. *Panel* **A**: Western blot analysis of Class I HDACs (HDAC-1,-2,-3,-8) and EZH2 knockdown status in MCF-7 and MDA-MB-231 generated using lentiviral vectors specific for EZH2 (ShEZH2), HDAC-1 (ShHDAC-1 4814), HDAC-2 (ShHDAC-2 4819), HDAC-3 (ShHDAC-3 4826), HDAC-8 (ShHDAC-8 4849). 40µg protein was loaded in each lane and β-actin was used as loading control. *Panel* **B**: Western blot analysis of TIMP-3 protein levels in MCF-7 and MDA-MB-231 cells following knockdown of I HDACs and EZH2. 40µg total protein was loaded in each lane and β- actin was used as loading control. Fold change represents ratio of TIMP-3 level (after normalization to loading control) in specific shRNA treated cells versus only vector (pLKO1) treated cells.The normalized band intensities obtained for control (pLKO1) have been assigned the value of 1 and those obtained for various treatments were plotted as fold increase with respect to control, respectively

ChIP experiments were performed to investigate the effect of TSA and DZNep treatments on H3K9/18 Ac, H3K27me3 and EZH2 levels at the *TIMP-3* promoter in both the cell lines. There was a marked increase in transcriptionally active H3K9/18Ac upon TSA treatment alone as well as in combination with DZNep in both MCF-7 and MDA-MB-231 cells (Figure 6.4). DZNep alone had no effect on H3K9/18Ac mark at the *TIMP-3* locus. A concomitant decrease in EZH2 and H3K27me3 at the *TIMP-3* promoter after DZNep treatment alone or in combination with TSA was also observed, which was more prominent in MCF-7 cells over MDA-MB-231 cells (Figure 6.4). Combined DZNep and TSA treatment was found to be more effective in decreasing EZH2 localization and H3K27me3 while concomitantly increasing H3K9/18 Ac at the *TIMP-3* locus in both cell lines.

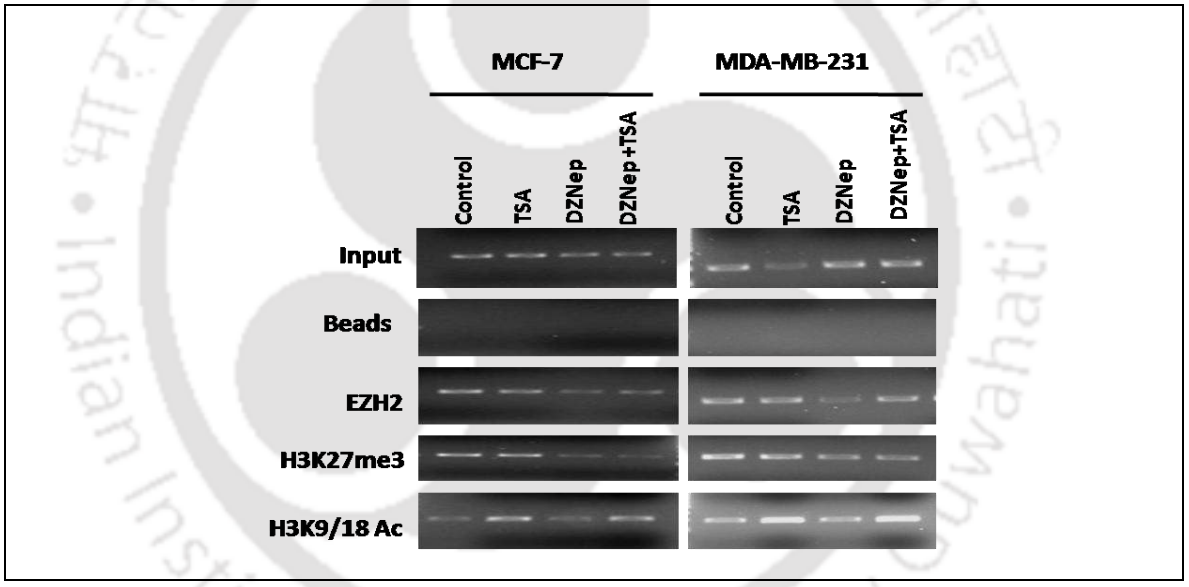


Figure 6.4. Changes in EZH2 occupancy and local histone modifications (H3K27me3 and H3K9/18 Ac) induced by various epigenetic drug treatments at the *TIMP-3* promoter. MCF-7 and MDA-MB-231 cells were treated with TSA (40ng/mL), DZNep (5μM) and combined DZNep (5μM) + TSA (40ng/mL), 72h treatment. Chromatin Immunoprecipitation (ChIP) was performed to analyze changes in EZH2 localization and other histone modifications at the *TIMP-3* promoter in MCF-7 and MDA-MB-231. Details of the experimental methodology used are discussed in Chapter III (Materials and Methods).

In conclusion, our data strongly suggest that Class I HDACs and EZH2 play important roles in regulating *TIMP-3* expression in MCF-7 and MDA-MB-231 cells. Elevated levels of transcriptionally repressive H3K27me3 and decreased active H3K9/18Ac at the *TIMP-3* promoter may contribute its repression independent of promoter DNA methylation in these cells.

Induction of *TIMP-3* steady state mRNA and protein levels by green tea polyphenols in human breast cancer cells

We tested the effect of GTP and EGCG on *TIMP-3* expression levels. MCF-7 and MDA-MB-231 cells were treated with 10µg/mL GTP and 20µM EGCG for 72 h. These doses have been previously used in other published studies (Thakur *et al*; 2012). Cells treated with other epigenetic drugs were used as controls for *TIMP-3* induction. RT-PCR analysis showed that GTP and EGCG treatment significantly upregulated ($p<0.05$) *TIMP-3* expression in both cell lines (Figure 6.5). TIMP-3 protein levels were determined in breast cancer cells treated with 10µg/mL GTP and 20µM EGCG for 3 days and 7 days. In MCF-7 cells, there was 4-5 folds and 3-5 folds induction in TIMP-3 protein levels after GTP and EGCG treatment respectively. Similarly in MDA-MB-231 cells, GTP and EGCG treated cells caused a 1.3-2 fold and 1.8-2.5 fold induction in TIMP-3 protein, respectively (Figure 6.6).

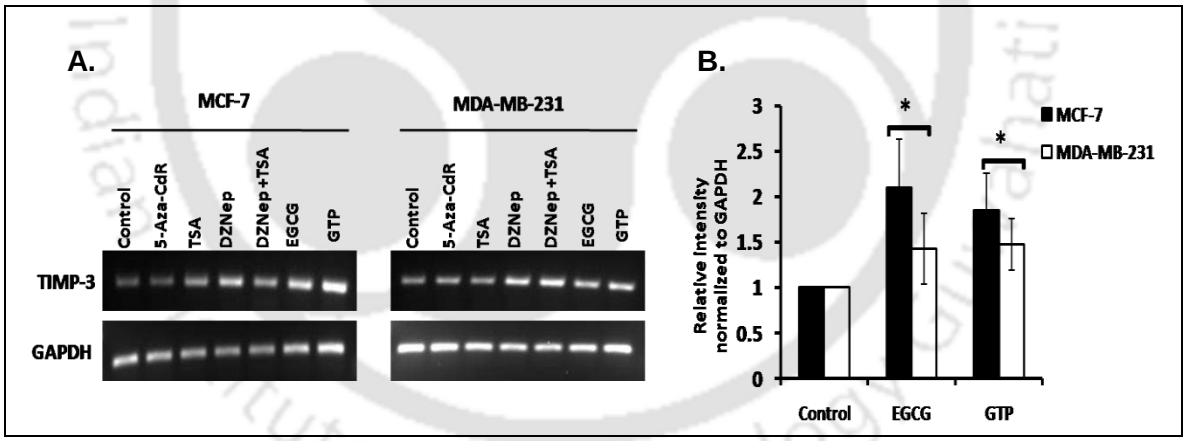


Figure 6.5. Green tea polyphenol mediated induction of *TIMP-3* steady state mRNA levels in human breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with 20µM EGCG and 10µg/mL GTP for 72h. Other epigenetic drug treatments i.e. 5-Aza-CdR (10µM), TSA (40ng/mL), DZNep (5µM) and combined DZNep (5µM) + TSA (40ng/mL) for 72h treatment were used as positive controls. *Panel* **A***:* Semi-quantitative RT-PCR analysis of TIMP-3 mRNA levels. GAPDH was used as internal control. *Panel* ***B****:* Graphical representation of the data in **A**. Densitometry based image analysis shows relative intensities of *TIMP-3* bands normalized to GAPDH in MCF-7 and MDA-MB-231. Bars represent the mean *TIMP-3* levels ± SD (n=3). Two-tailed Student's t-test was used to compare *TIMP-3* expression levels between individual treatment groups and control. *p< 0.05, **p< 0.01, ***p< 0.001

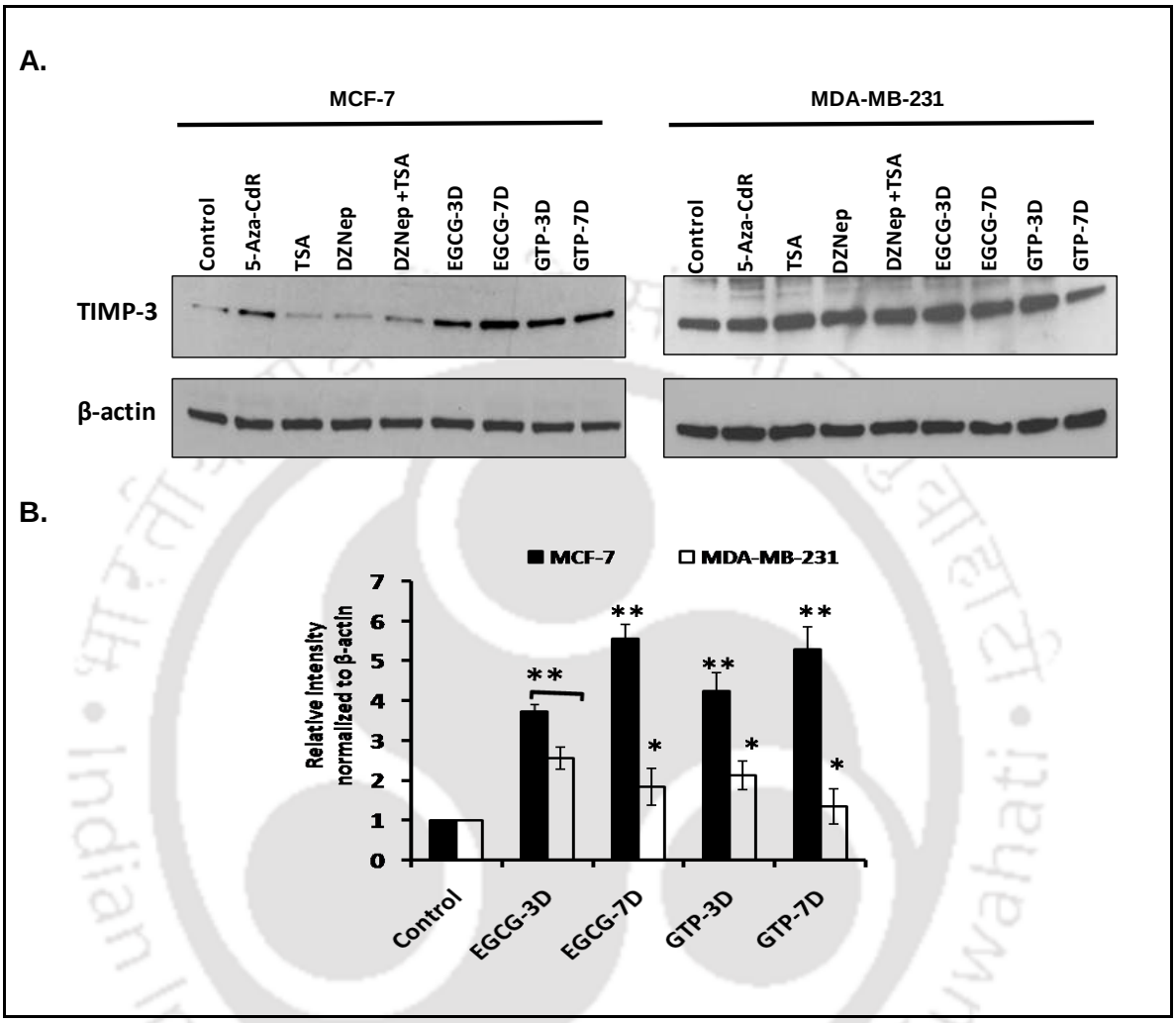


Figure 6.6. Green tea polyphenol mediated induction of TIMP-3 protein levels in human breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with 20μM EGCG and 10μg/mL GTP for 72h (3 Days) and 168h (7 Days). Other epigenetic drug treatments i.e. 5-Aza-CdR (10μM), TSA (40ng/mL), DZNep (5μM) and combined DZNep (5μM) + TSA (40ng/mL) for 72h (3D) treatment were used as positive controls. *Panel* **A**: Western blot analysis of TIMP-3 protein levels. 40μg total protein sample (whole cell lysates) was loaded in each lane and β-actin was used as loading control. *Panel* **B**: Densitometry based image analysis shows relative intensities of TIMP-3 bands normalized to β-actin. Bars represent the mean TIMP-3 levels ± SD (n=3). Two-tailed Student's t-test was used to compare TIMP-3 expression levels between individual treatment groups and control, *p< 0.05, **p< 0.01, ***p< 0.001.

Green tea polyphenols decrease protein levels of class I HDACs and histone methyltransferase EZH2 in human breast cancer cells

Earlier experiments demonstrated that, Class I HDACs and EZH2 mediate *TIMP-3* silencing (Figure 6.1-6.4) and also that the GTPs have the potential to induce *TIMP-3* expression in breast cancer cells (Figure 6.5-6.6). It was of interest to decipher the mechanism of *TIMP-3* induction by GTP and EGCG. First we determined the protein levels of class I HDACs (HDAC-1, -2, -3, -8) and EZH2 in MCF-7 and MDA-MB-231 cells treated with 10µg/mL GTP and 20µM EGCG which previously demonstrated elevated TIMP-3 levels (as shown in Figure 6.6) by Western blot analysis. Cells treated with 10µM 5-Aza-CdR, 40ng/mL TSA, 5µM DZNep and 40ng/mL TSA +5µM DZNep for 72h served as controls.

Compared to untreated MCF-7 cells, EGCG treatment caused a significant decrease in EZH2 levels to 50% (3 days) and 36% (7 days); whereas GTP treatment resulted in 69% and 65% decrease in EZH2 after 3 and 7 days respectively (Figure 6.7). Similarly in MDA-MB-231 cells, EZH2 levels were significantly reduced to 54% and 64% by EGCG after 3 and 7 days whereas 83% and 48% reduction in EZH2 was noted within 3 and 7 days of GTP treatment. In MCF-7, EGCG and GTP treatment for 3 days caused a significant decline in HDAC-2 protein levels while in MDA-MB-231, only prolonged exposure with EGCG for 7 days showed significant reduction of 68% in HDAC-2 levels. EGCG and GTP treatment caused no significant changes in HDAC-1 and HDAC-3 levels in MCF-7 cells which is in contrast to MDA-MB-231cells where HDAC-1 and HDAC-3 levels were found to be significantly reduced in EGCG (3-7 days) and GTP (3 days) treated cells. In both cell lines, there was a marked decrease in HDAC-8 levels following EGCG and GTP treatment. In summary, our results demonstrated that EGCG and GTP treatment cause a significant decrease in EZH2 and HDAC-8 protein levels in both breast cancer cell lines while the effect on other class I HDACs (HDAC-1,-2 and -3) was cell and time dependent.

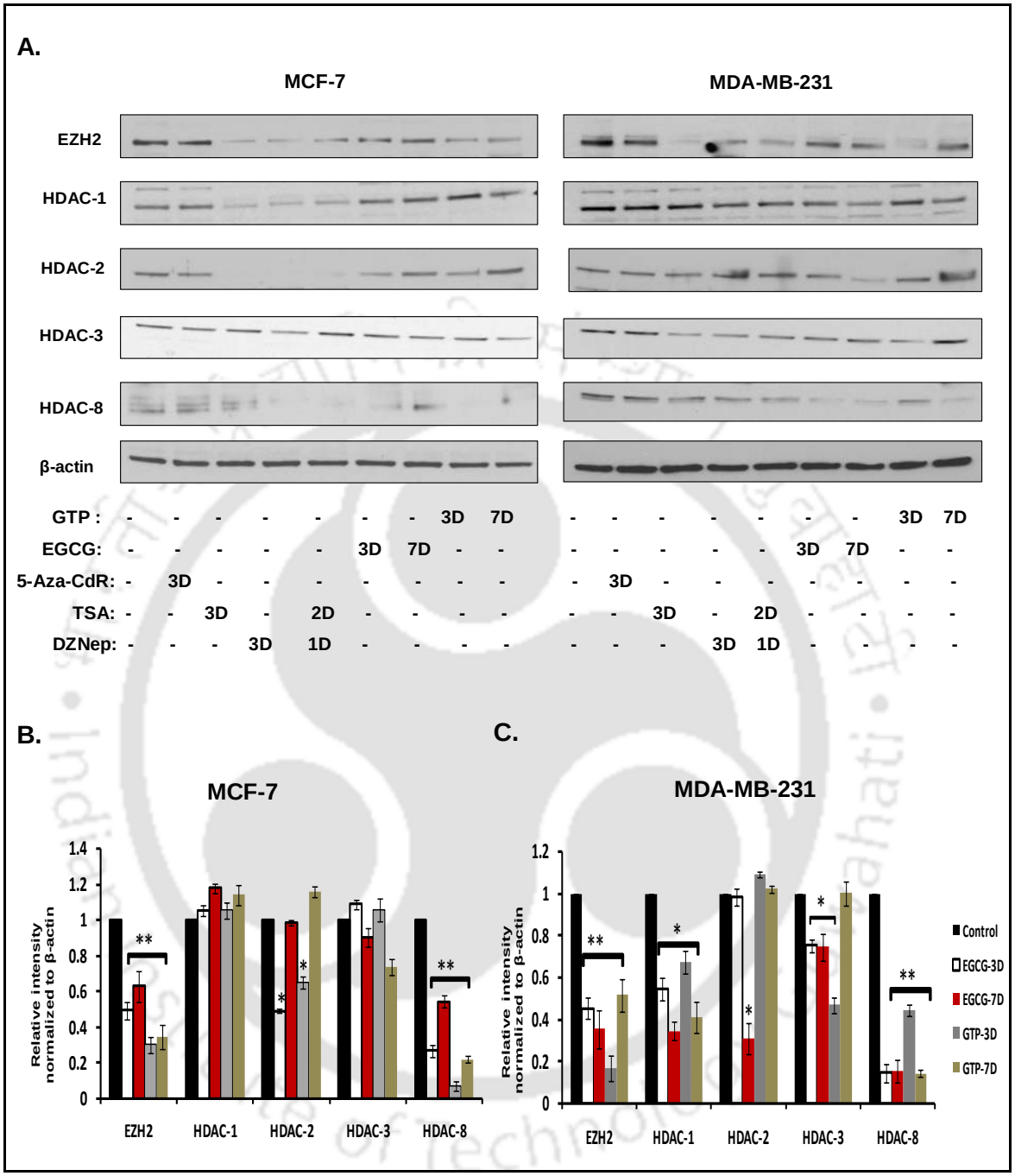


Figure 6.7. Green tea polyphenol mediated decrease in class I HDACs and histone methyltransferase EZH2 protein levels in human breast cancer cells. *Panel* **A** : Western blot analysis of class I HDACs (HDAC-1, -2, -3, and -8) and EZH2 protein levels in MCF-7 and MDA-MB-231 cells treated with 5-Aza-CdR (10μM), TSA (40ng/mL) , DZNep (5μM) and combined DZNep (5μM) + TSA (40ng/mL) , 20μM EGCG and 10μg/mL GTP for 3 days (3D) or 7 days (7D) as indicated. 40μg total protein sample was loaded in each lane and β-actin was used as loading control. *Panel* **B**: Graphical representation of data in **A** shows relative mean intensities of class I HDACs and EZH2 protein bands normalized to β-actin in MCF-7 and MDA-MB-231. Bars represent mean ± SD (n=3). Two-tailed Student's t-test was used to compare protein expression levels between individual treatment groups and control, *p< 0.05, **p< 0.01, ***p< 0.001.

