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4-Hydroxy Estradiol-Induced Oxidant-Mediated Signaling Is Involved In The Development Of Breast Cancer

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4-HYDROXY ESTRADIOL-INDUCED OXIDANT-MEDIATED SIGNALING IS

INVOLVED IN THE DEVELOPMENT OF BREAST CANCER

A dissertation submitted in partial fulfillment of the

requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PUBLIC HEALTH

by

Victor Onyishi Okoh

2011

To: Interim Dean Michele Ciccazzo
     College of Arts and Sciences

This dissertation, written by Victor Onyishi Okoh, and entitled 4-Hydroxy Estradiol-Induced Oxidant-Mediated Signaling is Involved in the development of Breast cancer, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Date of Defense: November 12, 2010

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Florida International University, 2011

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DEDICATION

I dedicate this thesis to my family (Stella, Onyenka, Isioma and Ifeoma) for their patience

and sacrifices during the course of this program. I also dedicate this thesis to my late

brother Ikechukwu Okoh for being a class act in his brief time on earth.

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ABSTRACT OF THE DISSERTATION

4-HYDROXY ESTRADIOL-INDUCED OXIDANT-MEDIATED SIGNALING IS

INVOLVED IN THE DEVELOPMENT OF BREAST CANCER

by

Victor Onyishi Okoh

Florida International University, 2011

Miami, Florida

Professor Deodutta Roy, Major Professor

Breast cancer is a disease associated with excess exposures to estrogens. While the mode of cancer causation is unknown, others have shown that oxidative stress induced by prolonged exposure to estrogens mediates renal, liver, endometrial and mammary tumorigenesis though the mechanism(s) underling this process is unknown. In this study, we show that 4-hydroxyl 17β-estradiol (4-OHE2), a catechol metabolite of estrogen, induces mammary tumorigenesis in a redox dependent manner. We found that the mechanism of tumorigenesis involves redox activations of nuclear respiratory factor-1 (NRF1); a transcriptions factor associated with regulation of mitochondria biogenesis and oxidative phosphorylation (OXPHOS), as well as mediation of cell survival and growth of cells during periods of oxidative stress. Key findings from our study are as follows: (i) Prolonged treatments of normal mammary epithelial cells with 4-OHE2, increased the formation of intracellular reactive oxygen species (ROS). (ii) Estrogen-induced ROS activates redox sensitive transcription factors NRF1. (iii) 4-OHE2 through activation of serine-threonine kinase and histone acetyl transferase, phosphorylates and acetylate NRF1 respectively. (iv) Redox mediated epigenetic modifications of NRF1 facilitates

mammary tumorigenesis and invasive phenotypes of breast cancer cells via modulations of genes involved in proliferation, growth and metastasis of exposed cells. (v) Animal engraftment of transformed clones formed invasive tumors. (vi) Treatment of cells or tumors with biological or chemical antioxidants, as well as silencing of NRF1 expressions, prevented 4-OHE2 induced mammary tumorigenesis and invasive phenotypes of MCF-10A cells. Based on these observations, we hypothesize that 4-OHE2 induced ROS epigenetically activate NRF1 through its phosphorylation and acylation. This, in turn, through NRF1-mediated transcriptional activation of the cell cycle genes, controls 4-OHE2 induced cell transformation and tumorigenesis.

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LIST OF ACRONYMS

2-Hydroxy estradiol	2-OHE2
2-Methoxy estradiol	2-MeO-E$_2$
4-Hydroxy estradiol	4-OHE$_2$
4-Methoxy estradiol	4-MeO-E2
Activator protein 1	AP-1
Adenosine-5'-triphosphate	ATP
5-Bromodeoxyuridine	BrdU
Catalase	Cata
Cell division cycle	CDC
Complementary deoxyribonucleic acid	cDNA
Chromatin immunoprecipitation	ChiP
Carbon dioxide	CO2
Acetyl group	COCH3
Catechol o methyl transferase	COMT
Copper	Cu
Copper ion	Cu2+
Cytochrome p450 1beta1	CYP450 1B1
Cyclic adenosine monophosphate response element binding protein	CREB
2', 7', -dichlorofluorescein	DCF
2', 7', -dichloro-dihydrofluorescein	DCFH

2', 7', -dichloro-dihydrofluorescein diacetate	DCFH-DA
Dulbecco's Modified Eagle Medium	DMEM
Dimethyl sulfoxide	DMSO
Deoxyribonucleic acid	DNA
17-β-Estradiol	E2
E2 Transcription Factor	E2F
Ebselen	Ebs
Electron Transport Chain	ETC
Eukaryotic initiation factor 2	eIF2
Estrogen receptor	ER
Estrogen receptor positive	ER^+
Estrogen receptor negative	ER^-
Estrogen response element	ERE
External regulated kinase	ERK
Estrogen receptor knock out	ERKO
Fetal bovine serum	FBS
Growth factor receptor positive	GFR+
Growth factor receptor negative	GFR^-
Hydrogen peroxide	H_2O_2
Hank's Balanced Salt Solution	HBSS
Horse serum	HS

Iron	Fe
Intergrin associated proteins	IAP
Knock down	Kd
Manganese	Mn
Manganese superoxide dismutase	MnSOD
Messenger ribonucleic acid	mtTFA, TFAM
Mitochondrial DNA	mtDNA
N-Acetyl-Cysteine	NAC
Nuclear respiratory factor 1	NRF1
Nuclear factor kappa beta	NFk B
Super oxide	$O_2^{.-}$
Super oxide dismutase	SOD
Optical density	OD
Oxidative phosphorylation	OXPHOS
Over expression	Ox
p300/CBP-associated factor	PCAF
Proliferating Cell Nuclear Antigen	PCNA
Polyacrylamide gel electrophoresis	PAGE
Phosphate buffered saline	PBS
Polymerase chain reaction	PCR
Phosphatase and tensin homolog	PTEN
Quantitative PCR	qPCR

Reduction oxidation	Redox
Ribonucleic acid	RNA
Ribonucleic acid degrading enzyme	RNase
Reactive oxygen species	ROS
Reverse transcription polymerase chain reaction	RT-PCR
Standard deviation	SD
Sodium dodecyl sulfate	SDS
Short hairpin RNA	shRNA
Small interfering RNA	siRNA
Tamoxifen	TAM
tertiarybutyl hydroperoxide	t-BOOH
Tumor necrotic alpha	TNF α
Tris(hydromethyl)methylamine	Tris
Zinc	Zn

CHAPTER I

INTRODUCTION

Estrogens are complete breast carcinogens in experimental models as they are capable of initiating and triggering growth and selection, resulting in palpable malignancy (1-3). Both epidemiological and animal studies indicate that estrogens are a known risk factor for breast cancer (1,3). However, the mechanisms by which estrogen initiate or stimulate the progression of cancer remain the subject of a long-standing controversy, because we have been not able to resolve whether estrogen or their metabolites are procarcinogenic.

We have previously shown that estrogen-induced tumor formation is decreased in animals exposed to inhibitors of estrogen metabolism or to hormonally potent estrogens with decreased metabolic conversion to catechol metabolites compared to E2 (2). Moreover, we have shown that chronic treatment of the hamsters with vitamin C reduced the incidence of E2-induced renal carcinogenesis by ~50% (2). Since vitamin C has no antiestrogenic activity, its strong inhibitory effect on estrogen-induced tumorigenesis may largely be derived from its protective effect against the oxidative damage caused by the estrogens and its metabolites. Most of the recent studies show that hydroxyestrogens and methoxy derivatives represent the majority of either urinary or intratumor estrogens in human breast cancer patients, whereas they are relatively scarce in urine or mammary glands of healthy women (4). These studies showed that 4-OH-E2 is elevated in breast tumors as compared to normal mammary tissues which implicates its role in breast cancer and strongly suggest the involvement of estrogen metabolites in the initiation and/or promotion steps of breast carcinogenesis.

Recent studies indicate that mammary tumors can develop despite a lack of a functional ER α [2]. Although tamoxifen and other antiestrogens are considered to prevent cancer through competition for the ER, other mechanisms cannot be ruled out as these compounds also block metabolism and redox cycling of estrogen, and are free radical scavengers (5). The 4-OH-E2 induces an estrogenic response in the uterus of ER null mice, and this response is not inhibited by ICI182780 (6). These findings suggest that, in addition, to nuclear ER-mediated genomic signaling pathways, non-genomic pathway(s) are involved in the estrogen-dependent growth of preneoplastic and neoplastic cells. We believe that to produce cell transformation both genomic and non-genomic actions of estrogen are required and these two complementary mechanisms through which estrogens produce transformed phenotype of cells. Formation of estrogen-DNA adducts in mammary tissues have been shown by combined LS-MS-MS and LC-nano ES tandem mass spectrometry (7). In addition, 4-hydroxycatechol estrogen conjugates with glutathione or its hydrolytic products (cysteine and N-acetylcysteine) have been detected in mammary tissues from ERKO/Wnt-1 mice (8). Although this type of DNA adduction may play a role in the generation of mutations, it appears to be a late event. In our recent studies, estrogen-induced a rapid increase of intracellular ROS formation in MCF-7 cells (9). Based on our preliminary study showing 4-OH-E2-induced ROS formation in MCF10A cells, the accumulation of 4-OH-E2 in estrogen target organ of the cancer will result in increased formation of ROS in the target cells. In addition, 4-OH-E2 is also strongly active at the estrogen receptor (10,11), which would add to the growth stimulation of the ER-positive cells. Our preliminary data revealed that 4-OH-E2 may also activate an additional signal transduction pathway that is different from the classical

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ER•signaling pathway. The contribution of this signal transduction system to breast carcinogenesis is the subject of the proposed study.

In pursuing mitochondrial regulators of cell cycle progression, we discovered that intracellular ROS levels increase as cells progress through G1 and into S phase upon E2 exposure (11). High ROS levels induce oxidative damage and arrests cells in G0/G1. However, low physiologic ROS levels are utilized by cells for cell-to-cell communication and in proliferation. Inhibition of estrogen-induced ROS by antioxidants prevents early G1 gene expression. We now know that the delicate intracellular interplay between oxidizing and reducing equivalents allows ROS to function as second messengers in the control of cell proliferation and cell transformation (11,12). Our preliminary study showed that MCF-10A cells treated with 4-OH-E2 rapidly produce ROS, which is preceded by an increase in the level of Ca2+. The accumulation of 4-OH-E2 as recently reported in the human breast tissue in cancer subjects (9) will allow increased formation of ROS and Ca2+ in the target organ of cancer in a sensitive subpopulation. Our preliminary study showed that the overexpression of catalase and MnSOD prevented 4-OH-E2-induced anchorage-independent growth of MCF-10A cells. These studies data indicate that 4-OH-E2-induced cell transformation may be mediated, in part, by ROS and Ca2+ signal transduction pathways that are different from the classical nuclear ER genomic signaling pathway. Therefore, how 4-OH-E2, through ROS signalings, produces malignant phenotype is an issue of fundamental importance to this proposal.

Recently, an NRF1 and CREB pair has been identified as a key regulator of the transcriptional program of the cell cycle in human cells because thes pair of transcription factors showed a significant co-occurrence rate on promoters of cell cycle regulated genes (13). NRF1 appears to play an important role as a regulator of cell cycle genes because NRF1 binding site has been identified by comparing genome-wide locations on genes involved in DNA replication, mitosis, and cytokinesis (14,15). Some of the genes which contain NRF1 binding sites on their promoters included Cdc2, Cdc25C, PCNA, and cyclin B1. In addition to NRF1's role in controlling mitochondrial biogenesis-related genes, these observations are consistent with a role for NRF1 in regulating genes involved in growth and development. The expression of early G1 genes, c-myc, c-fos, c-jun, cyclin D1, and NRF1 are regulated by ROS (2,16). The mRNAs of some of these genes are induced by estrogen-induced ROS and hydrogen peroxide as well as other inducers of oxidative stress (2,11). Based on these studies, we have proposed that 4-OH-E2-induced ROS through activating the transcription factor NRF1 leads to the induction of cell cycle genes that contain the binding site(s) for this transcription factor in their promoters. This, in turn, supports the growth of anchorage-dependent and -independent cells.

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CHAPTER II

HYPOTHESIS, SPECIFIC AIMS, AND RELEVANCE OF FINDINGS

Hypothesis

We hypothesize that 4-hydroxy estradiol (4-OH-E2), a catechol metabolite of 17β estradiol (E2)-induced ROS epigenetically activate NRF1 through its phosphorylation and acylation. This, in turn, through NRF1 mediated transcriptional activation of cell cycle genes, controls 4-OH-E2-induced cell transformation and tumorigenesis.

Specific Aim 1: To determine whether estrogen-induced RO/NS are involved in estrogen-induced in vivo growth of malignant breast epithelial cells. To achieve this aim, we would determine whether: a overexpresion of mt superoxide dismutase (SOD) or catalase that detoxify ROS; or co-treatment with the chemical antioxidant scavengers, N-acetylcysteine and ebselen, prevents estrogen-induced cell transformation and in vivo growth of malignant breast epithelial cells. b: Whether Knockdown of mitochondrial biogenesis through silencing of mtTFA will lead to attenuations of RO/NS production and whether reduced ROS production prevents estrogen-induced cell transformation and in vivo growth of malignant breast epithelial cells.

Specific Aims 2: To determine that 4-OH-E2-induced ROS through activation of NRF1 signaling transform normal breast epithelial cells to malignant cells. To accomplish these goals, we will determine whether the transformation of normal breast epithelial cells by 4-OH-E2 is prevented by: a) the overexpression of ROS modifiers catalase and MnSOD,

b) silencing of NRF1. Cell transformation will be assessed by determining colony efficiency, colony size, ductulogenic capacity, and invasiveness. We will also determine whether silencing of NRF1 and overexpression of MnSOD or catalase prevent 4-OH-E2-induced tumor formation in three-dimensional culture mimicking in vivo environment. The findings of this aim will reveal a new paradigm by showing that the carcinogenic activity of 4-OH-E2 requires NRF1.

Specific Aim 3: To determine whether 4-OH-E2 induced ROS through stimulating redox sensitive AKT kinase and histone acetyltransferase of CREB binding protein associated factor (P/CAF), respectively, control phosphorylation and acylation of the NRF1. The 4-OH-E2-induced epigenetic changes in turn activate NRF1. To test the postulate of this aim, we will determine that: i) NRF1 stability and transactivation are regulated by 4-OH-E2-induced ROS. This mechanism involves NRF1 phosphorylation by AKT kinase and NRF1 acylation by the histone acetyltransferase CREB binding protein-associated factor (P/CAF), respectively; and (ii) the 4-OH-E2-induced epigenetic changes have an additive or synergistic effect on NRF1 activation. We will determine whether NRF1 transctivating ability is modulated by controlling its phosphorylation and/or acylation; and (iii) transcriptional activation of cell cycle genes and cell cycle progression are prevented by silencing ofAKT, P/CAF, or NRF1. These observations will reveal the new paradigm of 4-OH-E2-induced cell cycle regulation of anchorage-independent cells by NRF1

Relevance of findings

This study addresses an important public health issue by studying whether phosphorylation and acetylation of NRF1 by 4-OH-E2-generated ROS promote malignant transformation of normal breast epithelial cells. The proposed studies are highly innovative, since they tested the novel concept that oxidants participate in 4-OH-E2-induced cellular transformation and tumorigenesis. Understanding if aberrant activation of NRF1 signaling by 4-OH-E2-generated ROS contributes to development of E2-dependent breast tumors provides new insights into cancer predisposition. Moreover, it provides a novel paradigm for understanding the molecular mechanisms underlying the carcinogenic effects of natural estrogen and other medicinal and environmental estrogenic agents. This is a new line of research that may well set the stage for the design and development of new and more effective NRF1 signaling-based drugs or gene therapies for the prevention and treatment of breast cancer.

CHAPTER III

LITERATURE REVIEW

ESTROGEN-INDUCED REACTIVE OXYGEN SPECIES-MEDIATED SIGNALINGS
CONTRIBUTE TO BREAST CANCER GROWTH

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ABSTRACT

Elevated lifetime estrogen exposure is a major risk factor for breast cancer.

Recent advances in the understanding of breast carcinogenesis clearly indicate that

induction of estrogen receptor (ER) mediated signaling is not sufficient for the

development of breast cancer. The underlying mechanisms of breast susceptibility to

estrogen's carcinogenic effect remain elusive. Physiologically achievable concentration

of estrogen or estrogen metabolites has been shown to generate reactive oxygen species

(ROS). Recent data indicates that these ROS can induce DNA synthesis, increased

phosphorylation of kinases, and activation of transcription factors, e.g., AP-1, NRF1,

E2F, NF_kB and CREB of non genomic pathways which are responsive to both oxidants

and estrogen. Estrogen-induced ROS by increasing genomic instability and by

transducing signal through influencing redox sensitive transcription factors play

important role (s) in cell transformation, cell cycle, migration and invasion of the breast

cancer. The present review discusses emerging data in support of the role of estrogen

induced ROS-mediated signaling pathways which may contribute in the development of

breast cancer. It is envisioned that estrogen induced ROS mediated signaling is a key complementary mechanism that drives the carcinogenesis process. ROS mediated signaling however occurs in the context of other estrogen induced processes such as ER-mediated signaling and estrogen reactive metabolite-associated genotoxicity. Importantly, estrogen-induced ROS can function as independent irreversible modifiers of phosphatases and activate kinases to trigger the transcription factors of downstream target genes which participate in cancer progression.

INTRODUCTION

Estrogen is a class of 18-carbon steroid. There are a total of 9 estrogens in humans of which the three major ones are 17β-Estradiol (E_2), Estrone (E_1), Estriol (E_3). These estrogens are synthesized primarily in the ovaries of premenopausal women. Other organs such as brain and adipose tissues also synthesize estrogens through aromatization of testosterone [1]. Estrogens are also synthesized in male species. A relatively high concentration of estrogen is found in rete testis fluid and in testicular vein and lymph of rodents [2;3]. The level of estrogens in peripheral blood of men averages 69 pg/ml compared to 199 pg/ml in non-ovulating women [4]. E_2 is the predominantly circulating and the most biologically active ovarian steroid. In addition to endogenous sources, the body burden of estrogen may increase from the intake of synthetic medicinal estrogens, such as 17α-ethinyl estradiol (used as oral contraceptive), mestranol, premarin (used in hormone replacement therapy), and diethylstilbestrol (used in the treatment of highly advanced breast and prostate cancers). Physiological estrogens are essential for the growth and maintenance of various reproductive and non-reproductive organs in the body

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where they elicit different growth responses in tissues depending on the cell-type, type of estrogen receptor (ER) present, dose and timing of exposure.

Elevated lifetime estrogen exposure has been shown to be a major risk factor for cancer in hormone-dependent organs, particularly breast and endometrium [5-9]. This may help explain the observation that breast cancer risk increases in the women who experience early menarche (onset of menstruation) and late menopause whereas breast cancer risk is reduced in the women who experience late menarche and early menopause [10;11]. A cohort study by the European Prospective Investigation into Cancer and Nutrition found that excess serum estrogens are associated with incidence of breast cancer in premenopausal women [12]. Similarly, estrogen replacement therapy in post menopausal women has been demonstrated to be associated with breast, cervical, and endometrial carcinoma [13]. In experimental models, estrogens are established breast carcinogens, as they are capable of initiating and triggering growth and selection to generate palpable malignancy [14-18]. These findings led the International Agency for Research on Cancer and National Toxicology Program to categorize estrogens as human carcinogens [19;20]. Post menopausal women exhibit the highest breast cancer incidence when serum estrogens are at their lowest level [6]. In contrast, the estrogen levels in mammary tissues are similar in pre and post menopausal women [21]. In addition, post-menopausal breast cancer patients have significantly higher estrogen levels in breast tissues than non disease pre or post menopausal women [22]. This finding indicates that site-specific estrogen synthesis or estrogen load may be the major source associated with breast carcinogenesis

and growth of breast cancer cells. However, the underlying mechanisms of breast cell susceptibility to estrogen's carcinogenic effect remain elusive.

The signaling pathway plays a critical role in mammary gland development. This pathway has also been shown to be essential in promoting growth of estrogen receptor positive (ER+) breast cancer cells. Whether this pathway alone is sufficient to induce breast carcinogenesis is still unresolved. Mammary tumors can develop in mice lacking functional ER alpha (ERα) [15;23]. Likewise, E_2 and its catechol metabolite, 4-hydroxy estradiol (4-OH-E_2) can induce an estrogenic response in the uterus of ERα null mice, where an antiestrogen (ICI182780) fails to inhibit such response [24]. Though tamoxifen (TAM) and other antiestrogens inhibit breast cancer growth through their actions at the ER, these antiestrogens also inhibit growth of cancer cells that are estrogen receptor negative [25-27]. In addition, estrogen undergoes oxidative metabolism and generates ROS which is implicated in carcinogenic conversion and growth of cancer cells [9;18]. TAM on the other hand can block estrogen metabolism and acts as a ROS scavenger [14]. These findings therefore suggest that other mechanism(s) independent of ER status exist that mediate estrogen induced cellsignaling leading to malignant transformation and growth of mammary epithelial cells. One such mechanism being investigated by us is the role of estrogen induced mitochondria ROS in mammary carcinogenesis. Using physiologically achievable estrogen concentrations corresponding to the estrogenic menstrual peak, we showed that estrogen induces rapid formation of mitochondrial ROS in MCF-7 cells [28]. Other reports support our observation that estrogen-induced ROS production relies on mitochondria, which are the main source of ROS in the epithelial

cell [29]. Some of the same mitogenic pathways that are sensitive to ROS levels are regulated by carcinogenic levels of estrogen. We have recently shown that inhibitors of mitochondrial ROS production prevent E_2-induced expression of cell cycle genes containing nuclear respiratory factor-1 (NRF1) binding sites (e.g., cyclin B1, PCNA, and PRC1), decrease E_2-induced NRF1 expression, and delay growth [30]. Together these studies indicate that in addition to ER mediated signaling, E_2 through ROS signaling can support initiation and progression of breast cancer by alternative pathways.

In this review, we discuss a novel concept that estrogen-induced ROS can produce genotoxic effects as well as support the development of estrogen-dependent breast tumors. This envisioned ROS mediated mechanism most likely contributes to the process that drives carcinogenesis, while this mechanism seems to occur in the context of other ER-mediated signaling and estrogen reactive metabolite-associated genotoxicity. To highlight the ROS mediated carcinogenesis mechanism, first, estrogen metabolism and redox cycling of hydroxylated estrogens leading to the generation of genotoxic estrogen metabolites is briefly discussed. Then, we elaborate on the role of mitochondria in estrogen-induced generation of oxidants and emerging data on how estrogen-induced ROS modifies redox-sensitive signal transduction pathways that contribute in the cell transformation, cell growth and cancer progression.

GENERATION OF GENOTOXIC METABOLITES OF ESTROGEN AS A POTENTIAL MECHANISM OF BREAST CARCINOGENICITY

Cells are protected from estrogen-mediated mitogenicity and genotoxicity through conjugation of parent estrogens to sulfate and glucuronide moieties. Estrogen metabolism and generation of genotoxic estrogen metabolites are summarized in Figure 1 and have been previously reviewed extensively [9;31-33], therefore we have limited our discussion in this area. Endogenous and synthetic estrogens, i.e. E_1, E_2, E_3, 17 α ethinyl estradiol (EE_2) and equilenin (Eq) are converted into catechol estrogens through aromatic hydroxylation by specific cytochrome P450 isoforms/peroxidase enzymes [9;34]. The catechol estrogens are inactivated by methylation, catalyzed by catechol methyltransferases, as well as by glucuronidation and sulfation, catalyzed by glucuronosyltransferases and sulfotransferases, respectively [35]. Oxidation of the catechol estrogens moieties gives rise to estrogen semi-quinones and quinines, i.e., estrogen-2,3-semi-quinone and estrogen-3,4-semi-quinone [36;37]. Catechol estrogens, particularly 4-OH-E_2, via nonenzymatic autoxidation, may undergo redox cycling to produce reactive semiquinone and quinone intermediates with concomitant production of ROS. However, this redox reaction of catechol estrogens is enhanced in the presence of Cu^{2+} or Fe^{3+} and by enzymatic catalysis by cytochrome P450 oxidases or peroxidases, which is accompanied with an increased generation of ROS. Catecholestrogens can reduce Cu^{2+} or Fe^{3+} ions to Cu^{+} or Fe^{2+}. The Cu^{+} or Fe^{2+} ions in turn, may reduce organic or inorganic peroxides, which are capable of initiating lipid peroxidation in the presence of oxygen. Both iron and copper are now recognized as major transition metals that may facilitate the formation of ROS through redox cycling of catechol compounds as well as

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by the Fenton reaction [38]. Estrogen quinones are conjugated with glutathione both in vivo and in vitro [37;39] by glutathione transferases [40]. 4-Hydroxylated estrogen metabolites or the diethylstilbestrol metabolite diethylstilbestrol-4, 4-quinone in a microsomal cytochrome P450 activation system, induce 8-hydroxylation of guanine bases of DNA. In addition to oxidant-induced damage to DNA, estrogens generate lipid peroxidation and oxygen radical-mediated oxidation of amino acid residues of proteins to carbonyl-containing moieties. The oxidant-induced oxidative DNA damage in estrogen-target tissue has been taken as evidence for the generation of oxygen radicals by metabolic redox cycling of catecholestrogens or diethylstilbestrol (DES) and for their participation in the induction of estrogen-induced cancer in animals or humans. Catechol estrogen semiquinone/quinone can also directly react with DNA to form adducts [31]. These adducts can either be stable DNA adducts that remain in DNA or can form depurinating adducts that destabilizes DNA's glycosyl bond [41]. DNA adduct formation and depurination at critical sites such as tumor suppressors or oncogenes can lead to mutations which have been postulated to be the initiator of breast cancer [41]. Rodent studies demonstrate that E_2 induces tumors in those tissues only where E_2 is predominantly converted to 4-OH-E_2 by 4-hydroxylase enzyme [42;43]. We have previously shown that E_2-induced tumor formation is decreased in animals exposed to inhibitors of estrogen metabolism [17;18] or to hormonally potent estrogens undergoing reduced metabolic conversion to catechol metabolites [44]. The rate of 4-OH-E_2 formation is also shown to be 4-fold higher than that of 2-OH-E_2 in human mammary fibroadenomas and adenocarcinomas [45]. The 4-OH-E_2 are the most prevalent estrogen metabolite in breast tumor tissues and have a significantly higher circulating level in

women with breast cancer [46]. Hydroxyestrogen and methoxy estrogen derivatives account for the majority of urinary or intra-tumor estrogens in human breast cancer patients but they are relatively scarce in the urine or mammary glands of healthy women [16]. Oxidative cellular damage such as 8-hydroxylation of guanine bases of DNA and lipid peroxidation have also been demonstrated in human breast and indicate the in vivo pro-oxidant effects of the estrogen and catecholestrogen metabolites formed in this tissue (reviewed in [9;31]). For example, elevated 4-OH-E_2 levels in breast tumors and in women with breast cancer compared to normal mammary tissues implicate 4-OH-E_2 in breast cancer and it strongly suggests that 4-OH-E_2 metabolite participates in development of breast carcinogenesis [47]. Our recent studies showed that E_2 or 4-OH-E_2 elicited transformed phenotypes in ERα negative MCF-10A cells through ROS-sensitive signal transduction pathways [48-51]. When exposed to E_2, 4-OH-E_2, 4-hydroxyequilenin, equilenin or its catechol metabolite, similar transformation in MCF-10F and MCF-10A were observed by other researchers [52;53]. Although neither $2\text{-OH-}E_2$ nor 2-OH-E_1 is carcinogenic in vitro or in vivo [42;54;55], both 2-OH-E_2 and 2-OH-E_1 are capable of producing ROS and undergo metabolic redox cycling like 4-OH-E_2. The lack of carcinogenicity of 2-hydroxylated estrogen metabolites in vivo may be due to their rapid inactivation by catechol-O-methyltransferase (COMT)-mediated O-methylation [55-57], rapid clearance [58], and weak estrogenic hormonal activity (compared with 4-OH-E_2) [59]. It may also be due to 2-MeO-E_2, the major product of COMT-mediated O-methylation of 2-OH-E_2 that possesses unique anti-tumorigenic activity. These studies support the concept that E_2 and mainly its metabolite 4-OH-E_2 are carcinogenic in breast epithelial cells.

ESTROGEN RECEPTOR MEDIATED CARCINOGENIC ACTIONS

The conventional paradigm of estrogen carcinogenic action is based on binding to ERα/β, which has been extensively reviewed; therefore, we limited our discussion in this area. Estrogen binding to its receptors, ERα and ERβ, initiates gene transcriptions by binding to estrogen response elements (ERE) of genes involved in cell growth. Therefore, increases in estrogen activity or exogenous estrogenic exposure may lead to increased cell proliferation [60]. It is speculated that rapidly proliferating cells are error prone due to the less time accorded for DNA proof reading and repairs. Errors incorporated into critical genes such as oncogenes and/or tumor suppressors may likely lead to mutations and neoplastic growth. Nevertheless, the classical concept of estrogen receptor mediated actions in cancer does not address the fact that the majority of normal mammary epithelial cells that transform into cancerous cells are ER negative (ER⁻) but are growth factor receptor positive (GFR⁺) [61;62]. On the other hand, about 7%-15% of normal mammary epithelial cells are ER positive (ER⁺) but lack growth factor receptors (GFR⁻) [63]. The predominant ER⁺ cells in normal mammary tissues are the stromal cells. Estrogen induced proliferative responses of normal ER⁻ mammary epithelial cells may be mediated by the induction of GFR⁺ stromal cells [64]. Interestingly, the majority of breast cancer cells in vivo are ER⁺ but GFR⁻ and do proliferate upon estrogen exposure [65]. These observations therefore implie that ER positive phenotypic expressions observed in majority of breast cancer cells may be manifestation of malignant transformation.