Green tea polyphenols mediated decrease in EZH2 localization, H3K27me3 and a concomitant increase in H3K9/18Ac at *TIMP-3* promoter

To address the direct association between green tea catechins mediated *TIMP-3* induction and decrease in the levels of various histone modifying enzymes or their activity, we next studied the effect of GTP and EGCG treatment on the pattern of histone modifications specifically at the *TIMP-3* promoter by ChIP assays using anti-EZH2, anti-H3K27me3 and anti-H3K9/18Ac antibodies. The immunoprecipitated DNA samples from MCF-7 and MDA-MB-231 cells were analyzed by PCR primers, designed to amplify YY1 binding site in *TIMP-3* promoter which is the site for recruitment of EZH2 containing PRC2 complex. Our results show that GTP and EGCG treatments decrease EZH2 binding and consequently reduce H3K27me3 at the *TIMP-3* promoter. Furthermore, decrease in repressive H3K27me3 chromatin mark was followed by a corresponding increase in transcriptionally active H3K9/18Ac enrichment at the *TIMP-3* promoter (Figure 6.8). These data suggest that GTP and EGCG treatment induce *TIMP-3* expression in breast cancer cells by decreasing class I HDACs and EZH2 protein levels which corroborates with their reduced promoter occupancy, the decline in repressive chromatin H3K27me3 mark and a concomitant increase in H3K9/18Ac levels at the *TIMP-3* gene locus.

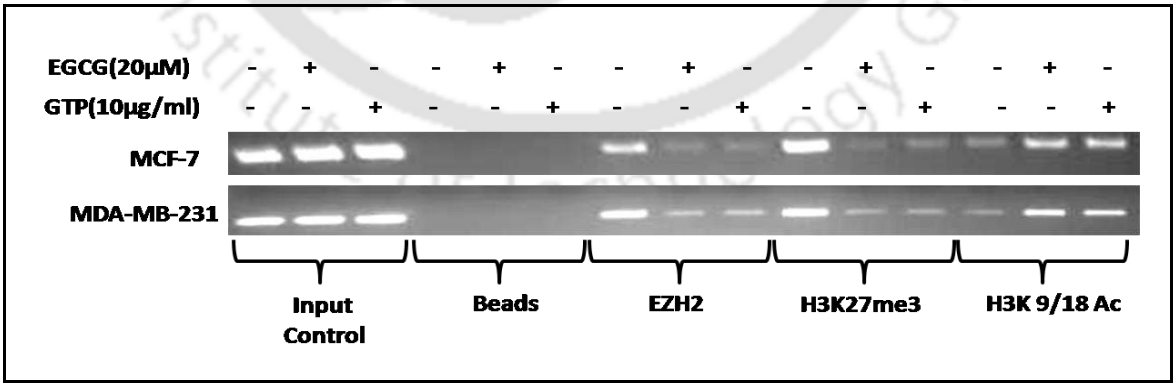


Figure 6.8. Green tea polyphenol mediated decrease in H3K27 trimethylation with a concomitant increase in H3K9/18 acetylation levels at the *TIMP-3* promoter in human breast cancer cells. Chromatin immuno-precipitation assay was performed to analyze the local histone modifications induced by 20μM EGCG and 10μg/mL GTP treatment for 72h at the *TIMP-3* promoter. EGCG and GTP treatment caused decreased association of EZH2 and therefore less H3K27me3 enrichment at the *TIMP-3* promoter with a concomitant increase in H3K9/18 Ac levels in MCF-7 and MDA-MB-231.

Green tea polyphenols cause decrease in global H3K27me3 with a concomitant increase in H3K9/18Ac levels in human breast cancer cells

The effect of GTP and EGCG mediated decrease in class I HDACs and EZH2 protein levels on global histone acetylation and methylation levels in breast cancer cells was also investigated. As shown in figure 6.9, analysis of acid-extracted histones from MCF-7 and MDA-MB-231 cells treated with GTP and EGCG for 72h show a marked global reduction in H3K27me3 compared to untreated cells.

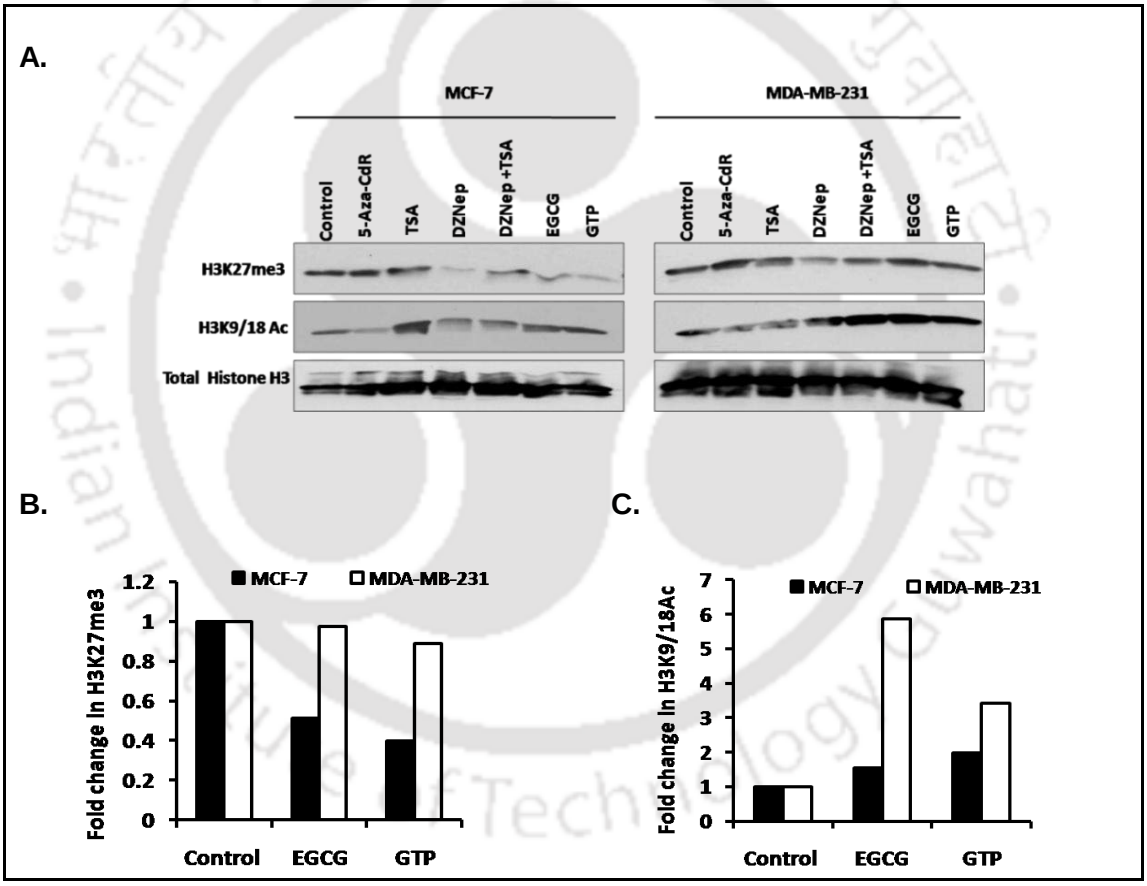


Figure 6.9. Effect of green tea polyphenols treatment on the global H3K27me3 and H3K9/18Ac levels in human breast cancer cells. *Panel* **A**: Western blot analysis of acid-extracted total histones from MCF-7 and MDA-MB-231 cells treated with 5-Aza-CdR (10μM), TSA (40ng/mL), DZNep (5μM) and combined DZNep (5μM) + TSA (40ng/mL), 20μM EGCG and 10μg/mL GTP for 72h. 20μg of total histone sample was loaded in each lane and total histone H3 served as internal control. *Panel* **B**: Graphical representation of ratio between relative intensities of histone H3 trimethylated at Lys 27 (H3K27me3) bands normalized to total histone H3 in both cell lines. *Panel* **C**: Graphical representation of ratio between relative intensities of histone H3 acetylated at Lys 9/18 (H3K9/18Ac) bands normalized to total histone H3 in both cell lines.

In MCF-7, 49% and 60% decrease in H3K27me3 was observed whereas in MDA-MB-231, 2.56% and 11.17% decrease in H3K27me3 was observed after EGCG and GTP treatment, respectively. There was concomitant increase in global H3K9/18Ac in the above EGCG and GTP treated breast cancer cells. In MCF cells, EGCG and GTP treatment cause 1.5 to 2 fold increase in H3K9/18Ac levels whereas in MDA-MB-231 cells, the increase was more prominent with 4-6 fold induction. GTP and EGCG mediated global reduction in histone methylation levels with a concomitant increase in histone acetylation levels support transcriptional activation of *TIMP-3* gene, which has been shown to be regulated by both these epigenetic mechanisms.

Green tea polyphenols suppress invasiveness and gelatinolytic activity in human breast cancer cells

Given the significance of TIMP-3 levels in regulating tumor invasion and metastasis, we assessed the functional effect of GTP and EGCG treatments on breast cancer cell migration and gelatinolytic (MMP-2/-9) activity. Wound healing experiments showed that in MCF-7 cells, 20µM EGCG and 10µg/mL GTP treatment for 48h significantly inhibited wound healing and caused only 33% and 27% wound closure respectively as compared to control (vehicle treated) cells (95%) (Figure 6.10). Similarly in MDA-MB-231 cells, which are highly invasive and migratory, 18% and 23% wound closure was observed with 20µM EGCG and 10µg/mL GTP treatment after 24h. Combined treatment with 5µM DZNep and 40ng/mL TSA also demonstrated a significant anti-migratory effect causing only 50% wound closure in MCF-7 and MDA-MB-231 cells after 48h and 24h treatment period respectively. Treatment with 5-Aza-CdR, TSA and DZNep caused 70-95% wound closure after 24-48h.

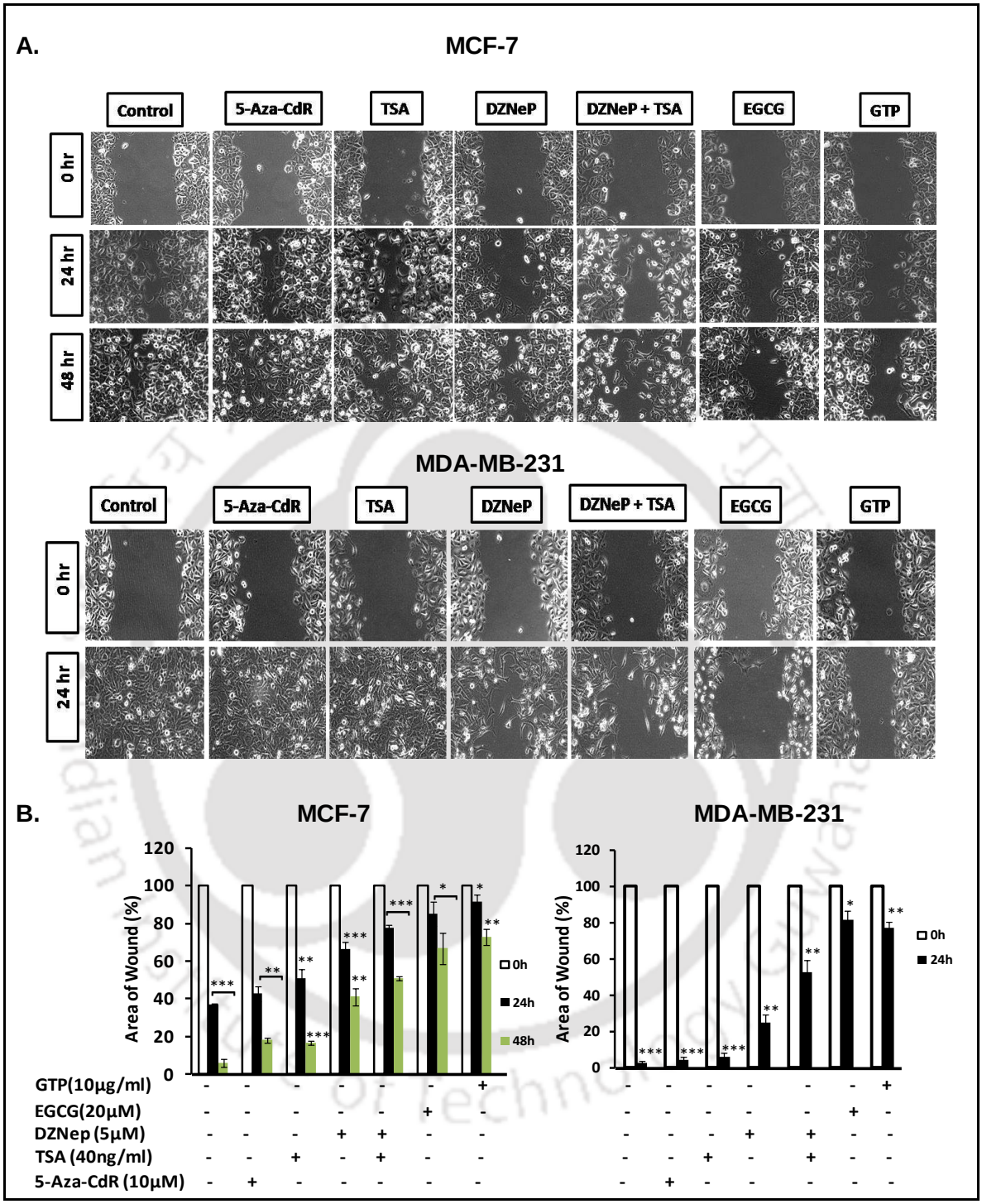


Figure 6.10. Green tea polyphenols treatment suppresses migration of human breast cancer cells. *Panel* **A**: Wound healing assay of MCF-7 and MDA-MB-231 cells treated with 5-Aza-CdR (10µM), TSA (40ng/mL), DZNep (5µM) and combined DZNep (5µM) + TSA (40ng/mL), 20µM EGCG and 10µg/mL GTP. Images were taken before (0h) and after wound closure (24h and 48h). *Panel* **B**: The results were expressed as the percentage of the remaining area determined by normalizing the area of wound after 24h or 48h (as indicated) to the initial wound area at 0h (set to 100%). Each bar represents the mean of five fields measured ± SD. Two-tailed Student's t-test was used to compare treatment groups and control. *p< 0.05, **p< 0.01, ***p< 0.001

TIMPs inhibit MMP activity by binding to them in 1:1 stoichiometry both in bound and secreted form and thus keep MMPs in an inactive state. Since MMP-2 and MMP-9 are prominent MMPs involved in ECM degradation and tumor cell migration that are regulated by TIMPs, we determined the enzymatic activity of these two MMPs using gelatin zymography. As shown in figure 6.11, a significant decrease in gelatinolytic activity was observed after EGCG and GTP treatment in MCF-7 cells.

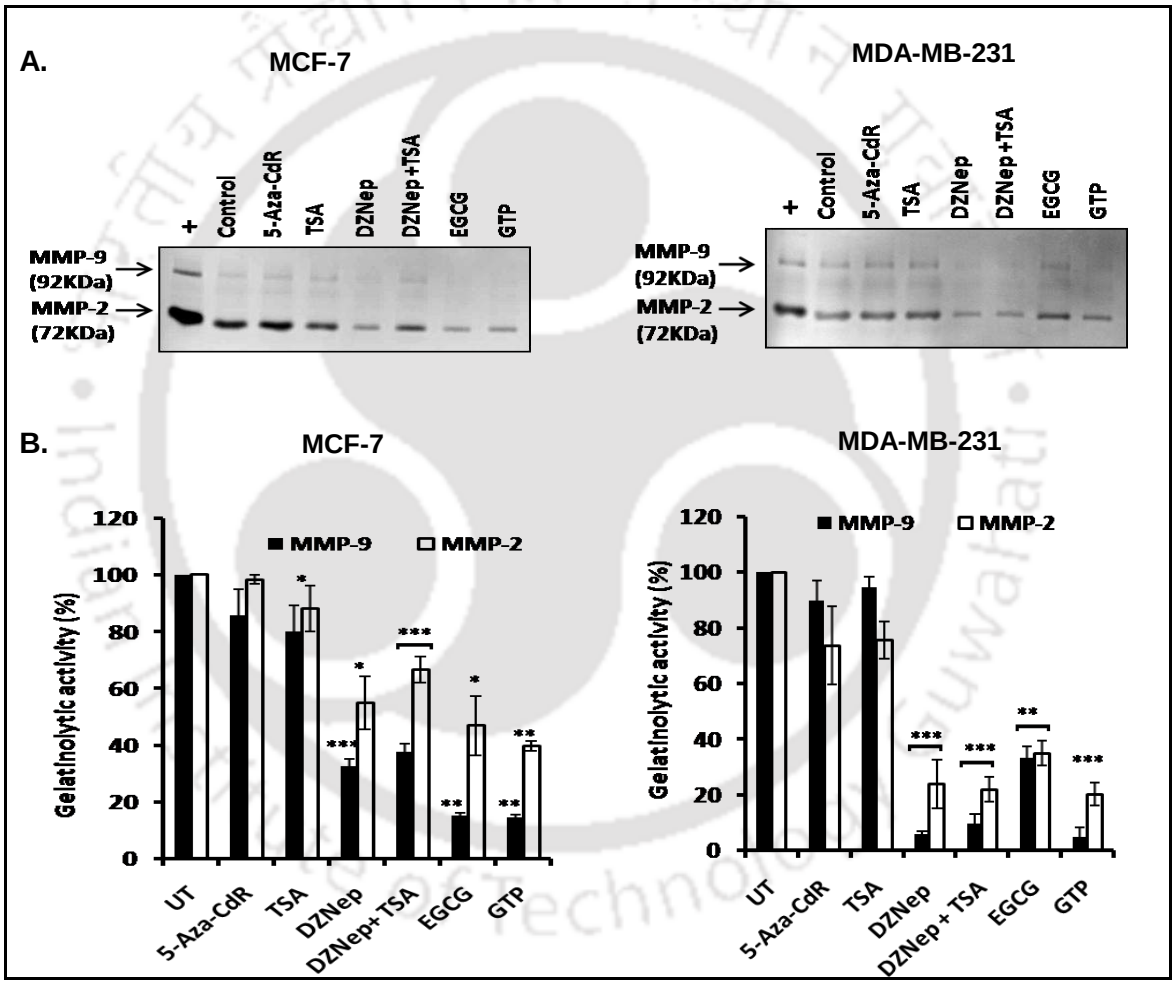


Figure 6.11. Green tea polyphenol treatment decrease gelatinolytic activity (Pro-MMP-2 and Pro-MMP-9) in human breast cancer cells. *Panel* **A** : Gelatin zymography assay for MMP-2 and MMP-9 activity in breast cancer cells treated with 5-Aza-CdR (10μM), TSA (40ng/mL), DZNep (5μM) and combined DZNep (5μM) + TSA (40ng/mL), 20μM EGCG and 10μg/mL GTP for 72h. Conditioned media was collected after 72h treatment and culture supernatant volume equivalent to 40μg total protein was used in the assay. HT1080 cell line conditioned medium was used as positive control and also served as molecular weight marker. *Panel* **B**: MMP-2 and MMP-9 band intensities (clearance zones) were quantified using Image J software. Bars represent mean ± SD (n=3) .Two-tailed Student's t-test was used to compare treatment groups and control. *p< 0.05, **p < 0.01, ***p< 0.001

In MDA-MB-231 cells, 20µM EGCG treatment for 72h caused 65-67% reduction in both MMP-2 and MMP-9 gelatinolytic activities. Treatment with 10µg/mL GTP resulted in a more pronounced inhibition of gelatinolytic activity as compared to EGCG treatment, causing 95% decrease in MMP-9 and 80% decrease in MMP-2 in MDA-MB-231. In both cell lines, 5µM DZNeP alone and in combination with 40ng/mL TSA significantly reduced gelatinolytic activities.

Next we studied the direct effect of EGCG and GTP treatment on MMP-2 and MMP-9 expression levels in MCF-7 and MDA-MB-231 cells. MMP-2 mRNA levels were found to be negligible in both cell lines (Figure 6.12). MMP-9 levels were relatively low in MCF-7 cells as compared to MDA-MB-231 cells. EGCG and GTP treatments had no significant effect on MMP-9 levels in either of the cell lines.