Furthermore, discrepancies between the binding affinity of various estrogens to ER and their potency both in vitro and in vivo have been reported (Figure 2). For example, 17α-estradiol has a very weak affinity for ER compared to that of DES and 17 α-

ethinylestradiol; but its ability to stimulate the growth of Leydig cells and other cells is equivalent to that of DES [66]. Similarly, the receptor binding affinity of 4-OH-E_2 is 50% that of DES or E_2, but its ability to stimulate cell growth in the mouse uterus is greater than either of these compounds [67]. In Noble rats, E_1 is more potent than E_2 in producing growth of mammary tissues [68;69]. In addition, estrogen-induced tumor formation is decreased in animals exposed to inhibitors of estrogen metabolism [17;18] or to hormonally potent estrogens with decreased metabolic conversion to catechol metabolites [44] compared to E_2. The ectopic expression of Wnt-1, a proto-oncogene in mice induced mammary hyperplasia and tumorigenesis in the absence of ERα in female and male mice [15;23]. All these studies indicate that estrogen induced cellular responses including carcinogenicity and growth of cancer cell are verycomplex and the conventional paradigm as described above cannot explain all observed discrepancies.

ESTROGEN, MITOCHONDRIA, AND ROS GENERATION

Both natural and synthetic estrogenic compounds containing a phenolic structure possess pro-oxidant and/or antioxidant activities. A balance of pro- and antioxidant effects of estrogens depends on the nature (structure) of the estrogen and on its concentrations. Under physiological conditions, the parent estrogen and the metabolic profile of estrogen metabolites in a specific tissue or fluid determine its pro- or antioxidant activities. In additionto the above-described metabolic redox cycling of hydroxylated estrogens producing ROS, we present here the evidence supporting the role of mitochondria in estrogen-induced generation of oxidants.

Estrogen and mitochondria: Mitochondria are an integral component of steroidogenesis. Key enzymes of estrogen biosynthesis (3 β-hydroxysteroid dehydrogenase and aromatase) have been identified in the mitochondria of ovarian tumor epithelial cells [70]. Exogenously added estrogen is preferentially and rapidly(> 50% within a few minutes) transported to mitochondria [71]. How estrogen functions in the mitochondria remains unclear; however, presence of ERα and ERβ in mitochondria indicates a potential role of estrogen in the regulation of mitochondrial genome transcription [72-74]. In the human mitochondrial genome, partial or ERE ½ sites in the D-loop region, CO II, tRNA-met, 12 S rRNA, 7 S rRNA, URF1, and URF5 were identified. We recently reported that mitochondria are a major target of estrogen within the cell [28;75]. Estrogen can also affect mitochondria at the protein level. Estrogen and ER agonists have been shown to inhibit mitochondrial respiratory complexes I, II, III, IV, and mitochondrial ATP synthase (F_0F_1-ATPase) [76;77], but most of these studies used cytotoxic dose of estrogen and any physiologic responses by mitochondria were probably overlooked. Mitochondrial proteins are encoded in the mitochondria and nuclear genomes. Mitochondrial transcription factor A (mtTFA), which controls mtDNA copy number, is essential for mitochondrial biogenesis [78]. When translocated into the mitochondria, mtTFA initiates transcription and replication of the mt genome. Translation is initiated by mitochondrial translation initiation factor 2 (MTIF2), which is encoded by the mt genome and contains a consensus binding site for ER. The tissue-specific coactivators of steroid receptors (including ER), such as peroxisome proliferator–activated receptor-coactivator-1 (PGC-1), and the PGC-1-related proteins, PRC and PERC, coactivate the expression of mtTFA [79-82]. Both transcriptional activators nuclear respiratory factor 1

and 2 (NRF1 and 2) and coactivators (PGC-1, PRC) act as important mediators in the maintenance and proliferation of mitochondria [83]. Steroid hormones, androgens and thyroxin, can induce changes in mitochndrial genome-derived mRNA and rRNA by increasing mtTFA mRNA expression from the nuclear genome [84]. At the post-translational level, an interaction between mtTFA and $_P$53 has been shown to control mitochondrial biogenesis [85], and $_P$53 interacts with ER [86]. Estradiol increased phosphorylation of a 76-kDa protein in the mitochondrial fraction of the rat corpus luteum [87]. This report, together with the presence of protein kinases within mitochondria, further suggests that estrogens regulate mitochondrial physiology at the post-translational level [77].

Mitochondria and generation of estrogen induced ROS: Several enzymatic systems and organelles produce ROS, such as NADPH oxidases at the plasma membrane, nitric oxide synthases, xanthine oxidase, peroxisomes at endoplasmic reticulum (cytochromes P450) and mitochondria. Mitochondria however, are the largest source of ROS in epithelial cells. Over 90% of oxygen is consumed by mitochondria, and up to 4% of the oxygen in the mitochondria is utilized to produce RO/NS. The respiratory chain produces superoxide radicals ($O_2^{.-}$). The major source of H_2O_2 in cells arises from the dismutation of $O_2^{.-}$ ($2O_2^{.-} + 2H^+ = H_2O_2 + O_2$). At physiologic pH, non-enzymatic dismutation is rapid ($\sim 10^5$ mol^{-1} sec^{-1}), but dismutation is markedly accelerated by the superoxide dismutases (SOD; $> 10^9$ mol-^{1} sec^{-1}). H_2O_2 freely diffuses through cellular membrane and is a precursor of OH$^.$. Mitochondrial nitric oxide synthase (NOS) produces nitric oxide (NO$^.$), which combines with $O_2^{.-}$ to generate peroxinitrite (ONOO$^.$). All these RO/NS may cause

mitochondrial and cellular damage if present in excess (Figure 3). The physiologic production of RO/NS by mitochondria can have profound effects on initiation of preneoplastic foci and its promotion as well as tumor cell growth both in vivo and in vitro (Figure 3). Multiple non-mitochondrion and mitochondrion-dependent defense system regulate intracellular RO/NS formation and maintain an appropriate redox environment [88]. Glutathione, a tripeptide thiol (cysteine-glutamine-glycine), and thioredoxin (Trx), a small protein that has two cysteines in its active site, are responsible for much of the antioxidant defense of cells but are also likely mediators of oxidative signaling. These thiols are used as substrates for glutathione peroxidases, Trx peroxidases (peroxiredoxins) [75;89], and glutathione-S-transferases, enzymes that regulate the steady state concentration of hydroperoxides (ROOH) including H_2O_2, and also used as electrophiles, such as 4-hydroxy-2,3-nonenal (4HNE), which is a lipid peroxidation product.

Earlier studies failed to identify that physiologic concentrations of estrogen stimulate mitochondrial ROS generation.That is why possibly cytotoxic doses of estrogen and isolated mitochondria were used. We and others have shown that mitochondria are significant targets of estrogen [28;30]. Recently, we reported that physiological concentrations of E_2 stimulate a rapid production of intracellular ROS in epithelial cells which depends on cell adhesion, the cytoskeleton, and integrins [28]. In our studies of E_2-induced ROS generation in MCF-7 and other cells, hydroxylated estrogen metabolites or adducts immediately after addition of E_2 were not detected which rules out the possibility of ROS generation by redox cycling of hydroxylated estrogens. These events occur

earlier than ER-mediated genomic actions. E_2-induced ROS production does not depend on the presence of ER on breast cancer cells as ER$^-$ cell line MDA-MB 468 produced ROS equal to or more than that of ER$^+$ MCF7, T47D, and ZR75 cell lines [28].

The ROS molecules, H_2O_2 and NO, also stimulate mitochondrial biogenesis, a process that depends on the flow of molecules into and out of the organelle [90;91]. It has been shown recently that oxidative stress causes an increase in mitochondrial mass in human lung cells. However, the molecular mechanism underlying the increase in mitochondrial mass and mtDNA is still unclear. Furthermore, the mitochondrial mass of mtDNA-less cells was also significantly increased by exposure to low concentrations of H_2O_2, suggesting that the increase in mitochondrial mass in replicating cells may result from an increase in ROS production. Thus, the delayed production of estrogen-induced ROS may occur via this mechanism, whereas the transient estrogen-induced increase in ROS may result from estrogen activation of the mtTFA complex leading to increased oxidative phosphorylation.

ESTROGEN-INDUCED ROS-MEDIATED OXIDATIVE DNA DAMAGES

Estrogen-induced oxidative DNA damage through ROS has been extensively reviewed [9;31-33], therefore we have limited our discussion in this area here. 8- Hydroxy-guanine (8-OHdG), an indicator of oxidative DNA damage, causes G->T and A->C substitutions in DNA templates. It has been shown that breast carcinoma DNA contains dramatically higher concentrations of base modifications, such as 8-hydroxy-guanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, and 8-hydroxyadenine [92-94]. An elevated level

(3.35-fold higher) of 8-OHdG was observed in ER$^+$ when compared with ER$^-$ malignant tissues. 8-OHdG level in ER$^+$ MCF-7 cell line was significantly higher (9.3-fold higher) than ER$^-$ MDA-MB 231 cell line [93]. There was a higher level of 8-OHdG in the DNA of early-stage cancer tissue than that of later-stage cancer tissue, which suggests that ROS may play an important role in the early phase of carcinogenesis. The urinary 8-OHdG level (1,827 +/- 1,500 pmol/kg/day) in 18 women with gynecologic cancer has been shown to be significantly higher than in 10 women (747 +/- 425 pmol/kg/day) without carcinoma [94]. Gene mutation induced by ROS has been observed in C-Ha-ras-1 and p53 [95;96]. Recently, it has been shown that methylation alone induced C:G-->T:A transitions in the p53 gene, whereas nitric oxide treatment produced more C:G-->A:T transversions at CpG sites and, thus, increased mutational yields. Alterations in the genome of breast cells is likely produced by oxidative attack of ROS generated by estrogen-induced oxidative stress in combination with receptor-mediated proliferation of oxidatively damaged cells. This view is supported by the findings of DNA damage induced in vitro or in vivo by estrogen-induced ROS, 4-OH-E$_2$ and both natural and synthetic estrogens in the target organs of cancer [31;32]. Catecholestrogen through redox cycling produce free radicals that generate various forms of free radical-induced DNA damage [31;32]. Cotreatment with an inhibitor of IL-1β and TNF-α, pentoxifylline, inhibited stilbene estrogen-induced increase in myeloperoxidase activities, 8-OHdG formation, mutations in the genome and prevented the estrogen induced testicular and uterine pre-neoplastic lesions [9]. Estrogen by stimulating nitric oxide synthase (NOS) and the subsequent generation of NO could affect methylation and expression of genes [97-99]. Thus, ROS/NO induced by estrogen may produce both gene mutations and gene

silencing, which, in turn, can predispose an individual to the development of cancer. Therefore, estrogen-induced ROS may play multiple roles in tumor initiation and progression. Compelling evidence for the transforming capacity of ROS is the finding that overexpression of Mox1 (the catalytic subunit of NADPH oxidase) induces superoxide generation and transforms NIH 3T3 cells. Furthermore, Mox1-transfected cells produce aggressive tumors in athymic mice similar in size to those produced by Ras-transformed NIH 3T3 cells [100], illustrating the critical role of this ROS regulator in vivo. These results are consistent with the concept that generation of genetic alterations is required for the initiation and progression of estrogen-induced tumors.

ESTROGEN-INDUCED ROS MEDIATED REDOX SIGNALINGS

ROS fulfill the characteristics of a second messenger since they are short-lived (rapidly generated and degraded), produced in response to a stimulus, highly diffusible (e.g., H_2O_2), and they are ubiquitously present in most cell types. In this section, we review the ROS-mediated signaling pathways which contribute to initiation, promotion and progression of estrogen-dependent breast tumors. Signaling proteins, including protein tyrosine phosphatases and several transcription factors, that contain critical cysteines are sensitive to redox changes and thus are potential targets of modifications by ROS (Figure 4). A common feature of redox signaling appears to be the oxidative modification of critical cysteines in protein phosphatases that act on protein kinases. Kinases in turn activate redox sensitive transcription factors that influence the expression of genes involved in cell transformation, cell growth, and cancer progression.

Regulation of phosphatases by estrogen-induced ROS: ROS reversibly regulate cysteine-based phosphatases, which include protein tyrosine phosphatases (PTPs), dual-specificity phosphatases (such as, Cdc25s), low-molecular-weight PTPs, and the lipid phosphatase, PTEN. Oxidation of PTPs can be reversed by thiol compounds, once the catalytic cysteine forms a disulfide bond with an N-terminal cysteine. This disulfide bond can originate from same site cysteines, as is the case with low molecular weight PTPS, from "distant" cysteines such as with PTEN and Cdc25, or from a bond between the PTP and a thiol, such as with PTP1B. The important step of the disulfide bond blocks the oxidation of the thiolate to Cys-SOH by the connection to another thiolate. Oxidation and phosphorylation have the potential of influencing each other in the signaling of PTPs and protein tyrosine kinase (PTKs). We have used Cdc25 as a major focus of this section to describe the redox regulation of phosphatase by estrogen-induced ROS. The Cdc25s, dual-specificity tyrosine phosphatases, possess the necessary elements to function directly in redox control of the cell cycle. Three homologues of Cdc25s exist in humans: Cdc25s A, B, and C. All contain a nucleophilic catalytic cysteine within a conserved motif that enables these enzymes to dephosphorylate phosphoproteins. The first suggestion that Cdc25 phosphatases act as potential redox controllers of the cell cycle was made more than 10 years ago by Mori laboratory which showed the direct involvement of ROS in the two-cell block of the mouse embryo [101]. Embryo development past the two-cell stage occurred when they injected the ROS modifiers SOD or Trx in vitro. Trx was shown to act through the phosphorylation state of Cdc2 kinase because the injection of Trx led to dephosphorylation and activation of the Cdc2 kinase during M-phase of the second cleavage. Other studies suggested that the oxidative stress

generated in human cells in response to UV or IR exposure degrades Cdc25s- A through its phosphorylation on serine-123 by check point kinase (chk) [102;103]. This leads to its ubiquitylation and its proteolytic degradation. In contrast, Finkel's laboratory has shown that Cdc25C undergoes rapid non proteasomal degradation in response to H_2O_2 treatment in HeLa cells [104]. This response is due to a decreased half-life for Cdc25C as a result of disulfide linkage formation between its active-site and backdoor cysteines. Cdc25C's response to H_2O_2 treatment does not depend on the Chk pathway, suggesting a direct interaction between ROS and Cdc25C. The active-site cysteines of the Cdc25's are highly susceptible to oxidation [105]. The rate of thiolate conversion to the sulfenic acid by H_2O_2 for Cdc25B is 15-fold and 400-fold faster than that for the protein tyrosine phosphatase PTP1B and the cellular reductant glutathione, respectively [105]. Cdc25A $(69M^{-1}S^{-1})$ and Cdc25C $(120M^{-1}S^{-1})$, with highly homologous catalytic domains to Cdc25B (~60% identity), are only slightly less reactive than Cdc25B $(164M^{-1}S^{-1})$. The sulfhydryl group on cysteine residues can be oxidized to form a disulfide bond (Cys-SSR), sulfenic acid (Cys-SOH), sulfinic acid (Cys-SO$_2$H) or sulfonic acid (Cys-SO$_3$H); the two latter being quite stable while disulfide bonds and protein sulfenic are relatively reactive and are often considered as the mediators in redox signaling. The active-site cysteine is protected by rapid intramolecular disulfide formation with back-door cysteines in wild-type enzymes and these intramolecular disulfides can then be rapidly and effectively reduced by Trx/TrxR2 but not glutathione [105]. Finkel and his associates fail to show oxidation of Cdc25A, because they used very a high concentration of H_2O_2 [106].

Rudolph's laboratory has shown that the oxidation of Cdc25's catalytic site cysteine residues decrease its activity via the formation of an intramolecular disulfide bond. In addition, the catalytic cys430 formed a disulfide bond with the invariant cys384 in some crystals suggesting that Cdc25 may be self inhibited during oxidative stress. The nucleophilic catalytic cysteines of phosphatases are known to be highly susceptible to oxidation, a property that permits redox regulation of this enzyme family. Recent studies have shown that O_2^- is a more efficient regulator of PTP1B than H_2O_2, with a calculated rate constant in vitro ($\sim$3 x 10^2 $M^{-1}sec^{-1}$) about eight times faster than for H_2O_2 [107]. Nevertheless, one can argue that the reaction rate would be more than 100 times faster with H_2O_2 than with O_2^- in cells, considering that the steady state concentration of H_2O_2 in the cytosol is approximately 10^3 times more than the concentration of O_2^- [108]. As described above, H_2O_2 exposure oxidized –SH group of Cdc25A faster than that of PTP1B [105;109]. Besides the widely believed concept that chk-mediated phosphorylation leads to Cdc25 proteosome-dependent degradation; its activity appears to be regulated by oxidation in the same way as oxidation regulates PTP1B activity.

A rapid decrease in Cdc25A activity was shown when MCF-7 cells were exposed to physiological concentrations of estrogen [51]. A similar decrease in Cdc25A activity was observed in H_2O_2 treated HeLa cells [110]. Further examination revealed a lower level of Cdc25A –SH residues in the estrogen treated group compared to the vehicle control [51]. In addition, cotreatment with antioxidants prevented the oxidation of –SH residues in estrogen treated cells [51] . These findings suggest that estrogen-induced ROS oxidatively inactivates Cdc25A. A recent study showed that the oxidation of the Cdc25

active cysteine site leads to enzyme inactivation [111;112]. We and others have also identified an estrogen mediated protein interaction between Cdc25A and ERK in MCF-7 and hepatoma cells respectively when treated with Cdc25A inhibitor Cpd [113]. Independent of ERα, estrogen activates the MAPK pathway via ROS -dependent inactivation of Cdc25A. An increase in Cdc25A mRNA has been reported after 6 hrs of E_2 treatment and an increased synthesis of Cdc25A protein was detected from 10 to 12 hrs after E_2 treatment [114;115]. The level of Cdc25A protein did not change immediately after E_2 exposure, however, an increased in the level of its protein as early as 3 hrs after E_2 exposure was detected. This observation suggests that E2 treatment increases Cdc25A stability. We further looked for any post-translational modification from E_2-exposure because full enzymatic activity of Cdc25A requires phosphorylation at multiple Ser and Thr residues in the amino-terminal of the protein. In contrast to UV or IR effects on Cdc25A in cells, we observed a rapid decrease in serine phosphorylation of Cdc25A in response to physiological concentrations of estrogen which paralleled the decrease in phosphatase activity. A similar decrease in the serine phosphorylation of Cdc25A and its activity was observed in response to the exposure of cells with H_2O_2. Cdc25A is also phosphorylated at tyrosine residues, and in contrast to serine phosphorylation, tyrosine phosphorylation of Cdc25A was enhanced in cells treated with estrogen or H_2O_2. Others have also shown that lower oxidant concentration increase the rate of tyrosine phosphorylation by tyrosine phosphatase [116]. These studies did not examine the oxidation of active site cysteines nor did they explain how tyrosine phosphorylation occurred and how tyrosine phosphorylation inactivated phosphatase activity. ROS activated ERK enhanced tyrosine phosphorylation of Cdc25A in E_2-treated

MCF-7 cells protects Cdc25A from degradation [117]. Tyrosine phosphorylation of increased the half-life and accumulation of Cdc25A which may contribute to Cdc25A - stimulated Cdk2 activity.

Besides the Cdc25, some of the other phophatases, such as, MKP3, PTEN, PTP1B1, PTP2A, and PP5 [118-120] are responsive to ROS and regulate estrogen-mediated signaling. For example, the activity of Ser/Thr protein phosphatase 5 (PP5) is responsive to oxidative stress and estrogen. Elevated levels of PP5 protein found in human breast cancer have been linked to the progression of human breast cancer [121]. ERK-specific phosphatase MKP3 is another ROS responsive phosphatse that may influence phosphorylation at Ser118 of ERα by regulation of pERK1/2 MAPK. Antioxidant treatment by increasing MKP3 phosphatase activity blocked tamoxifen resistance [122]. These phophatases are reversibly regulated by ROS. These oxidatively modified phophatases act on their corresponding kinases based on their temporal and spatial localization in the cells.

Activation of kinases by estrogen-induced ROS: Structural changes to PTPs, as well as serine/threonine and phospholipid phosphatases, are induced by ROS, and the altered protein conformation leads to signaling cascade upregulation. Included in these cascades are src/Abl kinase-dependent, MAPK-dependent, and PI3 kinase-dependent pathways, leading to activation of AP-1, NF-kB, NRF1, and other redox-regulated transcription factors.

We describe below some of the kinases which may be influenced by estrogen-induced ROS signaling via redox regulation of phosphatases.

A-Raf: The mitochondrial localization of protein kinases A-Raf, PKCδ, and PKCɛ is evidence that this subcellular compartment harbors redox sensors [123;124]. The role of the protein kinase A-Raf in the mitochondria is not clear, but A-Raf mRNA is highly expressed in normal murine tissue [125]. In HeLa cells, epidermal growth factor (EGF) rapidly and transiently activates A-Raf, which in turn phosphorylates the MAP kinase activator MEK1 [125]. Interestingly, E_2 can stimulate the phosphorylation of A-Raf and it appears to participate in cell cycle progression in ER^+ MCF-7 cells [126]. Thus, it appears that mitochondrial oxidants may modulate estrogen-induced cell proliferation via A-Raf pathway. In addition to A-Raf, other redox sensitive proteins associated with mitochondria, PKCδ and PKCζ, have been demonstrated to activate the Raf/MEK/ERK pathway or directly activate MAPKs, respectively [124;127]. As zinc finger domains of Raf and PKC are targets of oxidative stimuli and Raf and PKC are localized in mitochondria, estrogen-induced ROS may activate A-Raf and/or PKC via the MEK/ERK pathway. Signaling pathways involving JNK and p38 MAPK are very responsive to redox regulation. JNKs have been studied primarily in the context of stress responses and apoptosis; however, accumulating evidence indicates that the JNK pathway also contributes to proliferative responses. For example, $JNK1^{-/-}$ mouse embryonic fibroblasts (MEFs), as well as JNK1-JNK2-/ MEFs, proliferate more slowly than do wild-type MEFs and reach a lower saturation density, establishing that JNK is required for normal MEF proliferation [128]. A role for JNK in cancer development is supported by recent studies. It has been shown that skin tumorigenesis is suppressed in JNK2-deficient mice [129], and expression of an inactive variant of the JNK substrate c-Jun in immortalized fibroblasts expressing v-Src and v-Fos reduces tumorigenicity in nude mice [130]. The

oxidative enhancement of Src-family tyrosine kinases and certain PKC isoforms as well as the dissociation of the JNK/GSTp and ASK1/Trx complexes may together contribute to the redox sensitivity of these pathways. These and other data suggest that signaling through the A-Raf/JNK pathway is very responsive to redox regulation and mediates oncogenic signals and supports cell proliferation in the absence of stress.

ERK: In addition to the ability of E_2 to promote ER-dependent gene transcription, it rapidly activates ERK-1 and ERK-2 [130-134]. The activation mechanism by which this rapid signaling event occurs is unclear. The activation is extremely fast and it is believed that they are initiated at the plasma membrane and do not involve ER-mediated gene transcription. Several investigators have concluded that estrogen-induced MAPK activation is promoted by membrane bound ERα or ERβ [135-137]. There are no known functional motifs within the structure of the ER that can promote second messenger signaling [138]. Most of the studies investigating rapid MAPK activation by estrogen have employed estrogen responsive cells including MCF-7 breast cancer cells [133;139], osteosarcoma cells [140], and neuroblastoma cells [141]; yet these studies have not directly addressed the roles of ERα and ERβ in promoting estrogen-induced ERK activation. There are reports which show no correlation between ERβ expression patterns and the activation of ERK-1/-2 in estrogen treated breast cancer cell lines. Both estrogen and the pure antiestrogen ICI182780 activate ERK-1/-2 in human SKBR3 breast carcinoma cells, which lack ERα/β protein and m RNA [131]. Other researchers have suggested that a membrane-associated ER-like G protein is responsible for nongenomic estrogen signaling [142-144]. They showed evidence that cellular expression of the

orphan receptor, GPR30, is sufficient for estrogen-induced activation of ERK-1/-2. Based on these observations, it is not clear which membrane protein or how the membrane proteins (ER or GPR30) activate ERK signaling in estrogen exposed normal or cancer cells.

In many cells, extracellular H_2O_2 alone activates ERK-1/-2. As discussed previously, studies have shown that estrogen exposure rapidly activates ERK and our work indicates that both estrogen and H_2O_2 rapidly inactivate Cdc25A and activate the ERKs in MCF-7 cells. Inactivation of Cdc25A by its inhibitor Cpd5 results in the activation of EGFR tyrosine kinase and more recently was shown to activate ERK-1/2 in hepatoma cells [113]. Both EGFR and ERK have been shown to form protein complexes with Cdc25A in hepatoma cells [145]. Since ERK can interact with and phosphorylate EGFR, Cdc25A-ERK interaction could be responsible for EGFR activation. Moreover, napthoquinone based inhibitors inactivate Cdc25A by covalently binding to cysteine residues at the active sites of Cdc25A [146]. Thus, it is possible that ERK may be activated by inhibiting its association with Cdc25A or inactivating Cdc25A through its redox regulation by estrogen-induced ROS.

AKT: AKT (also called PKB) is a serine-threonine protein kinase. It is a downstream target of PI3-kinase. AKT binds to the products of PI3-kinase. AKT affects numerous downstream targets either directly or indirectly, including the pro-apoptotic proteins Bad and Caspase 9, the growth-inhibitory proteins glycogen synthase kinase-3β, the forkhead transcription factors, the kinase IKKα, and the kinase mTOR [147;148]. AKT has

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recently been identified to control NRF1 activity [149]. Several investigators have concluded that estrogen-induced AKT activation is promoted by membrane bound ERα or ERβ [150;151]. There are no known functional motifs within the structure of the ER that can promote second messenger signaling. There are reports which show no correlation between ERα/β expression patterns and the activation of AKT-1/-2 in estrogen treated breast cancer cell lines. 17α-estradiol, through an ER independent mechanism, activates PI3K-AKT signaling [152]. Recently Lee et.al., [151] reported that up-regulation of PI3K/AKT signaling by E$_2$ is mediated through activation of ERα, but not ERβ. In ovarian cancer cells, 4-OH-E$_2$ induces AKT phosphorylation while 2-OH-E$_2$ did not [153].Our study showed that 4-OH-E$_2$ increased AKT poshphorylation in ERα lacking MCF-10 cells. Based on these observations, it is not clear which membrane protein and/or how the membrane proteins activate AKT in estrogen exposed normal cells. AKT activity depends on its phosphorylation, which is positively regulated by PI3K and negatively regulated by a class of protein phosphatases (PPs) [154]. AKT can be activated by both E$_2$ and H$_2$O$_2$ [118;149-151]. ROS reversibly regulate cysteine-based phosphatases [119]. There are indirect evidences indicating that AKT associates with Cdc25. For example, AKT phosphorylates Cdc25s [155]. AKT activation suppresses Cdc25 inhibitor napthoquinone- and methylating agent temozolomide –induced G$_2$ arrest and cell death [156;157]. A recent study indicate that Cdc25A interacted with AKT [158]. The reversible inactivation of Cdc25A or PTEN by estrogen-induced ROS appears to be a potential key player in the activation of AKT.

<u>Activation of redox sensitive transcription factors by estrogen-induced oxidants signaling</u>: A significant number of transcription factors, including estrogen receptor, are redox sensitive. Some of these transcription factors contain critical cysteine residues that can be affected by ROS. As described above, signaling pathways are targeted by ROS, which result in the activation of kinases through inactivation of redox sensitive phosphatases. The active kinases, in turn, regulate phosphorylation of transcription factors. Regulation of AP-1, NF-kB, and CREB by both estrogen and ROS is widely accepted and their redox regulation by ROS has been discussed [9;118]. We have focused our discussion mainly on NRF1 in this section.