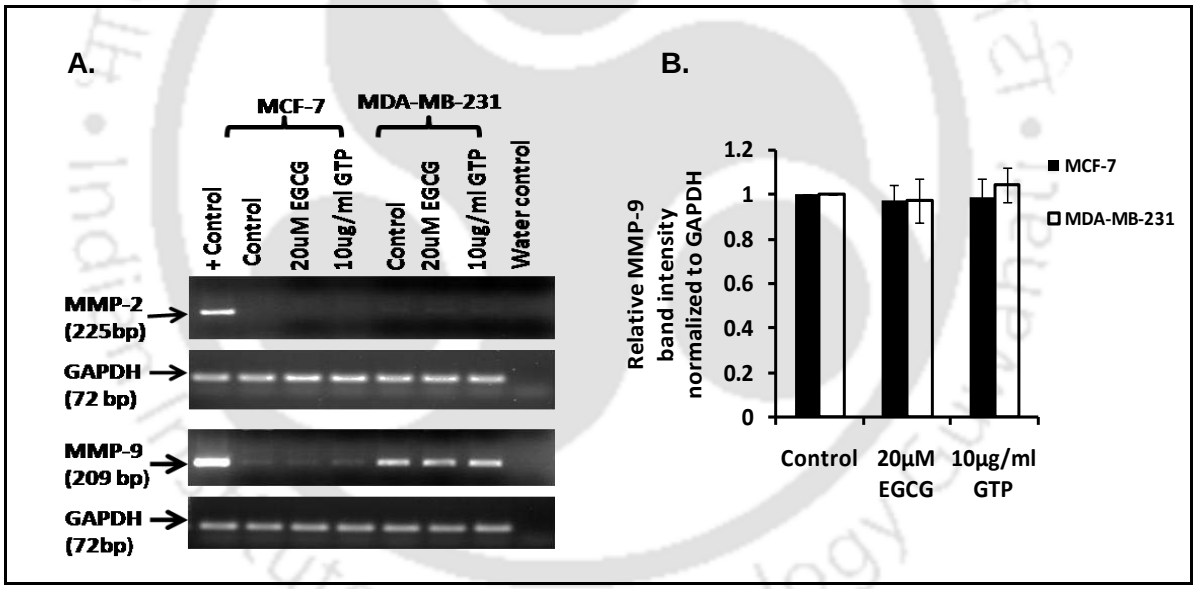


Figure 6.12. A semi-quantitative RT-PCR analysis of effect of EGCG and GTP treatment on MMP-2 and MMP-9 steady state mRNA levels in human breast cancer cells. *Panel* **A**: 2µg of total RNA was reverse transcribed using random hexamers. 100ng cDNA was used as template for PCR with gene specific primers for human MMP-2 and MMP-9. GAPDH was used as an internal control. cDNA from RWPE-1 cell line was used as positive control (+ control). Each experiment was repeated at least 3 times. *Panel* **B**: Graphical representation of MMP-9 band intensity in MCF-7 and MDA-MB-231 cells as determined using Image J. Bars represent mean ± SD (n=3).

DISCUSSION

TIMP-3 is a ubiquitously expressed, extracellular matrix bound protein that acts as an endogenous inhibitor of MMPs and inhibits apoptosis, invasion and angiogenesis (Brew and Nagase, 2010; Cruz-Munoz and Khokha, 2008; Visse and Nagase, 2003). Reduced expression of TIMP-3 protein was associated with poorly differentiated and aggressive breast carcinoma of higher nuclear and histological grades (Mylona *et al.*, 2006). Down-regulation of *TIMP-3* expression was predominantly attributed to promoter DNA hypermethylation however only 20% breast tumor specimens display *TIMP-3* methylation (Bachman *et al.*, 1999; Lui *et al.*, 2005).

In the studies presented in this chapter, we demonstrated that aberrant histone modification patterns at the *TIMP-3* promoter contributes to its epigenetic silencing, independent of promoter DNA hypermethylation, at least, in breast cancer cell lines. Hypermethylation in *TIMP-3* promoter was not detected in breast cancer cell lines used in our study and accordingly, there was no significant induction in *TIMP-3* mRNA levels after treatment with the demethylating agent 5-Aza-CdR. This observation is supported by another study which also reported that there was no *TIMP-3* hypermethylation in mammary cancer cell lines (Paz *et al.*, 2003). We showed that treatment of breast cancer cells with TSA (HDAC inhibitor) and DZNep (histone methylation inhibitor) could potentially reverse *TIMP-3* repression. Furthermore, genetic knockdown studies confirmed the involvement of class I HDACs in *TIMP-3* repression. EZH2, the core subunit of PRC2 complex catalyzes H3K27me3, which serves as repressive mark in maintaining the silenced state of several genes during development and establishing embryonic stem cell identity. Recent findings in breast cancer implicate that EZH2 protein levels increase through successive stages of neoplastic transformation, and enhanced EZH2 catalyzed H3K27me3 repressive mark results in the silencing of various critical tumor suppressor genes (Bachmann *et al.*, 2006; Collett *et al.*, 2006; Fujii *et al.*, 2008; Yang *et al.*, 2009). Similarly, abnormally high activity of HDACs has been linked to deacetylation and aberrant repression of several critical genes in breast cancer (Krusche *et al.*, 2005; Muller *et al.*, 2013; Stearns *et al.*, 2007). Our studies showed that decreased *TIMP-3* expression in breast cancer cells MCF-7 and MDA-MB-231 is

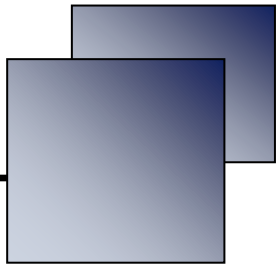
associated with elevated EZH2 and class I HDAC levels. ChIP studies demonstrated that decreased EZH2 and repressive H3K27me3 chromatin modification and a corresponding increase in H3K9/18Ac by combined DZNep and TSA treatment de-repress *TIMP-3* locus in both breast cancer cell lines. Recent studies in prostate cancer also demonstrated an inverse correlation between EZH2 and HDAC-1/2/3 levels to *TIMP-3* expression (Shinojima *et al.*, 2012).

In recent years, GTPs have been demonstrated to elicit a multifunctional effect in impeding various molecular pathways involved in neoplastic transformation and metastasis in various cancer types including breast cancer. To our knowledge, this is the first study to demonstrate that the epigenetic induction of *TIMP-3* may be one of the key mechanisms by which green tea polyphenols suppress gelatinolytic activity and tumor invasion in breast cancer cells. We also provided a novel mechanistic insight into the epigenetic regulation of *TIMP-3* gene in breast cancer cells. We demonstrated that GTP and EGCG treatment significantly decreased EZH2 protein levels and hence its localization and catalytic activity resulting in decreased H3K27me3 at the promoter region. Also, there was a significant decrease in HDAC-2 and HDAC-8 levels in MCF-7 and HDAC-1, HDAC-3 and HDAC-8 levels in MDA-MB-231 cells after treatment with GTP and EGCG, which may alter the balance between the histone acetyltransferases (HATs) and HDACs. This perhaps results in the deposition of increased transcriptionally active H3K9/18Ac at the *TIMP-3* promoter. The underlying mechanism by which green tea polyphenols decrease EZH2 and Class I HDACs in breast cancer cells was not the focus of the present study and requires further investigation. However the present findings are in line with previous studies which suggest that green tea catechins function as potent HDAC inhibitors and induce cell cycle arrest and apoptosis, by enhancing the proteasomal degradation of Class I HDACs in prostate cancer cells (Thakur *et al.*, 2012). Also, a recent study reported that EGCG treatment decreased the protein levels of polycomb group members EZH2 and Bmi1 in epidermal squamous cell carcinoma cells (Balasubramanian *et al.*, 2010; Choudhury *et al.*, 2011). It will be interesting to elucidate the effect of GTP or EGCG on HATs which may change the dynamic relationship between HDACs and HATs in the tumor cells.

Furthermore, we demonstrated by gelatin zymography that there was significant decrease in MMP-2 and MMP-9 activity in conditioned media collected from EGCG and GTP treated cells for 72h. Similar decrease in gelatinolytic activity was also observed in conditioned media collected from DZNep and TSA treated cells. EGCG has been shown to have a multifunctional effect on MMP-2 and MMP-9 including their expression, activation, secretion and the induction of some inhibitors such as RECK (Farabegoli *et al.*, 2011; Kato *et al.*, 2008; Sen *et al.*, 2010; Sen *et al.*, 2009). Based on previous studies it is arguable that the decrease in gelatinolytic activity might be due to direct effect of green tea polyphenols on MMP-2 and MMP-9 expression. But our finding suggests that MMP-2 mRNA was negligible (not detected) in MCF-7 and MDA-MB-231 cells, which is consistent with a previous study (Singer *et al.*, 2002). There was no further inhibitory effect of EGCG or GTP treatment on MMP-2 expression. MMP-9 expression levels also remained unaffected after EGCG or GTP treatment for 72h in both cell lines. The reason for the divergent findings might have to do with either variability in sub-clones of cell lines used or different doses of EGCG and treatment period used during the experiments. Intriguingly, though MMP-2 mRNA levels were found to very low in the cell lines used in our study, the gelatinolytic activity associated with MMP-2 was detectable in zymography. The possible reason may be that gelatin zymography can detect as low as 10pg of MMP-2 (Kleiner and Stetler-Stevenson, 1994). Here, we showed that green tea polyphenol mediated *TIMP-3* induction may play a crucial role in shifting MMP-TIMP balance towards TIMPs and decrease the elevated gelatinolytic activity (MMP-2/-9) frequently observed in breast tumors. However, further studies are needed to define the exact quantitative effect of green tea polyphenol mediated TIMP-3 induction on the inhibition of MMP-2/-9 activity and the suppression of cell invasiveness.

Based on our results, we conclude that the *in vitro* and *in vivo* anti-invasive effects of EGCG/GTP reported in breast cancer cells may be due to, in part, through epigenetic induction of endogenous MMP inhibitor, TIMP-3. Also, our results provide the first detailed insight into the epigenetic regulatory mechanism causing *TIMP-3* repression in breast cancer, highlighting the role of histone modifying enzymes EZH2 and HDACs.

Chapter VII



Conclusion

Breast cancer is one of the leading causes of cancer related deaths among women worldwide. In this endocrine related cancer, hormonal receptor status (ER, PR and Her2/neu) is a critical factor determining the clinical outcome of the disease. Combined estrogen blockade therapy using anti-estrogens and aromatase inhibitors, leads to tumor regression in the initial stages. However, over the course of treatment, tumors relapse due to acquisition of hormone refractory state, rendering the combined estrogen blockade therapy ineffective. The relapsed tumors eventually metastasize and are the major cause of breast cancer related mortality. Thus, endocrine resistance and tumor metastasis have emerged as two major barriers in breast cancer treatment.

In the past few decades, dietary phytochemicals such as EGCG have gained considerable attention as potential chemopreventive agents with minimum toxicity and fewer side effects. In breast cancer, the chemopreventive effects of EGCG were shown to be multifaceted, which involved modulation of a wide range of cellular targets. Given the central role of ER and estrogen signaling in breast tumorigenesis, it is of much relevance to study the effect of any potential therapeutic agent against breast cancer in the light of estrogen action on breast cancer cells. In this thesis, we addressed the combinatorial effect of EGCG and E2 on the proliferation, apoptosis, cell cycle, gelatinolytic activity, migration and invasion of breast cancer cells differing in their ER status. Our studies demonstrate that EGCG, in the presence or absence of E2, causes a significant decrease in proliferation, gelatinolytic activity, migration and invasion of breast cancer cells. EGCG mediated induction of apoptosis in breast cancer cells was also found to be independent of high exogenous E2 levels and ER status. Our findings highlight that the ER status or estrogenic stimulation of breast cancer cells does not affect the efficacy of EGCG.

Probing into the molecular mechanism of EGCG mediated decrease in gelatinolytic activity, we showed that EGCG significantly inhibits *in vitro* MMP-2 activity in a dose dependent manner. Furthermore, we showed that divalent cations have negative effect on EGCG mediated decrease in MMP-2 activity. We demonstrated that chelating divalent cations from the surrounding medium prior to treatment with EGCG, significantly improves EGCG's efficacy. In line with this, we found that pretreatment of HT1080 conditioned media or purified active MMP-

2 with EDTA followed by incubation with EGCG significantly enhanced the effect of EGCG in inhibiting MMP-2. In addition, incubation of EGCG with excess divalent cations partially reversed EGCG mediated MMP-2 inhibition, highlighting the fact that divalent cations present in the surrounding environment may mask the efficacy of EGCG. Known zinc chelators such as EDTA, glycine etc had no inhibitory effect on MMP-2 activity. Enzyme kinetic studies using MMP-2 specific fluorogenic substrate revealed that EGCG acts as an uncompetitive inhibitor of MMP-2 enzyme (Ki=11.69μM). This finding corroborates with the *in silico* molecular docking studies carried out by other colleagues in our laboratory (unpublished data). Molecular docking studies showed that EGCG does not interfere with the Zn^{+2} active site like other known MMP inhibitors. It was found to bind MMP-2 fibronectin type II repeat 3 region, which is required for the proper positioning of collagen substrate for catalysis. Our findings implicate that EGCG may emerge as a natural, non-toxic, selective MMP-2 inhibitor targeting sites which are structurally and functionally non-redundant among other MMP classes.

Aberrant epigenetic silencing of *TIMP-3* gene, that negatively modulates MMP activity, has been implicated in the pathogenesis and metastasis of breast cancer. In this thesis work, we demonstrated that GTPs and EGCG mediate epigenetic induction of TIMP-3 levels and play a key role in suppressing invasiveness and gelatinolytic activity of MMP-2 and MMP-9 in breast cancer cells. Treatment of MCF-7 and MDA-MB-231 breast cancer cells with 20μM EGCG and 10μg/ml GTP for 72h significantly induced *TIMP-3* mRNA and protein levels. Interestingly, investigations into the molecular mechanism revealed that *TIMP-3* repression in breast cancer cells is mediated by the epigenetic silencing mechanism(s) involving EZH2 and class I HDACs, independent of promoter DNA hypermethylation. Treatment with GTP and EGCG significantly reduced EZH2 and class I HDAC protein levels. Furthermore, transcriptional activation of *TIMP-3* was found to be associated with decreased EZH2 localization and H3K27 trimethylation deposition at the *TIMP-3* promoter with a concomitant increase in histone H3K9/18 acetylation. Our findings unravel the epigenetic mechanism underlying *TIMP-3* induction by GTPs and EGCG, which may at least in part be the molecular basis for the observed anti-invasive and anti-gelatinolytic effect of EGCG.

In summary, the present study provides a new insight into the green tea catechins mediated modulation of gelatinolytic activity in human breast cancer cells. To our knowledge, this is the first study to explore the combinatorial effect of EGCG and E2 on breast cancer cells. The data presented in this thesis provide strong evidence that the anti-proliferative, anti-gelatinolytic and anti-invasive effects of EGCG remain independent of the ER status or estrogenic stimulation of breast cancer cells. We also provided comprehensive insight into the modulation of *in vitro* MMP-2 activity by EGCG in a cell free environment. Our data highlight the negative effect of divalent cations on EGCG mediated inhibition of *in vitro* MMP-2 activity. This finding is particularly relevant in the context of *in vivo* effects of EGCG, which are observed at concentrations much higher than that measured in regular green tea consumers. Also, this is the first study to provide detailed molecular insight into epigenetic regulation of *TIMP-3* gene in breast cancer cells. Our findings implicate that the epigenetic induction of *TIMP-3* may be a key event modulated by green tea polyphenols in restoring the MMP:TIMP balance to delay breast cancer progression and invasion.

Bibliography

Abel, K. J., Brody, L. C., Valdes, J. M., Erdos, M. R., McKinley, D. R., Castilla, L. H., Merajver, S. D., Couch, F. J., Friedman, L. S., Ostermeyer, E. A., *et al.* (1996). Characterization of EZH1, a human homolog of Drosophila Enhancer of zeste near BRCA1. Genomics *37*, 161-171.

Al Saleh, S., Sharaf, L. H., and Luqmani, Y. A. (2011). Signalling pathways involved in endocrine resistance in breast cancer and associations with epithelial to mesenchymal transition (Review). International journal of oncology *38*, 1197-1217.

Ali, S., Metzger, D., Bornert, J. M., and Chambon, P. (1993). Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. The EMBO journal *12*, 1153-1160.

Bachman, K. E., Herman, J. G., Corn, P. G., Merlo, A., Costello, J. F., Cavenee, W. K., Baylin, S. B., and Graff, J. R. (1999). Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. Cancer research *59*, 798-802.

Bachmann, I. M., Halvorsen, O. J., Collett, K., Stefansson, I. M., Straume, O., Haukaas, S. A., Salvesen, H. B., Otte, A. P., and Akslen, L. A. (2006a). EZH2 Expression Is Associated With High Proliferation Rate and Aggressive Tumor Subgroups in Cutaneous Melanoma and Cancers of the Endometrium, Prostate, and Breast. Journal of Clinical Oncology *24*, 268-273.

Bachmann, I. M., Halvorsen, O. J., Collett, K., Stefansson, I. M., Straume, O., Haukaas, S. A., Salvesen, H. B., Otte, A. P., and Akslen, L. A. (2006b). EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *24*, 268-273.

Balasubramanian, S., Adhikary, G., and Eckert, R. L. (2010). The Bmi-1 polycomb protein antagonizes the (-)-epigallocatechin-3-gallate-dependent suppression of skin cancer cell survival. Carcinogenesis *31*, 496-503.

Balentine, D. A., Wiseman, S. A., and Bouwens, L. C. (1997). The chemistry of tea flavonoids. Critical reviews in food science and nutrition *37*, 693-704.

Baliga, M. S., Meleth, S., and Katiyar, S. K. (2005). Growth inhibitory and antimetastatic effect of green tea polyphenols on metastasis-specific mouse mammary carcinoma 4T1 cells in vitro and in vivo systems. Clinical cancer research : an official journal of the American Association for Cancer Research *11*, 1918-1927.

Banerji, S., Cibulskis, K., Rangel-Escareno, C., Brown, K. K., Carter, S. L., Frederick, A. M., Lawrence, M. S., Sivachenko, A. Y., Sougnez, C., Zou, L., *et al.* (2012). Sequence analysis of mutations and translocations across breast cancer subtypes. Nature *486*, 405-409.

Barrand, S., Andersen, I. S., and Collas, P. (2010). Promoter-exon relationship of H3 lysine 9, 27, 36 and 79 methylation on pluripotency-associated genes. Biochemical and Biophysical Research Communications *401*, 611-617.

Barski, A., Cuddapah, S., Cui, K., Roh, T.-Y., Schones, D. E., Wang, Z., Wei, G., Chepelev, I., and Zhao, K. (2007). High-Resolution Profiling of Histone Methylations in the Human Genome. Cell *129*, 823-837.

Bell, O., Wirbelauer, C., Hild, M., Scharf, A. N. D., Schwaiger, M., MacAlpine, D. M., Zilbermann, F., van Leeuwen, F., Bell, S. P., Imhof, A., *et al.* (2007). Localized H3K36 methylation states define histone H4K16 acetylation during transcriptional elongation in Drosophila. The EMBO journal *26*, 4974-4984.

Belotti, D., Paganoni, P., and Giavazzi, R. (1999). MMP inhibitors: experimental and clinical studies. The International journal of biological markers *14*, 232-238.

Bergers, G., Brekken, R., McMahon, G., Vu, T. H., Itoh, T., Tamaki, K., Tanzawa, K., Thorpe, P., Itohara, S., Werb, Z., and Hanahan, D. (2000). Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nature cell biology *2*, 737-744.

Bhide, S. V., Azuine, M. A., Lahiri, M., and Telang, N. T. (1994). Chemoprevention of mammary tumor virus-induced and chemical carcinogen-induced rodent mammary tumors by natural plant products. Breast cancer research and treatment *30*, 233-242.

Bigelow, R. L. H., and Cardelli, J. A. (2006). The green tea catechins, (-)-Epigallocatechin-3-gallate (EGCG) and (-)-Epicatechin-3-gallate (ECG), inhibit HGF//Met signaling in immortalized and tumorigenic breast epithelial cells. Oncogene *25*, 1922-1930.

Bjornstrom, L., and Sjoberg, M. (2004). Estrogen receptor-dependent activation of AP-1 via non-genomic signalling. Nuclear receptor *2*, 3.

Bode, W., Fernandez-Catalan, C., Grams, F., Gomis-Ruth, F. X., Nagase, H., Tschesche, H., and Maskos, K. (1999). Insights into MMP-TIMP interactions. Annals of the New York Academy of Sciences *878*, 73-91.

Boyer, L. A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L. A., Lee, T. I., Levine, S. S., Wernig, M., Tajonar, A., Ray, M. K., *et al.* (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature *441*, 349-353.

Bracken, A. P., Pasini, D., Capra, M., Prosperini, E., Colli, E., and Helin, K. (2003). EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. The EMBO journal *22*, 5323-5335.

Braicu, C., and Gherman, C. (2012). Epigallocatechin gallate induce cell death and apoptosis in triple negative breast cancer cells Hs578T. Journal of drug targeting.

Brew, K., and Nagase, H. (2010). The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. Biochimica et biophysica acta *1803*, 55-71.

Brglez, V., Pucer, A., Pungercar, J., Lambeau, G., and Petan, T. (2014). Secreted phospholipases A2 are differentially expressed and epigenetically silenced in human breast cancer cells. Biochemical and biophysical research communications *445*, 230-235.

Butt, M. S., and Sultan, M. T. (2009). Green tea: nature's defense against malignancies. Critical reviews in food science and nutrition *49*, 463-473.

Cabrera, C., Artacho, R., and Gimenez, R. (2006). Beneficial effects of green tea--a review. Journal of the American College of Nutrition *25*, 79-99.

Campbell-Thompson, M., Lynch, I. J., and Bhardwaj, B. (2001). Expression of estrogen receptor (ER) subtypes and ERbeta isoforms in colon cancer. Cancer research *61*, 632-640.

Campbell, R. A., Bhat-Nakshatri, P., Patel, N. M., Constantinidou, D., Ali, S., and Nakshatri, H. (2001). Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. The Journal of biological chemistry *276*, 9817-9824.

Cao, R., and Zhang, Y. (2004). The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Current opinion in genetics & development *14*, 155-164.

Cardoso, C., Mignon, C., Hetet, G., Grandchamps, B., Fontes, M., and Colleaux, L. (2000). The human EZH2 gene: genomic organisation and revised mapping in 7q35 within the critical region for malignant myeloid disorders. European journal of human genetics : EJHG *8*, 174-180.

Cavalieri, E., Chakravarti, D., Guttenplan, J., Hart, E., Ingle, J., Jankowiak, R., Muti, P., Rogan, E., Russo, J., Santen, R., and Sutter, T. (2006). Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. Biochimica et biophysica acta *1766*, 63-78.

Cavalieri, E., Frenkel, K., Liehr, J. G., Rogan, E., and Roy, D. (2000). Estrogens as endogenous genotoxic agents--DNA adducts and mutations. Journal of the National Cancer Institute Monographs, 75-93.

Cavalieri, E. L., and Rogan, E. G. (2011). Unbalanced metabolism of endogenous estrogens in the etiology and prevention of human cancer. The Journal of steroid biochemistry and molecular biology *125*, 169-180.

Cha, T. L., Zhou, B. P., Xia, W., Wu, Y., Yang, C. C., Chen, C. T., Ping, B., Otte, A. P., and Hung, M. C. (2005). Akt-mediated phosphorylation of EZH2 suppresses methylation of lysine 27 in histone H3. Science (New York, NY) *310*, 306-310.

Chambers, A. F., and Matrisian, L. M. (1997). Changing views of the role of matrix metalloproteinases in metastasis. Journal of the National Cancer Institute *89*, 1260-1270.

Chang, C. J., Yang, J. Y., Xia, W., Chen, C. T., Xie, X., Chao, C. H., Woodward, W. A., Hsu, J. M., Hortobagyi, G. N., and Hung, M. C. (2011). EZH2 promotes expansion of breast tumor initiating cells through activation of RAF1-beta-catenin signaling. Cancer cell *19*, 86-100.

Chase, A., and Cross, N. C. (2011). Aberrations of EZH2 in cancer. Clinical cancer research : an official journal of the American Association for Cancer Research *17*, 2613-2618.

Chaudhary, A. K., Pandya, S., Ghosh, K., and Nadkarni, A. (2013). Matrix metalloproteinase and its drug targets therapy in solid and hematological malignancies: an overview. Mutation research *753*, 7-23.

Chen, S., Bohrer, L. R., Rai, A. N., Pan, Y., Gan, L., Zhou, X., Bagchi, A., Simon, J. A., and Huang, H. (2010). Cyclin-dependent kinases regulate epigenetic gene silencing through phosphorylation of EZH2. Nature cell biology *12*, 1108-1114.

Cheng, S., Ding, L., Zhen, Y., Lin, P., Zhu, Y., Chen, Y., and Hu, X. (1991). Progress in studies on the antimutagenicity and anticarcinogenicity of green tea epicatechins. Chinese medical sciences journal = Chung-kuo i hsueh k'o hsueh tsa chih / Chinese Academy of Medical Sciences *6*, 233-238.

Cheng, X. W., Kuzuya, M., Kanda, S., Maeda, K., Sasaki, T., Wang, Q. L., Tamaya-Mori, N., Shibata, T., and Iguchi, A. (2003). Epigallocatechin-3-gallate binding to MMP-2 inhibits gelatinolytic activity without influencing the attachment to extracellular matrix proteins but enhances MMP-2 binding to TIMP-2. Archives of biochemistry and biophysics *415*, 126-132.

Chisholm, K., Bray, B. J., and Rosengren, R. J. (2004). Tamoxifen and epigallocatechin gallate are synergistically cytotoxic to MDA-MB-231 human breast cancer cells. Anti-cancer drugs *15*, 889-897.

Choi, J. D., and Lee, J. S. (2013). Interplay between Epigenetics and Genetics in Cancer. Genomics & informatics *11*, 164-173.

Choudhury, S. R., Balasubramanian, S., Chew, Y. C., Han, B., Marquez, V. E., and Eckert, R. L. (2011). (-)-Epigallocatechin-3-gallate and DZNep reduce polycomb protein level via a proteasome-dependent mechanism in skin cancer cells. Carcinogenesis *32*, 1525-1532.

Clarke, R., Leonessa, F., Welch, J. N., and Skaar, T. C. (2001). Cellular and molecular pharmacology of antiestrogen action and resistance. Pharmacological reviews *53*, 25-71.

Clarke, R., Skaar, T., Baumann, K., Leonessa, F., James, M., Lippman, J., Thompson, E. W., Freter, C., and Brunner, N. (1994). Hormonal carcinogenesis in breast cancer: cellular and molecular studies of malignant progression. Breast cancer research and treatment *31*, 237-248.

Collett, K., Eide, G. E., Arnes, J., Stefansson, I. M., Eide, J., Braaten, A., Aas, T., Otte, A. P., and Akslen, L. A. (2006). Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research *12*, 1168-1174.

Cooper, R., Morre, D. J., and Morre, D. M. (2005a). Medicinal benefits of green tea: Part I. Review of noncancer health benefits. Journal of alternative and complementary medicine (New York, NY) *11*, 521-528.

Cooper, R., Morre, D. J., and Morre, D. M. (2005b). Medicinal benefits of green tea: part II. review of anticancer properties. Journal of alternative and complementary medicine (New York, NY) *11*, 639-652.

Cox, A., Dunning, A. M., Garcia-Closas, M., Balasubramanian, S., Reed, M. W., Pooley, K. A., Scollen, S., Baynes, C., Ponder, B. A., Chanock, S., *et al.* (2007). A common coding variant in CASP8 is associated with breast cancer risk. Nature genetics *39*, 352-358.

Crespy, V., and Williamson, G. (2004). A review of the health effects of green tea catechins in in vivo animal models. The Journal of nutrition *134*, 3431s-3440s.

Cruz-Munoz, W., and Khokha, R. (2008). The role of tissue inhibitors of metalloproteinases in tumorigenesis and metastasis. Critical reviews in clinical laboratory sciences *45*, 291-338.

Csankovszki, G., Nagy, A., and Jaenisch, R. (2001). Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. The Journal of cell biology *153*, 773-784.

Curran, M., and Wiseman, L. (2001). Fulvestrant. Drugs *61*, 807-813; discussion 814.

Curran, S., and Murray, G. I. (1999). Matrix metalloproteinases in tumour invasion and metastasis. The Journal of pathology *189*, 300-308.

Curtis, C., Shah, S. P., Chin, S. F., Turashvili, G., Rueda, O. M., Dunning, M. J., Speed, D., Lynch, A. G., Samarajiwa, S., Yuan, Y., *et al.* (2012). The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature *486*, 346-352.

Dai, Q., Shu, X. O., Li, H., Yang, G., Shrubsole, M. J., Cai, H., Ji, B., Wen, W., Franke, A., Gao, Y. T., and Zheng, W. (2010). Is green tea drinking associated with a later onset of breast cancer? Annals of epidemiology *20*, 74-81.

Daniel, C. W., Silberstein, G. B., and Strickland, P. (1987). Direct action of 17 beta-estradiol on mouse mammary ducts analyzed by sustained release implants and steroid autoradiography. Cancer research *47*, 6052-6057.

Day, J. A., and Cohen, S. M. (2013). Investigating the selectivity of metalloenzyme inhibitors. Journal of medicinal chemistry *56*, 7997-8007.

Deb, G., Singh, A. K., and Gupta, S. (2014a). EZH2: Not EZHY (Easy) to Deal. Molecular cancer research : MCR.

Deb, G., Thakur, V. S., and Gupta, S. (2013). Multifaceted role of EZH2 in breast and prostate tumorigenesis: Epigenetics and beyond. Epigenetics : official journal of the DNA Methylation Society *8*, 464-476.

Deb, G., Thakur, V. S., Limaye, A. M., and Gupta, S. (2014b). Epigenetic induction of tissue inhibitor of matrix metalloproteinase-3 by green tea polyphenols in breast cancer cells. Molecular carcinogenesis

DeClerck, Y. A., Perez, N., Shimada, H., Boone, T. C., Langley, K. E., and Taylor, S. M. (1992). Inhibition of invasion and metastasis in cells transfected with an inhibitor of metalloproteinases. Cancer research *52*, 701-708.

Delany, A. M., Jeffrey, J. J., Rydziel, S., and Canalis, E. (1995). Cortisol increases interstitial collagenase expression in osteoblasts by post-transcriptional mechanisms. The Journal of biological chemistry *270*, 26607-26612.

Dell'Aica, I., Dona, M., Sartor, L., Pezzato, E., and Garbisa, S. (2002). (-)Epigallocatechin-3-gallate directly inhibits MT1-MMP activity, leading to accumulation of nonactivated MMP-2 at the cell surface. Laboratory investigation; a journal of technical methods and pathology *82*, 1685-1693.

Demeule, M., Brossard, M., Page, M., Gingras, D., and Beliveau, R. (2000). Matrix metalloproteinase inhibition by green tea catechins. Biochimica et biophysica acta *1478*, 51-60.

Deng, X., Bhagat, S., Dong, Z., Mullins, C., Chinni, S. R., and Cher, M. (2006). Tissue inhibitor of metalloproteinase-3 induces apoptosis in prostate cancer cells and confers increased sensitivity to paclitaxel. European journal of cancer (Oxford, England : 1990) *42*, 3267-3273.

Deng, Y. T., and Lin, J. K. (2011). EGCG inhibits the invasion of highly invasive CL1-5 lung cancer cells through suppressing MMP-2 expression via JNK signaling and induces G2/M arrest. Journal of agricultural and food chemistry *59*, 13318-13327.

Dimri, G., Band, H., and Band, V. (2005). Mammary epithelial cell transformation: insights from cell culture and mouse models. Breast cancer research : BCR *7*, 171-179.

Ding, L., and Kleer, C. G. (2006). Enhancer of Zeste 2 as a marker of preneoplastic progression in the breast. Cancer research *66*, 9352-9355.

Dreosti, I. E., Wargovich, M. J., and Yang, C. S. (1997). Inhibition of carcinogenesis by tea: the evidence from experimental studies. Critical reviews in food science and nutrition *37*, 761-770.

Du, J., Li, L., Ou, Z., Kong, C., Zhang, Y., Dong, Z., Zhu, S., Jiang, H., Shao, Z., Huang, B., and Lu, J. (2012). FOXC1, a target of polycomb, inhibits metastasis of breast cancer cells. Breast cancer research and treatment *131*, 65-73.

Duffy, M. J. (2006). Estrogen receptors: role in breast cancer. Critical reviews in clinical laboratory sciences *43*, 325-347.

Dworkin, A. M., Huang, T. H., and Toland, A. E. (2009). Epigenetic alterations in the breast: Implications for breast cancer detection, prognosis and treatment. Seminars in cancer biology *19*, 165-171.

Easton, D. F., Pooley, K. A., Dunning, A. M., Pharoah, P. D., Thompson, D., Ballinger, D. G., Struewing, J. P., Morrison, J., Field, H., Luben, R., *et al.* (2007). Genome-wide association study identifies novel breast cancer susceptibility loci. Nature *447*, 1087-1093.

Eisen, A., Lubinski, J., Klijn, J., Moller, P., Lynch, H. T., Offit, K., Weber, B., Rebbeck, T., Neuhausen, S. L., Ghadirian, P., *et al.* (2005). Breast cancer risk following bilateral oophorectomy in BRCA1 and BRCA2 mutation carriers: an international case-control study. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *23*, 7491-7496.

Ellis, M. J., Ding, L., Shen, D., Luo, J., Suman, V. J., Wallis, J. W., Van Tine, B. A., Hoog, J., Goiffon, R. J., Goldstein, T. C., *et al.* (2012). Whole-genome analysis informs breast cancer response to aromatase inhibition. Nature *486*, 353-360.

Ernst, T., Chase, A. J., Score, J., Hidalgo-Curtis, C. E., Bryant, C., Jones, A. V., Waghorn, K., Zoi, K., Ross, F. M., Reiter, A., *et al.* (2010). Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. Nature genetics *42*, 722-726.

Eskeland, R., Leeb, M., Grimes, G. R., Kress, C., Boyle, S., Sproul, D., Gilbert, N., Fan, Y., Skoultchi, A. I., Wutz, A., and Bickmore, W. A. (2010). Ring1B compacts chromatin structure and represses gene expression independent of histone ubiquitination. Molecular cell *38*, 452-464.

Fan, J., Yin, W. J., Lu, J. S., Wang, L., Wu, J., Wu, F. Y., Di, G. H., Shen, Z. Z., and Shao, Z. M. (2008). ER alpha negative breast cancer cells restore response to endocrine therapy by combination treatment with both HDAC inhibitor and DNMT inhibitor. Journal of cancer research and clinical oncology *134*, 883-890.

Fang, J., Shing, Y., Wiederschain, D., Yan, L., Butterfield, C., Jackson, G., Harper, J., Tamvakopoulos, G., and Moses, M. A. (2000). Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model. Proceedings of the National Academy of Sciences of the United States of America *97*, 3884-3889.

Farabegoli, F., Papi, A., and Orlandi, M. (2011). (-)-Epigallocatechin-3-gallate down-regulates EGFR, MMP-2, MMP-9 and EMMPRIN and inhibits the invasion of MCF-7 tamoxifen-resistant cells. Bioscience reports *31*, 99-108.

Faridi, J., Wang, L., Endemann, G., and Roth, R. A. (2003). Expression of constitutively active Akt-3 in MCF-7 breast cancer cells reverses the estrogen and tamoxifen responsivity of these cells in vivo. Clinical cancer research : an official journal of the American Association for Cancer Research *9*, 2933-2939.

Fernandez, S. V., Russo, I. H., and Russo, J. (2006). Estradiol and its metabolites 4-hydroxyestradiol and 2-hydroxyestradiol induce mutations in human breast epithelial cells. International journal of cancer Journal international du cancer *118*, 1862-1868.

Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., Bray, F. (2013). GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer. Available from http://globocan.iarc.fr, assessed on 18/3/2014

Filardo, E. J., Quinn, J. A., Frackelton, A. R., Jr., and Bland, K. I. (2002). Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. Molecular endocrinology (Baltimore, Md) *16*, 70-84.

Filardo, E. J., and Thomas, P. (2012). Minireview: G protein-coupled estrogen receptor-1, GPER-1: its mechanism of action and role in female reproductive cancer, renal and vascular physiology. Endocrinology *153*, 2953-2962.

Fisher, J. F., and Mobashery, S. (2006). Recent advances in MMP inhibitor design. Cancer metastasis reviews *25*, 115-136.

Fortunati, N., Becchis, M., Catalano, M. G., Comba, A., Ferrera, P., Raineri, M., Berta, L., and Frairia, R. (1999). Sex hormone-binding globulin, its membrane receptor, and breast cancer: a new approach to the modulation of estradiol action in neoplastic cells. The Journal of steroid biochemistry and molecular biology *69*, 473-479.

Foster, J. S., Henley, D. C., Bukovsky, A., Seth, P., and Wimalasena, J. (2001). Multifaceted regulation of cell cycle progression by estrogen: regulation of Cdk inhibitors and Cdc25A independent of cyclin D1-Cdk4 function. Molecular and cellular biology *21*, 794-810.

Foulkes, W. D., Metcalfe, K., Sun, P., Hanna, W. M., Lynch, H. T., Ghadirian, P., Tung, N., Olopade, O. I., Weber, B. L., McLennan, J., *et al.* (2004). Estrogen receptor status in BRCA1- and BRCA2-related breast cancer: the influence of age, grade, and histological type. Clinical cancer research : an official journal of the American Association for Cancer Research *10*, 2029-2034.

Frei, B., and Higdon, J. V. (2003). Antioxidant activity of tea polyphenols in vivo: evidence from animal studies. The Journal of nutrition *133*, 3275s-3284s.

Fujii, S., Ito, K., Ito, Y., and Ochiai, A. (2008). Enhancer of zeste homologue 2 (EZH2) down-regulates RUNX3 by increasing histone H3 methylation. The Journal of biological chemistry *283*, 17324-17332.

Fujii, S., Tokita, K., Wada, N., Ito, K., Yamauchi, C., Ito, Y., and Ochiai, A. (2011). MEK-ERK pathway regulates EZH2 overexpression in association with aggressive breast cancer subtypes. Oncogene *30*, 4118-4128.

Gaikwad, N. W., Yang, L., Muti, P., Meza, J. L., Pruthi, S., Ingle, J. N., Rogan, E. G., and Cavalieri, E. L. (2008). The molecular etiology of breast cancer: evidence from biomarkers of risk. International journal of cancer Journal international du cancer *122*, 1949-1957.

Gaikwad, N. W., Yang, L., Pruthi, S., Ingle, J. N., Sandhu, N., Rogan, E. G., and Cavalieri, E. L. (2009). Urine biomarkers of risk in the molecular etiology of breast cancer. Breast cancer : basic and clinical research *3*, 1-8.

Garbisa, S., Sartor, L., Biggin, S., Salvato, B., Benelli, R., and Albini, A. (2001). Tumor gelatinases and invasion inhibited by the green tea flavanol epigallocatechin-3-gallate. Cancer *91*, 822-832.

Gaughan, L., Stockley, J., Wang, N., McCracken, S. R. C., Treumann, A., Armstrong, K., Shaheen, F., Watt, K., McEwan, I. J., Wang, C., *et al.* (2011). Regulation of the androgen receptor by SET9-mediated methylation. Nucleic acids research *39*, 1266-1279.

Gehani, S. S., Agrawal-Singh, S., Dietrich, N., Christophersen, N. S., Helin, K., and Hansen, K. (2010). Polycomb Group Protein Displacement and Gene Activation through MSK-Dependent H3K27me3S28 Phosphorylation. Molecular cell *39*, 886-900.

Gonzalez, M. E., DuPrie, M. L., Krueger, H., Merajver, S. D., Ventura, A. C., Toy, K. A., and Kleer, C. G. (2011). Histone methyltransferase EZH2 induces Akt-dependent genomic instability and BRCA1 inhibition in breast cancer. Cancer research *71*, 2360-2370.