<u>ER</u>: A large class of DNA-binding proteins such as the glucocorticoid receptor (GR), ER, and early growth response factor-1 (egr-1), contain zinc finger domains [159], which consist of at least two zinc-coordinated thiolates. Oxidation of a zinc finger converts cysteine thiol groups to disulfide releasing zinc from the protein and resulting in a change in protein conformation that may affect protein function. Interestingly, oxidative stress has been reported to modulate ERα and ERβ expression in various cell lines. In human breast cancer cells MCF-7 and T-47D, exposure to 2.5µM of H_2O_2 increased the protein level of ERα while having a minimal effect on ERβ [160]. Endogenous oxidative stress induced by PMA (100 ng/ml) in the macrophage cell line, J774A1 also increased the expression of ERα [75] and it was found that Ras mitogenic activity is, in part, superoxide dependent. A recent study showed that at least 75 genes out of 891 estrogen/ER-regulated genes were responsive to oxidants and ERα silencing. The oxidant-sensitive subset of estrogen/ER-responsive breast cancer genes are linked to cell

growth and invasion pathways. Findings of this study suggest that oxidant sensitive signaling pathways contribute to the development of an aggressive subset of ER$^+$ breast cancers [161]. Although protein kinases are known to participate in phosphorylation signal cascades, they may also participate in redox signaling networks due to these zinc finger domains, e.g., ROS can activate PKC that triggers the release of zinc ions [162;163]. Another protein kinase, c-Raf, known to participate in the MAPK signal cascade was also demonstrated to be redox-activated at the zinc finger domain [162;164]. As zinc-finger domains within these kinases have been reported to act as oxidant sensors, a mode of cross-communication appears to exist between redox and phosphorylation networks. Like serine-thereonine phosphatases as discussed above, ROS can inhibit some of the protein tyrosine phosphatases by reversible oxidation of their cysteine residues and enhance protein kinases, such as Src, c-Jun amino-terminal kinase (JAK), Ras, PKC, and MAPK [21]. These kinases are involved in modulating ER signaling [165;166]. Thus, ROS-mediated signaling represents another means by which ER function can be modulated by oxidants, and may be responsible for the clinical phenotype of ER breast cancer. Rapid effects of estrogen have been demonstrated to mediate the DNA binding activity and phosphorylation of transcription factors. For instance, a 10 nM estradiol treatment of rat adipocytes doubles AP-1 DNA binding in 15 min [167]. In addition, CREB protein is phosphorylated rapidly by estradiol (10nM) treatment within 15 min. Given that estradiol can stimulate mitochondrial ROS generation and that the transcription factors AP-1, NF-kB, and CREB are targets of oxidative stimuli [167;168], it is possible that estrogen-specific effects at the level of the mitochondria can activate these transcription factors by above-descibed signal transduction pathways.

Mitochondrial generation of ROS leading to the activation of transcription factors AP-1, NF-kB, and CREB has downstream consequences for apoptosis, cell cycle progression, cell transformation, cell migration and cell invasion.

AP-1 and CREB: Growth factors and TPAactivate AP-1-dependent transcriptional activation and evidence is accumulating that ROS and 4-Hydroxy-2-nonenal (4HNE) increase the DNA binding activity of AP-1 [169-174]. The initial increase is not dependent upon protein synthesis and probably involves post–translational modifications of previously existing AP-1 complexes; however, increased synthesis of Fos and Jun also occurs rapidly upon stimulation [175]. Jun transcription is controlled by TRE and MEF2 sites while Fos transcription requires SRE and CRE sites that are regulated by all three MAPK cascades [176]. Binding of AP-1 to DNA can occur without phosphorylation, but increased binding and transcriptional activation are both signaled by phosphorylation of Fos and Jun [177]. Several transcription factors, including c-Fos and Elk-1 are phosphorylated by ERK1/2. The JNK and p38MAPK are more strongly activated by stress signals and their activation occurs through similar phosphorylation cascades. The c-Jun family of transcription factors are specifically phosphorylated by the JNK at Ser 63 and Ser 73 in a two-step process that requires binding to the delta subdomain prior to efficient phosphorylation at the NH_2 -terminal activation domain[178]. JNK also binds and phosphorylates the Elk-1 and ATF-2 transcription factors [179;180]. The phosphorylation of Jun at the N-terminus by JNK not only is required for transcriptional activation [181;182] but also seems to protect Jun against degradation by ubiquitination

[183]. This results in an increase in the half-life and accumulation of Jun that may contribute to the JNK-stimulated AP-1 transcriptional activity.

In addition to phosphorylation, the AP-1 complex is regulated by redox mechanisms. Fos and Jun have a single conserved cysteine residue located in the basic region of DNA–binding domains that must be reduced for DNA binding activity [184]. The transcription factor AP-1 is strongly responsive to redox regulation. The exact nature of the redox change is not clearly identified. Recent in vitro data indicate that reversible S-glutathiolation of the conserved cysteine may be involved [185]. Others suggest that the reversible redox regulation of AP-1 is mediated by Trx and the nuclear protein Ref1, which both increase AP-1 binding activity [186;187]. Direct association between Trx and Ref-1 has been reported [188]. Recently we have demonstrated the evidence for the involvement of redox signaling with estrogen-induced cell proliferation [28;75]. Physiological concentrations of E_2 stimulate a rapid production of intracellular ROS which lead to the phosphorylation of c-jun and CREB. Although direct ER transcription factor effects are required to promote DNA synthesis, our recent data showed that MCF-7 cell growth and cyclin D1 expression are suppressed by antioxidants and mitochondrial blockers. This observation supports our novel finding that nongenomic estrogen-induced mitochondrial ROS modulate the early stage of cell cycle progression [28]. It has become evident that regulation of the cellular redox environment is critical for estrogen-mediated signaling. Thus, ROS may regulate AP-1 activity through several targets. ROS can modulate effector molecules such ERK and the c-fos/c-jun heterodimer (AP-1); and these effector molecules are known to participate in growth signal transduction [28;30]. It

is possible that JNK pathway may be involved in the AP-1 binding and transcriptional activation induced by mitochondrial oxidants produced upon exposure to estrogen. Although the ERK pathway has been shown to be involved in AP-1 activation by other agonists, we think that it may not participate in this case, based on the inability of H_2O_2 alone to stimulate the ERK pathway in MCF-7 cells [189]. JNK is activated by stress stimuli through an activation module that may include the MAP3K apoptosis-signal regulating kinase (ASK-1) [85].Interestingly, recent data suggest that ASK-1 is activated by ROS either through Trx oxidation [190] or through dimerization [191]. Thus, ROS can either activate the JNK module or induce the formation of 4HNE, which may directly activate JNK [173].

The expression of early G1 genes, c-myc, c-fos, c-jun, cyclin D1, and NRF1 are regulated by ROS [192;193]. The mRNAs of some of these genes are induced by estrogen-induced ROS and H_2O_2 as well as other inducers of oxidative stress [114;130;192;194]. As described above c-Fos and c-Jun comprise part of the transcription factors AP-1; and AP-1 binding activates the expression of down-stream target genes. In this regard, the induction of c-Fos, c-Jun and cyclin D1 is an ideal paradigm for studying the role of early response genes in response to oxidative stress-induced by estrogen. The mRNAs of c-Fos and c-Jun are induced by estrogen and also by relatively small amounts of H_2O_2, superoxide, NO, and other inducers of oxidative stress [114;192]. The earliest known growth-regulatory gene activated by E_2 is c-Myc. Increased transcription of myc, as measured by accumulation of mRNA, occurs within 30 min, and peak protein levels are observed within 1–3 h of treatment with E_2 of MCF-7 cells [114]. Recent studies indicate

that Myc protein is stabilized by E_2, up to 12 h after its addition, well past the decline in mRNA levels. Myc expression elicits active cyclin E-Cdk2 and S-phase entry in growth-arrested MCF-7 cells. Although Myc is clearly implicated in E_2 action, it was not found in a genetic screen for E_2-responsive genes in MCF-7 cells. Myc is induced by exposure to ROS [114]. Cyclin D1 is a well-defined target of E_2 action in MCF-7 cells, and in rodent breast epithelial tissue, ovary and uterine endometrium. In MCF-7 cells, addition of E_2 to G0/G1-arrested cells results in a two- to three-fold induction of cyclin D1 mRNA within 1-3 h and a three- to five-fold increase in synthesis and steady-state levels of cyclin D1 protein within 3-6 h. Given that cyclin D1-knockout mice are fertile, the physiologic relevance of ER as a target of cyclin D1 is uncertain. Cyclin D has a half ERE. Planas-Silva et. al., demonstrated that E_2 transiently upregulates synthesis of c-Fos and c-Jun in MCF-7 cells, but not in hER-transfected HaCa T nontransformed human keratinocytes [195]. Earlier studies indicated, however, that E_2 increases AP-1 activity without increased synthesis of c-Jun or c-Fos and without the synthesis of any new proteins. Foster and Wimalasena demonstrated that ligand-activated ER is fully active at 8–10 h after E_2 treatment [114]. The products of at least two E_2 target genes, c-Myc and cyclin D1, can independently promote G1/S transition in MCF-7 cells and activate cyclin E–Cdk2 coincident with formation of high molecular mass cyclin E–Cdk2–p130 complexes depleted in p21CIP1 and p27KIP1. The expression of c-fos and c-myc did not depend on the presence of growth factors, suggesting that integrins alone through ROS could regulate the Go/G1 transition. Furthermore, integrin clustering in the absence of cell spreading was sufficient to induce expression of early genes (jun B and ras) involved in the Go/G1 transition and were able to trigger G1/S transition through modulating

cyclin D1 expression [196;197]. Integrins, through ROS, control the expression of these cell cycle genes, and it is now obvious that cell adhesion and integrin ligation are obligatory processes for regulation of cdks and cyclins. JNK and p38 also have been shown to cooperate with ERK in pp60(v-src)-induced cyclin D1 expression in breast cancer cells [114]. However, an unresolved issue regarding E_2-mediated signaling is whether E_2 receptors themselves actually initiate signal that can intervene in the Go/G1 and G1/S transitions. The expression of early G1 genes, c-myc, c-fos, c-jun , cyclin D1 are regulated by ROS [192]. Low and intermediate levels of RO/NS regulate cellular signaling and play an important role in normal cell proliferation [9;196]. Constant activation of AP-1 transcription factor appears to be one functional role of elevated ROS levels during tumor progression [198]. Based on these data, estrogen-induced ROS may control the expression of genes involved in cell cycle progression.

NF-kB: Activation of NF-kB is another well-studied model of redox regulation. The oxidation of a conserved cysteine residue in the DNA-binding region adjacent to the leucine zipper in AP-1 abolishes its binding to DNA [185]. The DNA binding activity of AP-1 is restored by Trx and the nuclear signaling protein redox factor-1 (Ref-1) [188] and enhanced by transient overexpression of Trx in vivo. Compared with NF-kB, AP-1 is less sensitive to oxidative inhibition by glutathione disulfide in vitro but more sensitive to oxidized Trx. Thus, in intact cells, the elevation of intracellular oxidized glutathione (GSSG) levels by inhibition of glutathione reductase inhibits selectively the DNA-binding activity of NF-kB and not that of AP-1 [199]. The protein NF-kB is regulated by its interactions with the inhibitory co-factor, which sequesters NF-kB to the cytoplasm.

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AKT is known to phosphorylate an upstream kinase, IKKβ, which stimulates the degradation of Ik-B [130]. As AKT can participate in the activation of IKKβ, it may be possible for estrogen-induced mitochondrial ROS to stimulate the transcription activity of NF-kB via the AKT pathway.

NRF1: The human alpha-palindromic nuclear respiratory factor -1 binding protein (NRF1) is a 503 amino acid (aa) transcription factor [200;201], located on chromosome 7q32.2 [202] and codes for 68 kDa nuclear proteins [200;203]. Several compounds that induce cellular oxidative stress have been shown to increase NRF1 expression and increase in mitochondria proliferation. For example, the bacterial lipopolysaccharide and tertiary butyl hydroperoxide have both been demonstrated to induce cellular oxidative stress which upregulates NRF1 expression and mitochondria biogenesis in rat liver cells [149;193]. Likewise, rat liver cells overexpressing NRF1 have been shown to induce ROS production and increase in mitochondria proliferation [149]. Similarly, exposures of human lung fibroblast cell line, MRC-5, with H_2O_2 at concentrations of 90-360 μM induced increases in mtDNA and mitochondria biogenesis in exposed cells [204]. The role of oxidative stress and NRF1 expression were further corroborated by works done by Miranda et. al., which revealed that HeLa cells depleted of mtDNA were under oxidative stress which induced expression of NRF1 and mitochondrial biogenesis [205]. Lastly, estrogen exposure has been reported to increase NRF1 overexpression which increased mitochondria biogenesis in MCF-7 malignant mammary epithelial cells, and human bronchial epithelial cells from female patients [206;207].

Oxidants modulate NRF1 expression and mitochondria biogenesis; antioxidants conversely inhibit intracellular ROS and NRF1 expressions. For example, oral administration of vitamin C has been demonstrated to inhibit NRF1 expressions with reduction in mitochondria biogenesis and diminished endurance training capacity [208]. In addition, catalase overexpressions have been shown to reduce NRF1 expressions and mitochondria content in heart muscles of aged mice which attenuated age related diseases observed in mice [209]. These studies imply that cellular oxidative status modulates NRF1 expression. Activated NRF1 expressions in response to ROS can lead to enhanced mitochondria biogenesis and increased bioenergetics, as well as regulation of genes involved in apoptosis and cell cycle progression.

NRF1 expression in MCF-7 (or any ER$^+$ cell types) may not be entirely due to cell's ER status. Estrogens concentration of 10 nM has been reported to induce oxidative stress in MCF-7 cells [210;211]. In a similar manner, the pro-oxidative compound sodium butyrate [212;213] has also been shown to induce NRF1 expression in MCF-7 cells [214]. Sodium butyrate lacks estrogenic property. In addition, oxidative stress has been reported to induce NRF1 expressions in ER$^-$ breast cancer cell line such as MDA-MB-468 as well as in ER$^-$ human bone osteosarcoma (U2OS) cells [215]. Likewise, SERMs such as TAM, RAL and Fulvestrant which are also pro-oxidant upon chronic exposures [216], also increase NRF1 expressions in SERM resistant MCF-7 cells [217]. These studies suggest that estrogen inductions of NRF1 in estrogen responsive breast cancer cells are not exclusive to ER status. It implies that perhaps estrogen induced oxidants also mediate NRF1 expressions in breast cancer cells irrespective of ER status. This point is

important when considering mechanisms by which estrogen induce mammary carcinogenesis and growth of breast cancer cells.

This observation is supported by several findings. For example it has been reported that exposures of rat cardiomyocytes and liver cells to sublethal levels of pro-oxidative compounds such as LPS or t-BOOH induced oxidative stress, NRF1 expressions, mitochondria biogenesis, and promoted cell's survival and growth [193;218]. Conversely, silencing of complex II, one of the primary genes regulated by NRF1, inhibited OXPHOS, ROS production and augmented cell growth [219]. In similar manner, exogenous addition of oxidants to hepatoma cells depleted of mitochondria induced NRF1 expressions and cell proliferation [149]. These findings imply that oxidant induced NRF1 regulation promotes cell growth by coupling mitochondria bioenergetic with cell proliferation. This is further supported by findings in our laboratory that chronic exposures of non-tumorigenic, ER$^-$ mammary cells (MCF-10A) with 100 ng/ml estradiol or its catechol derivatives, 4-OH-E$_2$ but not 2-OH-E$_2$, induced oxidative stress, mammary transformations as well as up-regulations of NRF1. However silencing of NRF1 inhibited estrogen induced oxidative stress and inhibition of cell's transformation [49;50]. Similar observations were also reported in pre-malignant primary fibroblast cells. In these cell lines, it was observed that NRF1 and other mitochondria genes were among the transcription factors over-expressed during malignant transformation [220]. However in this study, high NRF1 expressions were likewise linked to oxidative stress and energy demand by proliferating cells. These studies imply that for cells to survive in elevated oxidative environment, they must be able to up-regulate NRF1 expression, which in turn

increases mitochondria biosynthesis and OXPHOS. However, it is counterintuitive to up-regulate pro-oxidative mechanisms during periods of oxidative stress. It is therefore speculated that high ROS levels serve other purposes such as clonal selection of neoplastic cells, and induction of apoptosis in non-neoplastic cells [221;222]. Similar mechanism might be in play during estrogen induced mammary carcinogenesis. In population of initiated cells, oxidative stress and NRF1 expression may serve to provide a pro-tumorigenic selective environment as well as the energy necessary for these cells to proliferate and grow, irrespective of ER status.

Though estrogens have been reported to modulate NRF1 expressions via E_2/ER binding to ERE on NRF1 promoter regions in a number of cell lines [206;207], we demonstrated that prolonged treatment of MCF-7 cells with estrogen also induced NRF1 expression, increased cell proliferation and colony formation in soft agar assays (Okoh et al, unpublished data). Interestingly, the silencing of NRF1 or pretreatment of cells with NAC significantly reduced NRF1 expression and inhibited estrogen induced cell proliferation and anchorage independent growth (Okoh et al, unpublished data). Conversely, overexpression of NRF1 in the same cell line increased cell proliferation independently of estrogen action. These findings from our laboratory therefore imply that NRF1 expression observed in MCF-7 cells treated with estrogen is redox based as antioxidants abolish NRF1 expressions. It also suggests that redox expressions of NRF1 due to estrogen exposure may contribute to breast cancer cell growth independent of E_2/ER/ERE mechanisms. If this is the case, it can be surmised that estrogen induced ROS contributes in part to growth of malignant cells in a manner unrelated to ER activations. This

assertion may therefore explain why oxidative stress is positively correlated with tumorigenesis [223;224], aggressive breast cancer phenotypes [225], and therapeutic resistance of breast cancer patients [226].

ROLE OF ESTROGEN-INDUCED REDOX SIGNALING IN THE DEVELOPMENT OF BREAST CANCER

ROS affects generation of new phenotypes of cells during the development of breast cancer through its influence on the regulation of protein kinases and signal transduction pathways. Here we describe how estrogen-induced ROS may contribute in the generation of different cell phenotypes required for the development of breast cancer.

Estrogen-induced ROS as potential mediator of cell transformation and generators of human breast cancer stem cells: A delicate intracellular interplay between oxidizing and reducing equivalents allows ROS to function as second messengers in the control of cell proliferation and cell transformation [227;228]. Many studies implicate ROS in cell transformation and in the uncontrolled growth potential of tumor cells [224;229-233]. We also find that estrogen stimulates the growth of TM3 and HEK 293 cells in an ER-independent manner [9;30]. In another study estradiol-induced kidney tumor incidence in Syrian hamsters treated with antioxidants was effectively reduced by ebselen, sodium 2-mercaptoethanesulfonate (Mesna, a cytoprotective thiol-containing agent), NAC and vitamin-C administration [17;18]. Also, cotreatment with pentoxifylline, an inhibitor of IL-1β and Tumor necrosis factor (TNFα) synthesis, prevents estrogen-induced testicular and uterine cancers [9]. Superoxide dismutases (SODs) are the first and the most

important line of antioxidant enzyme defense against ROS and particularly superoxide (O_2^-) radical. Overexpression of the enzyme responsible for mitochondrial superoxide detoxification, manganese superoxide dismutase (MnSOD), inhibits the malignant phenotype [234-236]. MnSOD knockout mice exhibit increased oxidative DNA damage [237]. Soini et al.,2001 reported that MnSOD expression is less frequent in the tumor cells of invasive breast carcinomas than in in situ carcinomas or non-neoplastic breast epithelial cells. Several epidemiologic studies have shown an increased risk of breast cancer in the MnSOD Ala-Val polymorphic populations [238;239]. In a recent study it was shown that genetic polymorphism in the MnSOD gene may be associated with increased risk of breast cancer among Chinese women with high levels of oxidative stress or low intake of antioxidants [240]. Studies also show that there is no positive relation between genetic variation in MnSOD and breast cancer [241]. Our preliminary study showed that MCF-10A cells treated with 4-OH-E_2 rapidly produce ROS, which is preceded by an increase in the level of Ca^{2+}. The accumulation of 4-OH-E_2 as recently reported in the human breast tissue in cancer subjects [46;242] will allow increased ROS and Ca^{2+} levels in the target organ of cancer in a sensitive subpopulation. Later, overexpression of catalase and MnSOD prevented 4-OH-E_2-induced anchorage-independent growth of MCF-10A cells. These data together indicate that 4-OH-E_2-induced cell transformation may be mediated, in part, by ROS signal transduction pathways that are different from the classical nuclear ER genomic signaling pathway (Fig. 4).

Estrogen-induced ROS as mediators of cell cycle progression: As shown in Figure 3, high ROS levels induce oxidative damage and arrests cells in G0/G1. However, low physiologic ROS levels are utilized by cells for cell-to-cell communication and in proliferation [28]. The rapid stimulation of intracellular ROS by PDGF, EGF, and NGF suggests that this underlying mechanism of cell growth may be shared by other growth factors or mitogens, including estrogen [243]. Exogenous addition of low concentrations of H_2O_2 and/or O_2^- has been demonstrated to stimulate cell growth in a variety of cell types including fibroblasts and prostate cancer cells [159]. Although these phenomena have been overshadowed by studies concentrating on the role of ROS in the pathology of various diseases and apoptosis, they suggest that outcomes other than apoptosis may be coupled to the signaling event represented by the estrogen-induced generation of ROS. Cell cycle progression is regulated by an intricate web of signaling pathways mostly requiring growth factor stimulation and ultimately involving cyclins, cyclin-dependent kinases (cdks), and cell cycle inhibitors. Constant activation of redox sensitive transcription factors appears to be one functional role of elevated ROS levels during tumor progression [198]. The results of our more recent studies show that ebselen (a substance with glutathione peroxidase-like activity), and inhibitors of mitochondrial replication and protein synthesis prevent estrogen-induced G1 to S transition. Low and intermediate levels of ROS regulate cellular signaling and play an important role in normal cell proliferation [196]. These data suggest that redox signaling supports cell proliferation in the presence of estrogen-induced ROS.

Several lines of indirect evidence support a role for mitochondrial oxidants in estrogen-induced cell proliferation. Isoforms of Trx, Trx-2 and thioredoxin reductase (TrxR2), which regenerates the reductant form of Trx, have been detected in mitochondria [89;244]. Dominant-negative inhibition of TrxR2 causes an increase in H_2O_2 accumulation, which activates cell proliferation [245] and increased expression of cell cycle genes involved in the G_1 and S phases of the cell cycle [244-247]. In vitro treatments of primary human endometrial stromal cells with estrogen at different concentrations (10nM-100nM) result in an increase in Trx protein and mRNA expression which implicates Trx in cell growth and differentiation of estrogen responsive tissue [247]. Alterations in the cellular redox status due to increased expression of TrxR2 have been suggested to play a role in the growth of hepatocellular carcinomas [248]. Whether estrogen can specifically increase the expression or activity of Trx2 and/or TrxR2 is not known, but these findings suggest that estrogen may modulate signal transduction of mitochondrial derived ROS via the thioredoxin system. Indirect evidence is also provided by the overexpression of the MnSOD enzyme responsible for mitochondrial ROS detoxification, which inhibits breast tumor growth [235]. As H_2O_2 is a highly diffusible signaling molecule and both MnSOD and NOS are modulated by estrogen [249;250], it is plausible that estrogen-induced ROS signaling at the level of mitochondria contributes to cell proliferation.

Many of the genes associated with high-risk breast tumors appeared to be involved in cell-cycle regulation, including Cdc2 and PRC1 [251]. Integrins through ROS control the expression of cell cycle genes; and cell adhesion and integrin ligation are obligatory

processes for regulation of cdks and cyclins [182-184]. Recently, an NRF1 and CREB pair has been identified as a key regulator of the transcriptional program of the cell cycle in human cells because this pair of transcription factors show a significant co-occurrence rate on promoters of cell cycle regulated genes [252;253]. NRF1 appears to play an important role as a regulator of cell cycle genes because NRF1 binding site has been identified by comparing genome-wide locations on genes involved in DNA replication, mitosis, and cytokinesis. Some of the genes which contain NRF1 binding sites on their promoters include Cdc2 [254], guanine-nucleotide exchange factor, RCC1, DNA polymerase-α, ornithine decarboxylase, GADD153, growth-arrest and DNA-damage-inducible protein153 [252]. Mammary carcinogenesis is characterized by an increased expression of NRF1, which may be linked, with increased energy demand by rapidly proliferating cells [255;256]. However, NRF1's role in mammary carcinogenesis may be more than mere energy modulators. NRF1 has been reported to be over-expressed in a number of malignant tissues including breast cancer tissues where its levels are higher in disease regions compared to adjacent normal regions suggesting that NRF1 may play a role in mammary carcinogenesis [256]. For example microarray study of genes expressed during mammary tumorigenesis in MMTV-neu mouse model reveals that NRF1 were among the transcription factors over-expressed in tumor tissues compared to adjacent normal tissue [257]. Similarly, another microarray analysis of NRF1 expression in human tissues reveals that while normal mammary tissue had more NRF1 expression than most normal tissues analyzed, (except for lung tissues), the levels of this transcription factor were highest in breast cancer tissues compared to other cancer types [256]. Lastly, NRF1 is amongst a few sets of transcription factors whose overexpression is correlated with

progression of mammary carcinogenesis [255]. While these findings were attributable to increased energy demand, other evidence exist that suggest that these transcription factors may directly or indirectly regulate genes involved in cell proliferation in manners unrelated to mitochondria biogenesis. Whether NRF1's role in these malignant cells is indicative of metabolically active cells or whether it also regulates growth related genes that promotes malignancy is yet to be investigated. NRF1 may also play major roles in modulating cell proliferation in a manner not related to mitochondria biogenesis.

NRF1 regulates many other genes that may be involved in the development of breast cancer than that described above. For instance, there is a striking overlap in target genes between E2F4 and NRF1 [252;255]. E2Fs are target of both estrogen and oxidative stress [258;259]. There is a functional interaction of E2Fs with other transcription factors, such as FOXO1, hypoxia-inducible factor 1 (HIF-1), nuclear factor B (NFB), and peroxisome proliferator-activated receptor (PPAR), including NRF1, during oxidative responses in cells. NRF1 has been reported to transcriptionally regulate the expression of E2F1 [260] and E2F6 [215] genes. Both genes belong to E2F family of transcription factors which functions in cell cycle controls. While E2F1 is generally considered as a transcription factor activator and E2F6 as a transcription factor repressor, the coordinated activities of these genes are essential in regulating cell cycle control and to have a major role during carcinogenic process [261]. Overexpression of E2F1 in mammalian cells has been reported to induce neoplastic transformation of rat embryo fibroblasts, induce colony formation in soft agar assay and induce tumor in nude mice [262;263]. Conversely, under-expression of these same genes has likewise been shown to induce broad range of

mice tumors as well [264;265]. This seemingly contradictory observation of E2F1 expressions highlights the tight regulations NRF1 must undertake to keep E2F1 gene expression at 'normal' physiological levels. E2F6 is a member of the E2F family of transcription factor involved in regulation of cell proliferation and differentiation via target genes involved in DNA replication, DNA repair, cell cycle control and apoptosis [261;266;267]. E2F6 expressions in cells induces growth by inhibiting cell's exit from the S phase, and delays re-entry into the cell cycle in quiescent cells [268;269]. Growth arrested cells due to delayed cell cycle could accumulate pre-malignant genetic lesions and cell transformations potentials as DNA repairs and proof reading also ceases during quiescence.

Findings that the proliferative growth of breast cancer cells exposed to estrogens activate E2F1 expressions an ER independent manner provide further support to the above concept [270]. Of interest in this study is the finding that overexpression of ER in ER⁻ breast cancer cells (MDA-MB-231) did not induce cell proliferation upon estrogen treatment even though E2F1 was mildly activated [270]. Similarly, it was shown in ER⁺ ZR-75 cells that ER/Sp1/NFY interactions are not necessary for estrogen-induced transactivation [259]. These findings indicate that ER status is not the sole determinant of breast cancer progression and growth due to estrogen exposures. It should be noted also that another study with similar conditions had observed that MCF-7 cells exposed to estrogen increased NRF1 and mitochondrial expressions via ER activation [206]. Both of these in vitro studies with MCF-7 cells may therefore serve to elucidate the mechanism by which estrogen induces growth of breast cancer cells, i.e. estrogens induced oxidants

mediate NRF1 expression which may regulate E2F1 expression and promote breast cancer cell growth.

Other than binding to promoter regions of target genes involved in mitochondria biogenesis and OXPHOS, NRF1 also modulates genes involved in mitochondria regulations. For example, Nrf-2 (NFE2L2), a redox sensitive gene known to regulate antioxidants responses, has been reported to bind to antioxidant response elements on NRF1 promoter region to regulate ROS production as well as induction of antioxidant responses in cardiomyocytes overexpressing heme oxygenase (HO)-1 [271]. Likewise, cMyc overexpressions and induction of apoptosis in NIH 3T3 cells depleted of serum is also reported to be mediated by NRF1 activations [272]. Hormones such as Thyroid hormone (T_3) and estrogens (E_2) have also been reported to upregulate NRF1 expressions and mitochondria biogenesis through binding to responsive elements in NRF1 promoter regions [273;274]. NRF1 has also been reported to transcriptionally regulate expression of myocyte enhancer factor 2A (MEF2A) which is a muscle specific gene [275]. MEF2A is a DNA-binding transcription factor that activates muscle-specific, growth factor-induced, and stress-induced genes such as the cytochrome c oxidase (COX) subunits 6A (COX6A). Interestingly, MEF2A also functions in skeletal and cardiac muscle development as well as in neuronal cell differentiation and survival. They also play diverse roles in the control of cell growth, survival and apoptosis via p38 MAPK signaling pathway in muscle cells [276]. Dysregulation of MEF2A is implicated in Chinese patients with coronary artery disease [277;278] and in hepatocellular carcinoma [279].Our recent study showed that co-exposure of inhibitors of mitochondria shown to

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control mitochondrial oxidant production prevents E_2-induced expression of cell cycle genes, cyclin B1, PCNA, and PRC1 containing NRF1 binding sites, and caused loss of NRF1 expression and growth delay. The E_2-induced stimulation of the expression of NRF1 and these cell cycle genes, and growth were restored by removing exogenous mitochondrial inhibitors that diminished oxidant exposure indicating that oxidative stress stimulates these cell cycle genes in part via NRF1 activation. Cyclin D1 has recently been reported to inhibit NRF1 expression which leads to reduced mitochondria biogenesis in mouse embryo fibroblasts (MEFs) [280]. Overexpression of cyclin D1 is usually associated with cancer cell growth [281]. While it is counter intuitive that rapidly proliferating cell such as MEF would inhibit mitochondria biogenesis which is a major source of energy production, proliferating cells do generate excess ROS because they are metabolically active. Excess cellular ROS are known to have adverse effects of cell's fate ranging from apoptosis to growth retardation [228]. Therefore it is plausible that cyclin D1 expression that drives cell cycle progression could also function to reduce mitochondria activity during periods of excess ROS. Hence the inhibition of NRF1 expression by cyclin D1 may be a negative feedback mechanism that protects the cell from harmful effect of mitochondrial derived ROS. This assertion is supported by the fact that cyclin D1 expression is likewise upregulated by mitogenic E_2 [282;283] as well as by ROS [284].