Gonzalez, M. E., Li, X., Toy, K., DuPrie, M., Ventura, A. C., Banerjee, M., Ljungman, M., Merajver, S. D., and Kleer, C. G. (2009). Downregulation of EZH2 decreases growth of estrogen receptor-negative invasive breast carcinoma and requires BRCA1. Oncogene *28*, 843-853.

Goodin, M. G., Fertuck, K. C., Zacharewski, T. R., and Rosengren, R. J. (2002). Estrogen receptor-mediated actions of polyphenolic catechins in vivo and in vitro. Toxicological sciences : an official journal of the Society of Toxicology *69*, 354-361.

Gruber, C. J., Tschugguel, W., Schneeberger, C., and Huber, J. C. (2002). Production and actions of estrogens. The New England journal of medicine *346*, 340-352.

Gustafsson, J. A. (1999). Estrogen receptor beta--a new dimension in estrogen mechanism of action. The Journal of endocrinology *163*, 379-383.

Hadler-Olsen, E., Winberg, J. O., and Uhlin-Hansen, L. (2013). Matrix metalloproteinases in cancer: their value as diagnostic and prognostic markers and therapeutic targets. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine *34*, 2041-2051.

Hakim, I. A., Harris, R. B., and Weisgerber, U. M. (2000). Tea intake and squamous cell carcinoma of the skin: influence of type of tea beverages. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology *9*, 727-731.

Hall, J. M., Couse, J. F., and Korach, K. S. (2001). The multifaceted mechanisms of estradiol and estrogen receptor signaling. The Journal of biological chemistry *276*, 36869-36872.

Hanahan, D., and Weinberg, Robert A. (2011). Hallmarks of Cancer: The Next Generation. Cell *144*, 646-674.

Hasty, K. A., Pourmotabbed, T. F., Goldberg, G. I., Thompson, J. P., Spinella, D. G., Stevens, R. M., and Mainardi, C. L. (1990). Human neutrophil collagenase. A distinct gene product with homology to other matrix metalloproteinases. The Journal of biological chemistry *265*, 11421-11424.

Hatziapostolou, M., and Iliopoulos, D. (2011). Epigenetic aberrations during oncogenesis. Cellular and molecular life sciences : CMLS *68*, 1681-1702.

Hayes, D. F. (2000). Do we need prognostic factors in nodal-negative breast cancer? Arbiter. European journal of cancer (Oxford, England : 1990) *36*, 302-306.

Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M., Strom, A., Treuter, E., Warner, M., and Gustafsson, J. A. (2007). Estrogen receptors: how do they signal and what are their targets. Physiological reviews *87*, 905-931.

Hervouet, E., Cartron, P. F., Jouvenot, M., and Delage-Mourroux, R. (2013). Epigenetic regulation of estrogen signaling in breast cancer. Epigenetics : official journal of the DNA Methylation Society *8*, 237-245.

Hidalgo, M., and Eckhardt, S. G. (2001). Development of matrix metalloproteinase inhibitors in cancer therapy. Journal of the National Cancer Institute *93*, 178-193.

Higdon, J. V., and Frei, B. (2003). Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. Critical reviews in food science and nutrition *43*, 89-143.

Hirose, M., Hoshiya, T., Akagi, K., Futakuchi, M., and Ito, N. (1994). Inhibition of mammary gland carcinogenesis by green tea catechins and other naturally occurring antioxidants in female Sprague-Dawley rats pretreated with 7,12-dimethylbenz[alpha]anthracene. Cancer letters *83*, 149-156.

Hirose, M., Mizoguchi, Y., Yaono, M., Tanaka, H., Yamaguchi, T., and Shirai, T. (1997). Effects of green tea catechins on the progression or late promotion stage of mammary gland carcinogenesis in female Sprague-Dawley rats pretreated with 7,12-dimethylbenz(a)anthracene. Cancer letters *112*, 141-147.

Hirose, M., Nishikawa, A., Shibutani, M., Imai, T., and Shirai, T. (2002). Chemoprevention of heterocyclic amine-induced mammary carcinogenesis in rats. Environmental and molecular mutagenesis *39*, 271-278.

Hockel, M., and Vaupel, P. (2001). Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. Journal of the National Cancer Institute *93*, 266-276.

Hofseth, L. J., Raafat, A. M., Osuch, J. R., Pathak, D. R., Slomski, C. A., and Haslam, S. Z. (1999). Hormone replacement therapy with estrogen or estrogen plus medroxyprogesterone acetate is associated with increased epithelial proliferation in the normal postmenopausal breast. The Journal of clinical endocrinology and metabolism *84*, 4559-4565.

Holinka, C. F., Hata, H., Kuramoto, H., and Gurpide, E. (1986). Responses to estradiol in a human endometrial adenocarcinoma cell line (Ishikawa). Journal of steroid biochemistry *24*, 85-89.

Howells, L. M., Moiseeva, E. P., Neal, C. P., Foreman, B. E., Andreadi, C. K., Sun, Y. Y., Hudson, E. A., and Manson, M. M. (2007). Predicting the physiological relevance of in vitro cancer preventive activities of phytochemicals. Acta pharmacologica Sinica *28*, 1274-1304.

Hu, Z., Fan, C., Oh, D. S., Marron, J. S., He, X., Qaqish, B. F., Livasy, C., Carey, L. A., Reynolds, E., Dressler, L., *et al.* (2006). The molecular portraits of breast tumors are conserved across microarray platforms. BMC genomics *7*, 96.

Huang, E., Cheng, S. H., Dressman, H., Pittman, J., Tsou, M. H., Horng, C. F., Bild, A., Iversen, E. S., Liao, M., Chen, C. M., *et al.* (2003). Gene expression predictors of breast cancer outcomes. Lancet *361*, 1590-1596.

Hunter, D. J., Kraft, P., Jacobs, K. B., Cox, D. G., Yeager, M., Hankinson, S. E., Wacholder, S., Wang, Z., Welch, R., Hutchinson, A., *et al.* (2007). A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer. Nature genetics *39*, 870-874.

Ikeda, K., and Inoue, S. (2004). Estrogen receptors and their downstream targets in cancer. Archives of histology and cytology *67*, 435-442.

Imren, S., Kohn, D. B., Shimada, H., Blavier, L., and DeClerck, Y. A. (1996). Overexpression of tissue inhibitor of metalloproteinases-2 retroviral-mediated gene transfer in vivo inhibits tumor growth and invasion. Cancer research *56*, 2891-2895.

Inoue, M., Robien, K., Wang, R., Van Den Berg, D. J., Koh, W. P., and Yu, M. C. (2008). Green tea intake, MTHFR/TYMS genotype and breast cancer risk: the Singapore Chinese Health Study. Carcinogenesis *29*, 1967-1972.

Isemura, M., and Timmermann, B. N. (2013). Plant polyphenols and health benefitts. Current pharmaceutical design *19*, 6051-6052.

Iwasaki, M., Inoue, M., Sasazuki, S., Sawada, N., Yamaji, T., Shimazu, T., Willett, W. C., and Tsugane, S. (2010). Green tea drinking and subsequent risk of breast cancer in a population-based cohort of Japanese women. Breast cancer research : BCR *12*, R88.

Jakacka, M., Ito, M., Weiss, J., Chien, P. Y., Gehm, B. D., and Jameson, J. L. (2001). Estrogen receptor binding to DNA is not required for its activity through the nonclassical AP1 pathway. The Journal of biological chemistry *276*, 13615-13621.

Jang, E. R., Lim, S. J., Lee, E. S., Jeong, G., Kim, T. Y., Bang, Y. J., and Lee, J. S. (2004). The histone deacetylase inhibitor trichostatin A sensitizes estrogen receptor alpha-negative breast cancer cells to tamoxifen. Oncogene *23*, 1724-1736.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., and Thun, M. J. (2009). Cancer statistics, 2009. CA: a cancer journal for clinicians *59*, 225-249.

Jenuwein, T., and Allis, C. D. (2001). Translating the histone code. Science *293*, 1074-1080.

Jenuwein, T., Laible, G., Dorn, R., and Reuter, G. (1998). SET domain proteins modulate chromatin domains in eu- and heterochromatin. Cellular and molecular life sciences : CMLS *54*, 80-93.

Jones, P. A., and Baylin, S. B. (2002). The fundamental role of epigenetic events in cancer. Nature reviews Genetics *3*, 415-428.

Jones, P. A., and Takai, D. (2001). The role of DNA methylation in mammalian epigenetics. Science (New York, NY) *293*, 1068-1070.

Jones, P. L., Jan Veenstra, G. C., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J., and Wolffe, A. P. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nature genetics *19*, 187-191.

Jordan, V. C., and Brodie, A. M. (2007). Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids *72*, 7-25.

Jovanovic, J., Ronneberg, J. A., Tost, J., and Kristensen, V. (2010). The epigenetics of breast cancer. Molecular oncology *4*, 242-254.

Kamangar, F., Dores, G. M., and Anderson, W. F. (2006). Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *24*, 2137-2150.

Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E., and Sasaki, H. (2004). Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature *429*, 900-903.

Katiyar, S., and Mukhtar, H. (1996). Tea in chemoprevention of cancer. International journal of oncology *8*, 221-238.

Katiyar, S. K., Agarwal, R., and Mukhtar, H. (1992). Green tea in chemoprevention of cancer. Comprehensive therapy *18*, 3-8.

Kato, K., Long, N. K., Makita, H., Toida, M., Yamashita, T., Hatakeyama, D., Hara, A., Mori, H., and Shibata, T. (2008). Effects of green tea polyphenol on methylation status of

RECK gene and cancer cell invasion in oral squamous cell carcinoma cells. British journal of cancer *99*, 647-654.

Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., *et al.* (1995). Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science (New York, NY) *270*, 1491-1494.

Kavanagh, K. T., Hafer, L. J., Kim, D. W., Mann, K. K., Sherr, D. H., Rogers, A. E., and Sonenshein, G. E. (2001). Green tea extracts decrease carcinogen-induced mammary tumor burden in rats and rate of breast cancer cell proliferation in culture. Journal of cellular biochemistry *82*, 387-398.

Khan, S. I., Aumsuwan, P., Khan, I. A., Walker, L. A., and Dasmahapatra, A. K. (2012). Epigenetic events associated with breast cancer and their prevention by dietary components targeting the epigenome. Chemical research in toxicology *25*, 61-73.

Khoi, P. N., Park, J. S., Kim, J. H., Xia, Y., Kim, N. H., Kim, K. K., and Jung, Y. D. (2013). (-)-Epigallocatechin-3-gallate blocks nicotine-induced matrix metalloproteinase-9 expression and invasiveness via suppression of NF-kappaB and AP-1 in endothelial cells. International journal of oncology *43*, 868-876.

Kim, H., Kang, K., and Kim, J. (2009). AEBP2 as a potential targeting protein for Polycomb Repression Complex PRC2. Nucleic acids research *37*, 2940-2950.

King, M. C., Wieand, S., Hale, K., Lee, M., Walsh, T., Owens, K., Tait, J., Ford, L., Dunn, B. K., Costantino, J., *et al.* (2001). Tamoxifen and breast cancer incidence among women with inherited mutations in BRCA1 and BRCA2: National Surgical Adjuvant Breast and Bowel Project (NSABP-P1) Breast Cancer Prevention Trial. JAMA : the journal of the American Medical Association *286*, 2251-2256.

Kittaneh, M., Montero, A. J., and Gluck, S. (2013). Molecular Profiling for Breast Cancer: A Comprehensive Review. Biomarkers in cancer *5*, 61-70.

Kleer, C. G., Cao, Q., Varambally, S., Shen, R., Ota, I., Tomlins, S. A., Ghosh, D., Sewalt, R. G., Otte, A. P., Hayes, D. F., *et al.* (2003). EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proceedings of the National Academy of Sciences of the United States of America *100*, 11606-11611.

Kleiner, D. E., and Stetler-Stevenson, W. G. (1994). Quantitative zymography: detection of picogram quantities of gelatinases. Analytical biochemistry *218*, 325-329.

Kleiner, D. E., and Stetler-Stevenson, W. G. (1999). Matrix metalloproteinases and metastasis. Cancer chemotherapy and pharmacology *43 Suppl*, S42-51.

Klinge, C. M. (2001). Estrogen receptor interaction with estrogen response elements. Nucleic acids research *29*, 2905-2919.

Klose, R. J., and Bird, A. P. (2006). Genomic DNA methylation: the mark and its mediators. Trends in biochemical sciences *31*, 89-97.

Ko, S., Ahn, J., Song, C. S., Kim, S., Knapczyk-Stwora, K., and Chatterjee, B. (2011). Lysine Methylation and Functional Modulation of Androgen Receptor by Set9 Methyltransferase. Molecular Endocrinology *25*, 433-444.

Kohlmaier, A., Savarese, F., Lachner, M., Martens, J., Jenuwein, T., and Wutz, A. (2004). A chromosomal memory triggered by Xist regulates histone methylation in X inactivation. PLoS biology *2*, E171.

Kondo, Y., Shen, L., Cheng, A. S., Ahmed, S., Boumber, Y., Charo, C., Yamochi, T., Urano, T., Furukawa, K., Kwabi-Addo, B., *et al.* (2008). Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. Nature genetics *40*, 741-750.

Krusche, C. A., Wulfing, P., Kersting, C., Vloet, A., Bocker, W., Kiesel, L., Beier, H. M., and Alfer, J. (2005). Histone deacetylase-1 and -3 protein expression in human breast cancer: a tissue microarray analysis. Breast cancer research and treatment *90*, 15-23.

Ku, M., Koche, R. P., Rheinbay, E., Mendenhall, E. M., Endoh, M., Mikkelsen, T. S., Presser, A., Nusbaum, C., Xie, X., Chi, A. S., *et al.* (2008). Genomewide analysis of PRC1 and PRC2 occupancy identifies two classes of bivalent domains. PLoS genetics *4*, e1000242.

Kumamoto, M., Sonda, T., Nagayama, K., and Tabata, M. (2001). Effects of pH and metal ions on antioxidative activities of catechins. Bioscience, biotechnology, and biochemistry *65*, 126-132.

Kuruto-Niwa, R., Inoue, S., Ogawa, S., Muramatsu, M., and Nozawa, R. (2000). Effects of tea catechins on the ERE-regulated estrogenic activity. Journal of agricultural and food chemistry *48*, 6355-6361.

Kushima, Y., Iida, K., Nagaoka, Y., Kawaratani, Y., Shirahama, T., Sakaguchi, M., Baba, K., Hara, Y., and Uesato, S. (2009). Inhibitory effect of (-)-epigallocatechin and (-)-epigallocatechin gallate against heregulin beta1-induced migration/invasion of the MCF-7 breast carcinoma cell line. Biological & pharmaceutical bulletin *32*, 899-904.

Kushner, P. J., Agard, D. A., Greene, G. L., Scanlan, T. S., Shiau, A. K., Uht, R. M., and Webb, P. (2000). Estrogen receptor pathways to AP-1. The Journal of steroid biochemistry and molecular biology *74*, 311-317.

Lakhani, S. R., Van De Vijver, M. J., Jacquemier, J., Anderson, T. J., Osin, P. P., McGuffog, L., and Easton, D. F. (2002). The pathology of familial breast cancer: predictive value of immunohistochemical markers estrogen receptor, progesterone receptor, HER-2, and p53 in patients with mutations in BRCA1 and BRCA2. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *20*, 2310-2318.

Lambert, J. D., and Elias, R. J. (2010). The antioxidant and pro-oxidant activities of green tea polyphenols: a role in cancer prevention. Archives of biochemistry and biophysics *501*, 65-72.

Lapidus, R. G., Nass, S. J., Butash, K. A., Parl, F. F., Weitzman, S. A., Graff, J. G., Herman, J. G., and Davidson, N. E. (1998). Mapping of ER gene CpG island methylation-specific polymerase chain reaction. Cancer research *58*, 2515-2519.

Lau, P. N. I., and Cheung, P. (2011). Histone code pathway involving H3 S28 phosphorylation and K27 acetylation activates transcription and antagonizes polycomb silencing. Proceedings of the National Academy of Sciences *108*, 2801-2806.

Lauer-Fields, J. L., Whitehead, J. K., Li, S., Hammer, R. P., Brew, K., and Fields, G. B. (2008). Selective modulation of matrix metalloproteinase 9 (MMP-9) functions via exosite inhibition. The Journal of biological chemistry *283*, 20087-20095.

Lechner, M., Boshoff, C., and Beck, S. (2010). Cancer epigenome. Advances in genetics *70*, 247-276.

Lee, S. T., Li, Z., Wu, Z., Aau, M., Guan, P., Karuturi, R. K., Liou, Y. C., and Yu, Q. (2011). Context-specific regulation of NF-kappaB target gene expression by EZH2 in breast cancers. Molecular cell *43*, 798-810.

Levine, S. S., Weiss, A., Erdjument-Bromage, H., Shao, Z., Tempst, P., and Kingston, R. E. (2002). The core of the polycomb repressive complex is compositionally and functionally conserved in flies and humans. Molecular and cellular biology *22*, 6070-6078.

Lewis, E. B. (1978). A gene complex controlling segmentation in Drosophila. Nature *276*, 565-570.

Li, Y., Yuan, Y. Y., Meeran, S. M., and Tollefsbol, T. O. (2010). Synergistic epigenetic reactivation of estrogen receptor-alpha (ERalpha) by combined green tea polyphenol and histone deacetylase inhibitor in ERalpha-negative breast cancer cells. Molecular cancer *9*, 274.

Liang, Y. C., Lin-Shiau, S. Y., Chen, C. F., and Lin, J. K. (1999). Inhibition of cyclin-dependent kinases 2 and 4 activities as well as induction of Cdk inhibitors p21 and p27 during growth arrest of human breast carcinoma cells by (-)-epigallocatechin-3-gallate. Journal of cellular biochemistry *75*, 1-12.

Liao, S., Umekita, Y., Guo, J., Kokontis, J. M., and Hiipakka, R. A. (1995). Growth inhibition and regression of human prostate and breast tumors in athymic mice by tea epigallocatechin gallate. Cancer letters *96*, 239-243.

Liotta, L. A., and Stetler-Stevenson, W. G. (1990). Metalloproteinases and cancer invasion. Seminars in cancer biology *1*, 99-106.

Lui, E. L., Loo, W. T., Zhu, L., Cheung, M. N., and Chow, L. W. (2005). DNA hypermethylation of TIMP3 gene in invasive breast ductal carcinoma. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie *59 Suppl 2*, S363-365.

Maenner, S., Blaud, M., Fouillen, L., Savoye, A., Marchand, V., Dubois, A., Sanglier-Cianferani, S., Van Dorsselaer, A., Clerc, P., Avner, P., *et al.* (2010). 2-D structure of the A region of Xist RNA and its implication for PRC2 association. PLoS biology *8*, e1000276.

Margueron, R., Li, G., Sarma, K., Blais, A., Zavadil, J., Woodcock, C. L., Dynlacht, B. D., and Reinberg, D. (2008). Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. Molecular cell *32*, 503-518.

Margueron, R., and Reinberg, D. (2011). The Polycomb complex PRC2 and its mark in life. Nature *469*, 343-349.

Marino, M., Acconcia, F., Bresciani, F., Weisz, A., and Trentalance, A. (2002). Distinct nongenomic signal transduction pathways controlled by 17beta-estradiol regulate DNA synthesis and cyclin D(1) gene transcription in HepG2 cells. Molecular biology of the cell *13*, 3720-3729.

Marquez, D. C., and Pietras, R. J. (2001). Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. Oncogene *20*, 5420-5430.

Martignone, S., Menard, S., Bufalino, R., Cascinelli, N., Pellegrini, R., Tagliabue, E., Andreola, S., Rilke, F., and Colnaghi, M. I. (1993). Prognostic significance of the 67-kilodalton laminin receptor expression in human breast carcinomas. Journal of the National Cancer Institute *85*, 398-402.

Massarweh, S., and Schiff, R. (2007). Unraveling the mechanisms of endocrine resistance in breast cancer: new therapeutic opportunities. Clinical cancer research : an official journal of the American Association for Cancer Research *13*, 1950-1954.