Estrogen-induced ROS as a mediators of breast cancer progression: E2F1 is over-expressed in breast tumors when compared with normal mammary tissue, and that patients with high E2F1-expressing tumors have significantly reduced disease-free status

and overall survival compared with patients with low-expressing E2F1 tumors [285-289]. It should be also noted that NRF1 expressions are positively correlated with oxidative stress and progressions of mouse or human mammary carcinogenesis [255-257]. E2F6 on the other hand are transcription factor repressors and function mainly in growth inhibition [261;268]. Interestingly, one of the target gene of E2F6 is the breast cancer gene BRCA1 [290]. BRCA1 regulates cell cycle progression, apoptosis, and DNA repair, and loss of BRCA1 expression is linked to cell proliferation and transformation [291]. While BRCA1 overexpression has been reported to confer therapeutic resistance, BRCA1 deficiency confers sensitivity to oxidative stress [292]. In addition, BRCA1 mutations are associated with breast cancer progression [290]. Therefore, NRF1 dysregulation of E2F6 can lead to BRCA1 dysfunctions with adverse consequences ranging from apoptotic cell death to malignant transformation of breast epithelial cells.

In addition to regulation of E2F family of transcription factors, NRF1 is also known to regulate genes that favor breast growth and metastasis. For example NRF1 tightly regulates the expression of calpain proteases in normal cells [293]. Overexpression of this protolytic enzyme mediates tumorigenesis, metastasis and angiogenesis in breast cancer and other cancer types [294;295]. NRF1 also regulates expression of integrin-associated protein [296]. Integrins are essential for a cell's adhesion during normal development. Dysregulation of intergrin has been shown to promote carcinogenesis in mammary cells as well as other cells types [297]. Lastly, NRF1 has also been reported to regulate proliferation and growth of breast cancer in response to estrogen via activations of p115, membrane tethering protein involved in mammary development [298].

The DNA binding domain of redox sensitive NRF1 protein has been found in the regulatory regions of over 600 human genes [254]. Interestingly, while NRF1 expression is usually associated with modulating energy demand in proliferating cells, it is equally expressed in quiescence cells albeit by different mechanisms [254]. NRF1 is ubiquitously expressed in skeletal muscles, heart muscle, brown adipose fat, kidney and liver cells, and all cells with high energy demand [82;299;300]. NRF1 has been implicated in regulating genes unrelated to mitochondria biogenesis as well. For example, NRF1 transcriptionally regulates expression of CXCR4, a co-receptor for human immunodeficiency virus infection [301], GluR2, a neural subunit that controls Ca^{2+} permeability of AMPA receptors [302], CD155, a human polio virus receptor that functions in cell adhesion [201], the X gene of hepatitis B virus (HPV) which functions in HBV replication in vivo [303], integrin-associated protein which plays significant role in neurite outgrowth [296], GalNAc-T3 genes that function in glycosylation of mucin proteins in epithelial derived tumors [214], fragile X mental retardation 1 (FMR1) genes [304], and visinin-like protein-1 (VSNL1), a neuronal Ca^{2+} sensor protein [305] whose dysregulation is implicated in Alzheimer disease and schizophrenia [306], as well as cancer promotion and metastasis [307] .

These findings suggest that redox sensitive NRF1 directly or indirectly participates in the processes that favor tumorigenic conversion and aberrant cell growth either by modulating energy demand and/or modulating expressions of genes involved in cell cycle controls. While NRF1's actions on these genes are associated with normal physiological function, their dysregulation may lead to increased apoptosis or cell growth. In situations

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where NRF1 expressions or functions are dysregulated by estrogen or estrogen induced oxidative stress, downstream genes likewise can be modulated and manifest adverse effects on cell's fate. These effects may range from induction of apoptosis, to malignant transformation and growth of cancer cells. In mammary cells where aberrant cell growth or apoptosis that have been pre-initiated during estrogen metabolism and are under oxidative stress, oxidant induced NRF1 expression could further enhance excess growth in subsets of initiated cells.

Estrogen induced ROS, NRF1 and therapeutic resistance: Redox sensitive NRF1 may play an important role in therapeutic resistance of breast cancer cells. Drug resistance of a number of cancer cells are characterized by oxidative stress and metastasis [224;308;309]. Tumors contain a subset of cells that both self renew and give rise to differentiated progeny. The self renewal properties of these cells are the real driving force behind tumor growth, metastasis and recurrence as they are responsible for tumor escape from conventional therapies resulting in disease relapse. Oxidative stress induces apoptosis in a number of cells but in drug resistant cancer cell, the opposite is true; oxidative stress facilitates malignant growth and metastasis of breast cancer cells by activating MMPs such as collagenases (MMP-2) and inhibiting antiproteases such as tissue inhibitory metalloproteases TIMP [310;311]. Interestingly high mitochondria content and oxidative stress are not only associated with breast cancer risk, they are also reported to be positively correlated with stages of cancer and therapeutic resistance [308;312;313]. Whether excess NRF1 expression mediates chemoresistance and malignant growth of cancer cells or whether increased oxidative state observed in cancer

cells are the manifestation of metabolically active cells is unknown. For example, treatment of several cancer cells lines with natural or synthetic flavonoids have been shown to restore chemo-sensitivity of these cells to cancer drugs [314;315]. Similarly, antioxidants have also been demonstrated to improve efficacies of chemo and radio-therapies in several types of human cancers [316-320]. While some epidemiological studies confirm the effectiveness of antioxidant supplementation [321], many studies see no effect [322]. Similarly several in vitro and in vivo studies have shown that antioxidants can improve drug efficacies [323]. Antioxidants when enhance the anti cancer drug effects in vitro and in animal models, implies that lowering cancer redox status may be pertinent to its therapeutic response. If that is the case, then NRF1 modulation of cellular redox status via mitochondria regulation may be central in oxidant mediated chemoresistance and malignant growth of cancer cells.

The MDR1 gene (multidrug resistance gene 1) is a membrane glycoprotein that functions in effluxing drugs from within cells in a number of malignant cell types [324]. This gene is expressed in 42% of breast cancer cases and patients with tumors expressing MDR1 gene are three times more likely to experience chemotherapy failures than patients whose tumors were MDR1 negative [325]. TAM, one of the most popular drugs used to treat breast cancer and which have a high failure rate after prolong usage has been reported to up regulate MDR1 gene expression in breast cancer cells [326]. TAM exposures have been shown to increase NRF1 expression in MCF-7 breast cancer cells as well [327]. Interestingly, NRF1 is a transcriptional regulator of MDR1 gene expression in resistant oral cancer cells and in acute lymphoblastic leukemia (ALL) [328;329]. This could be a

mechanism that integrates drug induced oxidative stress, NRF1 expression and chemotherapeutic resistance in a number of cancers including breast cancer. While evidence of such association has not been reported in drug resistant human breast cancer cases, the rationales for such investigations are merited. There are indications that NRF1 expression may correlate with different stages of breast cancer causing possible therapeutic failures [255;330].

SUMMARY

Until recently, it has been argued that hydroxylated metabolites of estrogen (catechol estrogens) participate in redox cycling in the endoplasmic reticula of cells and during this redox cycling of hyroxylated estrogens, ROS are generated. These redox reactions may occur in vivo, if free hydroxylated metabolites are in close proximity of activating enzymes within the target cells. Studies recently showed that estrogens or estrogen metabolites directly acting on mitochondria of epithelial or immune cells generate ROS. Based on the dose and time of induction, the ROS, seem to play dual functions in epithelial cells. While higher doses of ROS induce oxidative damage in the genome of cells leading to cell apoptosis, exposure of low levels of ROS produce genomic instability as well as transduce signals for cell growth, cell transformation and cell invasion. This view is consistent with findings that (i) estrogen-induced ROS can lead to increased phosphorylation of kinases, such as ERK, AKT, with this effect being attributed to the redox regulation of redox-sensitive phosphatses. Some of the nongenomic pathways by which estrogen activates MAPK and AKT pathways can be explained based on estrogen-induced ROS transducing signal to their respective specific phosphatses. (ii) The

transcription factors AP-1, NRF1, E2F, NFkB and CREB are responsive to both oxidants and estrogen. It is possible that estrogen-induced ROS transduce signals to the nucleus for the activation of transcription factors such as AP-1, CREB, E2F, NF-kB, and NRF1 to regulate their downstream target genes involved in cell transformation, cell cycle, migration and invasion (Figure 4). (iii) There is growing evidence that estrogen-induced regulation of breast cancer cell cycle is coupled to rates of cellular metabolism, ROS levels and mitochondrial mass. (iv) Inhibition of cell transformation, growth of breast cancer cells and tumor formation by overexpression of ROS detoxifying genes and co-treatment with antioxidants supports the role of ROS in estrogen-dependent breast cancer. (v) Estrogen-induced ROS by increasing genomic instability and by transducing signal through influencing redox sensitive transcription factors contribute to initiation as well as progression of breast cancer. It is envisioned that this is an important mechanism that drives the carcinogenesis process, but that it occurs in the context of other processes such as ER-mediated signaling and estrogen reactive metabolite-associated genotoxicity under investigations that may also contribute to the process. Taken together, estrogen-induced ROS mediated redox signaling seems to be complementary to the process of ER mediated cell transformation, growth and carcinogenesis. On the other hand, estrogen-induced ROS can function as independently reversible modifiers of phosphatases and activate kinases to trigger the transcription factors of target genes which participate in cancer progression. Further elaboration however, points to a convergence of both ER mediated signaling and E_2 induced ROS signaling effects on the transcription factors which possibly act in synergy and contribute to the growth and cancer progression.

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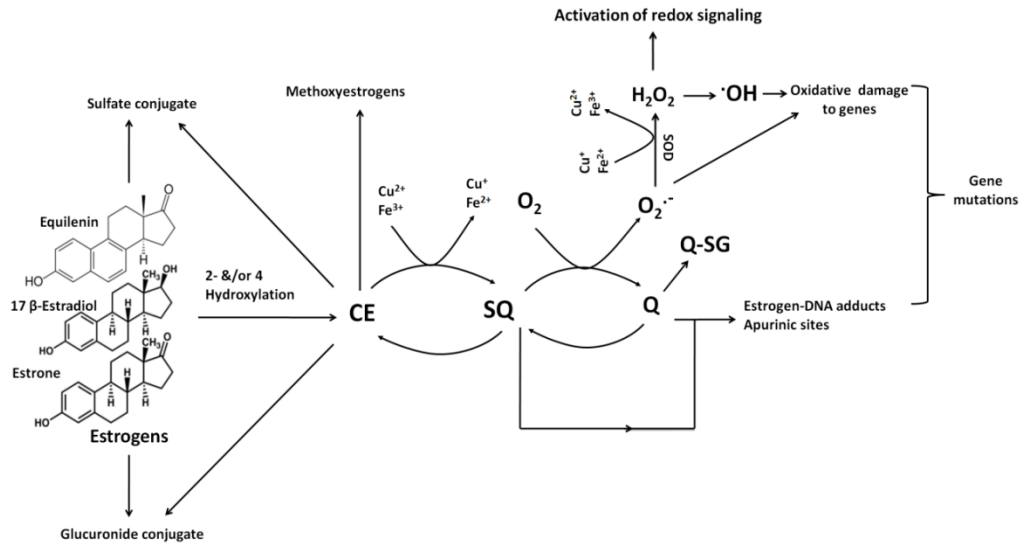
Figure 1. Scheme showing metabolism of estrogen and redox cycling of its metabolites. CE = Catechol estrogen, SQ = Semi quinone, Q = Quinone, Q-SG = Quinone-glutathione conjugate.
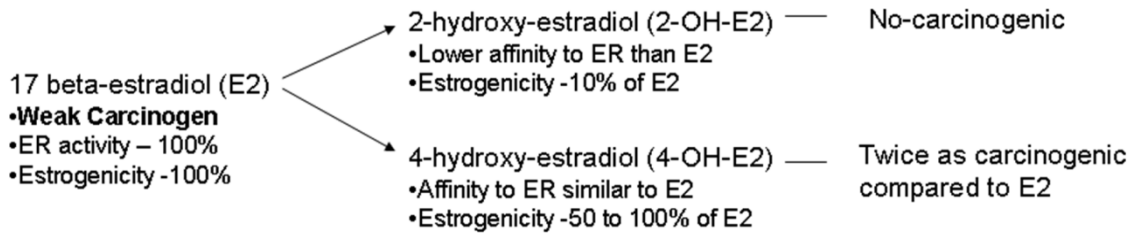

Figure 2. Estrogen induced cellular responses including carcinogenicity and growth of cancer cell do not   correlate with the binding affinity of various estrogens to estrogen receptor (ER) and their potency both in vitro and *in vivo*
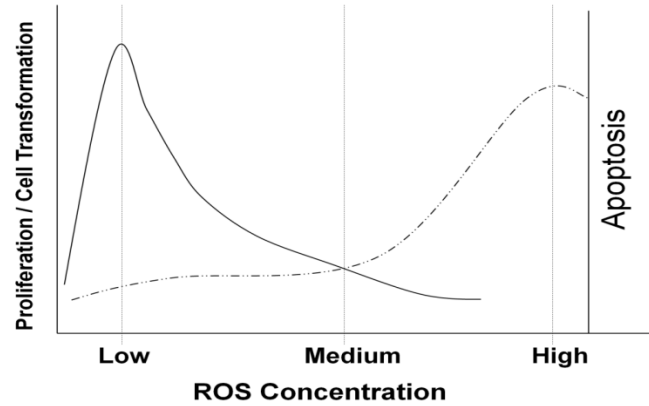
Figure 3. Dual Roles of ROS: High ROS levels induce oxidative damage and produce cell death, where as low physiologic ROS levels facilitate cell-to-cell communication and cell proliferation
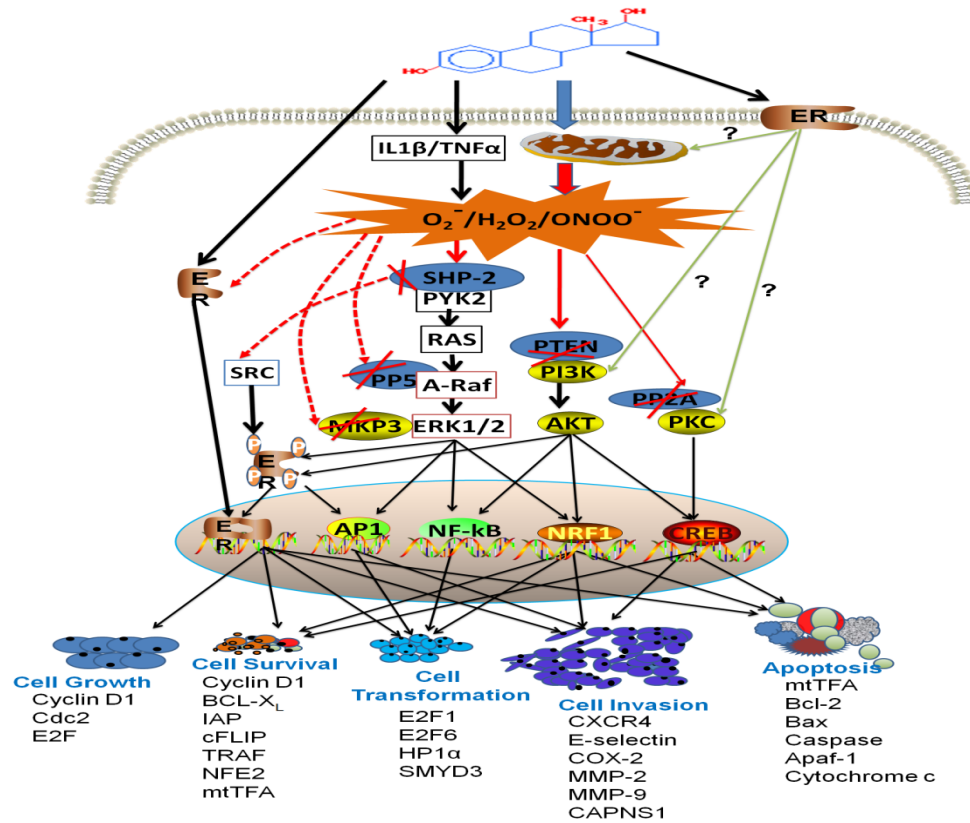

Figure 4. Mechanism of ROS signaling in coordinated inactivation of phosphatases and activation of kinases leading to estrogen-mediated changes in the expression of genes involved in growth, apoptosis, transformation or invasion of cells

CHAPTER IV

REACTIVE OXYGEN SPECIES CAUSES NEOPLASTIC TRANSFORMATION
AND XENOGRAFT GROWTH OF 4-HYDROXY ESTRADIOL-TRANSFORMED
MAMMARY EPITHELIAL CELLS VIA TRANSDUCTION OF REDOX SIGNALS
TO PI3K/AKT PATHWAY

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ABSTRACT

Unopposed free estrogen (estrogen without progesterone) is a major risk factor for breast

tumors. The mechanism by which estrogen is involved in the development of malignant

breast lesions is not clear. We found that reactive oxygen species (ROS)- induced by

repeated exposures to 4-hydroxy-estradiol, a predominant catechol metabolite of 17 β-

estradiol, caused transformation of immortalized human mammary epithelial cells. This

was evident from inhibition of growth of tumors by overexpression of catalase as well as

co-treatment with ebselen. Assessment of 4-hydroxy-estradiol transformed clones in 2-D

and 3-D cell cultures showed that they formed spheroid structures in various matrices as

well acquired invasive properties. During 4-OH-E2-mediated malignant transformation

process increased AKT phosphorylation through PI3K activation was observed. This

PI3K-mediated phosphorylation of AKT in 4-OH-E2-induced malignant cells was

inhibited by ROS modifiers as well as by silencing of AKT expression. RNA interference

of AKT markedly inhibited 4-OH-E2-induced in vitro tumor formation. Among the genes

being significantly up-regulated during the 4-OH-E2-mediated malignant transformation

process well known proliferating cell nuclear antigen (PCNA) as well as the cell cycle

genes cyclinB1 and Cdc25c9 were identified. The expression of these genes was

inhibited by ROS modifiers as well as by silencing of AKT expression and NRF1, a transcription factor that controls mitochondrial biogenesis (a major source of ROS). These studies demonstrate that: (i) 4-hyroxy-estradiol is one of the main estrogen metabolites that induce mammary tumorigenesis and (ii) ROS-mediated redox signaling leading to the activation of PI3K/AKT signaling plays an important role in 4-hydroxy-estradiol-induced malignant phenotype of breast epithelial cells. In conclusion, ROS are important signaling molecules in the development of estrogen-induced malignant breast lesions that may be useful as therapeutic targets for prevention and treatment of breast cancer.

INTRODUCTION

Elevated lifetime estrogen exposure is a well-known major risk factor for breast cancer. A large body of epidemiological and experimental evidence points to a role for estrogen in the etiology of human breast cancer (1-9). In experimental models, estrogens are complete breast carcinogens, as they are capable of initiating and triggering growth and selection to generate palpable malignancy (8-14). However, the signaling mechanisms by which estrogen contributes in the initiation of breast cancer remain the subject of a long-standing controversy. This is due, in part, to the inability to resolve whether estrogen or estrogen metabolites are procarcinogenic. 17β-estradiol (E2) is metabolized to 2- and 4-hydroxy-estradiols by cytochrome p450s. We have previously shown that E2-induced renal tumor formation is decreased in animals exposed to inhibitors of estrogen metabolism or to hormonally potent estrogens undergoing reduced metabolic

conversion to catechol metabolites compared to E2 (10-12, 15). Russo and others have shown that E2 or 4-OH-E2 transform normal ERα negative breast epithelial MCF-10F cells (17-20). 17ß-estradiol-induced transformed MCF10F cells formed tumors in SCID mice. 4-OH-E2 is twice as capable of producing anchorage-independent growth as E2 in MCF10F cells (18,20). In contrast, neither 2-OH-E2 nor 2-OH-E1 are carcinogenic in vitro or in vivo (15). Similar results have been reported in MCF-10A cells exposed to 17 beta-estradiol, equilenin or its catechol metabolite, 4-hydroxyequilenin (21,22). Recently, Parks et al (2009) demonstrated that treatment of MCF-10A cells with 4-OHE2 induced in vitro transformation of cells (23). However, these studies in MCF-10A failed to show in vivo tumorigenicity, invasiveness or display other salient neoplastic properties. In the present study we have established that repeated exposure of 4-OH-E2 to MCF-10A produced tumors both in vitro and in vivo.

Induction of estrogen receptor (ER) upon estrogen exposure is not sufficient for the development of breast cancer. Recent studies indicate that mammary tumors can develop in the absence of a functional ERα (24). Although tamoxifen and other antiestrogens are thought to prevent cancer through their actions at the ER, other mechanisms cannot be ruled out as these compounds also block metabolism and redox cycling of estrogen and are free radical scavengers (25). 4-OH-E2 induces an estrogenic response in the uterus of ERα null mice, and this response is not inhibited by the antiestrogen ICI182780 (26). These findings suggest that estrogen-dependent growth of cells is regulated not only by nuclear ER-mediated genomic signaling pathways, but also by non-ER pathway(s). We believe that genomic and non-genomic actions of estrogen produce complementary

effects that are required for cellular transformation. Physiologically achievable concentrations of estrogen or estrogen metabolites directly acting on mitochondria of mammary epithelial or immune cells generate reactive oxygen species (ROS) (27). We previously showed that 17-β estradiol (E2)-induced DNA synthesis in breast cancer MCF-7 cells depends on mitochondrial oxidant signaling (28). In this study, we have extended our efforts on understanding how an E2 metabolite, 4-OH-E2 produces malignant phenotype through ROS signaling. We investigated whether the susceptibility of normal breast epithelial MCF-10A cells to neoplastic transformation by estrogens depends on ROS-mediated redox signalings. We present here for the first time that oxidants induced by E2 and 4-OH-E2 but not 2-OH-E2 exposures mediate in vitro transformation of MCF-10A cells. 4-OH-E2 transformed cells are not only tumorigenic in mice but also display invasive properties as well as proliferate independent of growth factors. Co-treatments of 4-OH-E2 transformed cells with biological or chemical ROS scavengers, or silencing of Akt1 prevented tumorigenic conversion of MCF-10A cells. We propose a mechanism of tumorigenic conversion that involves oxidant-mediated activation of redox sensitive PI3K/AKT signaling.

MATERIALS AND METHODS

Reagents

17β-Estradiol (E2), 2-hydroxyestradiol (2-OH-E2), 4-hydroxyestradiol (4-OH-E2), Ebselen, N-acetyl-cysteine (NAC), and dimethylsulfoxide (DMSO) were all purchased from Sigma (St Louis, MO, USA). All antibodies; PI3K (p110), phospho PI3K (p85),

phospho-AKT (ser 473) and total AKT antibodies were purchased from Cell Signaling Technology Inc. (Boston, MA). All tissue cultures reagents were purchased from Invitrogen Corporation (CA) unless otherwise specified.

Culture of MCF-10A cells and Adenovirus gene transfer

Human mammary epithelial cells (MCF-10A) were obtained from American Type Culture Collection (ATCC) and were routinely cultured in phenol red-free DMEM-F12 media (1:1) supplemented with 5% horse serum, hydrocortisone (0.5 μg/ml), insulin (10 μg/ml), epidermal growth factor (20 ng/ml), 100 ng/ml cholera toxin and penicillin-streptomycin (100 μg/ml each) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cell culture media, serum, antibiotics, and growth supplements except cholera toxin (Calbiochem, La Jolla, CA) were purchased from Invitrogen Corp CA). For experimental purposes, culture media were changed to starvation media (serum free media + antibiotics) and allowed to incubate for 48 hrs prior to commencement of most experiments, unless otherwise indicated. Serum deprivation synchronizes cells in the G_0/G_1 phase of the cell cycle.

The Adenovirus-CMV (empty vector), Adenovirus-MnSOD (AdCMVMnSOD), and Adenovirus-Catalase (AdCMVCat) constructs were purchased from ViraQuest, Inc. (North Liberty, IA, USA). The adenovirus constructs used were replication-defective, E1- and E3-deleted recombinant adenovirus (29). Inserted into the E1 region of the adenovirus genome was either the human MnSOD or catalase gene, both of which are driven by a cytomegalovirus promoter. Cells were seeded in plates at 15%-70%

confluence. The following day, cells were infected with adenoviruses over-expressing MnSOD or catalase or vector at 100 MOI in serum free media. Control cells were treated with 100 MOI of the adenovirus-CMV construct. This viral load was determined to achieve greater than 50% growth arrests of MCF-10A cells without significant cell death for the duration of experiment. Infected cells were cultured for 48 hrs after which cells were used for experiments.

Akt$_1$ RNAi transfections

Pre-designed and verified human shRNA for Akt$_1$ and corresponding null vectors were purchased from OriGene (OriGene Technologies, Inc. Rockville, MD). Transfections of cells were carried out in a sub-confluent cell population using FuGENE 6 (Roche) transfection reagents according to the manufacturer's protocol. Briefly, MCF10A cells were seeded in 6 well plates with growth factor supplemented media (SM) overnight. Post seeding, cells were transfected with 2 µl of Fugene-6 (Roche) preincubated for 20 min at room temperature with 0.5 µg plasmid RNAi or its null controls (sham). Forty eight hrs post transfection, media were changed to serum-free (starvation media) media and incubated for an additional 48 hrs, after which cells were used for various experiments. Transfection efficiencies ranged between 60-80% as quantified by decreased protein expression levels.

Cell viability assay

CellTiter-Fluor™ Cell Viability kit was purchased from the Promega Corporation and used according to manufacturer's instructions. Briefly, cells were seeded in 96 well plate

at a density of 1.0×10^4 cells/well, serum starved for 48 hrs and treated with estrogens or ROS modifiers. At the end of treatment procedure, substrate reagents (GF-AFC) were mixed with substrate buffer and dispensed into wells. This assay measures protease activity in live cells as opposed to MTT or MTS assay kits that measure formation of formazon crystals by mitochondrial enzymes. Plates were read on a fluorescence plate reader at 380–400 nm excitation and 505 nm emission and data is expressed as mean of three experiments +/- SD.

Cell transformation

The cell transformation was carried out by a modified protocol of Russo's group (18). Briefly, MCF-10A cells were seeded at 30% density in a 10 cm dish. After 24 hrs of seeding, media were replaced with stavation media and allowed to culture for 48 hrs, and then cells were subjected to two treatment cycles with E2 or its catechol metabolites. A treatment cycle includes a 48 hr starvation period, 48 hr treatment period (100 ng/ml of either E2, 2-OHE2, and 4-OHE2), and 48 hr recovery period in growth media containing 10% horse serum (HS) and no growth supplements. At the end of two treatment cycles, cells that would be used for immunoprecipitation and Western blot analysis were treated for an additional 30 mins with estrogens, lysed with RIPA buffer, immunoprecipitated and processed for western analysis. For anchorage independent growth assay 5000 cells/well were used for colony formation assays in soft agar.

Anchorage independent growth

Anchorage independent growth, an indicator of neoplastic transformation of cells, was assessed as previously described by Zhou et al (30). Briefly, base support agar were made fresh by diluting 1.0% molten agarose mixed with 1:1 2x culture media (2x DMEM/F12 media, 20% HS, 2x Penicillin-Streptomycin and 200 pg/ml estrogens) to a final 0.5%. Molten agar was left at 42 ^{o}C in a water bath until dispensed at 200 ul/well in 48 well plates, then allowed to solidify for 4 hrs at room temperature. Top agarose overlay was made fresh by mixing 0.7% molten agarose with 2x culture media containing appropriate 5000 cells/well, then gently overlaid over base agar. Cells were incubated for a minimum of 21 days in a 37°C incubator with 5% CO_2. Cultures were fed every week with top agar layer and colony formation was assumed when cell masses were 100 micron or greater as measured on a Nikon TE2000U inverted microscope (Nikon Corp., USA) with Metamorph software (Universal Imaging, USA). Images were acquired by using an Olympus C-5060 digital camera attached to the Nikon TE2000U inverted microscope with a 4x objective. Four wells were enumerated for each group and data expressed as mean of five wells +/- SD.