Masuda, M., Suzui, M., Lim, J. T., Deguchi, A., Soh, J. W., and Weinstein, I. B. (2002). Epigallocatechin-3-gallate decreases VEGF production in head and neck and breast

carcinoma cells by inhibiting EGFR-related pathways of signal transduction. Journal of experimental therapeutics & oncology *2*, 350-359.

Masuda, M., Suzui, M., Lim, J. T., and Weinstein, I. B. (2003). Epigallocatechin-3-gallate inhibits activation of HER-2/neu and downstream signaling pathways in human head and neck and breast carcinoma cells. Clinical cancer research : an official journal of the American Association for Cancer Research *9*, 3486-3491.

McDonnell, D. P., Clemm, D. L., Hermann, T., Goldman, M. E., and Pike, J. W. (1995). Analysis of estrogen receptor function in vitro reveals three distinct classes of antiestrogens. Molecular endocrinology (Baltimore, Md) *9*, 659-669.

McGarvey, K. M., Greene, E., Fahrner, J. A., Jenuwein, T., and Baylin, S. B. (2007). DNA methylation and complete transcriptional silencing of cancer genes persist after depletion of EZH2. Cancer research *67*, 5097-5102.

McKay, D. L., and Blumberg, J. B. (2002). The role of tea in human health: an update. Journal of the American College of Nutrition *21*, 1-13.

Mikhailova, M., Xu, X., Robichaud, T. K., Pal, S., Fields, G. B., and Steffensen, B. (2012). Identification of collagen binding domain residues that govern catalytic activities of matrix metalloproteinase-2 (MMP-2). Matrix biology : journal of the International Society for Matrix Biology *31*, 380-388.

Mohan, C. M., Deb, G., and Limaye, A. M., (2014). Epigallocatechin gallate induces the steady state mRNA levels of pS2 and PR genes in MCF-7 breast cancer cells. Indian Journal of Experimental Biology *52,* 312-316.

Morgunova, E., Tuuttila, A., Bergmann, U., and Tryggvason, K. (2002). Structural insight into the complex formation of latent matrix metalloproteinase 2 with tissue inhibitor of metalloproteinase 2. Proceedings of the National Academy of Sciences of the United States of America *99*, 7414-7419.

Morin, R. D., Johnson, N. A., Severson, T. M., Mungall, A. J., An, J., Goya, R., Paul, J. E., Boyle, M., Woolcock, B. W., Kuchenbauer, F., *et al.* (2010). Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. Nature genetics *42*, 181-185.

Mueller, S. O., Clark, J. A., Myers, P. H., and Korach, K. S. (2002). Mammary gland development in adult mice requires epithelial and stromal estrogen receptor alpha. Endocrinology *143*, 2357-2365.

Mukhtar, H., and Ahmad, N. (1999). Green tea in chemoprevention of cancer. Toxicological sciences : an official journal of the Society of Toxicology *52*, 111-117.

Mukhtar, H., Wang, Z. Y., Katiyar, S. K., and Agarwal, R. (1992). Tea components: antimutagenic and anticarcinogenic effects. Preventive medicine *21*, 351-360.

Muller, B. M., Jana, L., Kasajima, A., Lehmann, A., Prinzler, J., Budczies, J., Winzer, K. J., Dietel, M., Weichert, W., and Denkert, C. (2013). Differential expression of histone deacetylases HDAC1, 2 and 3 in human breast cancer--overexpression of HDAC2 and HDAC3 is associated with clinicopathological indicators of disease progression. BMC cancer *13*, 215.

Munaut, C., Noel, A., Hougrand, O., Foidart, J. M., Boniver, J., and Deprez, M. (2003). Vascular endothelial growth factor expression correlates with matrix metalloproteinases MT1-MMP, MMP-2 and MMP-9 in human glioblastomas. International journal of cancer Journal international du cancer *106*, 848-855.

Murphy, G., Nguyen, Q., Cockett, M. I., Atkinson, S. J., Allan, J. A., Knight, C. G., Willenbrock, F., and Docherty, A. J. (1994). Assessment of the role of the fibronectin-like domain of gelatinase A by analysis of a deletion mutant. The Journal of biological chemistry *269*, 6632-6636.

Mylona, E., Magkou, C., Giannopoulou, I., Agrogiannis, G., Markaki, S., Keramopoulos, A., and Nakopoulou, L. (2006). Expression of tissue inhibitor of matrix metalloproteinases (TIMP)-3 protein in invasive breast carcinoma: relation to tumor phenotype and clinical outcome. Breast cancer research : BCR *8*, R57.

Nagano, J., Kono, S., Preston, D. L., and Mabuchi, K. (2001). A prospective study of green tea consumption and cancer incidence, Hiroshima and Nagasaki (Japan). Cancer causes & control : CCC *12*, 501-508.

Nagase, H., and Woessner, J. F., Jr. (1999). Matrix metalloproteinases. The Journal of biological chemistry *274*, 21491-21494.

Nan, X., Ng, H.-H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature *393*, 386-389.

Nekrasov, M., Klymenko, T., Fraterman, S., Papp, B., Oktaba, K., Kocher, T., Cohen, A., Stunnenberg, H. G., Wilm, M., and Muller, J. (2007). Pcl-PRC2 is needed to generate high levels of H3-K27 trimethylation at Polycomb target genes. The EMBO journal *26*, 4078-4088.

Nikoloski, G., Langemeijer, S. M. C., Kuiper, R. P., Knops, R., Massop, M., Tonnissen, E. R. L. T. M., van der Heijden, A., Scheele, T. N., Vandenberghe, P., de Witte, T., *et al.* (2010). Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. Nature genetics *42*, 665-667.

O'Meara, M. M., and Simon, J. A. (2012). Inner workings and regulatory inputs that control Polycomb repressive complex 2. Chromosoma *121*, 221-234.

Ogunleye, A. A., Xue, F., and Michels, K. B. (2010). Green tea consumption and breast cancer risk or recurrence: a meta-analysis. Breast cancer research and treatment *119*, 477-484.

Ohm, J. E., McGarvey, K. M., Yu, X., Cheng, L., Schuebel, K. E., Cope, L., Mohammad, H. P., Chen, W., Daniel, V. C., Yu, W., *et al.* (2007). A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nature genetics *39*, 237-242.

Osborne, C. K., Schiff, R., Fuqua, S. A., and Shou, J. (2001). Estrogen receptor: current understanding of its activation and modulation. Clinical cancer research : an official journal of the American Association for Cancer Research *7*, 4338s-4342s; discussion 4411s-4412s.

Overall, C. M. (2002). Molecular determinants of metalloproteinase substrate specificity: matrix metalloproteinase substrate binding domains, modules, and exosites. Molecular biotechnology *22*, 51-86.

Overall, C. M., Wrana, J. L., and Sodek, J. (1991). Transcriptional and post-transcriptional regulation of 72-kDa gelatinase/type IV collagenase by transforming growth factor-beta 1 in human fibroblasts. Comparisons with collagenase and tissue inhibitor of matrix metalloproteinase gene expression. The Journal of biological chemistry *266*, 14064-14071.

Pandey, M., Shukla, S., and Gupta, S. (2010). Promoter demethylation and chromatin remodeling by green tea polyphenols leads to re-expression of GSTP1 in human prostate cancer cells. International journal of cancer Journal international du cancer *126*, 2520-2533.

Pandey, R. R., Mondal, T., Mohammad, F., Enroth, S., Redrup, L., Komorowski, J., Nagano, T., Mancini-Dinardo, D., and Kanduri, C. (2008). Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. Molecular cell *32*, 232-246.

Park, J. W., Hong, J. S., Lee, K. S., Kim, H. Y., Lee, J. J., and Lee, S. R. (2010). Green tea polyphenol (-)-epigallocatechin gallate reduces matrix metalloproteinase-9 activity following transient focal cerebral ischemia. The Journal of nutritional biochemistry *21*, 1038-1044.

Pasini, D., Malatesta, M., Jung, H. R., Walfridsson, J., Willer, A., Olsson, L., Skotte, J., Wutz, A., Porse, B., Jensen, O. N., and Helin, K. (2010). Characterization of an antagonistic switch between histone H3 lysine 27 methylation and acetylation in the transcriptional regulation of Polycomb group target genes. Nucleic acids research *38*, 4958-4969.

Paz, M. F., Fraga, M. F., Avila, S., Guo, M., Pollan, M., Herman, J. G., and Esteller, M. (2003). A systematic profile of DNA methylation in human cancer cell lines. Cancer research *63*, 1114-1121.

Pei, D., and Weiss, S. J. (1995). Furin-dependent intracellular activation of the human stromelysin-3 zymogen. Nature *375*, 244-247.

Peng, J., Sengupta, S., and Jordan, V. C. (2009). Potential of selective estrogen receptor modulators as treatments and preventives of breast cancer. Anti-cancer agents in medicinal chemistry *9*, 481-499.

Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., *et al.* (2000). Molecular portraits of human breast tumours. Nature *406*, 747-752.

Pianetti, S., Guo, S., Kavanagh, K. T., and Sonenshein, G. E. (2002). Green tea polyphenol epigallocatechin-3 gallate inhibits Her-2/neu signaling, proliferation, and transformed phenotype of breast cancer cells. Cancer research *62*, 652-655.

Pike, M. C., Spicer, D. V., Dahmoush, L., and Press, M. F. (1993). Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiologic reviews *15*, 17-35.

Place, A. E., Jin Huh, S., and Polyak, K. (2011). The microenvironment in breast cancer progression: biology and implications for treatment. Breast cancer research : BCR *13*, 227.

Plass, C., Pfister, S. M., Lindroth, A. M., Bogatyrova, O., Claus, R., and Lichter, P. (2013). Mutations in regulators of the epigenome and their connections to global chromatin patterns in cancer. Nature reviews Genetics *14*, 765-780.

Plath, K., Fang, J., Mlynarczyk-Evans, S. K., Cao, R., Worringer, K. A., Wang, H., de la Cruz, C. C., Otte, A. P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. Science *300*, 131-135.

Polyak, K. (2007). Breast cancer: origins and evolution. The Journal of clinical investigation *117*, 3155-3163.

Polyak, K., and Metzger Filho, O. (2012). SnapShot: breast cancer. Cancer cell *22*, 562-562.e561.

Preston-Martin, S., Pike, M. C., Ross, R. K., Jones, P. A., and Henderson, B. E. (1990). Increased cell division as a cause of human cancer. Cancer research *50*, 7415-7421.

Qin, J., Wang, Y., Bai, Y., Yang, K., Mao, Q., Lin, Y., Kong, D., Zheng, X., and Xie, L. (2012). Epigallocatechin-3-gallate inhibits bladder cancer cell invasion via suppression of NF-kappaBmediated matrix metalloproteinase-9 expression. Molecular medicine reports *6*, 1040-1044.

Rebbeck, T. R., Kauff, N. D., and Domchek, S. M. (2009). Meta-analysis of risk reduction estimates associated with risk-reducing salpingo-oophorectomy in BRCA1 or BRCA2 mutation carriers. Journal of the National Cancer Institute *101*, 80-87.

Ren, G., Baritaki, S., Marathe, H., Feng, J., Park, S., Beach, S., Bazeley, P. S., Beshir, A. B., Fenteany, G., Mehra, R., *et al.* (2012). Polycomb protein EZH2 regulates tumor invasion via the transcriptional repression of the metastasis suppressor RKIP in breast and prostate cancer. Cancer research *72*, 3091-3104.

Riising, E. M., Boggio, R., Chiocca, S., Helin, K., and Pasini, D. (2008). The polycomb repressive complex 2 is a potential target of SUMO modifications. PloS one *3*, e2704.

Rinn, J. L., Kertesz, M., Wang, J. K., Squazzo, S. L., Xu, X., Brugmann, S. A., Goodnough, L. H., Helms, J. A., Farnham, P. J., Segal, E., and Chang, H. Y. (2007). Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell *129*, 1311-1323.

Roodi, N., Bailey, L. R., Kao, W. Y., Verrier, C. S., Yee, C. J., Dupont, W. D., and Parl, F. F. (1995). Estrogen receptor gene analysis in estrogen receptor-positive and receptor-negative primary breast cancer. Journal of the National Cancer Institute *87*, 446-451.

Roy, A. M., Baliga, M. S., and Katiyar, S. K. (2005). Epigallocatechin-3-gallate induces apoptosis in estrogen receptor-negative human breast carcinoma cells via modulation in protein expression of p53 and Bax and caspase-3 activation. Molecular cancer therapeutics *4*, 81-90.

Rush, M., Appanah, R., Lee, S., Lam, L. L., Goyal, P., and Lorincz, M. C. (2009). Targeting of EZH2 to a defined genomic site is sufficient for recruitment of Dnmt3a but not de novo DNA methylation. Epigenetics *4*, 404-414.

Sak, K., and Everaus, H. (2004). Nongenomic effects of 17beta-estradiol--diversity of membrane binding sites. The Journal of steroid biochemistry and molecular biology *88*, 323-335.

Sartippour, M. R., Heber, D., Ma, J., Lu, Q., Go, V. L., and Nguyen, M. (2001). Green tea and its catechins inhibit breast cancer xenografts. Nutrition and cancer *40*, 149-156.

Sauvageau, M., and Sauvageau, G. (2010). Polycomb group proteins: multi-faceted regulators of somatic stem cells and cancer. Cell stem cell *7*, 299-313.

Scaling, A. L., Prossnitz, E. R., and Hathaway, H. J. (2014). GPER Mediates Estrogen-Induced Signaling and Proliferation in Human Breast Epithelial Cells and Normal and Malignant Breast. Hormones & cancer.

Schlesinger, Y., Straussman, R., Keshet, I., Farkash, S., Hecht, M., Zimmerman, J., Eden, E., Yakhini, Z., Ben-Shushan, E., Reubinoff, B. E., *et al.* (2007). Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nature genetics *39*, 232-236.

Schmitges, Frank W., Prusty, Archana B., Faty, M., Stützer, A., Lingaraju, Gondichatnahalli M., Aiwazian, J., Sack, R., Hess, D., Li, L., Zhou, S., *et al.* (2011). Histone Methylation by PRC2 Is Inhibited by Active Chromatin Marks. Molecular cell *42*, 330-341.

Schoeftner, S., Sengupta, A. K., Kubicek, S., Mechtler, K., Spahn, L., Koseki, H., Jenuwein, T., and Wutz, A. (2006). Recruitment of PRC1 function at the initiation of X inactivation independent of PRC2 and silencing. The EMBO journal *25*, 3110-3122.

Schor, S. L., and Schor, A. M. (2001). Phenotypic and genetic alterations in mammary stroma: implications for tumour progression. Breast cancer research : BCR *3*, 373-379.

Schramm, L. (2013). Going Green: The Role of the Green Tea Component EGCG in Chemoprevention. Journal of carcinogenesis & mutagenesis *4*, 1000142.

Schuettengruber, B., and Cavalli, G. (2009). Recruitment of polycomb group complexes and their role in the dynamic regulation of cell fate choice. Development (Cambridge, England) *136*, 3531-3542.

Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B., and Cavalli, G. (2007). Genome regulation by polycomb and trithorax proteins. Cell *128*, 735-745.

Schwartz, Y. B., Kahn, T. G., Stenberg, P., Ohno, K., Bourgon, R., and Pirrotta, V. (2010). Alternative Epigenetic Chromatin States of Polycomb Target Genes. PLoS genetics *6*, e1000805.

Seely, D., Mills, E. J., Wu, P., Verma, S., and Guyatt, G. H. (2005). The effects of green tea consumption on incidence of breast cancer and recurrence of breast cancer: a systematic review and meta-analysis. Integrative cancer therapies *4*, 144-155.

Sen, T., Dutta, A., and Chatterjee, A. (2010). Epigallocatechin-3-gallate (EGCG) downregulates gelatinase-B (MMP-9) by involvement of FAK/ERK/NFkappaB and AP-1 in the human breast cancer cell line MDA-MB-231. Anti-cancer drugs *21*, 632-644.

Sen, T., Moulik, S., Dutta, A., Choudhury, P. R., Banerji, A., Das, S., Roy, M., and Chatterjee, A. (2009). Multifunctional effect of epigallocatechin-3-gallate (EGCG) in downregulation of gelatinase-A (MMP-2) in human breast cancer cell line MCF-7. Life sciences *84*, 194-204.

Shah, S. P., Roth, A., Goya, R., Oloumi, A., Ha, G., Zhao, Y., Turashvili, G., Ding, J., Tse, K., Haffari, G., *et al.* (2012). The clonal and mutational evolution spectrum of primary triple-negative breast cancers. Nature *486*, 395-399.

Shao, Z., Raible, F., Mollaaghababa, R., Guyon, J., Wu, C., Bender, W., and Kingston, R. (1999). Stabilization of Chromatin Structure by PRC1, a Polycomb Complex. Cell *98*, 37-46.

Shi, B., Liang, J., Yang, X., Wang, Y., Zhao, Y., Wu, H., Sun, L., Zhang, Y., Chen, Y., Li, R., *et al.* (2007). Integration of estrogen and Wnt signaling circuits by the polycomb group protein EZH2 in breast cancer cells. Molecular and cellular biology *27*, 5105-5119.

Shin, Y. J., and Kim, J. H. (2012). The role of EZH2 in the regulation of the activity of matrix metalloproteinases in prostate cancer cells. PloS one *7*, e30393.

Shinojima, T., Yu, Q., Huang, S. K., Li, M., Mizuno, R., Liu, E. T., Hoon, D. S., and Lessard, L. (2012). Heterogeneous epigenetic regulation of TIMP3 in prostate cancer. Epigenetics : official journal of the DNA Methylation Society *7*, 1279-1289.

Simon, J. A., and Lange, C. A. (2008). Roles of the EZH2 histone methyltransferase in cancer epigenetics. Mutation research *647*, 21-29.

Sims, R. J., and Reinberg, D. (2008). Is there a code embedded in proteins that is based on post-translational modifications? Nat Rev Mol Cell Biol *9*, 815-820.

Singer, C. F., Kronsteiner, N., Marton, E., Kubista, M., Cullen, K. J., Hirtenlehner, K., Seifert, M., and Kubista, E. (2002). MMP-2 and MMP-9 expression in breast cancer-derived human fibroblasts is differentially regulated by stromal-epithelial interactions. Breast cancer research and treatment *72*, 69-77.

Sonoo, H., Kurebayashi, J., Iino, Y., Inaji, H., Watanabe, T., Toi, M., Kobayashi, S., Sato, B., and Yoshimoto, M. (1999). Current Status and Controversial Issues concerning Endocrine Therapy for Patients with Recurrent Breast Cancer in Japan. Breast cancer (Tokyo, Japan) *6*, 344-350.

Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., *et al.* (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proceedings of the National Academy of Sciences of the United States of America *98*, 10869-10874.

Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J. S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., *et al.* (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. Proceedings of the National Academy of Sciences of the United States of America *100*, 8418-8423.

Sotgia, F., Rui, H., Bonuccelli, G., Mercier, I., Pestell, R. G., and Lisanti, M. P. (2006). Caveolin-1, mammary stem cells, and estrogen-dependent breast cancers. Cancer research *66*, 10647-10651.

Sparmann, A., and van Lohuizen, M. (2006). Polycomb silencers control cell fate, development and cancer. Nature reviews Cancer *6*, 846-856.

Stacey, S. N., Manolescu, A., Sulem, P., Rafnar, T., Gudmundsson, J., Gudjonsson, S. A., Masson, G., Jakobsdottir, M., Thorlacius, S., Helgason, A., *et al.* (2007). Common variants on chromosomes 2q35 and 16q12 confer susceptibility to estrogen receptor-positive breast cancer. Nature genetics *39*, 865-869.

Stearns, V., Zhou, Q., and Davidson, N. E. (2007). Epigenetic regulation as a new target for breast cancer therapy. Cancer investigation *25*, 659-665.

Steffensen, B., Hakkinen, L., and Larjava, H. (2001). Proteolytic events of wound-healing--coordinated interactions among matrix metalloproteinases (MMPs), integrins, and extracellular matrix molecules. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists *12*, 373-398.

Stephens, P. J., Tarpey, P. S., Davies, H., Van Loo, P., Greenman, C., Wedge, D. C., Nik-Zainal, S., Martin, S., Varela, I., Bignell, G. R., *et al.* (2012). The landscape of cancer genes and mutational processes in breast cancer. Nature *486*, 400-404.