Invasion and ductulogenic assays

In order to determine invasiveness of transformed cells, several colonies were aseptically picked, dissociated with trypsin and cultured for 20-30 passages in a low growth factor media (DMEM F12, 5% FCS, 4 ng/ml EGF, 2.0 ng/ml insulin, 100 ng/ml hydrocortisone, 1x Penicillin-Streptomycin) and eventually DMEM/F12 with 5% HS media, and 1x Penicillin-Streptomycin. Cells (5.0 x 10^3 cells/ml) for invasion assays were seeded over

8 μm pore transwell filter insert (Transwell, Coastar Cambridge, MA) precoated with Matrigel (Collaborative Research, Bedford, MA). Chemoattractants used were reduced growth factor supplemented media or media with 10% FBS media positive control cells MDA MB 231. Matrix invasion was allowed for 16 hrs at 37 °C in a CO_2 incubator. The non-invaded cells inside chambers were wiped off with a cotton swab, and the filters were fixed, stained by Diff Quick (Sigma, St. Louis, MO), cut out and mounted onto glass slides. The total number of cells that crossed the membrane were counted under a light microscope, enumerated and expressed as fold increase compared to parent cell line. The experiments were repeated five times and results are expressed as the mean ±S.D.

For ductulogenesis, 1.0×10^3 cells/ml of transformed cells (p121 and screened from 3x Matrigel), MDA MB 231 and parental MCF-10A cells were mixed with collagen (Collagen Co., Palo Alto, CA, USA) and seeded in chamber slides precoated also with collagen. Cells were incubated for three weeks with bi-weekly feeding with 5% HS media. To confirm spheroid formation from collagen matrix, we diluted HuBiogel, a human matrix mimetic (VIVO Biosciences Inc.), 1:3 with media and coated 0.22 micron pore transwell filter inserts for 6 hrs at 37 °C incubator. Cells (1.0×10^3) were seeded into each insert and chemoattractant media (DMEM/F12, 5% HS, 1x Penicillin-Streptomycin) were added at bottom of insert. Cells were cultured for 14 days with media changed twice weekly. Images were acquired with a Olympus C-5060 digital camera under an inverted microscope with 4x objective as described above.

Chemical antioxidant treatments

The treatment procedure for Ebselen (a glutathione peroxidase mimetic which also removes both H_2O_2 and peroxynitrite) or NAC (a precursor of glutathione and scavenger of ROS) (31, 32) varies according to the experiments design. For all experiments, 40 μM Ebselen and 1.0 mM NAC were used for cell treatments. For example, in DCF assays, antioxidants were pre-loaded onto cells for 2-4 hrs before ROS measurement commences. For BrdU assays, cells were cultured with the chemical antioxidants throughout the experimental procedure. For transformation regimen, antioxidants were applied to cells each time cells were treated with estrogens. For anchorage independent growth assays, antioxidants were added to soft agar matrix media and during weekly feeding of colonies.

Measurement of reactive oxygen species (ROS)

Cellular ROS were measured on a 96 well plate reader and confocal fluorescence microscopy as previously described by Felty et al (27). Briefly MCF-10A cells were seeded at a concentration of 1.0×10^4 cells per well in black 96-well flat bottom plates (ThermoFisher Scientific Inc. USA) and allowed to adhere overnight. Post seeding, cells were serum starved for 48 hrs after which they were pretreated for 4 hrs with chemical antioxidants Ebselen or NAC (Sigma USA) diluted in Hank's balanced salt solution (HBSS) followed by incubation with 10 μM 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Invitrogen Corp) for 20 min. Cells were rinsed with HBSS followed by various estrogen treatments as described in the figure legends. DCFH-DA is a non-fluorescent cell-permeable compound, which is acted upon by endogenous esterase that removes the acetate groups generating DCFH. In the presence of intracellular ROS, DCFH is rapidly

oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF). The oxidative products were measured with a Tecan Genios 96 well microplate reader using 485 and 535 nm as excitation and emission filters respectively or fluorescence images were acquired on a Nikon TE2000U inverted fluorescence microscope equipped with a Nikon D-Eclipse C1 laser scanning confocal microscope system (Nikon Corp., USA). The built-in Nikon EZ-C1 software was used for confocal image acquisition and analyses. DCFH-DA stock solutions were diluted at a 1:1 ratio with Pluronic F-127 (20% w/v). Data are expressed as mean of three experiments +/- SD.

Immunoprecipitation and Western Blot Analysis

After the respective treatments, cells were rinsed twice with ice cold phosphate buffered saline (PBS), harvested with lysis buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% Nonidet P-40, 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (Roche). Samples were diluted to 500 µg of protein in 1 ml of lysis buffer, and pre-cleared for 1 hr at 4 °C with 10 µl of 1:1 slurry of protein A-agarose beads (Invitrogen Corp) in lysis buffer. After a brief centrifugation to remove pre-cleared beads, 2 µg of desired capture antibodies were added to each supernatant and incubated on a rocking platform at 4 °C overnight and captured proteins were precipitated with 40 µl of protein A-agarose beads for 2 hr. The beads were washed five times with lysis buffer and resuspended in 40 µL sample loading buffer, subjected to electrophoresis and electro-blotted onto a PVDF nylon membrane. Primary antibodies used for Western blots were diluted 1:1000 in phosphate buffered saline Tween-20, PBST and horseradish peroxidase-conjugated secondary antibodies were diluted 1:50,000 in PBST. Blots were

treated with ECL reagents (Amersham Biotech), and proteins were detected by autoradiography. Band intensity was quantified with Bio-Rad Gel Doc Imaging System.

Immunofluorescence labeling

MCF-10A cells were seeded and treated in chamber slides as indicated in legends to the figures. Post treatment, cells were fixed with ice cold methanol for 15mins, and permeabilized with 0.5% Triton X-100 for 30 mins. Cells were blocked with 1% normal goat sera for 1 hr after which they were probed with antibodies diluted 1:500 for Akt and 1:500 for phospho AKT. Alexa Fluor labeled secondary antibody directed against AKT antibody was diluted 1:1000. The confocal fluorescence images were scanned on a Nikon TE2000U inverted fluorescence microscope equipped with a Nikon D-Eclipse C1 laser scanning confocal microscope system (Nikon Corp., USA). The z-series scanning was done at every 1 μm up to a z-depth of 10 μm by using a Nikon 40 x 1.30 NA DIC H/N2 Plan Fluor oil immersion objective. The built-in Nikon EZ-C1 software was used for confocal image acquisition and analyses.

RESULTS

Exposure of MCF-10A cells to 17β-estradiol (E2) and its metabolites produces a rapid increase in ROS levels: Before carrying out cell transformation, we characterized normal human mammary epithelial cells for their ability to produce ROS in response to 17 beta-estradiol (E2) exposure. These cells respond to E2 in terms of producing ROS very similar to breast cancer cells. ROS production by E2 and its metabolites, 2-OH-E2 and 4-

OH-E2 in normal human mammary epithelial MCF-10A cells was dose-dependent (Fig. 1 A,B). 4-OH-E2 induced significantly more ROS in these cells compared to E2 and 2-OHE2. The abilities of these estrogens to produce ROS were inhibited by overexpression of catalase or treatment with Ebselen or NAC (Fig. 1C). When MnSOD were over-expressed in these cells, ROS levels increased significantly compared to cells treated with estrogen alone (Fig. 1C). We also measured the cellular protease activities to rule out the possibility that the differential ROS levels were not as a result of differential cell densities or viability. Our results indicate that 4-OH-E2 is the most effective in generating intracellular ROS in MCF-10A cells. Mitochondria may be the major source of estrogen induced intracellular ROS, because overexpression of MnSOD, a mitochondria superoxide dismutase that converts super oxides to hydrogen peroxide, increased the ROS content maximally.

Exposure of MCF-10A cells to 17β-estradiol (E2) and its catechol metabolites induced dose-dependent colony formation: Both MCF-10F and MCF-10A cells are spontaneously immortalized, ER negative normal human breast epithelial cell lines that are non tumorigenic in vivo. We used the anchorage independent growth (AIG) assay to examine cell transforming ability of E2 by detecting AIG positive colony formation. E2 exposure to MCF-10A cells produced dose dependent increase in colony formation. We found that repeated treatments of MCF-10A cells with various doses of E2 or its catechol metabolites induced in vitro transformation of MCF-10A cells in a dose dependent manner (Fig 2). 4-OH-E2 is more potent in transforming normal mammary epithelial

cells compared to E2, while 2-OHE2 is a weakly transforming metabolite of MCF-10A cells (Fig 2).

Clonogenic expansion and invasiveness of 4-OH-E2 transformed MCF-10A cells: Since 4-OH-E2 induced the highest transforming frequency, we further examined whether 4-OH-E2 induced colonies are clonogenic. We picked several colonies from each soft agar at the end of 21days and cultured them in media with 10% FBS (designated as a regular media -RM). Several of these clones did not survive beyond the 10th passage in RM. However, of the 5 that survived up to the 21st passage, we determined whether these cells have acquired anchorage independent growth properties, a hallmark for transformed cells and whether these clones also respond to estrogens. Cells were fed twice per week and cultured for 21days. Colonies were counted from quadruplicate wells, (± SD). One of our clones was highly clonogenic (P21) and responsive to E2. We labelled this clone as MCF-10T15 (Figure 3A). Analysis of the invasive property of this clone MCF-10T15 by invasion assay showed that it is highly invasive (Fig. 3B).

 3-D Spheroid formation of 4-OH-E2 transformed clone: To assess whether 4-OHE2 transformed MCF-10A cells are neoplastic, we picked few colonies from anchorage independent growth assay and cultured them repeatedly in growth factor reduced media, then assessed cells periodically for their ability to form spheroid structures in collagen coated 0.22 μm transwell inserts, or in a rotary vessel using HuBiogel, a memetic of human stromal matrix. We found that over progressive passages, the clones in collagen

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matrix assumed a more heterogeneous population with small masses (p0) as opposed to homogenous population with aggressive phenotype (p200) (Fig. 4).

The tumorigenic conversion ability of 4-OH-E2-transformed MCF-10A cells was further investigated by 3-D culture using HuBiogel^{TM}. For 3-D culture, anchorage-independent MCF-10A human mammary gland epithelial cells transformed by 4-OH-E2 treatment were mixed with 3D HuBiogel^{TM} matrix containing DMEM-F12, seeded into 55 ml rotating-wall vessels and incubated at 37°C for 16 days. These conditions allow for the spontaneous formation of tissue-like spheroids (Fig 4).

Loss of ductulogenicity in 4-OH-E2 transformed cells: The ability to form ductile structures are characteristic of normal mammary epithelial in collagen matrix. Loss of this ability is a hallmark of transformed cells. Assessment of transformed cells in a collagen matrix indicates that these cells have indeed lost their ability to form ductile structures upon repeated treatment with 4-OHE2 (Fig 5A). This phenotypic change was evident right from p0 cell population and continued even at p121.

Aggressive phenotype enrichment: Initial invasion assessment of fifteen colonies from soft agar assay indicates that three colonies have acquired the ability to invade Matrigel matrix. These clones were subsequently cultured in growth factor reduced media over several generations and assessed periodically for invasive phenotype. We observed that successive passage of these clones increased their ability to invade Matrigel matrix. One of the clones termed clone c (MCF-10Ac) had actually acquired invasion capability that

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is about 30% that of MDA MB 231 cell line at passage (Fig 5B). The other clones had lesser invasive abilities compared to MCF-10Ac (data not shown).

4-OH-E2 treatment causes epithelial to mesenchymal transition (EMT) in MCF10A cells: We found that the 4-OH-E2 transformed clones looked very different from WT both in morphology, size and time it takes to form a sheet upon seeding. Figs. 5C, 1,2 shows that transformed clones were bigger than their WT counterpart in monolayer culture just after seeding (3 hrs). In addition, transformed cells displayed an abnormal differentiation pattern and loss of cell polarity, all phenotypes of cancer cells (Fig. 5C 3,4). The majority of clones exhibited morphological changes that resembled epithelial to mesenchymal transition. As shown in Figure 8b, MCF10A cells showed highly organized cell-cell adhesion and cell contact, whereas 4-OH-E2 transformed-MCF10A cells had an elongated and refractive appearance with cell scattering and loss of cell-cell contacts. The cobblestone-like morphology of MCF10A cells at confluency was replaced in 4-OH-E2 transformed-MCF10A cells by a spindle-like fibroblastic morphology.

Inhibition of 4-OH-E2-induced cell transformation by ROS modifiers: In cells overexpressed with adenovirus construct containing catalase and MnSOD that lowers oxidant production as well as in mtTFA silenced cells, E2 produced fewer colonies compared to E2 alone (Fig 6). Treatment of cells with chemical ROS scavenger (Ebselen or NAC) significantly inhibited the abilities of E2 or 4-OHE2 to induce neoplastic transformation of MCF-10A cells as assessed by inability to form colonies and grow in soft agar assays (Fig 6). This implies that oxidants induced by E2 and 4-OHE2 are

necessary for tumorigenic transformation of MCF-10A cells and when oxidant levels were scavenged by biological and chemical antioxidants, estrogen induced transformation of mammary cells was inhibited.

The growth of the E2-induced transformed clone was highly responsive to E2 and was inhibited by Ebselen and N-acetyl cysteine. Antioxidants reduce E2-induced DNA synthesis in MCF-10A transformed cells (Figure 7). As shown in Figure 8, treatment with E2 produced spheroids (Left Upper Panel). Overexpression of catalase and Ebselen inhibited E2-induced tumor spheroid formation. Cells were labeled with CFSE using the Vybrant kit for checking viability. All spheroids showing the green fluorophore (Fig. 8 Right Upper Panel) indicate that cells in tumor spheroids are alive.

4-OHE2 induced ROS activates PI3K/AKT signaling pathway: Consequences of elevated ROS in cells are apoptotic cell death, quiescence or cell transformation and neoplastic growth (33,34) The signaling pathway associated with survival of cells under oxidative stress is attributed in part to activation of PI3K and AKT signaling pathways (34, 35). Therefore, we determined whether estrogen induced oxidants in normal mammary epithelial cells activate PI3K and AKT signaling pathways during neoplastic transformations of MCF-10A. We found that repeated treatments of MCF-10A cells with E2 and 4-OHE2 increased phosphorylation of both PI3K and AKT in cells treated with regimen of estrogen which produced cell transformation (Fig 9). Phosphorylation of both PI3K and AKT was attenuated by co-treatment with either biological or chemical ROS modifiers (Fig 5). The activation of PI3K and AKT activation by 4-OHE2 were about 30% and 120% higher than E2 or 2-OHE2 respectively (Fig 10). Interestingly, we also

observed that silencing of Akt_1 (Fig 11A), the downstream recipient of PI3K activation and Akt1 regulates proliferation and growth of mammalian cells significantly diminished 4-OHE2 induced neoplastic transformations of MCF-10A cells (Fig 11B). These data support that intracellular ROS induced by 4-OHE2 may activate AKT signaling pathway which favors survival and proliferation of cells, both required for malignant transformation.

4-OH-E2-induced ROS modulates cell cycle genes through a nongenomic, ER independent signaling pathway: We investigated whether 4-OH-E2-induced ROS signaling is involved in the modulation of cell cycle genes in the conversion of normal breast epithelial cells to malignant cells. Using a normal cell line (MCF-10A) that develop transformed clones in response to 4-OH-E2 or E2, the expression of cell cycle genes, cyclin D and pcna were measured by real time RT-PCR (Fig. 12). After exposure of 8h following two 48 hr treatments with 4-OH-E2; we observed an approximately 1.34-, and 1.54- fold increase in the mRNA expression of pcna and cyclin D1. Overexpression of MnSOD and catalase as well as co-treatment with Ebselen and NAC markedly decreased 4-OH-E2 induced pcna and cyclin D1 expression compared to E2 treated cells.

DISCUSSIONS

Our results showed that E2 and its metabolite, 4-OH-E2, elicited transformed phenotypes in MCF-10A cells, which are estrogen receptor-α (ERα) negative. Similar results have been reported in MCF-10A cells exposed to E2 or its catechol metabolite and 4-hydroxyequilenin (21-23). Russo and others have shown that E2 or 4-OH-E2 transformed

MCF-10F cells were also ERα negative (17-20). Our results are in agreement with previous studies that 4-OH-E2 is twice as carcinogenic as E2. In contrast, neither 2-OH-E2 is carcinogenic in vitro or in vivo (15). Interestingly, both 2-OH-E2 and 2-OH-E1 are capable of producing ROS and undergo metabolic redox cycling like 4-OH-E2. The lack of carcinogenicity of 2-hydroxylated estrogen metabolites in vivo may be due to their rapid inactivation by COMT-mediated O-methylation, rapid clearance, and weak estrogenic hormonal activity as compared with 4-OH-E2 (10-12, 36,37). Most importantly, it may be due to 2-MeO-E2, the major product of COMT-mediated O-methylation of 2-OH-E2 that possesses unique anti-tumorigenic activity. These studies support the concept that E2 and its main metabolite 4-OH-E2 are carcinogenic in breast epithelial cells.

Recent studies indicate that mammary tumors can develop in the absence of a functional ERα (24). Although tamoxifen and other antiestrogens are thought to prevent cancer through their actions at the ER, other mechanisms cannot be ruled out as these compounds also block metabolism and redox cycling of estrogen and are free radical scavengers (25). 4-OH-E2 induces an estrogenic response in the uterus of ERα null mice, and this response is not inhibited by the antiestrogen ICI182780 (26). These findings suggest that estrogen-dependent growth of cells is regulated not only by nuclear ER-mediated genomic signaling pathways, but also by non-genomic pathway(s). Genomic and non-genomic actions of estrogen may produce complementary effects that are required for cellular transformation. Estrogen is genotoxic, as seen by the presence of DNA adducts in mammary tissues from ERKO/Wnt-1 mice (38,39). Although the

formation of DNA adducts may lead to gene mutation, this type of DNA damage appears to be a late event arising from E2 metabolism. We have recently shown that estrogen induces rapid formation of ROS in MCF-7 cells (27,40). 4-OH-E2 has been implicated in transforming MCF-10A cells via ROS formation based on inhibition of anchorage independent growth of MCF-10A cells (23). This study did not show in vivo tumor formation of transformed cells. The main difference between of our work and previous reports is that our transformed clones are tumorigenic in mice and ROS modifiers. The overexpression of catalase that converts hydrogen peroxide to water and Ebselen, a gluthione peroxidase mimic, inhibited cell transformation and tumor formation. This is important because MCF-10A cells are easily transformed in an in vitro system, even by mild stress such as reduced growth factor media or hypoxic conditions (41).

Both E2 and 4-OH-E2 treatment of MCF-10A cells, increased the formation of ROS as compared to untreated cells, whereas 2-OHE2 induced the minimum increase in ROS formation in MCF-10A cells. Over-expression of biological ROS modifiers and chemical scavengers of ROS prevented 4-OH-E2-induced anchorage independent growth of MCF-10A cells. We observed similar results with 3-D culture of transformed cells using HuBiogel and xenograft tumor growth. These findings suggest that ROS induced by repeated exposures to 4-OH-E2, a predominant catechol metabolite of E2, cause transformation of immortalized human mammary epithelial cells with malignant growth in nude mice. Since 4-OH-E2 induces more ROS formation compared to E2 in MCF10A cells, the accumulation of 4-OH-E2 in the breast is expected to augment ROS formation here as well. 4-OH-E2 strongly binds to ER (42,43) and it takes longer to dissociate from the ER than E2 (44). The greater ROS production, ER action, and breast tissue

accumulation of 4-OH-E2 compared to E2 may account for its greater carcinogenicity in MCF-10A cells.

We and others have shown that mitochondria are significant targets of estrogen (27,40). Recently, we reported that physiological concentrations of E2 stimulate a rapid production of intracellular ROS in epithelial cells which depends on cell adhesion, the cytoskeleton, and integrins (27,40). These events occur earlier than ER-mediated genomic actions. E2-induced ROS production does not depend on the presence of ER on breast cancer cells as ER$^-$cell lines MDA-MB 468 produced ROS equal to or more than that of ER$^+$ MCF7, T47D, and ZR75cell lines (27).

It has been wrongly concluded by Parks et al (23) that redox cycling of catechol estrogen is the source of ROS. Catechol estrogens, particularly 4-OH-E2, via nonenzymatic autoxidation, may undergo redox cycling to produce reactive semiquinone and quinone intermediates with concomitant production of ROS (10-12). However, this redox reaction of catechol estrogens is enhanced in the presence of Cu^{2+} or Fe^{3+} ions and by enzymatic catalysis by cytochrome P450 oxidases or peroxidases, which is accompanied with an increased generation of ROS. Furthermore, Parks et al (23) implied the contribution of redox cycling of catechol estrogen generating ROS based on indirect evidence using a non-specific inhibitor of cytochromes P450, SKF525A and dicumarol, an inhibitor of quinine reductase. Dicumarol can also inhibit mitochondrial diaphorase, which is involved in reduction of Coenzyme Q10 in the mitochondria (45). Similarly, SKF-525A inhibits mitochondrial oxidative metabolism in intact cells and isolated mitochondria

(46). Lower ROS formation observed in the presence of SKF525A and dicumarol may be as a result of inhibition of the mitochondrial electron transport chain. Increased ROS formation is observed within 30 seconds of E2 treatment (40). Due to the speed of ROS production as observed in our study, it is unlikely that redox cycling of 4-OHE2 is the source of these oxidants. Furthermore, in our studies of E2-induced ROS generation in MCF-7 and other cells, hydroxylated estrogen metabolites or adducts immediately after addition of E2 were not detected which also rules out the possibility of ROS generation by redox cycling of hydroxylated estrogens.

Little is known about the potential direct involvement of ROS signaling in breast cancer development. However, we and others have recently discovered that estrogen-induced oxidative bursts occur exclusively in perinuclear regions. This surge in ROS production may target inducible promoters, signaling transcription-initiation complex assembly and subsequently, driving estrogen-induced gene expression (28). While studying mitochondrial regulators of cell cycle progression, we discovered that E2-induced G1 to S phase transition is associated with an increase in intracellular ROS levels (28). These findings strongly support the idea that both E2-induced ROS and ER activity are required for breast cancer cell proliferation (28). When produced at high levels, ROS levels induce oxidative damage and G0/G1 arrest. However, when present at physiologic levels, ROS mediate essential cellular functions such as cell-to-cell communication and proliferation. For example, estrogen-generated ROS mediate early G1 gene expression, as seen by the ability of antioxidants to block this effect (28). We now know that the delicate intracellular interplay between oxidizing and reducing equivalents allows ROS to

function as second messengers in signaling pathways controlling cellular proliferation and transformation (47,48). Recent studies implicate a role for ROS in cell transformation and several lines of indirect evidence support a role for ROS in the development of breast cancer (49,50), We recently found that, in Syrian hamsters, estradiol-induced kidney tumor formation was reduced by the antioxidants N-acetylcysteine, vitamin C, sodium 2-mercaptoethanesulfonate (cytoprotective thiol-containing agent), and Ebselen (51,52). Consistent with this finding, estrogen-induced testicular and uterine cancers are prevented by pentoxifylline, a compound with antioxidant effects stemming from its ability to block synthesis of the inflammatory mediators, IL-1β and TNFα (49). Overexpression of manganese superoxide dismutase (MnSOD), the mitochondrial enzyme responsible for superoxide detoxification, blocks the appearance of malignant phenotypes (53), and the loss of this enzyme partly contributes to malignant phenotypes (54,55). Not surprisingly, MnSOD knockout mice exhibit increased oxidative DNA damage (56). MnSOD expression is less frequently found in tumor cells of invasive breast carcinomas than in non-neoplastic breast epithelial cells (57). Several epidemiological studies have shown that MnSOD polymorphic populations have an increased risk of breast cancer (59-60). The recent findings that 4-OH-E2 accumulates in the breast tissue of cancer subjects (61-63) and predominant 4-hydroxylation of E2 occurs in the target organs of cancers (64-66) suggest that the target organ of cancer would be particularly sensitive to 4-OH-E2-induced ROS formation. In our studies, overexpression of catalase and antioxidants (N-acetyl cysteine and Ebselen) prevented 4-OH-E2-induced anchorage independent growth of MCF-10A cells. These

results indicate that 4-OH-E2-induced cell transformation may be mediated, in part, by redox-sensitive signal transduction pathways.

Both AKT and ERK are redox sensitive kinases. Thus, 4-OH-E2-generated ROS may activate ERK or AKT, which could then directly phosphorylate and activate transcription factors controlling cell cycle, cell migration or cell invasion genes. Several investigators have concluded that estrogen-induced AKT activation is promoted by membrane bound ERα or ERβ (67,68). There are no known functional motifs within the structure of the ER that can promote second messenger signaling. There are reports which show no correlation between ERα/β expression patterns and the activation of AKT-1/-2 in estrogen treated breast cancer cell lines. 17α-estradiol, through an ER independent mechanism, activates PI3K-AKT signaling (69). Recently, Lee et.al. (67) reported that up-regulation of PI3K/AKT signaling by E2 is mediated through activation of ERα, but not ERβ. In ovarian cancer cells, 4-OH-E2 induces AKT phosphorylation while 2-OH-E2 did not (70). Our study showed that 4-OH-E2 increased AKT poshphorylation in ERα lacking MCF-10 cells, while 2-OH-E2 did not increase AKT phosphorylation. The PI3K inhibitor, LY294002, and ROS modifiers blocked 4-OH-E2-induced AKT phosphorylation. AKT activity depends on its phosphorylation, which is positively regulated by PI3K and negatively regulated by a class of protein phosphatases (PPs) (71). AKT can be activated by both E2 and H_2O_2 (67-73). ROS reversibly regulate cysteine-based phosphatases (reviewed in 47). The ability of E2 and H_2O_2 to activate AKT may be attributable to inactivation of cysteine-based phosphatases by ROS (47, 67-73). The

reversible inactivation of phosphatases, such CDC25A and PTEN, by estrogen-induced ROS may be a key component of AKT activation (47,72).

The PI3K/Akt signaling pathway seems ubiquitous to carcinogenic conversions (74-76). Oxidant mediated hyperactivation of AKT can phosphorylate and inhibit pro-apoptotic proteins such as BAD and caspase 9 while phosphorylating and activating pro-growth transcription factors such as ASK1 and GSK3. The outcome of this hyperactivation could therefore be cells surviving and proliferating in a high oxidative state. If these cells have been initiated by acquisition of pre-tumorigenic lesions by 4-OHE2 metabolism, oxidant mediated growth of these cells could be the basis for malignant transformation of mammary cells. The loss of PTEN activity, hyperactivation of PI3K/AKT signaling pathway, excess estrogen exposure and oxidative stress have been implicated in breast carcinogenesis (47). In this study, we also observed that PI3K/AKT signaling proteins were hyperactivated in MCF-10A cells treated repeatedly with estradiol and 4-OHE2 though the activations of 4-OHE2 were more than those of E2 and 2-OHE2 respectively. Importantly, chemical and biological antioxidant mitigated PI3K/AKT activations and inhibited estrogen induced mammary tumorigenesis. To rule out the possibility that antioxidant regulation of PI3K/AKT activations are not related to estrogen induced mammary tumorigenesis, we silenced AKT_1 expression, the AKT isoform implicated in survival, growth and tumorigenesis of cells including mammary cells (74-76). We found that silencing of this gene prevented estrogen's ability to transform MCF-10A cells. These data indicates that estrogen induced redox activation of PI3K/AKT signaling pathway is essential for mammary tumorigenesis.

A substantial number of experimental and epidemiological studies support an important role for AKT in tumorigenesis. PI3-kinase and AKT act as oncogenic determinants in several human cancers. AKT genes are amplified or overexpressed in gastric, ovarian, breast, pancreatic, and prostate cancers (77,78). AKT1 levels are higher in a panel of human breast carcinoma cell lines than in breast epithelial cells, particularly those with higher HER2 expression. AKT1 activity is increased by either E2 or IGF-I in estrogen-dependent MCF-7 cells, and both factors act synergistically to increase AKT1 activity and promote cell proliferation (79). Transgenic mice expressing AIB1 (ER co-activator) in the mammary gland develop mammary hyperplasia and mammary carcinomas. Increased activation of the PI3K/AKT pathway is implicated in the development of mammary carcinoma in AIBI mice (80). AKT activation amplifies the proliferation induced by cyclin D1 or HPV E7 during morphogenesis and cooperates with these oncoproteins to promote proliferation and morphogenesis in the absence of growth factors (81). H-ras transformation of MCF-10A cells results in upregulation of MAP kinase and PI3-kinase signals (82). Similarly benzo(a)pyrene quinone is reported to induce anchorage-independent growth of MCF-10A cells which depends on the activation of PI3K/AKT activation (83). Chronic activation of AKT2 leads to an increase of the events associated with tumorigenesis (84). Most importantly, AKT activation disrupts mammary acinar architecture and enhances proliferation in an mTOR-dependent manner (80). Our study showed that the exposure of 4-OH-E2 or E2 to normal human breast epithelial MCF-10A cells produced transformed phenotypes. These cells show increased AKT and increased cell number in the absence of EGF or insulin. The 4-OH-E2-induced cell proliferation was attenuated by the antioxidant N-acetylcysteine. Overexpression of

catalase and MnSOD also reduced the extent of 4-OH-E2-dependent increased cell growth and AKT activation. Taken together, these data indicate that 4-OH-E2-induced ROS activates the AKT pathway in MCF-10A cells to support the growth of cells in growth factor deficient conditions.

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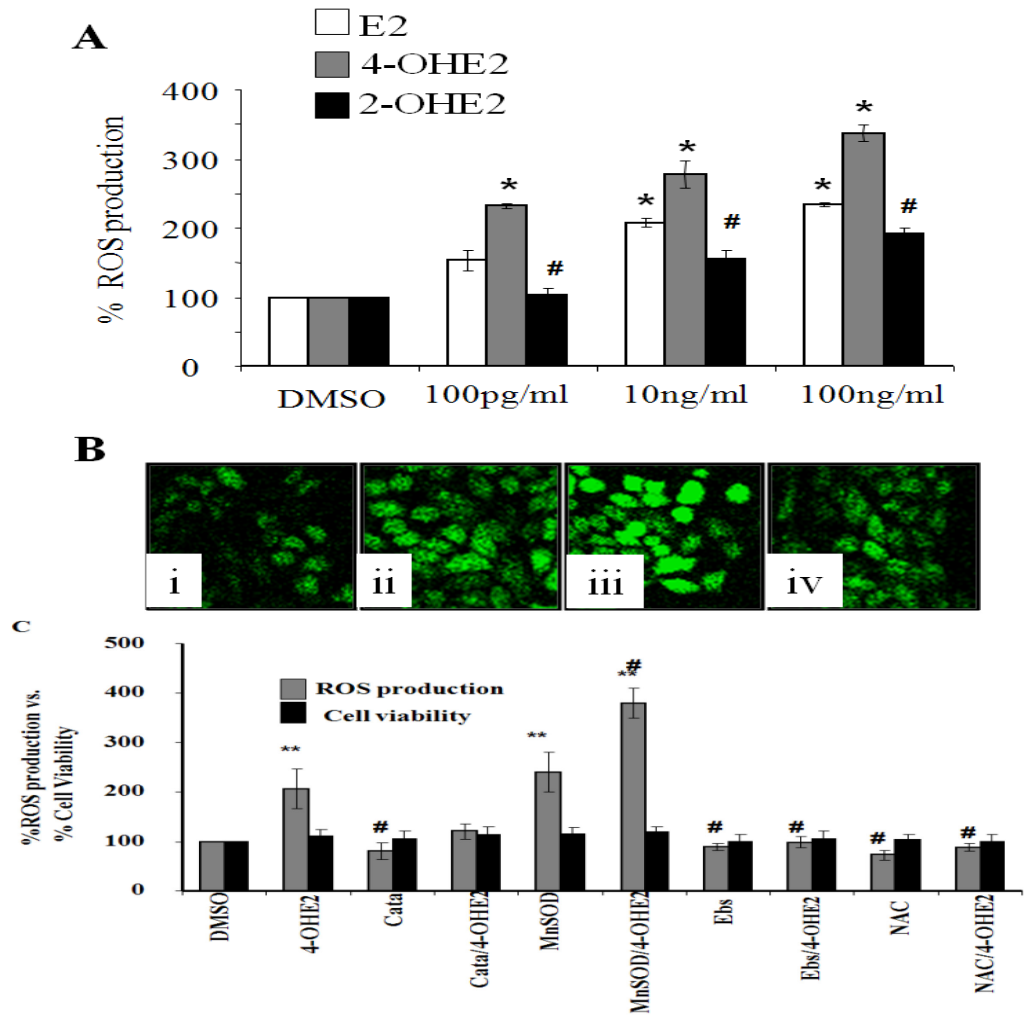
Fig. 1. 17β-estradiol and its catechol metabolites induce ROS production in MCF-10A cells in a dose dependent manner. Cells were seeded at 1.0×10^4 cells per well. 24 hrs post seeding, cells were serum starved for 48 hrs in DMEM/F12 (1:1) with 1x penstrep. For the groups that were treated with NAC or ebselen, these cells were pre-treated with 1.0 mM NAC, pH 7.0 or 40 μM ebselen for 4 hrs before DCF assay. For the groups that were treated with catalase or manganese super dismutase (MnSOD), these cells were infected with adenovirus over-expressing these ROS modifiers at moi 100 for 48 hrs prior to seeding for DCF assay. At end of starvation and antioxidant treatments, cells were preloaded with 10 μM 2',7'-dichlorofluorescin diacetate (DCFH-DA) diluted in Hanks Balance Saline Solution (HBSS), and incubated for 20 mins in 37°C incubator. Post DCF treatment, cells were washed with HBSS and treated with various concentrations of estrogens diluted in HBSS and fluorescence emission read on Tecan Genios microplate reader using 485 and 535 nm as excitation and emission filters respectively. Fluorescence

images were acquired on confocal microscopy. To determine whether observed ROS modification by estrogens or antioxidant were due to differential cell density or viability, duplicates plates were seeded and treated with ROS modifiers as described above, then challenged with 100 ng/ml 4-OHE2. Instead of loading DCF, cells were treated with CellTiter-Fluor™ as described by manufacturer. This reagent measures protease activity as an indication of cell viability. Plates were read on fluorescence plate reader at 400nm excitation and 505nm emission and data expressed as mean of three-five experiments +/- SE. (A) Histogram of fluorescence emitted by the oxidation of DCFH-DA by estrogens and read on Tecan plate reader in 5 min. Data is expressed as mean of +/- SE of five experiments. (*) Indicates significantly different from control (DMSO) groups while (#) indicates significant different from 4-OHE2, (P<0.05). (B) Confocal images of cells preloaded with 1.0 μM DCF and treated with 100 pg/ml of various estrogens, and fluorescence image acquired 5 min after treatment. (I) DMSO, (II) E2, (III) 4-OHE2, (IV) 2-OHE2. (C) Histogram of ROS modulation by 4-OHE2 versus viable cells. (**) Indicates significantly different from control (DMSO) groups while (#) indicates significant different from 4-OHE2, (P<0.05)
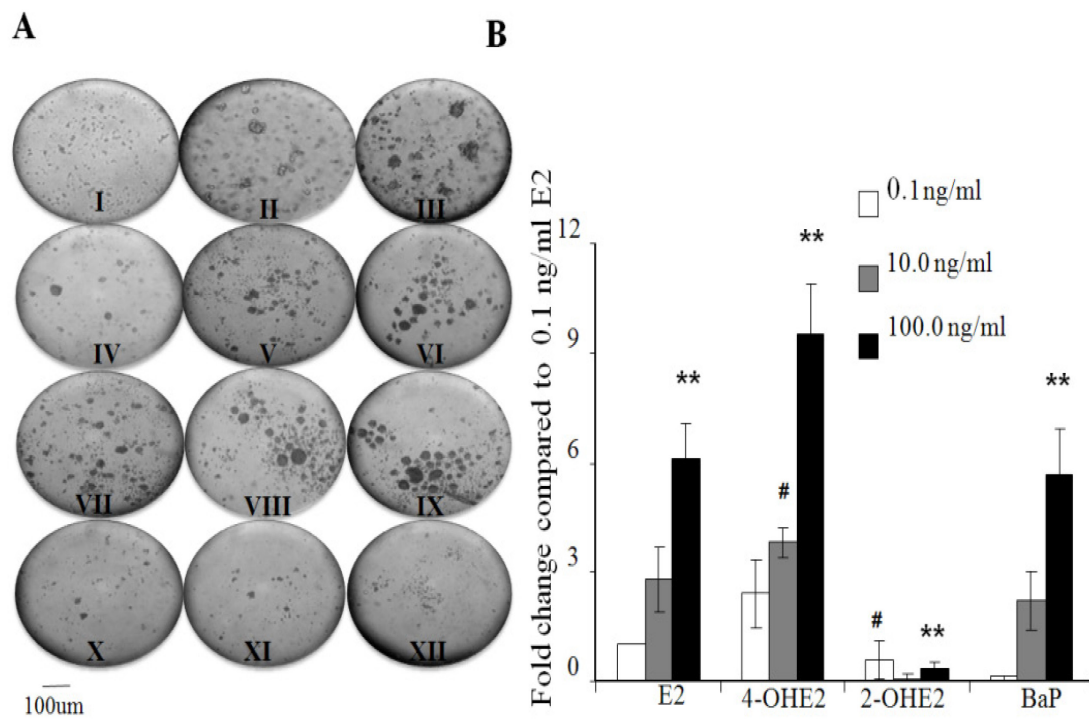

Fig. 2. E2 and 4-OHE2, but not 2-OHE2 transforms MCF-10A cells in a dose dependent manner. MCF-10A cells were seeded at sub-confluence and serum deprived for 48 hrs after which they were exposed to various doses of E2 or its catechol metabolites for two treatment cycles of 48 hrs each, interspersed with 48 hrs recovery period. (I) DMSO, (II-III) 5.0 and 50.0 μg/ml BaP as positive controls respectively. We used estrogen concentrations of 0.1, 10.0 and 100.0 ng/ml respectively for transformation experiments;

IV-VI) E2; VII-IX) 4-OHE2 and X-XII) 2-OHE2 treatments. Fig 2B) Fold differences in colony formations were compared to colonies from 0.1ng/ml estrogen treatments. Data expressed as mean of four wells +/- SD. (**) Indicates significantly different from control 0.1 ng/ml groups while (#) indicates significant different from 100.0 ng/ml 4-OHE2 treated group, (P<0.05)
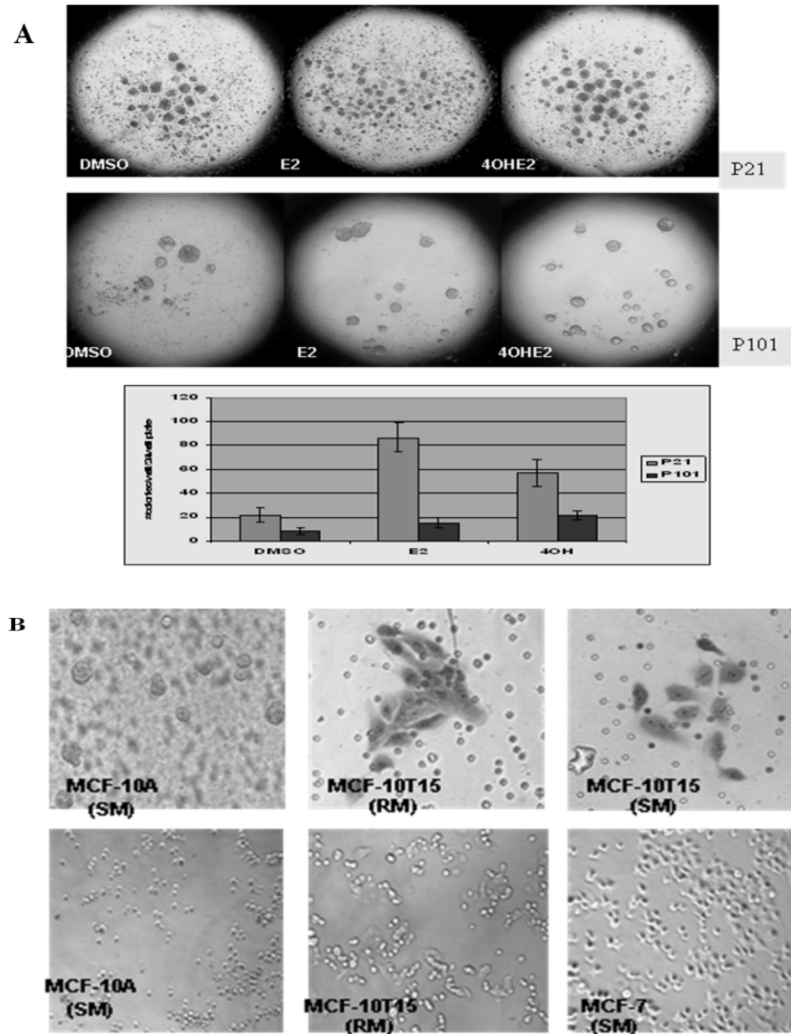

Fig. 3. Clonogenic expansion and invasiveness of 4-OH-E2 transformed MCF-10A cells: A. For colonogenic expansion analysis, we picked several colonies from each soft agar at the end of 21days and cultured them in media with 10% FBS, designated regular media (RM). Several of these clones did not passage beyond 10th passage in RM. However, of the 5 that survived up to the 21st passage, we seeded these cells at 50 k cells/well in soft agar to determine whether these cells have acquired anchorage independent growth properties, a hallmark for transformed cells and whether these clones also respond to 100pg/ml estrogens. Cells were fed twice per week and cultured for 21days. Colonies were counted from quadruplicate wells, (± SD). B. To assess invasive properties of the

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clones derived from 4-OH-E2 induced MCF-10A transformed cells, we seeded 10K MCF-10T15 cells, a clone from MCF-10A transformation in BD BioCoat™ Matrigel™ Invasion Chambers. We also seeded these cells in glass chamber as we previously found that MCF-10A cells don't attach very well to glass in the first 16-24hrs. For the invasion assay (a), the chemotractant is either growth supplemented media (SM) or media with only 10% FBS (RM).
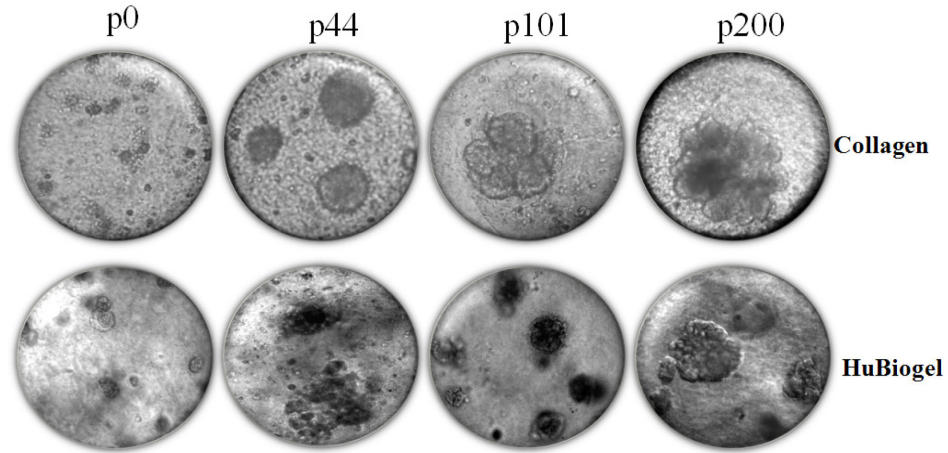
Fig. 4. Spheriod Formation in Collagen and HuBiogel
 Single colony from anchorage independent growth were aseptically picked at end of 21st day of culture. Clones were grown in growth factor reduced media (DMEM F12, 5% FCS, 4 ng/ml EGF, 2.0 ng/ml insulin, 100 ng/ml hydrocortisone, 1x penstrep) for 40-60 generations, then in growth factor depleted media (DMEM F12, 5% FCS, 1x penstrep) onward. Clones were routinely assessed on collagen and HuBiogel for phenotypic evolutions. p0 indicates transformed cells prior to colony assay, p44-p200 are phenotypes of clone over several passages. Images were acquired with hand held Nikon digital camera over an inverted microscope with 20x objective.
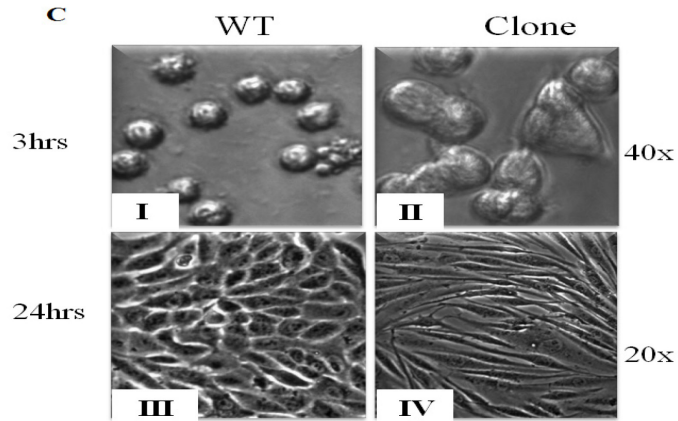

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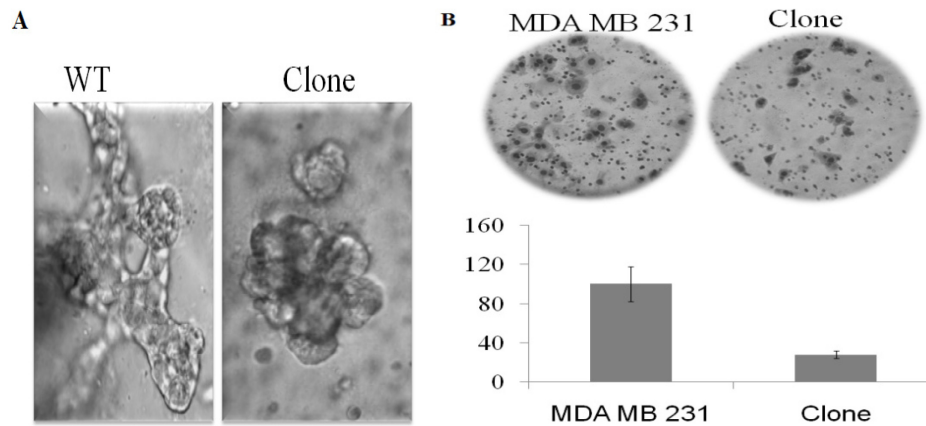
Fig.5. Phenotypic Assessment of Transformed Cells

Clones from p121 cells were assessed for ductulogenesis, seeding phenotype and invasiveness. A) Ductulogenic assay on collagen matrix. B) Invasive characteristics of clone compared to MDA-MB 231 on matrigel 16 hrs post seeding. Data normalized to MDA-MB, +/- SD. Histogram of invaded cells expressed as mean invaded cells per field. C 1-II) Phenotypic assessment of clone versus wild type - 3 hrs post seeding and III-IV) 24 hrs post seeding.
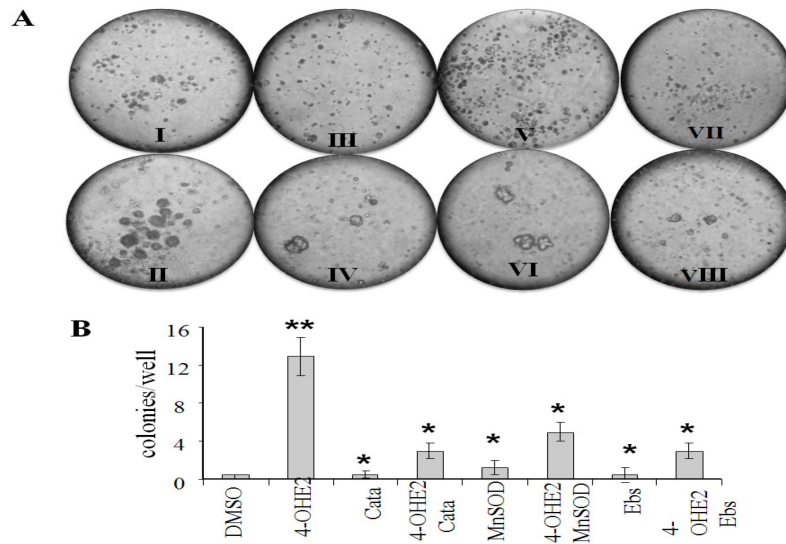

Fig . 6. ROS Scavengers Attenuates Cell Transformation. To determine whether ROS scavenger attenuates in vitro transformations of MCF-10A cells, we infected cells with adenovirus over-expressing catalase or MnSOD at moi 100. Post infections, cells were subjected to transformation regimen. For group that were treated with chemical modifiers, this group were treated continuously with 40.0 uM ebselen throughout transformation period except during recovery times. At end of transformations procedures, cells were seeded for soft agar colony formation at 7.5 x 10^4 cells/well in 48 well plate. Assays were fed weekly for 3 weeks. Colonies formed in 4 wells were

enumerated and scored as mean +/- SD. Images were captured with Nikon digital camera over in inverted microscope with 4x objective. Fig 3A: (I) DMSO, (II) 4-OHE2, (III) catalase infected cells, (IV) Catalase+4-OHE2, (V) MnSOD infected cells, (VI) MnSOD infection+4OHE2, (VII) Ebselen treated cells (VIII) Ebselen treated cells + 4-OHE2. Fig 3B: Number of colonies per well. Data are means +/- SD of five plates. (**) Indicates significantly different from control (DMSO) groups while (*) indicates significant different from 4-OHE2, (P<0.05)
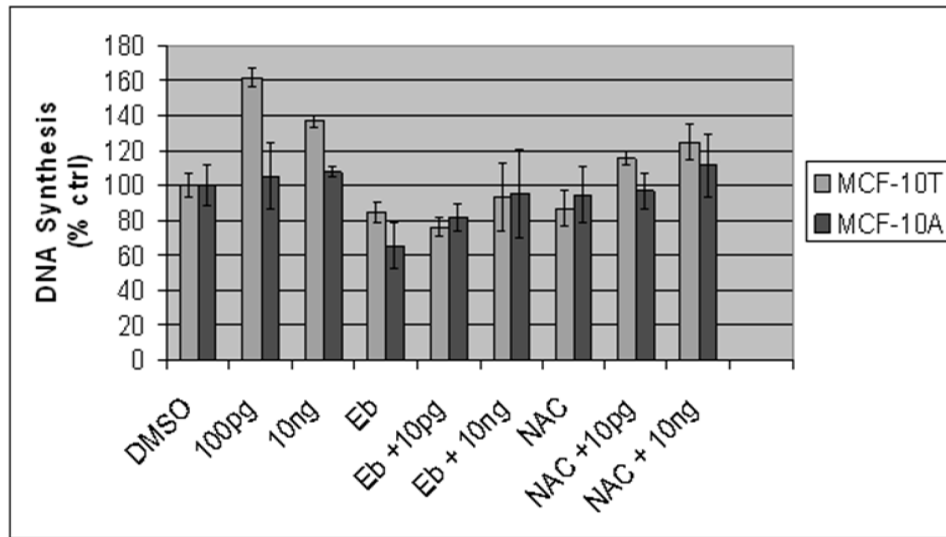

Figure 7. The growth of E2-induced transformed clone was inhibited by both antioxidants, ebselen and N-acetyl cysteine. Cells were grown in 96-well plates for 2 days in 10% FBS DMEM/F12 and serum starved 2 days prior to addition of E2 for 18 h-48 h unless specified otherwise. Bromodeoxy uridine (BrdU) incorporation assay was used to measure DNA synthesis as indicator of apoptosis in transformed cells. Antioxidants ebselen, NAC, and catalase were pretreated for 2 h prior to the addition of E2. Colorimetric BrdUrd incorporation was measured at 450 nm with a plate reader. Results are expressed as mean OD ± SD of three separate experiments with control set as 100% DNA synthesis. (**) Indicates treatment significantly different from control. (*) Indicates treatment significantly different from E2. (P<0.05)
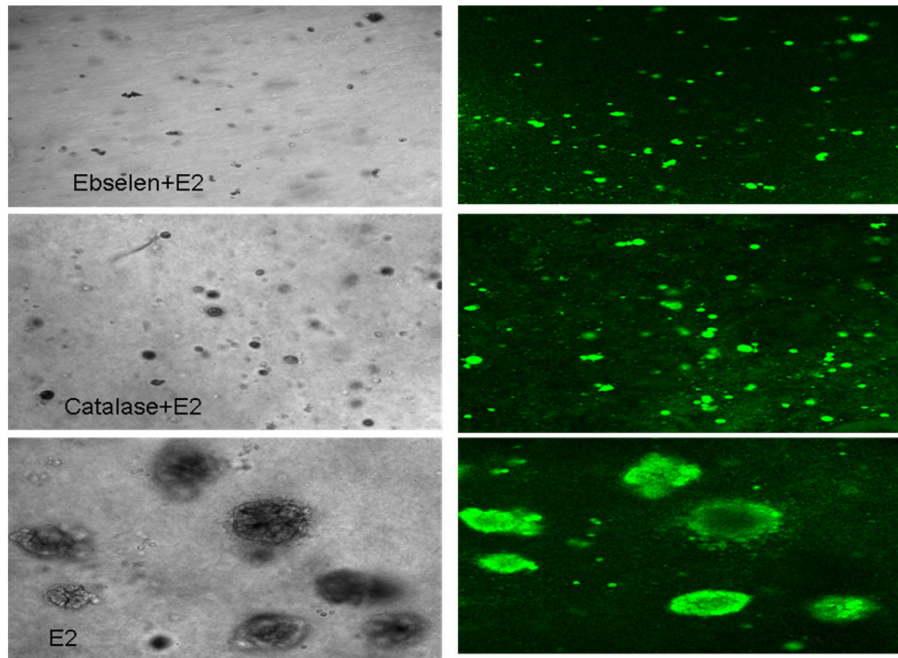
Figure 8. E2-induced 3-D tumor spheroid formation was inhibited by ROS modifiers. Spheroids were grown in a rotary vessel using HuBiogel, a memetic of human stromal matrix in catalase over-expressing cells or ebselen treated cells. Cells were labeled with CFSE using the Vybrant kit for checking viability. All spheroids showing the green fluorophore (Right Upper Panel) indicate that cells in tumor spheroids are alive.
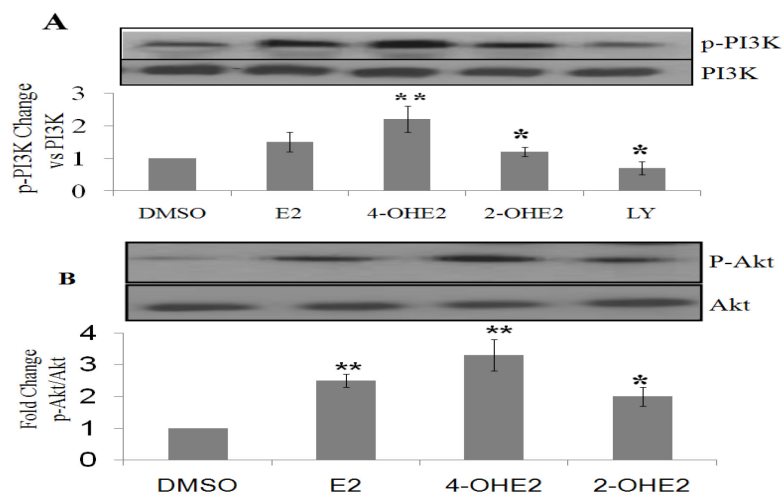

Fig. 9. Estrogen and its metabolites differentially activates PI3K/Akt signaling pathway during mammary transformation. MCF-10A cells were seeded for transformation as described in methods. At the end of transformation process, cells were treated for additional 30 min with 100.0 ng/ml estrogens or its catechol metabolites. Cells were harvested and lysed with RIPA buffer and PI3K and Akt proteins were

immunoprecipitated with respective antibodies and fractionated on 12% SDS-PAGE electrophoresis gel. Proteins were blotted onto PVDF membrane. Membrane were then probed with (A) p85 (p-PI3K) and p110 subunits of PI3K respectively. (B) Phosphorylated Akt (pAkt) and total Akt. Band intensity were quantified with Biorad GelDoc 2000 and normalized to vehicle. (**) Indicates that E2 and 4-OHE2 treatment group is significantly different from control (DMSO) groups while (*) indicates that other treatments groups are significantly different from 4-OHE2. (P<0.05). Results are expressed as mean fold change of three separate experiments.
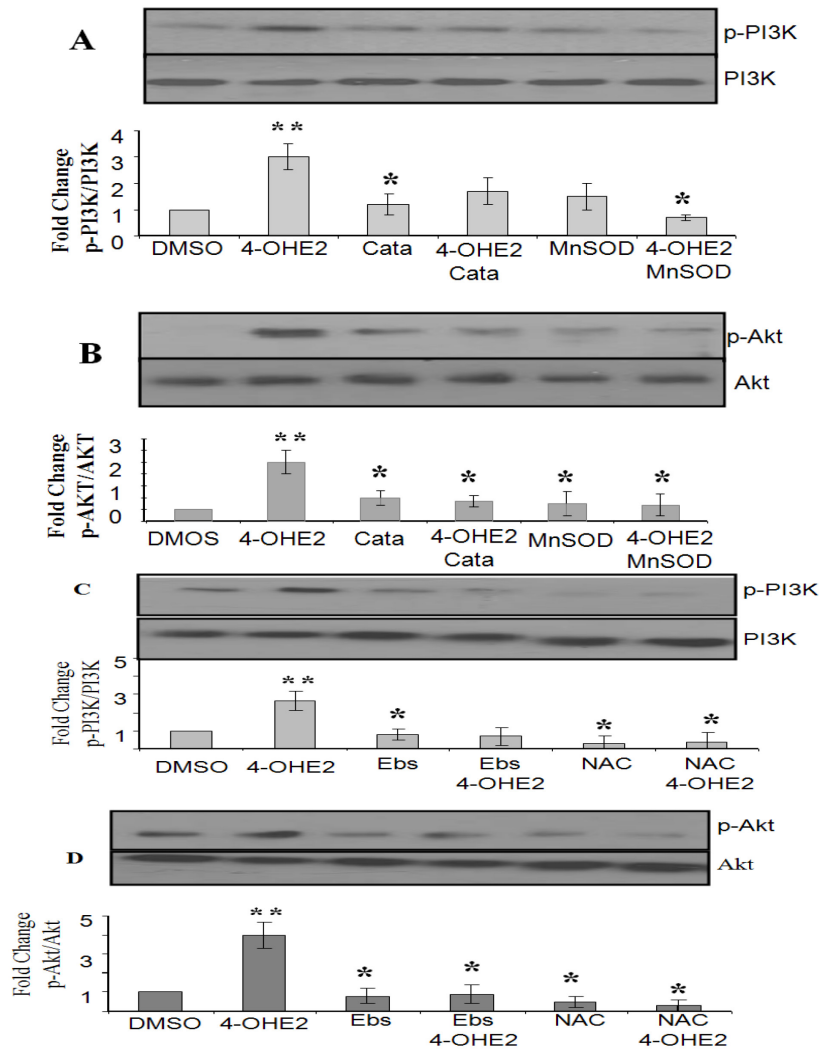