Sternlicht, M. D., and Werb, Z. (2001). How matrix metalloproteinases regulate cell behavior. Annual review of cell and developmental biology *17*, 463-516.

Stetler-Stevenson, W. G. (1990). Type IV collagenases in tumor invasion and metastasis. Cancer metastasis reviews *9*, 289-303.

Stetler-Stevenson, W. G., Liotta, L. A., and Brown, P. D. (1992). Role of type IV collagenases in human breast cancer. Cancer treatment and research *61*, 21-41.

Stewart, M. D., Li, J., and Wong, J. (2005). Relationship between histone H3 lysine 9 methylation, transcription repression, and heterochromatin protein 1 recruitment. Molecular and cellular biology *25*, 2525-2538.

Stoner, M., Saville, B., Wormke, M., Dean, D., Burghardt, R., and Safe, S. (2002). Hypoxia induces proteasome-dependent degradation of estrogen receptor alpha in ZR-75 breast cancer cells. Molecular endocrinology (Baltimore, Md) *16*, 2231-2242.

Strahl-Bolsinger, S., Hecht, A., Luo, K., and Grunstein, M. (1997). SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. Genes & development *11*, 83-93.

Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1995). Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. The Journal of biological chemistry *270*, 5331-5338.

Stuart, E. C., Jarvis, R. M., and Rosengren, R. J. (2010). In vitro mechanism of action for the cytotoxicity elicited by the combination of epigallocatechin gallate and raloxifene in MDA-MB-231 cells. Oncology reports *24*, 779-785.

Sun, M., Paciga, J. E., Feldman, R. I., Yuan, Z., Coppola, D., Lu, Y. Y., Shelley, S. A., Nicosia, S. V., and Cheng, J. Q. (2001). Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor alpha (ERalpha) via interaction between ERalpha and PI3K. Cancer research *61*, 5985-5991.

Suzuki, Y., Miyoshi, N., and Isemura, M. (2012). Health-promoting effects of green tea. Proceedings of the Japan Academy Series B, Physical and biological sciences *88*, 88-101.

Suzuki, Y., Tsubono, Y., Nakaya, N., Suzuki, Y., Koizumi, Y., and Tsuji, I. (2004). Green tea and the risk of breast cancer: pooled analysis of two prospective studies in Japan. British journal of cancer *90*, 1361-1363.

Tachibana, H. (2009). Molecular basis for cancer chemoprevention by green tea polyphenol EGCG. Forum of nutrition *61*, 156-169.

Tachibana, H., Koga, K., Fujimura, Y., and Yamada, K. (2004). A receptor for green tea polyphenol EGCG. Nature structural & molecular biology *11*, 380-381.

Tachibana, M., Ueda, J., Fukuda, M., Takeda, N., Ohta, T., Iwanari, H., Sakihama, T., Kodama, T., Hamakubo, T., and Shinkai, Y. (2005). Histone methyltransferases G9a and GLP form heteromeric complexes and are both crucial for methylation of euchromatin at H3-K9. Genes & development *19*, 815-826.

Taniguchi, H., Jacinto, F. V., Villanueva, A., Fernandez, A. F., Yamamoto, H., Carmona, F. J., Puertas, S., Marquez, V. E., Shinomura, Y., Imai, K., and Esteller, M. (2012). Silencing of Kruppel-like factor 2 by the histone methyltransferase EZH2 in human cancer. Oncogene *31*, 1988-1994.

Tay, W. M., da Silva, G. F., and Ming, L. J. (2013). Metal binding of flavonoids and their distinct inhibition mechanisms toward the oxidation activity of Cu2+-beta-amyloid: not just serving as suicide antioxidants! Inorganic chemistry *52*, 679-690.

Thakur, V. S., Deb, G., Babcook, M. A., and Gupta, S. (2014). Plant phytochemicals as epigenetic modulators: role in cancer chemoprevention. The AAPS journal *16*, 151-163.

Thakur, V. S., Gupta, K., and Gupta, S. (2012). Green tea polyphenols causes cell cycle arrest and apoptosis in prostate cancer cells by suppressing class I histone deacetylases. Carcinogenesis *33*, 377-384.

Thangapazham, R. L., Singh, A. K., Sharma, A., Warren, J., Gaddipati, J. P., and Maheshwari, R. K. (2007). Green tea polyphenols and its constituent epigallocatechin gallate inhibits proliferation of human breast cancer cells in vitro and in vivo. Cancer letters *245*, 232-241.

Tie, F., Banerjee, R., Stratton, C. A., Prasad-Sinha, J., Stepanik, V., Zlobin, A., Diaz, M. O., Scacheri, P. C., and Harte, P. J. (2009). CBP-mediated acetylation of histone H3 lysine 27 antagonizes Drosophila Polycomb silencing. Development *136*, 3131-3141.

Trimarchi, M. P., Mouangsavanh, M., and Huang, T. H. (2011). Cancer epigenetics: a perspective on the role of DNA methylation in acquired endocrine resistance. Chinese journal of cancer *30*, 749-756.

Truax, A. D., Thakkar, M., and Greer, S. F. (2012). Dysregulated recruitment of the histone methyltransferase EZH2 to the class II transactivator (CIITA) promoter IV in breast cancer cells. PloS one *7*, e36013.

Tsai, M. C., Manor, O., Wan, Y., Mosammaparast, N., Wang, J. K., Lan, F., Shi, Y., Segal, E., and Chang, H. Y. (2010). Long noncoding RNA as modular scaffold of histone modification complexes. Science *329*, 689-693.

Tyson, J. J., Baumann, W. T., Chen, C., Verdugo, A., Tavassoly, I., Wang, Y., Weiner, L. M., and Clarke, R. (2011). Dynamic modelling of oestrogen signalling and cell fate in breast cancer cells. Nature reviews Cancer *11*, 523-532.

Umeda, D., Yano, S., Yamada, K., and Tachibana, H. (2008). Green tea polyphenol epigallocatechin-3-gallate signaling pathway through 67-kDa laminin receptor. The Journal of biological chemistry *283*, 3050-3058.

Uria, J. A., Jimenez, M. G., Balbin, M., Freije, J. M., and Lopez-Otin, C. (1998). Differential effects of transforming growth factor-beta on the expression of collagenase-1 and collagenase-3 in human fibroblasts. The Journal of biological chemistry *273*, 9769-9777.

Van Wart, H. E., and Birkedal-Hansen, H. (1990). The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proceedings of the National Academy of Sciences of the United States of America *87*, 5578-5582.

Varambally, S., Dhanasekaran, S. M., Zhou, M., Barrette, T. R., Kumar-Sinha, C., Sanda, M. G., Ghosh, D., Pienta, K. J., Sewalt, R. G., Otte, A. P., *et al.* (2002). The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature *419*, 624-629.

Vincenti, M. P. (2001). The matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) genes. Transcriptional and posttranscriptional regulation, signal transduction and cell-type-specific expression. Methods in molecular biology (Clifton, NJ) *151*, 121-148.

Vire, E., Brenner, C., Deplus, R., Blanchon, L., Fraga, M., Didelot, C., Morey, L., Van Eynde, A., Bernard, D., Vanderwinden, J. M., *et al.* (2006). The Polycomb group protein EZH2 directly controls DNA methylation. Nature *439*, 871-874.

Visse, R., and Nagase, H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circulation research *92*, 827-839.

Walker, E., Chang, W. Y., Hunkapiller, J., Cagney, G., Garcha, K., Torchia, J., Krogan, N. J., Reiter, J. F., and Stanford, W. L. (2010). Polycomb-like 2 associates with PRC2 and

regulates transcriptional networks during mouse embryonic stem cell self-renewal and differentiation. Cell stem cell *6*, 153-166.

Wang, L., Brown, J. L., Cao, R., Zhang, Y., Kassis, J. A., and Jones, R. S. (2004). Hierarchical recruitment of polycomb group silencing complexes. Molecular cell *14*, 637-646.

Weatherman, R. V., and Scanlan, T. S. (2001). Unique protein determinants of the subtype-selective ligand responses of the estrogen receptors (ERalpha and ERbeta) at AP-1 sites. The Journal of biological chemistry *276*, 3827-3832.

Weigel, N. L., and Zhang, Y. (1998). Ligand-independent activation of steroid hormone receptors. Journal of molecular medicine (Berlin, Germany) *76*, 469-479.

Weihua, Z., Andersson, S., Cheng, G., Simpson, E. R., Warner, M., and Gustafsson, J.-Å. (2003). Update on estrogen signaling. FEBS Letters *546*, 17-24.

Weis, K. E., Ekena, K., Thomas, J. A., Lazennec, G., and Katzenellenbogen, B. S. (1996). Constitutively active human estrogen receptors containing amino acid substitutions for tyrosine 537 in the receptor protein. Molecular endocrinology (Baltimore, Md) *10*, 1388-1398.

Widschwendter, M., Fiegl, H., Egle, D., Mueller-Holzner, E., Spizzo, G., Marth, C., Weisenberger, D. J., Campan, M., Young, J., Jacobs, I., and Laird, P. W. (2007). Epigenetic stem cell signature in cancer. Nature genetics *39*, 157-158.

Wilkinson, F. H., Park, K., and Atchison, M. L. (2006). Polycomb recruitment to DNA in vivo by the YY1 REPO domain. Proceedings of the National Academy of Sciences of the United States of America *103*, 19296-19301.

Wu, A. H., and Butler, L. M. (2011). Green tea and breast cancer. Molecular nutrition & food research *55*, 921-930.

Wu, A. H., Yu, M. C., Tseng, C. C., Hankin, J., and Pike, M. C. (2003). Green tea and risk of breast cancer in Asian Americans. International journal of cancer Journal international du cancer *106*, 574-579.

Wu, C. D., and Wei, G. X. (2002). Tea as a functional food for oral health. Nutrition (Burbank, Los Angeles County, Calif) *18*, 443-444.

Xi, H., Yu, Y., Fu, Y., Foley, J., Halees, A., and Weng, Z. (2007). Analysis of overrepresented motifs in human core promoters reveals dual regulatory roles of YY1. Genome research *17*, 798-806.

Xu, K., Wu, Z. J., Groner, A. C., He, H. H., Cai, C., Lis, R. T., Wu, X., Stack, E. C., Loda, M., Liu, T., *et al.* (2012). EZH2 oncogenic activity in castration-resistant prostate cancer cells is Polycomb-independent. Science (New York, NY) *338*, 1465-1469.

Xu, X., Wang, Y., Lauer-Fields, J. L., Fields, G. B., and Steffensen, B. (2004). Contributions of the MMP-2 collagen binding domain to gelatin cleavage. Substrate binding via the collagen binding domain is required for hydrolysis of gelatin but not short peptides. Matrix biology : journal of the International Society for Matrix Biology *23*, 171-181.

Yager, J. D., and Davidson, N. E. (2006). Estrogen carcinogenesis in breast cancer. The New England journal of medicine *354*, 270-282.

Yang, X., Karuturi, R. K., Sun, F., Aau, M., Yu, K., Shao, R., Miller, L. D., Tan, P. B., and Yu, Q. (2009). CDKN1C (p57) is a direct target of EZH2 and suppressed by multiple epigenetic mechanisms in breast cancer cells. PloS one *4*, e5011.

Yap, K. L., Li, S., Munoz-Cabello, A. M., Raguz, S., Zeng, L., Mujtaba, S., Gil, J., Walsh, M. J., and Zhou, M. M. (2010). Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. Molecular cell *38*, 662-674.

Yiannakopoulou, E. (2014). Effect of green tea catechins on breast carcinogenesis: a systematic review of in-vitro and in-vivo experimental studies. European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation (ECP) *23*, 84-89.

Yoo, K. H., and Hennighausen, L. (2012). EZH2 methyltransferase and H3K27 methylation in breast cancer. International journal of biological sciences *8*, 59-65.

Yu, M., Mazor, T., Huang, H., Huang, H. T., Kathrein, K. L., Woo, A. J., Chouinard, C. R., Labadorf, A., Akie, T. E., Moran, T. B., *et al.* (2012). Direct recruitment of polycomb repressive complex 1 to chromatin by core binding transcription factors. Molecular cell *45*, 330-343.

Yuan, J. M., Koh, W. P., Sun, C. L., Lee, H. P., and Yu, M. C. (2005). Green tea intake, ACE gene polymorphism and breast cancer risk among Chinese women in Singapore. Carcinogenesis *26*, 1389-1394.

Yuan, W., Xu, M., Huang, C., Liu, N., Chen, S., and Zhu, B. (2011). H3K36 Methylation Antagonizes PRC2-mediated H3K27 Methylation. Journal of Biological Chemistry *286*, 7983-7989.

Yue, W., Yager, J. D., Wang, J. P., Jupe, E. R., and Santen, R. J. (2013). Estrogen receptor-dependent and independent mechanisms of breast cancer carcinogenesis. Steroids *78*, 161-170.

Zeidler, M., Varambally, S., Cao, Q., Chinnaiyan, A. M., Ferguson, D. O., Merajver, S. D., and Kleer, C. G. (2005). The Polycomb Group Protein EZH2 Impairs DNA Repair in Breast Epithelial Cells. Neoplasia *7*, 1011-1019.

Zhang, L., Zhao, L., Zhao, D., Lin, G., Guo, B., Li, Y., Liang, Z., Zhao, X. J., and Fang, X. (2010). Inhibition of tumor growth and induction of apoptosis in prostate cancer cell lines by overexpression of tissue inhibitor of matrix metalloproteinase-3. Cancer gene therapy *17*, 171-179.

Zhang, M., Holman, C. D., Huang, J. P., and Xie, X. (2007). Green tea and the prevention of breast cancer: a case-control study in Southeast China. Carcinogenesis *28*, 1074-1078.

Zucker, S., Cao, J., and Chen, W. T. (2000). Critical appraisal of the use of matrix metalloproteinase inhibitors in cancer treatment. Oncogene *19*, 6642-6650.

Appendix

LIST OF ABBREVIATIONS

µg	Microgram
µL	Microliter
5-Aza-CdR	5-aza-2'-deoxycytidine
67LR	67kDa Laminin receptor
AF-1	Activation function 1
AF-2	Activation function -2
ANOVA	Analysis of variance
AP-1	Activator protein-1
AR	Androgen receptor
ATCC	American type culture collection
bp	Base pairs
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
COMT	Catechol-O-methyltransferase
CRPC	Castration resistant prostate cancer
CXC	Cysteine rich domain
DBD	DNA binding domain
DCIS	Ductal carcinoma *in situ*
DMEM	Dulbecco's Modified Eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNMT	DNA methyltransferase
DPBS	Dulbecco's phosphate buffered saline
DZNep	3-Dezaneplanocin-A

E2	17β- Estradiol
EC	(-)-Epicatechin
ECG	(-)- Epicatechin-3-gallate
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
EED	Embryonic ectoderm development
EGC	(-) Epigallocatechin
EGCG	Epigallocatechin-3-gallate
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERE	Estrogen response element
EZH1/2	Enhancer of zeste homolog 1/2
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
GAPDH	Glyceraldehyde-3–phosphate dehydrogenase
GPR30	G-protein coupled receptor 30
GTP	Green tea polyphenols
h	Hours
H3K27me3	Histone H3 trimethylated at lysine 27
H3K9/18Ac	Histone H3 acetylated at lysine 9/18
H3K9me2	Histone H3 dimethylated at lysine 9
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HER	Human epidermal receptor
HGF	Hepatocyte growth factor
HMTase	Histone methyltransferase

HSP	Heat shock protein
IARC	International agency for research on cancer
IDC	Infiltrating ductal carcinoma
IGF	Insulin like growth factor
ILC	Invasive lobular carcinoma
IP	Immunoprecipitation
Kb	Kilobases
kDa	Kilo Dalton
LBD	Ligand binding domain
LCIS	Lobular carcinoma *in situ*
M	Molar
MAPK	Mitogen Activated Protein Kinase
mbER	Membrane bound estrogen receptor
MCAM	Methylated CpG island microarrays
mg	Milligram
Min	Minutes
mL	Milliliter
mm	Millimeter
mM	Millimolar
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MSP	Methylation specific PCR
NCCS	National centre for cell sciences
ncRBD	Non-coding RNA binding domain and a DNA binding domain
ng	Nanogram
nM	Nanomolar

PAGE	Polyacrylamide gel electrophoresis
PcG	Polycomb group
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PET	Polyethylene terephthalate
PI	Propidium iodide
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PPO	Polyphenol oxidase
PR	Progesterone receptor
PRC	Polycomb repressive complex
PRE	Polycomb response element
RbAp46/48	Retinoblastoma associated protein 46/48
RFU	Relative fluorescence units
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SERM	Selective estrogen receptor modulator
SET	Su(var)3-9 enhancer of zeste trithorax domain
SHBG	Sex hormone binding globulin
SiRNA	Small interfering RNA
SNP	Single nucleotide polymorphism

SUZ12	Suppressor of zeste 12
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitor of matrix metalloproteinase
TNFα	Tumor necrosis factor α
TSA	Trichostatin A
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

LIST OF FIGURES

LIST OF TABLES

List of Publications and Presentations

Publications

1. **Gauri Deb**, Anup K Singh and Sanjay Gupta (2014)
EZH2: Not EZHY (Easy) to Deal. *Molecular Cancer Research;* in press. (PMID: 24526064)

2. **Gauri Deb**, Vijay S Thakur, Anil M Limaye and Sanjay Gupta (2014)
Epigenetic induction of tissue inhibitor of matrix metalloproteinase-3 by green tea polyphenols in breast cancer cells *Molecular Carcinogenesis.,* in press. (PMID: 24481780)

3. Vijay S Thakur, **Gauri Deb**, Melissa Babcook and Sanjay Gupta (2014)
Plant phytochemicals as epigenetic modulators: Role in cancer chemoprevention. *The AAPS journal* 16, 151-163. (PMID: 24307610)

4. C M Mohan, **Gauri Deb** and Anil M Limaye (2014)
Epigallocatechin -3-gallate induces steady state mRNA levels of PS2 and PR genes in MCF-7 breast cancer cells. *Indian Journal of* Experimental *Biology 52*, 312-116.

5. **Gauri Deb**, Vijay S Thakur and Sanjay Gupta (2013)
Multifaceted role of EZH2 in breast and prostate tumorigenesis: Epigenetics and beyond. *Epigenetics: official journal of the DNA Methylation Society 8*, 464-476. (PMID: 23644490)

Manuscripts under preparation

1. **Gauri Deb**, Shalinee Jha, Sahil Batra, Shankar Prasad Kanaujia and Anil M Limaye.
EGCG inhibits MMP-2 activity *in vitro* and *in silico* via a Zinc chelation independent mechanism.

2. **Gauri Deb** and Anil M Limaye.

EGCG inhibits growth, proliferation, invasion and gelatinolytic activity in breast cancer cells independent of estrogenic stimulation or the estrogen receptor status.

3. **Gauri Deb**, Vijay S Thakur, Anil M Limaye and Sanjay Gupta.
Histone modifications caused by green tea polyphenols lead to epigenetic reactivation of *TIMP-3* in human prostate cancer cells.

4. Vijay S Thakur, **Gauri Deb**, Mike Jackson and Sanjay Gupta.
Green tea polyphenols induce *GSTP1* activation by acetylating p53 in prostate cancer.

Book Chapters

1. **Gauri Deb** and Sanjay Gupta
Natural phytochemicals as epigenetic modulators.
Invited book chapter in "Genomics, proteomics and metabolomics in nutraceuticals & functional foods (2nd Edition)", edited by Debasis Bagchi, Anand Swaroop and Manashi Bagchi. (Under preparation)

Poster presentations

1. **Gauri Deb**, Vijay S Thakur, Anil M Limaye and Sanjay Gupta
"Green tea polyphenol-mediated epigenetic reactivation of TIMP-3 reduces invasiveness and gelatinolytic activity in human breast cancer cells"- presented at the AACR Annual Meeting, held in San Diego, CA, USA (2014).

2. Dixcy Jaba Sheeba JM, Mohan C Manjegowda, Marine Hussain, **Gauri Deb**, Neeraj Kumar and Anil M Limaye
"Estrogen regulation of ECM remodeling and associated genes"- presented at the 33rd Annual Convention of Indian Association for Cancer Research held in Kerala, India (2014).