Fig. 10. 4-OHE2 Activation of PI3K/Akt Signaling Pathway is Abrogated by Chemical and Biological ROS Modifiers. MCF-10A were seeded and treated with Ebselen or NAC during transformation regimen, or pre-infected with adenovirus expressing Catalase or MnSOD, then subject to transformation regimen as described in methods. At the end of transformation process, cells were treated for additional 30 minutes with 100 ng/ml 4-

OHE2. Cells were lysed with RIPA buffer and immunoprecipitated with PI3K and Akt antibodies respectively. IP products were fractionated on 12% SDS-PAGE electrophoresis gel. Proteins were blotted onto PVDF membrane. Membranes were then probed with phosphorylated PI3K (p-PI3K) and PI3K, or phosphorylated Akt (pAkt) and total Akt. Band intensity were quantified with Biorad GelDoc 2000 and normalized to vehicle. (A) Immunoblot of activated PI3K and its modulation by biological ROS modifiers at the end of transformation regimen. (B) Immunoblot of activated Akt and its modulation by biological ROS modifiers at the end of transformation regimen. (C) Immunoblot of activated PI3K and its modulation by chemical ROS modifiers at the end of transformation regimen. (D) Immunoblot of activated Akt and its modulation by chemical ROS modifiers at the end of transformation regimen. (**) Indicates that 4-OHE2 treatment group is significantly different from control (DMSO) treatments groups while (*) indicates treatment is significantly different from 4-OHE2, (P<0.05). Results are expressed as mean fold change of three separate experiments.
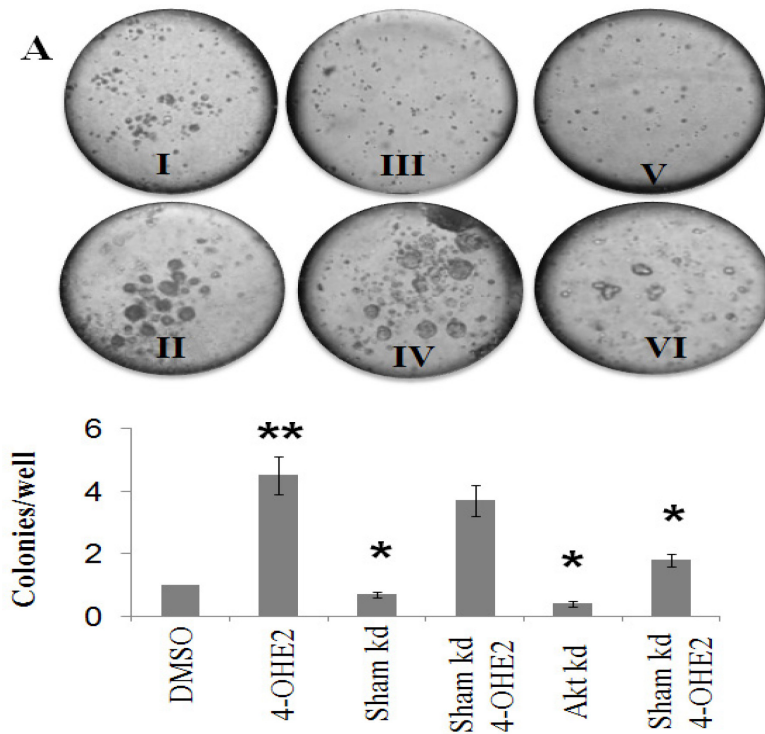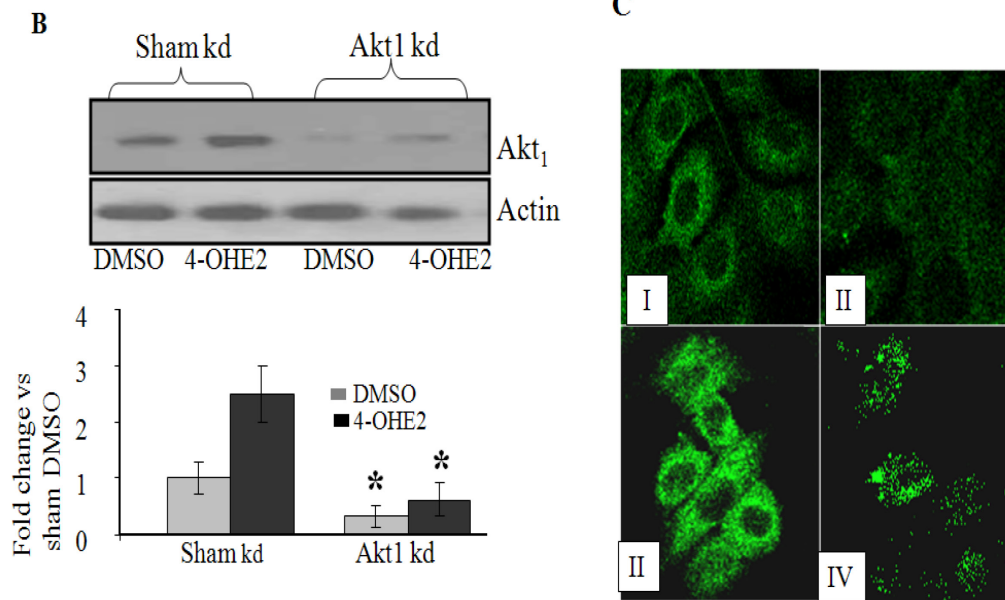
Fig. 11. 4-OHE2 induced Activation of Akt1 Mediates MCF-10A Neoplastic Transformation . To ascertain whether Akt activations are involved during estrogen induced mammary transformation, cells were transfected with shRNA for Akt1 or it corresponding vector, and subsequently subjected to transformation regimen. After transformation process, cells were used for soft agar colony assay, or treated for additional 30 minutes then used for immunoblot. Cells were also seeded for immunofluorescence labeling with anti Akt antibodies for confocal microscopy. Panel A: I) DMSO, II) 4-OHE2, III) Sham Akt1 knockdown, IV) Sham Akt1 knockdown + 4-OHE2, V) Akt1 knockdown, VI) Akt1 knockdown + 4-OHE2. (**) Indicates that 4-OHE2 treatment group is significantly different from control (DMSO) groups while (*) indicates that other treatments groups are significantly different from 4-OHE2, (P<0.05). Panel B: Immunoblot of transformed cells expressing total Akt. Data is expressed as fold difference of 3 experiments, OD ± SD. (*) indicates treatment is significantly different from 4-OHE2, (P<0.05). Panel C: Immunofluorescence label of transformed cells expressing total Akt, I) sham kd + DMSO, II) Sham kd + 4-OHE2, III) Akt1 kd + DMSO, IV) Akt1 kd + 4-OHE2.
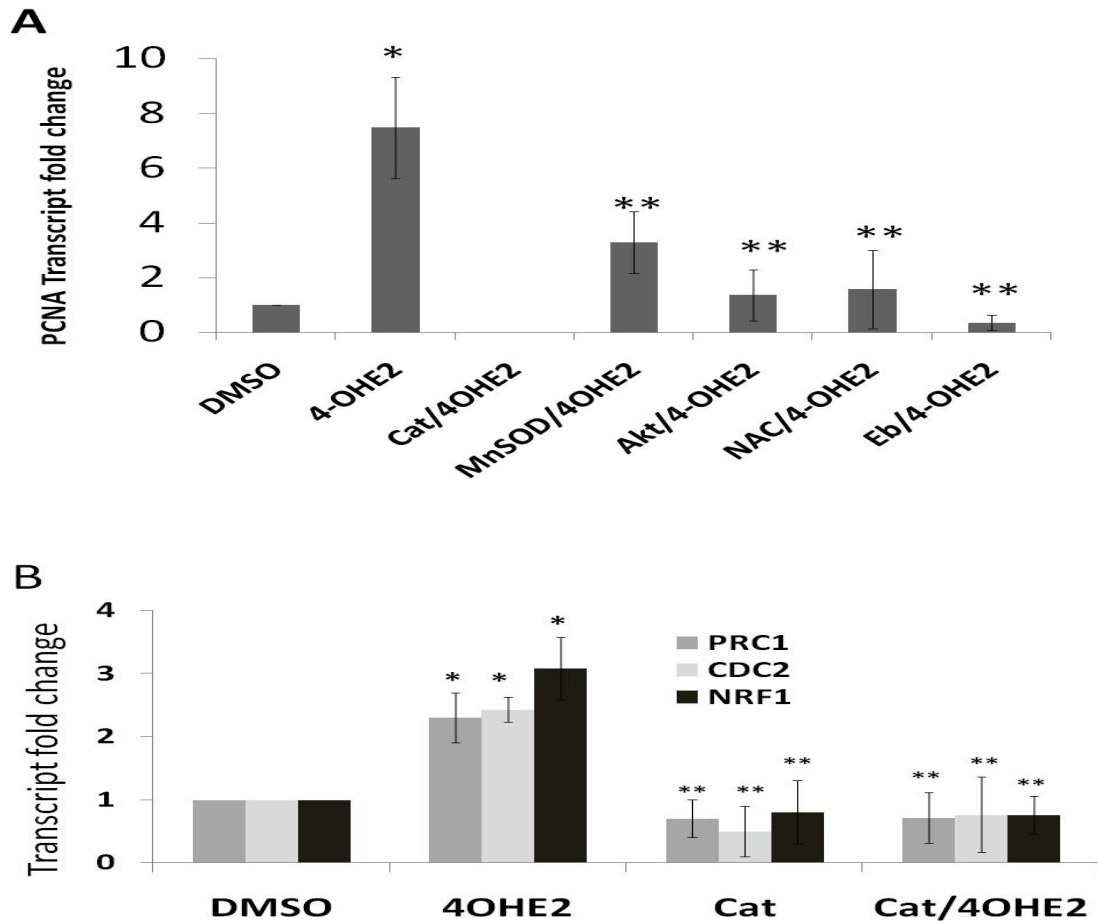
Fig. 12. 4-OHE2 induced up-regulation of cell cycle gene during neoplastic transformation of mammary cells is inhibited by ROS modulators

MCF-10A cells were seeded for transformation as described in methods. At end of transformation period, cells were treated for additional 18 hours with vehicles or estrogens after which they were washed with cold PBS containing protease inhibitors. Cells were detached with trypsin and RNA isolated from 2.0 x106 cells, and 10ng RNA were used for qRT-PCR analysis of cell cycle genes primers on ABI Real time PCR machine. Data represents mean of 3 different experiments +/-SE, (P<0.05). (*) indicates treatment significantly different from DMSO while (**) indicates significant difference from 4-OHE2. A) Fold change of PCNA transcript in 4-OHE2 transformed cells treated with ROS modulators, or transformed cells transfected with Akt1 RNAi. B) Fold change of cell cycle genes in 4-OHE2 transformed cells overexpressing catalase.

CHAPTER V

ESTROGEN-INDUCED REDOX SIGNALING OF NRF1 MEDIATES IN VITRO
GROWTH AND METASTASIS OF BREAST CANCER CELLS

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ABSTRACT

We have shown earlier that chronic exposure to estrogen induces ROS production and

promote growth and metastasis of breast cancer cells via ER related pathways. Recently

nuclear respiratory factor 1 (NRF1), a transcription factor, known to regulate cell's redox

status via mitochondria biogenesis has been implicated to play a role in breast

carcinogenesis. In this study we demonstrate that estrogen induced ROS mediated

signaling activates AKt activation which phosphorylates NRF1, a transcription factor,

known to play a critical role in mitochondrial biogenesis.   This is the first report to

demonstrate that exposure of MCF-7 cells to estradiol induced phosphorylation of AKt to

activate NRF1. Activated NRF1 then modulates estrogen induced growth and invasion of

MCF-7 cells in a redox dependent manner. Treatments of cells with ROS modulators or

knockdown of NRF1 diminished NRF1 expression as well as estrogen induced growth of

MCF-7 cells. These findings suggest that estrogen-induced redox signaling of NRF1 may

be required for in vitro growth and metastasis of breast cancer cells.

INTRODUCTION

Excess estrogen (also referred to as E_2 or estrogen) exposure is associated with breast, endometrial and testicularovarian cancer [1-3]. Estrogens promote proliferation and growth of cancer cells by activation of estrogen receptors (ER) and non-ER activation pathways [1;2] While ER and regulatory gene altered by estrogen exposure may promote growth of breast cancer cells, recent evidence suggest that reactive oxygen species (ROS) may contribute in regulating survival, proliferation, growth and metastasis of breast cancer [4-8].

The role of ROS in breast cancer is not new. Both in vitro and in vivo studies have suggested that malignant breast cells compared to normal cells are under intrinsic oxidative pressures [9-12], which may correlate positively with excess estrogen exposure [13;14] and inefficient antioxidant systems [15]. These redox imbalances result in accumulations of oxidants, such as, hydrogen peroxide (H_2O_2) and hydroxyl ions (-OH) [16;17]. These oxidants can have significant consequences on the fate of cancer cells ranging fromfrom apoptotic cell death, to proliferation, growth, metastasis and therapeutic resistance [18-21]. However, the mechanism (s) involved in oxidative stress and fate of breast cancer is complex and poorly understood. Several studies however suggest that impaired redox mechanism induces dysregulated phosphorylation and dephosphorylation of signaling proteins which activate or deactivate redox sensitive transcription factors [22]. NRF1 (nuclear respiratory factor-1/alpha-palindrome-binding protein) is one of the main transcription factors which are modulated in response to

oxidative stress. , NRF1 is a redox sensitive transcription factor known to regulate mitochondria biogenesis as well as oxidative phosphorylation (OXPHOS) [23;24]. OXPHOS is involved in the production of most of the mitochondria ROS. Dysregulated and increased OXPHOS activity may result in excess ROS production which can have deleterious effects onmtDNA integrity and ETC. [25]. Simultaneously, estrogens have been shown to induce increased NRF1 expressions,OXPHOS activity [26] and ROS production in mammary cancer cells [27]. Therefore, breast cancer tissues with significant oxidative damages [5;14], possibly, are the source of mitochondria derived oxidants via estrogen induced NRF1. This may also explain why ER[+] breast cancer have high mitochondria copy numbers [28], exhibit significant mtDNA damages [29-31] and positively correlate with NRF1 expressions in higher grades [32;33]. .

In contrast, sublethal levels of ROS induce NRF1 expression which regulates the mitochondria/DNA retrograde communication. If dysregulated, NRF1 can turn on genes involved in carcinogenesis and metastasis of cancer cells [34]. That is why antiestrogenic compounds such as raloxifene and tamoxifen likely fail upon prolong usage because these compounds act as pro-oxidant upon chronic exposures [44-47] as well as inducers of NRF1 [48].

NRF1 is predominantly associated with mitochondria regulation in response to oxidative stress, genome wide in silico analysis however, revealed that this gene also regulates over 400 genes involved in cell cycle progression, metabolism, DNA replication, and

transcriptional regulation [35], as well as genes involved in cell survival and growth [36], invasion and metastasis [37;38].

In this study, we investigate whether estrogen-induced oxidants mediate in vitro growth and metastasis of malignant breast epithelial cells and the mechanisms involved. Our study revealed that estrogen induced NRF1 expression and its phosphorylation and dephosphorylation activated by redox dependent cellular signaling mediate proliferation, anchorage independent growth and metastasis of MCF-7 cells. Further, over-expression of biological ROS scavengers (MnSOD and Catalase) or treatments of cells with chemical antioxidant, (N-acetylcysteine (NAC) and ebselen), inhibited estrogen induced NRF1 expression and growth of malignant breast epithelial cells.

MATERIALS AND METHODS

Cell Line and Cell Culture

Human mammary adenocarcinoma cell line (MCF-7) were obtained from American Type Culture Collection (ATCC) and were routinely cultured in phenol red-free DMEM/F12 media with 10% (vol/vol) FBS, 1x penstrep. DMEM/F12 Media. FBS and antibiotics were purchased from Invitrogen corp. For experiments , cells were seeded at 10-75% confluency depending on the experimental designs and incubated at 37°C in a humidified incubator containing 5% CO_2 and 85% humidity. Cells were allowed to adhere for 24 hrs. after which culture medium were replaced with serum-free media (SFM) containing penicillin-streptomycin (100 μg/ml each) and cells were allowed to grow for 48 hrs.

Serum deprivation was used to synchronize cells in the G_0/G_1 phase of the cell cycle followed by treatments with estrogens or antioxidants, as described in the figure legends.

Measurement of Reactive Oxygen Species (ROS)

Cellular ROS were measured as previously described [27]. Briefly MCF-7 cells were seeded at a concentration of 1.0×10^4 cells per well in black 96-wells plate with clear flat bottom (ThermoFisher Scientific Inc. USA) and allowed to adhere overnight. Next day, cells were cultured in SFM for 48 hrs. They were then pretreated for 4 hrs with chemical antioxidants [ebselen and N-acetyl cysteine (NAC)] diluted in Hank's balanced salt solution (HBSS), followed by incubation with 10 μM 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Invitrogen Corp.) for 20 min. Cells were rinsed with HBSS followed by various treatments with 100 pg/ml estrogens as described in the figure legends. DCFH-DA is a non-fluorescent cell-permeable compound, which is acted upon by endogenous esterases that remove the acetate groups generating DCFH. In the presence of intracellular ROS, DCFH is rapidly oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF). The oxidative products is then measured with a Tecan Genios microplate reader using 485 and 535 nm as excitation and emission filters, respectively. DCFH-DA stock solutions were diluted at a 1:1 ratio with Pluronic F-127 (20% w/v).

RNAi transfections

Pre-designed and tetracycline inducible human shRNA for mitochondria transcription factor A (TFAM) and Akt_1 shRNA with corresponding vectorcontrol were purchased from OriGene (OriGene Technologies, Inc. Rockville, MD). Pre-designed NRF1 siRNA

and corresponding scrambled constructs were purchased from Ambion (Applied Biosystems/Ambion, Austin, TX). Plasmid over-expressing NRF1 were custom made by OriGene (Rockville MD) and FLAG tagged DN-NRF1 is a kind gift from Dr. Liu. All purified plasmids were transfected into 35% confluent MCF-7 cells using FuGENE 6 (Roche) transfection reagents according to the manufacturer's protocol. Transfection efficiencies for all plasmid were determined by protein expression levels which ranged from 50% to 80% reductions in respective gene expressionsThe concentration of plasmids used for transfections in all experiments was as recommended by Roche and were based on surface area of dishes used for each experiment. Post transfection, cells and their lysates were used for western blot analysis, BrdU, soft agar assays and invasion assays as described in subsequent sections.

BrdU cell proliferation assay

MCF-7 cells were seeded in 96-well plates at a density of 3,500 cells/well and incubated overnight. Cells were plated in quadruplicate for each experimental group. After 24 hrs of incubation cells were cultured in SFM for 48 hrs. Cells cells were the treated with E2 (100 pg/ml) in the presence or absence of antioxidants for 18 hrs. Cell proliferations were measured by colorimetric immunoassay of BrdU incorporation into the DNA using Roche BrdU cell labeling kits as recommended by manufactures (Roche Molecular Biochemical, Indianapolis, IN). Briefly, cells were pulsed with BrdU labeling reagent for 3 hrs followed by fixation in FixDenat solution for 30 min at room temperature. Thereafter, cells were incubated with 1:100 dilution of anti- BrdU-POD for 1 h at room

temperature. Finally, the immunoreaction was detected by adding the substrate solution and the color developed was read at 370 nm with a Tecan Genios microplate reader.

Adenovirus Transduction

Adenoviruses over-expressing MnSOD (SOD), Catalase (Cata) or control vectors were purchased from ViraQuest, Inc. (North Liberty, IA, USA). MCF-7 cells were seeded into appropriate dishes at 15-70% confluence depending on the experimental design. Next day, cells were infected with Adenovirus over-expressing SOD, Catalase or vector at either moi 50 to 400 in SFM. Cells were cultured for 48 hrs after which they were used for various experiments.

Chemical antioxidant treatments

The treatment procedure for Ebselen (a glutathione peroxidase mimetic which also removes both H_2O_2 and peroxynitrite) and NAC (a precursor of glutathione and scavenger of ROS [49;50] differs according to the experiments to be performed. For example, in DCFH assays used to measure intracellular ROS in live cells, 40 μM and 1.0 mM ebselen and NAC respectively were pre-loaded onto cells 2-4 hrs before ROS measurement were commenced. For BrdU assays, cells were treated with 40 uM and 1.0 mM ebselen and NAC respectively throughout the culture periods. For anchorage independent growth assays, antioxidants were mixed with cells and agar matrix before assay commenced, and added to media during bi-weekly feeding of colonies. For immunoprecipitations (IP) and western blot analysis, cells were exposed to a single treatment with these antioxidants during 48 hr culture in SFM before western analysis. .

Cell viability assay

CellTiter-Fluor™ Cell Viability kit were purchased from promega corporation and according to manufacturer's instructions assay was conducted. Briefly, cells were seeded in 96 well plate at 1.0×10^4 cells/well, culyuired in SFM for 48 hrs post seeding, and treated accordingly with estrogens and antioxidants. At the end of the treatments , substrate reagent (GF-AFC) were mixed with substrate buffer and dispensed into well. This assay measures cell's protease activities as quiescent and growth arrested cells are still viable even if mitochondria activities or oxidant levels are diminished. Plates were read on fluorescence plate reader at 380–400 nm excitations and 505 nm emissions.

Western Blot Assays

Cells for western blot analysis were seeded in T-75 flasks and grown to 70% confluence and manipulated according to experimental designs. Post treatments, cells were harvested in radioimmune precipitation buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% Nonidet P-40, 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (Roche). Equal amounts of total cellular protein were mixed with loading buffer (25% glycerol, 0.075% SDS, 1.25% 2-mercaptoethanol, 10% bromphenol blue, 3.13% stacking gel buffer) and fractionated by electrophoresis on 12% polyacrylamide, 0.1% SDS resolving gels. Rainbow marker (Biorad USA) was used as the molecular weight standard. Proteins were transferred to PVDF Immobolin-P transfer membranes (Millipore) using transfer buffer [25 mM glycine, 25 mM ethanolamine, and 20% methanol]. The membranes were blocked one hour at RT with blocking buffer [1×phosphate buffered saline, 0.1% Tween-

20 with 5% (w/v) nonfat dry milk (PBS-T)]. Blots were subsequently incubated for 2 hr at room temperature with various antibodies as indicated in legends.

Immunoprecipitation

For experiments to determine epigenetic modifications of NRF1, cells were seeded in T-75 and grown to 70% confluence. Post treatments, cells were rinsed twice with PBS, harvested with lysis buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% Nonidet P-40, 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (Roche). Whole cell lysates (WCL) were diluted to 500 µg of protein in 1 ml of lysis buffer, and samples were pre-cleared for 1 hr at 4 °C with 40 µl of a 1:1 slurry of protein A-agarose beads (Invitrogen Corp) in lysis buffer and 1 µg of rabbit IgG. After a brief centrifugation to remove pre-cleared beads, 0.5 µg of anti-NRF1 antibody was added to each sample and incubated on a rocking platform at 4 °C overnight. Captured proteins were pulled down (precipitated??) by the addition of 10 µl of protein A-agarose beads to each sample and the slurries were incubated on the rocking platform at 4 °C for 2hrs. The beads were then washed five times with lysis buffer and resuspended in 40 µL of 1×SDS electrophoresis sample buffer [50 mM Tris–HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol], resolved on 12% SDS-PAGE electrophoresis and transferred onto a PVDF nylon blotting membrane. To determine whether serine residues of NRF1 are phosphorylated due to treatment, blot was probed with 1-5000 mouse monoclonal antibody against phosphoserine residue (Santa Cruz Biotech). To determine whether treatments induced modulations of NRF1 correlates with TFAM, one of its effector, WCL from same group were immunoprecipitated with rabbit anti mtTFA and

probed with mouse against mtTFA. All primary antibodies were diluted 1:1000 and detected after incubation with horseradish peroxidase(HRPO)-conjugated secondary antibody diluted 1:50,000 PBS-T. Blots were treated with ECL reagents (Amersham Biotech), and all proteins were detected by autoradiography.

Anchorage independent Growth

Anchorage-independent growth assays were performed as previously described [51]. Briefly, base support agar were made fresh by diluting 1.0% molten agarose mix with 1:1 with 2x culture media (2x DMEM/F12 media, 20% HS, 2x Penstrep antibiotics and 200 pg/ml estrogens) to a final of 0.5%. molten agar were maintained at 42 $^{\circ}$C water bath until dispensed at 200 □l/well in 48 well plates, then allowed to solidify for 4 hrs at room temperature. Top agarose overlay were made fresh by mixing 0.7% molten low melting point (LMP) agarose with 2x culture media containing appropriate 5000 cells/well, then gently overlaid over preformed base agar. Cells were incubated for a minimum of 21 days in 37°C incubator with 5% CO_2. Cultures in 48 well plate were fed once every week with 200□l of 0.5% melted agar in growth media. Colonies of separate cell aggregates at 100□M or greater in size, as measured with metamorph software (Nikon Corporation, USA) were counteds. Images were acquired by Olympus C-5060 digital camera under an inverted microscope with 4x objective.

Invasion Assay

The in vitro invasion assay was carried out to examine tumor cell invasiveness as described previously [52] with some modifications. Briefly, 24-well Transwell unit with

8 μm polycarbonate Nucleopore filters (Corning) were coated with 60 μL of 0.8 Matrigel. MCF-7 cells (1×10^5) transfected with plasmid overexpressing NRF1 or null were placed in the upper compartment, and the medium containing 10% fetal bovine serum was added to the lower compartment. For group treated with NAC, the chemical ROS modifier was added to media in the lower chamber to a concentration of 1.0 mM. The Transwell plates were incubated at 37°C for 48 and 72 hrs accordingly. Cells invaded to the lower surface of the membrane were stained with Giemsa staining and observed using light microscope. The invading cells were stained and counted per insert and photographed. Experiment were conducted in triplicates and repeated three times.

Statistical analysis

Results are expressed as mean ± S.D. Differences between means were evaluated by two-tailed Student's t-test. ANOVA was used to determine differences between groups.

RESULTS

Estrogen induced ROS levels mediate proliferation and growth of MCF-7 cells in vitro

Estrogens have been reported to modulate mitochondria ROS production in MCF-7 cells and other cell types [27;53]. These ROS are postulated by us and other [20;47;54;55] to mediate proliferation and growth of subsets of breast cancer cells. To test this hypothesis, biological ROS modulators (MnSOD and catalase) were oveerexpressed in MCF-7 cells , or cells were pretreated with chemical ROS modulators, 40 μM ebselen or 1.0 mM NAC). These cells were then challenged with 100 pg/ml estrogens. Data reveals that treatment of cells with estrogen alone increased ROS production and DNA synthesis

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whereas treatment with catalase alone (moi 50) diminished ROS production and DNA synthesis. However, co-treatment of cells with catalase (moi 50) and estrogen significantly increased ROS level and DNA synthesis beyond catalase alone infected groups. Interestingly, similar trends were observed in MnSOD infected cells at moi 50 though the magnitude in ROS production and DNA synthesis is significantly more than that observed in estrogen or catalase treated groups (Fig. 1A). Both chemical ROS modulators used in this experiment also diminished ROS production and DNA synthesis. However, when cells were infected with adenoviruses at moi of 200, catalase diminished estrogen induced ROS production and DNA synthesis while MnSOD significantly increased ROS production and dramatically reduced DNA synthesis compared to cells treated with moi 50 (Fig 1B). To resolve the inverse correlation of MnSOD over-expression at moi 200 and DNA synthesis, we infected cells with various viral loads of MnSOD expressing viruses; and as control, we also infected cells transfected with control adenovirus vectors at moi 50. Infected cells were then used for colony assay and monitored for anchorage independent growth for three weeks. We observed that low viral MnSOD load (moi 5-50) promoted increased number and size of colonies (Fig. 2B, C) whereas viral load higher than moi 50 induced diminished cell growth (Fig 2D-G). Adenovirus containing control vectors at moi 50 did not induce growth advantage over corresponding MnSOD expressing adenoviruses indicating that it's the overexpressed MnSOD that is modulating cell growth. However, to ascertain whether the observed cancer growth retardation of MnSOD infection is higher than moi 50 is due to cell lethality, we stained single cell population from all virus infected groups (control, catalase and MnSOD) with trypan blue solution and found that over 70% of the cells

infected with virus up to moi 200 were viable whereas 90% of moi 400 and above

infected cells were not viable after 21 days of cultures in soft agar assay (data not

shown). Since moi 200 infected cells were viable up to 21 days, we chose this viral load

for subsequent experiments. The effects of ROS modulators on estrogen induced growth

of breast cancer were further evaluated byco-treatment of MCF-7 cells with estrogen and

biological or chemical ROS modulator. It wasfound that these antioxidants significantly

inhibited estrogen induced growth of MCF-7 cells in vitro (Fig 3).