3. **Gauri Deb**, Sahil Batra and Anil M Limaye

"EGCG inhibits MMP-2 and MMP-9 activity by direct inhibition"- Presented at the 31th Annual Convention of IACR and International symposium on "Cancer genomics and its impact in the clinics" held in Mumbai, India (2012).

4. **Gauri Deb** and Anil M Limaye

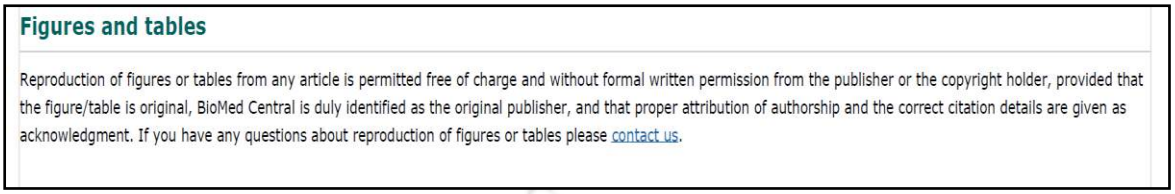
"Effect of EGCG on MCF-7 cell proliferation and gelatinolytic activities"-presented at the 30th Annual Convention of Indian Association for Cancer Research and International symposium on "signaling network and cancer", held in Kolkata, India (2011).

Platform presentations

1. **Gauri Deb**, Vijay S Thakur, Anil M Limaye and Sanjay Gupta
Molecular insights into the epigenetic induction of tissue inhibitor of matrix metalloproteinase-3 (TIMP-3) by green tea polyphenols in breast cancer cells presented at the 33rd Annual Convention of Indian Association for Cancer Research held in Kerala, India (2014).

Copyrights and Permissions

1. Figure 2.1 – Publisher – BioMed Central, Citation provided in the text and bibliography section.

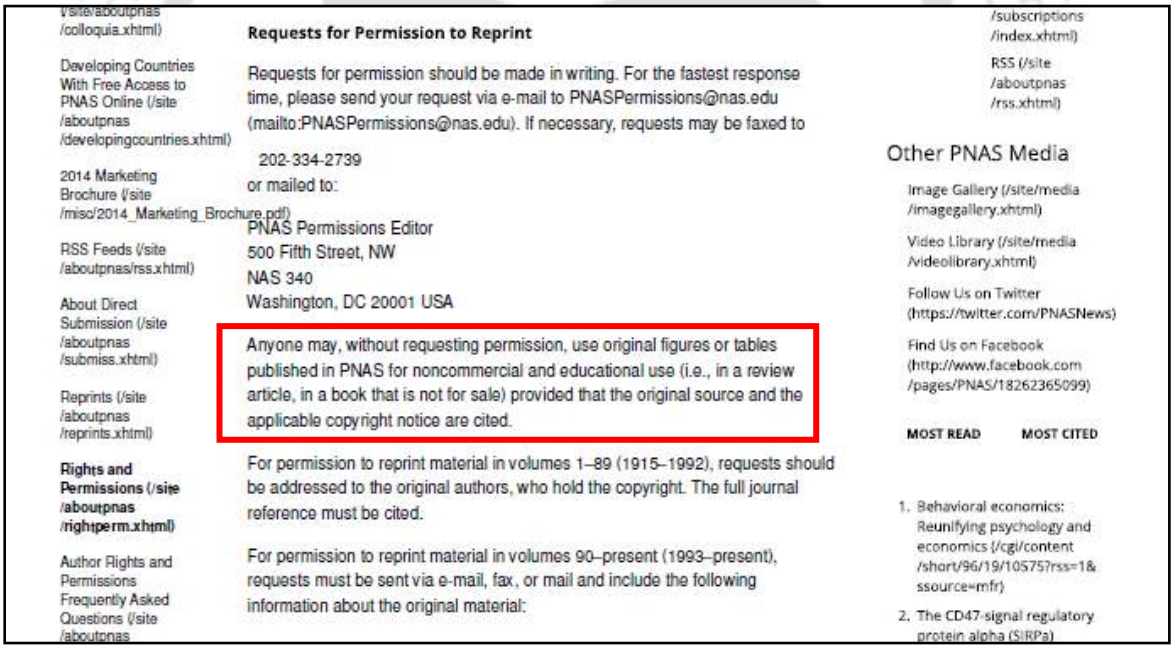


Figures and tables

Reproduction of figures or tables from any article is permitted free of charge and without formal written permission from the publisher or the copyright holder, provided that the figure/table is original, BioMed Central is duly identified as the original publisher, and that proper attribution of authorship and the correct citation details are given as acknowledgment. If you have any questions about reproduction of figures or tables please contact us.

2. **Figure 2.2 and 2.3** - Citation for incidence and mortality data provided in the text and bibliography. Online analysis done at http://globocan.iarc.fr, accessed on 18/3/2014

3. **Figure 2.6 –** Citation given and no permission required for academic use



/site/aboutpnas /colloquia.xhtml)

Developing Countries With Free Access to PNAS Online (/site /aboutpnas /developingcountries.xhtml)

2014 Marketing Brochure (/site /misc/2014_Marketing_Brochure.pdf)

RSS Feeds (/site /aboutpnas/rss.xhtml)

About Direct Submission (/site /aboutpnas /submiss.xhtml)

Reprints (/site /aboutpnas /reprints.xhtml)

Rights and Permissions (/site /aboutpnas /rightsperm.xhtml)

Author Rights and Permissions Frequently Asked Questions (/site /aboutpnas

Requests for Permission to Reprint

Requests for permission should be made in writing. For the fastest response time, please send your request via e-mail to PNASPermissions@nas.edu (mailto:PNASPermissions@nas.edu). If necessary, requests may be faxed to

202-334-2739

or mailed to:

PNAS Permissions Editor
500 Fifth Street, NW
NAS 340
Washington, DC 20001 USA

Anyone may, without requesting permission, use original figures or tables published in PNAS for noncommercial and educational use (i.e., in a review article, in a book that is not for sale) provided that the original source and the applicable copyright notice are cited.

For permission to reprint material in volumes 1–89 (1915–1992), requests should be addressed to the original authors, who hold the copyright. The full journal reference must be cited.

For permission to reprint material in volumes 90–present (1993–present), requests must be sent via e-mail, fax, or mail and include the following information about the original material:

/subscriptions /index.xhtml)

RSS (/site /aboutpnas /rss.xhtml)

Other PNAS Media

Image Gallery (/site/media /imagegallery.xhtml)

Video Library (/site/media /videolibrary.xhtml)

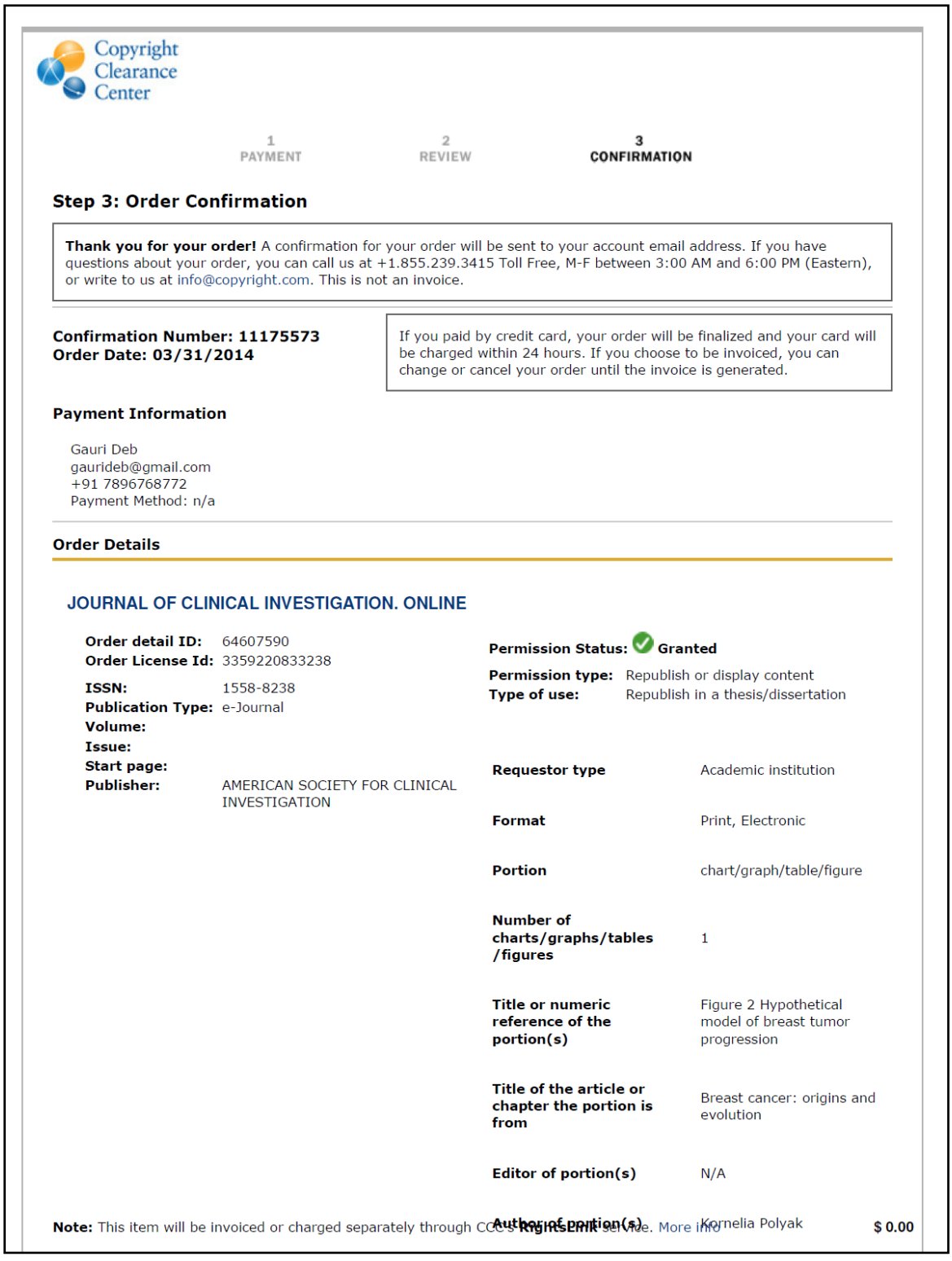
Follow Us on Twitter (https://twitter.com/PNASNews)

Find Us on Facebook (http://www.facebook.com /pages/PNAS/18262365099)

MOST READ **MOST CITED**

1. Behavioral economics: Reunifying psychology and economics (/cgi/content /short/96/19/10575?rss=1&ssource=mfr)
2. The CD47-signal regulatory protein alpha (SIRPa)

4. Figure 2.7 – Copy of permission for reuse in thesis in this thesis.



Copyright Clearance Center

1 PAYMENT 2 REVIEW 3 CONFIRMATION

Step 3: Order Confirmation

Thank you for your order! A confirmation for your order will be sent to your account email address. If you have questions about your order, you can call us at +1.855.239.3415 Toll Free, M-F between 3:00 AM and 6:00 PM (Eastern), or write to us at info@copyright.com. This is not an invoice.

Confirmation Number: 11175573
Order Date: 03/31/2014

If you paid by credit card, your order will be finalized and your card will be charged within 24 hours. If you choose to be invoiced, you can change or cancel your order until the invoice is generated.

Payment Information

Gauri Deb
gaurideb@gmail.com
+91 7896768772
Payment Method: n/a

Order Details

JOURNAL OF CLINICAL INVESTIGATION. ONLINE

Order detail ID:	64607590
Order License Id:	3359220833238
ISSN:	1558-8238
Publication Type:	e-Journal
Volume:	
Issue:	
Start page:	
Publisher:	AMERICAN SOCIETY FOR CLINICAL INVESTIGATION

Permission Status: Granted
Permission type: Republish or display content
Type of use: Republish in a thesis/dissertation

Requestor type	Academic institution
Format	Print, Electronic
Portion	chart/graph/table/figure
Number of charts/graphs/tables /figures	1
Title or numeric reference of the portion(s)	Figure 2 Hypothetical model of breast tumor progression
Title of the article or chapter the portion is from	Breast cancer: origins and evolution
Editor of portion(s)	N/A
Author of portion(s)	Kornelia Polyak

Note: This item will be invoiced or charged separately through CCC's **RightsLink** service. More info **$ 0.00**

5. Table 2.1- Copy of license agreement for reuse in this thesis.

ELSEVIER LICENSE
TERMS AND CONDITIONS

Apr 01, 2014

This is a License Agreement between Gauri Deb ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Gauri Deb
Customer address	Dept of Biotechnology Guwahati, 781039
License number	3360060225580
License date	Apr 01, 2014
Licensed content publisher	Elsevier
Licensed content publication	Cancer Cell
Licensed content title	SnapShot: Breast Cancer
Licensed content author	Kornelia Polyak,Otto Metzger Filho
Licensed content date	16 October 2012
Licensed content volume number	22
Licensed content issue number	4
Number of pages	2
Start Page	562
End Page	562.e1
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No

Title of your thesis/dissertation	Molecular Insights Into The Modulation Of Gelatinases By The Green Tea Polyphenol, (-) Epigallocatechin-3-gallate (EGCG), In Human Breast Cancer Cells
Expected completion date	Apr 2014
Estimated size (number of pages)	160
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.00 USD / 0.00 GBP
Total	0.00 USD

6. Figure 2.11 – Copy of license for reuse in this thesis

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

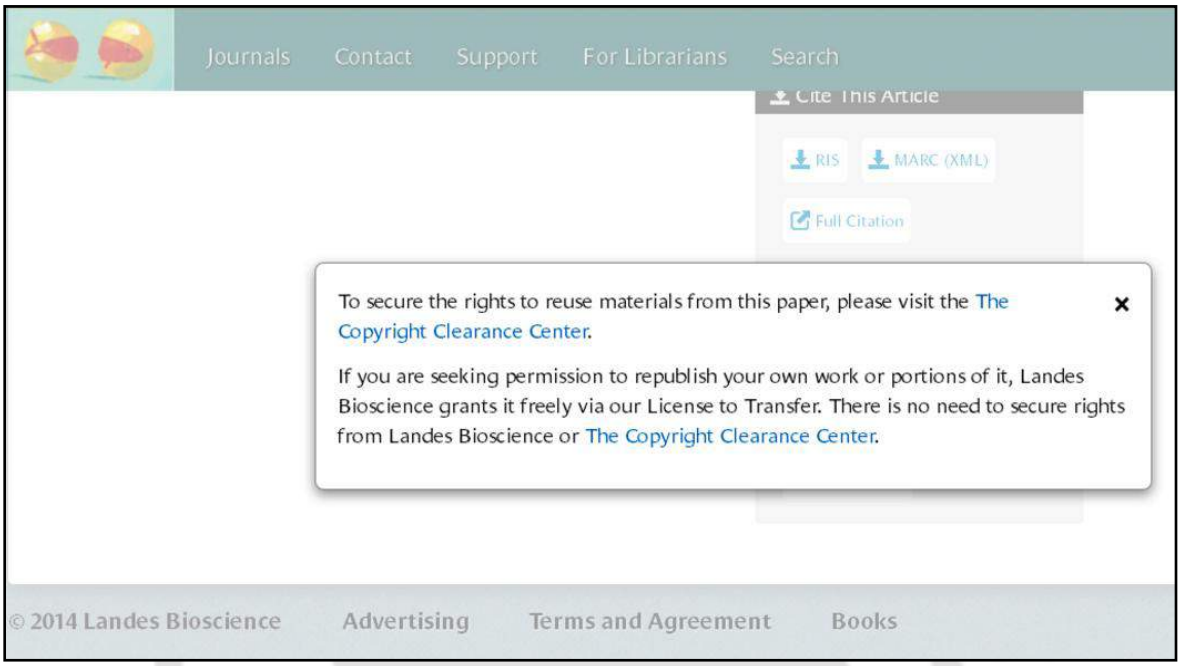
Mar 26, 2014

This is a License Agreement between Gauri Deb ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

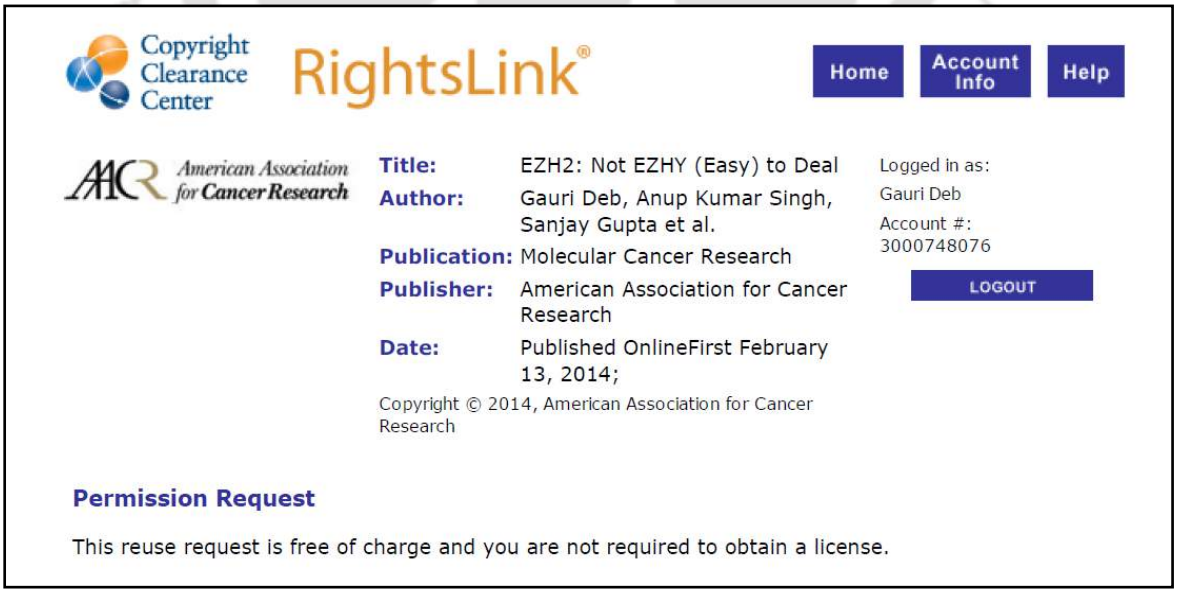
All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3356350724975
License date	Mar 26, 2014
Licensed content publisher	Nature Publishing Group
Licensed content publication	Oncogene
Licensed content title	The green tea catechins, (-)-Epigallocatechin-3-gallate (EGCG) and (-)-Epicatechin-3-gallate (ECG), inhibit HGF//Met signaling in immortalized and tumorigenic breast epithelial cells
Licensed content author	R L H Bigelow and J A Cardelli
Licensed content date	Jan 30, 2006
Volume number	25
Issue number	13
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	1
High-res required	no
Figures	Figure 1 Structure of green tea catechins
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	Molecular Insights Into The Modulation Of Gelatinases By The Green Tea Polyphenol, (-) Epigallocatechin-3-gallate (EGCG), In Human Breast Cancer Cells
Expected completion date	Apr 2014
Estimated size (number of pages)	160
Total	0.00 USD

7. Text (Chapter II), Table 2.2 and figures (2.8, 2.9 and 2.10) – Author of the publication and no permission required for reuse in this thesis.



8. Text (Chapter II) - Author of the publication and no permission required for reuse in this thesis.



9. Chapter VI – Author of the publication and copy of license for reuse of text and figures in this thesis.

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Feb 06, 2014

This is a License Agreement between Gauri Deb ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3323411499636
License date	Feb 06, 2014
Licensed content publisher	John Wiley and Sons
Licensed content publication	Molecular Carcinogenesis
Licensed content title	Epigenetic induction of tissue inhibitor of matrix metalloproteinase-3 by green tea polyphenols in breast cancer cells
Licensed copyright line	© 2014 Wiley Periodicals, Inc.
Licensed content author	Gauri Deb,Vijay S. Thakur,Anil M. Limaye,Sanjay Gupta
Licensed content date	Jan 31, 2014
Start page	n/a
End page	n/a
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	Molecular Insights Into The Modulation Of Gelatinases By The Green Tea Polyphenol, (-) Epigallocatechin-3-gallate (EGCG), In Human Breast Cancer Cells
Expected completion date	Apr 2014
Expected size (number of pages)	160
Total	0.00 USD
Terms and Conditions	