Estrogen induced oxidants activate Akt signaling pathway which contributes to in vitro
growth of MCF-7 breast cancer cells

The mechanisms by which estrogen induce cell proliferations and growth of estrogen

responsive breast cancer cells are attributable to ER mediated process. It has been shown

that upon estrogen stimulations, cells secrete growth factors which activate receptor

tyrosine kinases and initiation of complex signaling cascade of phosphorylation-

dephosphorylation reactions that subsequently leads to cancer cell growth and metastasis

[56]. One such signaling pathway activated by estrogen exposure that leads to survival

and growth of MCF-7 cells is the PI3K/Akt signaling pathways [57;58]. Oxidants such as

superoxides and hydrogen peroxides have likewise been reported to activate this

signaling pathway which mediates survival and growth of exposed cells [18-20]. To

determine whether E2 induced oxidants can activate PI3K/Akt signaling pathway and

whether ROS scavengers or inhibitors of PI3K would mitigateAkt phosphorylation,

MCF-7 cells were either infected with biological antioxidants, or pretreated with

chemical ROS scavengers (ebselen, NAC) prior to estrogen treatments as described in

methods. Whole cell lysate were resvolved on 12% SDS-PAGE gel, and probed with appropriate antibodies. Data reveals that 100 pg/ml estrogen treatment induced Akt activation which were inhibited by chemical or biological antioxidants as well as by LY294002, an inhibitor of PI3K which regulates Akt activations (Fig 4). These findings support the hypothesis that oxidant (ROS) induced by estrogen exposures can activate Akt signaling proteins and activated Akt has been demonstrated to favor survival, proliferation and growth of cells during periods of oxidative stress [59;60].

Estrogen induced Akt activation phosphorylates NRF1 which promotes growth of responsive cells

Cellular responses to oxidants are mediated in part, by redox activation of NRF1 which regulates mitochondria biogenesis and cellular antioxidant responses [61;62]. NRF1 has been reported to be trans-activated by Akt phosphorylation of its serine/threonine residue in response to growth factors and oxidative stress [62-64]. Trans-activated NRF1 are believed to mediate cellular responses to oxidative stress such as mitochondria biogenesis, restoration of redox homeostasis and induction of survival and proliferation mechanisms of cells [42].

Similarly, treatments of MCF-7 cells with 100 pg/ml estrogen for 45 minutes induced rapid serine phosphorylation of NRF1 (Fig 5) which were abrogated by both chemical and biological ROS modulators. In addition, silencing of Akt_1, the Akt isoform implicated in mediating cell survival and growth [65-67] likewise reduced NRF1 phosphorylation, and anchorage independent growth of MCF-7 cells (Fig 6). These

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findings imply that estrogen induced oxidants modulate NRF1 expressions. It also implies that redox activations of Akt signaling in response to estrogen exposures phosphorylate and tran-sactivates NRF1. The absence of Akt1 expression leads to reduced NRF1 phosphorylation and reduced growth of MCF-7 cells exposed to estrogen.

NRF1 expression promotes in vitro proliferation and growth of estrogen responsive breast cancer cells

In order to show that NRF1 are implicated in the mediation of growth of breast cancer cells in vitro, NRF1 was silenced in MCF-7 cells. Cells were then cultured with or without estrogens for 21days. Colony formation, growth and viability of cells in soft agar assays were measured. Data reveals that estrogen increased colony formation growth in control knockdown groups (Null kd) comparable to the untransfected cells (Fig 7B i-iv). However, estrogen fails to induce colony formation or colony growth of NRF1 silenced cells implying that NRF1 are essential in the mediation of estrogen induced in vitro growth of MCF-7 cells (Fig 7B v,vi). To test whether reduction in growth is due to cell death because of NRF1 knockdown, cells from soft agar plates were counted for cell viability per 1000 cells using trypan blue exclusion assays. We found that while there were differences in cell death due to NRF1 status, differences were not significant to account for observed lack of colony formations or growth of cells in NRF1 knockdown versus Null kd cells (data not shown). In addition, immunoprecipitation analysis of NRF1 expression and phosphorylation status revealed that Null kd cells expresses increased NRF1 protein and exhibit increased phosphorylation in response to prolonged estrogen exposure (Fig 7A,B). Silencing of NRF1 however, inhibits its expression or phosphorylation in response to chronic exposures to estrogens (Fig 7C). To confirm that

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NRF1 is indeed responsible for this observation, the expression levels of mtTFA, one of the genes targeted by NRF1 expression [68] was assessed. Western blot analysis of cell lysates from Null kd vs NRF1 knock down revealed a positive correlation between NRF1 and mtTFA expressions; i.e. up regulation of NRF1 leads to up regulation of mtTFA and vice versa (Fig 7). To confirm NRF1's role in growth regulation of MCF-7 exposed to estrogens, we overexpressed NRF1 (NRF1ox) or its DN-NRF1 and assessed in vitro proliferation and growth in response to estrogens. We observed that while over-expressing NRF1 enhanced estrogen induced proliferation and growth of MCF-7 cells, over-expressing DN-NRF1 actually reduced estrogen mediated proliferation and growth of MCF-7 cells (Fig. 8). Interestingly however, the effects of NRF1ox alone were significantly greater than E2 treated control cells in both soft agar and BrdU assays.

NRF1 over-expression modulates invasion of MCF-7 cells

NRF1 has been implicated in the modulation of various protease expressions involved in cell migration and spreading. Proteases include the calpains [37] whose over-expression is implicated in tumorigenesis, metastasis and angiogenesis in breast cancer and other cancer types [69;70], GalNAc-T3 genes that function in glycosylation of mucin proteins in epithelial derived tumors [71] which is also plyas a central role in invasive mammary [72] and colorectal [73] carcinoma. In this study, in vitro invasion assay of MCF-7 cells overexpressing NRF1 displayed a threefold increase in invading cells compared to vector or non transfected (NT) group (Fig. 9A). NRF1ox cells when transfected with DN-NRF1 or treated with ebselen showed significantly higher number of cells compared to the control MCF-7 cells trasnfected with vector/DN-NRF1 alone and also treated with

ebselen. (Fig. 9B). In addition, groups treated with estrogen also had significantly more

invading cells and mucosal environment compared with groups treated with vehicle (Fig

9B). These findings indicate that NRF1 participates in cell invasion in a redox dependent

manner.

DISCUSSIONS

Estrogens are chemicals capable of promoting growth of breast cancer cells via ER and

non-ER mediated pathways. Estrogens induce ROS via mitochondria biogenesiss. At

sublethal levels, these ROS induce redox signaling that participate in growth of estrogen

exposed breast cancer cells [27;74;75]. In this study, we provide evidence that estrogen

induced ROS are essential for proliferation and malignant growth of estrogen responsive

breast cancer cells. We also report that the mechanism involves redox activation and

phosphorylation of NRF1 because over-expression of biological ROS scavengers or

treatment of cells with chemical antioxidants inhibited NRF1 activation which in turn

reduced estrogen mediated growth and metastasis of breast cancer cells.

The role of estrogen induced oxidants in breast carcinogenesis has long been established

[76-79]. However, the role of these oxidants in mediation of growth of cancer cell

remainscontroversial. In this study, we observed that treatments of MCF-7 cells with a

dose comparable to physiological level of estrogen induced intracellular ROS which

promoted in vitro growth of breast cancer cells. Conversely pre-treatment of cells with

chemical or biological antioxidant prior to estrogen exposure inhibited induced ROS

formation and in vitro growth of cells. These observations implies that the growth

promoting properties of estrogen in breast cancer cells are mediated in part by estrogen induced oxidants as ROS scavengers abolished estrogen induced growth of breast cancer cells. These findings are in line with other reports which indicate that sublethal levels of oxidants induce significant mitogenic effects on a number of gynecological cancers [80-82] including breast cancer [4;54;83]. It may also explain the aggressive nature of breast cancer attained after therapeutic failures because these therapeutic agents also act as pro-oxidant upon their prolonged use [44-47]. Nonetheless, the initial antiproliferative responses of these chemotherapeutic agents may be due to their antioxidant properties [84-87].

Our group and others have reported that mitochondria is a major source of estrogen induced ROS [27;75] in breast cancer cells. We investigated whether altering mitochondria MnSOD activity would modulate estrogen induction of intracellular ROS and growth of breast cancer cells. We found that infection of MCF-7 cells with adenovirus overexpressing MnSOD at moi 50 increased intracellular ROS production, as well as increased proliferation and growth of MCF-7 cells. However, when cells were infected with adenovirus at moi 200, ROS production increased significantly compared to ROS from moi 50 infected cells, but cell proliferation and growth decreased significantly. ROS production from moi 50 infected cells correlated positively with DNA synthesis and growth of MCF-7 cells, however, the effects of estrogen exposure on MnSOD expressing cells were statistically insignificant. This implies that the mitogenicity observed in these cells are due entirely to mitochondria ROS and not by estrogens exposures. Therefore, while we were able to show a direct correlation between intracellular ROS production

and cell growth in response to estrogen exposure, we were unable to show any significant combinational effects between estrogen exposures, MnSOD overexpression and cell growth even though our data does indicates a correlation between MnSOD expression and cell proliferation.

It has been reported that ROS mediates mitogenic activation of the PI3K/Akt signaling pathway which promotes survival and growth of exposed cells [88;89]. Estrogens has likewise been demonstrated to activate PI3K/Akt signaling pathway in endometrial and breast cancer cells, in an ER dependent and independent manner [90;91]. However, whether oxidants mediate estrogen induced activation of PI3K/Akt signaling in breast cells and whether Akt activation is linked with growth of these cells is not clear. In our study, we observed that estrogen induced Akt activation in breast cancer cells were diminished by catalase over-expression and by chemical ROS scavengers. Whether oxidant activation of Akt in estrogen exposed cells is associated with proliferation and growth regulation is not known. To investigate this possible link, we silenced Akt1, an Akt isoform known to be involved in regulating mammary tumorigenesis and growth of cancer cells [65;92]. Wefound that knocking down this gene diminished estrogen induced proliferation and growth of MCF-7 cells. While Akt is a global activator of a number of transcription factors with diverse physiological functions, our data suggest that estrogen activation of Akt in breast cancer cells are also ROS mediated and activation of Akt signaling cascades are essential in transducing signaling processes that favors survival and growth of breast cancer cells.

Activated Akt promotes cell survival and growth via a number of mechanisms. For example, Akt phosphorylates and deactivates pro-apoptotic factors such as BAD, Caspase-9, and Forkhead transcription factors (FKHR) as well as other pro-survival transcription factors [93]. Other targets of activated Akt in response to oxidative stress includes NRF1, a redox sensitive gene known to regulate transcription of genes involved in antioxidant responses, apoptosis, as well as regulation of mitochondrial biogenesis [62;94-96]. Oxidant mediated phosphorylation of NRF1 are thought to induce nuclear translocation and transcriptional regulation of various cellular response such as restoration of redox homeostasis, enhancement of cell survival and growth, as well as regulation of mitochondria biogenesis [97]. Prolong estrogen exposure has likewise been reported to up-regulate NRF1 expressions via ER alpha (ERα) mediated mechanisms in MCF-7 cells which leads to increased mitochondria biogenesis in these cells [26]. While this observation is novel, others have reported that sodium butyrate, a non estrogenic but pro-oxidative compound [98] also induces NRF1 expression in MCF-7 cells [71]. Furthermore, SERMs such as TAM, RAL and Fulvestrant which are all pro-oxidant upon chronic exposures [99], also increases NRF1 expression in SERM resistant MCF-7 cells [100]. These studies all suggest that estrogen inductions of NRF1 in estrogen responsive or resistant breast cancer cells are not exclusive to ER status. It implies that perhaps estrogen induced oxidants may also mediate NRF1 expression in breast cancer cells irrespective of ER status.

Here, we report that exposure of MCF-7 cells to estrogen not only up-regulates NRF1 expressions, but it also induces NRF1 phosphorylation by Akt in a redox dependent

manner. Treatment of cells with ROS modulators or silencing of NRF1 fail to mediate estrogen induced NRF1 expressions and phosphorylation, and inhibited growth of breast cancer cells. While ERα is believed to regulate estrogen induced NRF1 expression in MCF-7 cell line [26], our data indicates that estrogen induced oxidants are essential for NRF1 expressions and phosphorylation. Upon phosphorylation, this transcription factor is believed to translocate into the nucleus where they regulate mitochondria biogenesis, oxidative phosphorylation and mediates oxidant induced survival and growth of breast cancer cells, by unknown mechanism. These observation are in agreement with reports that have shown NRF1 is phosphorylations by oxidants in rat hepatoma cells promoting survival and growth of during periods of oxidative stress [62]. Our data may also explain the observation that despite the high oxidative environment of breast cancer cells in vivo [11], these cells survive, proliferate and grow as opposed to undergoing apoptosis and cell death. Activated NRF1 may also cause cancer cell metastasis, therapeutic failures and poor prognosis of breast cancer patients as oxidative stress are associated with metastasis and drug failures in a number of malignancies including breast cancer [101;102]. In addition, it has been reported that estrogen and insulin induce proliferation and growth of MCF-7 cells via NRF1 activation [103], but insulin, with estrogens also, act as pro-oxidants [104;105] and activate Akt signaling pathways [106;107] in a variety of cells types. It is therefore feasible that the pro-oxidative properties of estrogen alone or in combination with insulin promote survival and growth of breast cancer cells through persistent expressions and phosphorylation of NRF1.

In summary, overexpression of MnSOD and Catalase or treatment with antioxidant ebselen selectively inhibited cell proliferation and invasion of MCF-7 cells that were transfected with NRF1. Also, knocking down Akt isoform in estrogen treated cells that has been shown to phsphorylate NRF1 reduced the rate of cell proliferation. It appears therefore, that the level of NRF1 expression indicates a redox state of breast cancer cells and activation of NRF1 via Akt pathway is a key modulator of estrogen induced breast cancer cell proliferation and metastasis.

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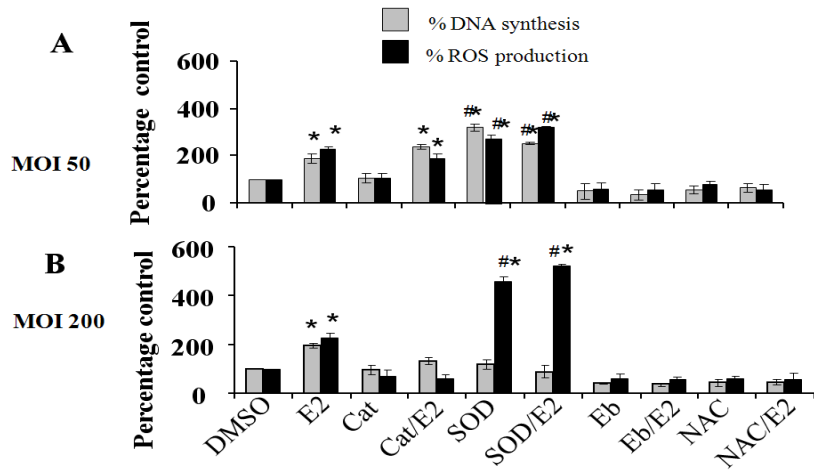
Fig. 1. Estrogen induced ROS mediates in vitro proliferation of MCF-7 breast cancer Cells. 3500 cells/well were seeded in 96well plate for BrdU cell proliferation assays and 1.0x10^4cells/well for ROS production assays. Next day, cells were infected with Adenovirus over-expressing MnSOD or Catalase at either moi 50 or 200 in serum free media. Other group of cells was treated with 40μm ebselen and 1mM NAC diluted also in serum free media. Cells were cultured for 48hrs and assayed for ROS production or BrdU assays. For ROS assays, cells were pretreated for 4hrs before assay with ebselen and NAC after which 10μM DCF were loaded onto the cells for 20mins, then cells were challenged with 100pg/ml estrogen, and ROS readings were taken every 5mins on Tecan plate reader. For cell proliferation assays, 48hrs post infections, wells were challenged with 100pg/ml estrogen and allowed to incubate for additional 18hrs after which BrdU assay were carried out with Roche BrdU assay kit. (A) MCF-7 cells infected with catalase or MnSOD at moi 50, or treated chemical antioxidant, then challenged with estrogen. (B) MCF-7 cells infected with catalase or MnSOD at moi 200, or treated chemical antioxidant, then challenged with estrogen. ROS production upon E2 challenge was compared with DNA synthesis. Data is expressed as standard error of mean of three experiments, (+/- SE). (*) indicates significant different from control group. (*#) indicates treatment significantly different from E2 treated group. (P<0.05)
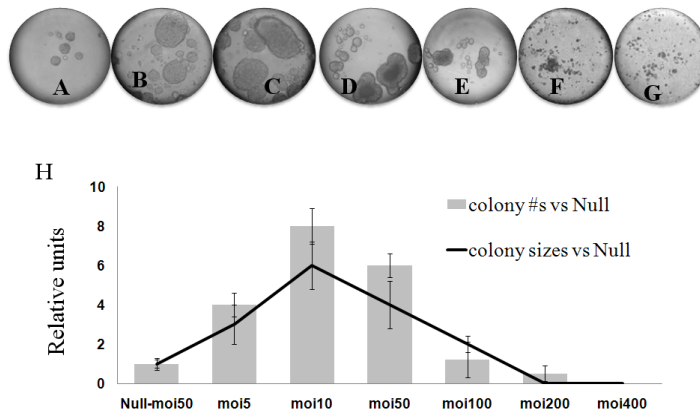
Fig. 2. Differential effects of ROS production on in vitro growth of MCF-7. MCF-7 cells were infected with various concentrations of MnSOD over-expressing viruses for 24hrs after which cells were detached and seeded for soft agar assays such that each well in 48 well plate had 5000cells/well. Cells were incubated for 5weeks with weekly feeding. Colony numbers and sizes were scored using Nikon metamorph software and expressed as mean of five wells, +/- SD. (*) indicates significant difference from control group (null moi 50), (P<0.05). Representative Images were acquired with hand held Nikon camera over an inverted microscope with 4x objective. A) AdEmpty (Null) moi50, B) AdMnSOD Moi 5, C) AdMnSOD moi 10, D) AdMnSOD moi 50, E) AdMnSOD moi 100, F) AdMnSOD moi 200, G) AdMnSOD moi 400, H) Mean colony and size numbers compared to null vector.
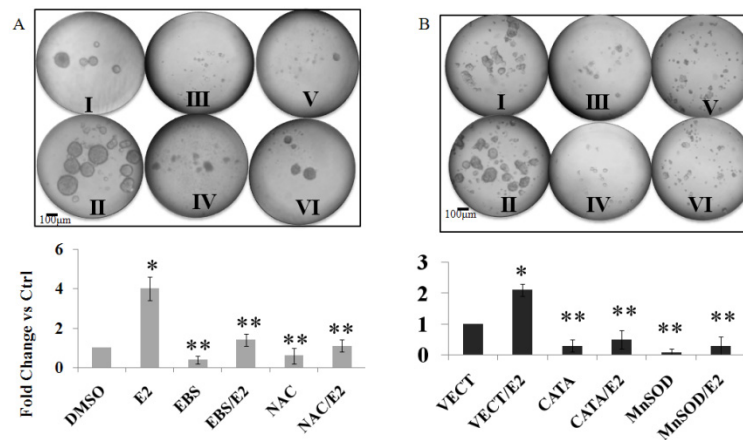
Fig 3. ROS Scavengers inhibits E2 induced growth of MCF-7 cells. Cells were infected with biological antioxidants for 24hrs in serum free media and allowed to recover afterwards. 1.0x10^4 cell pellet was resuspended with 500ul 2x culture media containing 100 pg/ml E2 or 40 µM ebselen. Cell suspensions were then mixed 1:1 with 0.5% molten agarose and over layered on precast bottom agarose in 48 well plate. Cultures were allowed to incubate for 3-5wks with weekly feeding and images were acquired with a hand-held Nikon digital camera over 4x inverted microscope. Qudruplicate wells were

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counted and expressed as mean three experiments +/- SE. (Panel A: Chemical antioxidant treatment group) (I) DMSO, (II) 100pg/ml E2, (III) Ebs, (IV) Ebs/E2, (V) NAC, (VI) NAC/E2. (Panel B: Biological antioxidant treatment group) (I) DMSO, (II) 100pg/ml E2, (III) Cata, (IV) Cata/E2, (V) MnSOD, (VI) MnSOD/E2. (*) indicates significant difference from control group. (**) indicates significant difference from E2 treated group, ($P<0.05$).
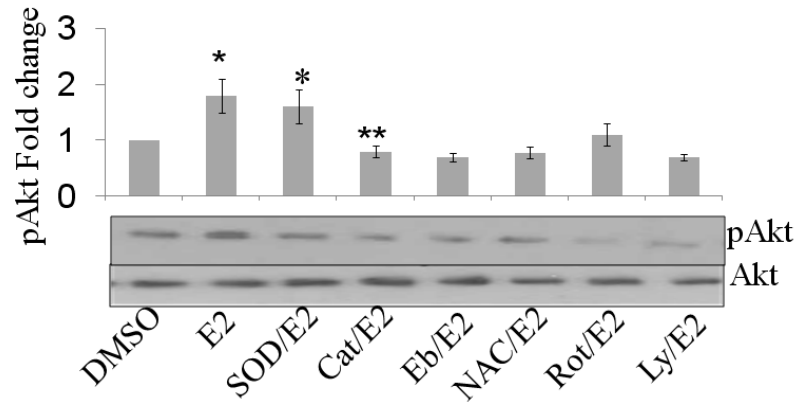

Fig. 4. Estrogen induced activation of Akt is abrogated by ROS scavengers. MCF-7 cells were either pre-infected with MnSOD or catalase at moi 50 and cells seeded for experiment, or cells were seeded and treated with 40 μM ebselen, 10μmol/L LY294002 (LY) and 1.0μM Rotenone for 48 hrs in starvation media. At end of starvation periods, cells were harvested and lysed with RIPA buffer, and 100 ug total proteins were fractionated in SDS PAGE electrophoresis gel. Blots were probed for phosphorylated Akt (pAkt) and total Akt and band intensity were quantified with Biorad GelDoc 2000 and normalized to vehicle. (A) Representative Immunoblot probed for pAkt and total Akt. (B) Densitometry of pAkt normalized with total Akt and expressed as fold change vs. DMSO. (**) indicates significant difference from control group. (*) indicates significant difference from E2 treated group, ($P<0.05$).
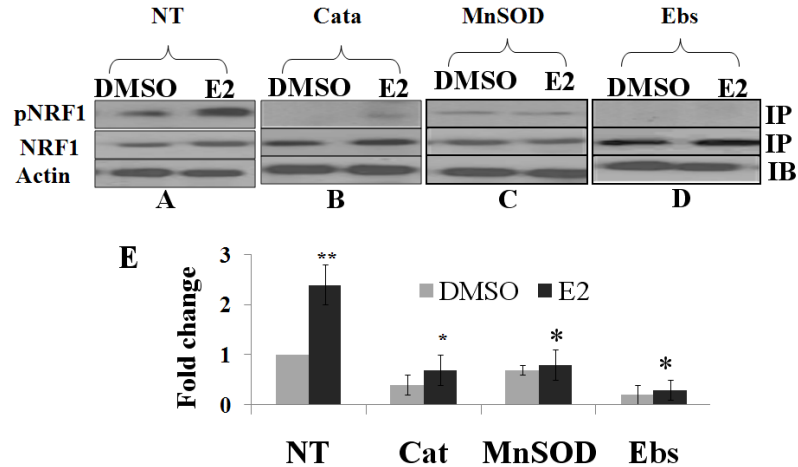
Fig. 5. Estrogen induced NRF1 activation is abrogated by ROS scavengers. To determine whether ROS scavengers mitigates estrogen induced activations of NRF1 in breast cancer, MCF-7 cells were seeded, infected with catalase or MnSOD, or with 40uM ebselen and estrogen as previously described. Lysate were immunoprecipitated with NRF1 and immunoblot probed with anti serine antibody (pNRF1) or NRF1 antibody. For loading control, 50 ug whole cell lysate (WCL) were fractionated and blot probed with beta actin (actin). Band intensity of pNRF1 was compared with NRF1, and then normalizes with DMSO from NT group. Representative data of 3 experiments is presented above. (Panel A) Non transfected group (NT) treated DMSO or E2, (Panel B, C) Catalase or MnSOD infected groups respectively, and then treated with DMSO or E2. (Panel D) Ebselen and E2 co-treated group. (E) Densitometry of pNRF1 normalized with total NRF1 and expressed as fold change vs. DMSO in NT group. (**) Indicates that E2 treatment group is significantly different from control (DMSO) groups while (*) indicates that other treatments groups are significantly different from E2 treatment groups. Data is expressed as mean of 3 experiments, OD $\pm$ SE, (P<0.05).
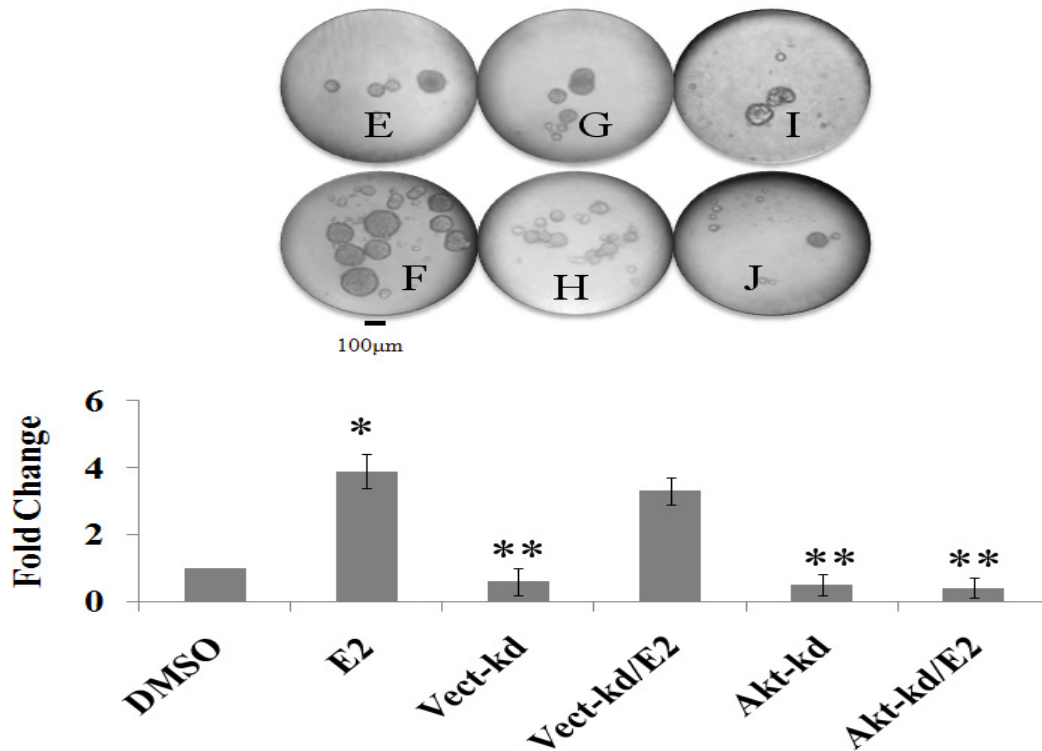

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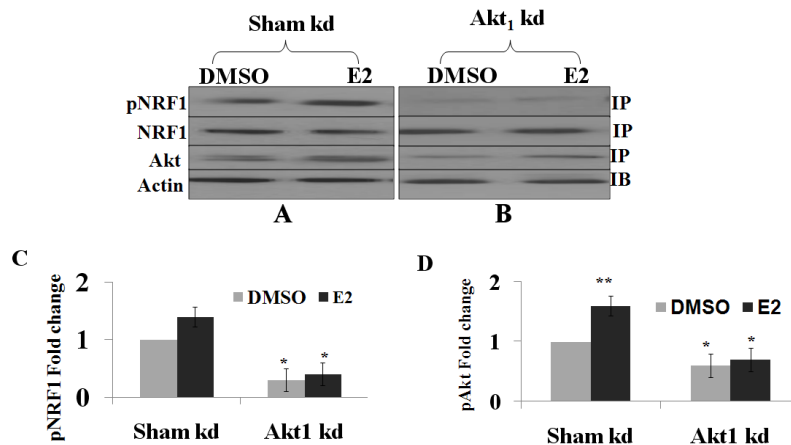
Fig. 6. Akt phosphorylate and activate NRF1 which mediate in vitro growth of MCF-7 cells. To determine role of Akt and NRF1 phosphorylation in estrogen induced growth of breast cancer cells, MCF-7 cells were transfected with pre-designed and verified Akt1 shRNA plasmid or its vector for 72 hrs with FuGENE 6 transfection reagent without change of media. Post transfection, cells were seeded for colony assay, or challenged with 100 pg/ml estrogen or DMSO for 30 mins. Cells were then harvested with RIPA buffer and 500 ug total protein was IP with NRF1, fractionated on SDS-PAGE and blots probed with serine antibody. 50 ug of WCL was also fractionated on SDS-PAGE gel and probed for total Akt, and β actin as experimental loading control. Band intensity were measured with Biorad versadoc 2000 software and expressed as fold change from DMSO. (Panels A and B) Western blot analysis of vector and Akt$_1$ shRNA plasmid transfected into MCF-7 cells then treated with DMSO or E2. (C D) Band intensity of NRF1 phosphoserine (pNRF1) normalized to total NRF1, and Akt normalized to actin respectively. (E-J) Representative pictures colonies from soft agar assay after 3 weeks incubation, and expressed as mean +/- SE of three experiments. (E) DMSO, (F) E2, (G) vector- kd/DMSO, (H) vector- kd/E2, (I) Akt$_1$ kd/DMSO, (J) Akt$_1$ kd/E2, (K) Fold increase of colony formation versus DMSO. (**) Indicates that E2 treatment group is significantly different from control (DMSO) groups while (*) indicates that other treatments groups are significantly different from E2 treatment groups. Data is expressed as mean of 3 experiments, OD ± SE, (P<0.05).
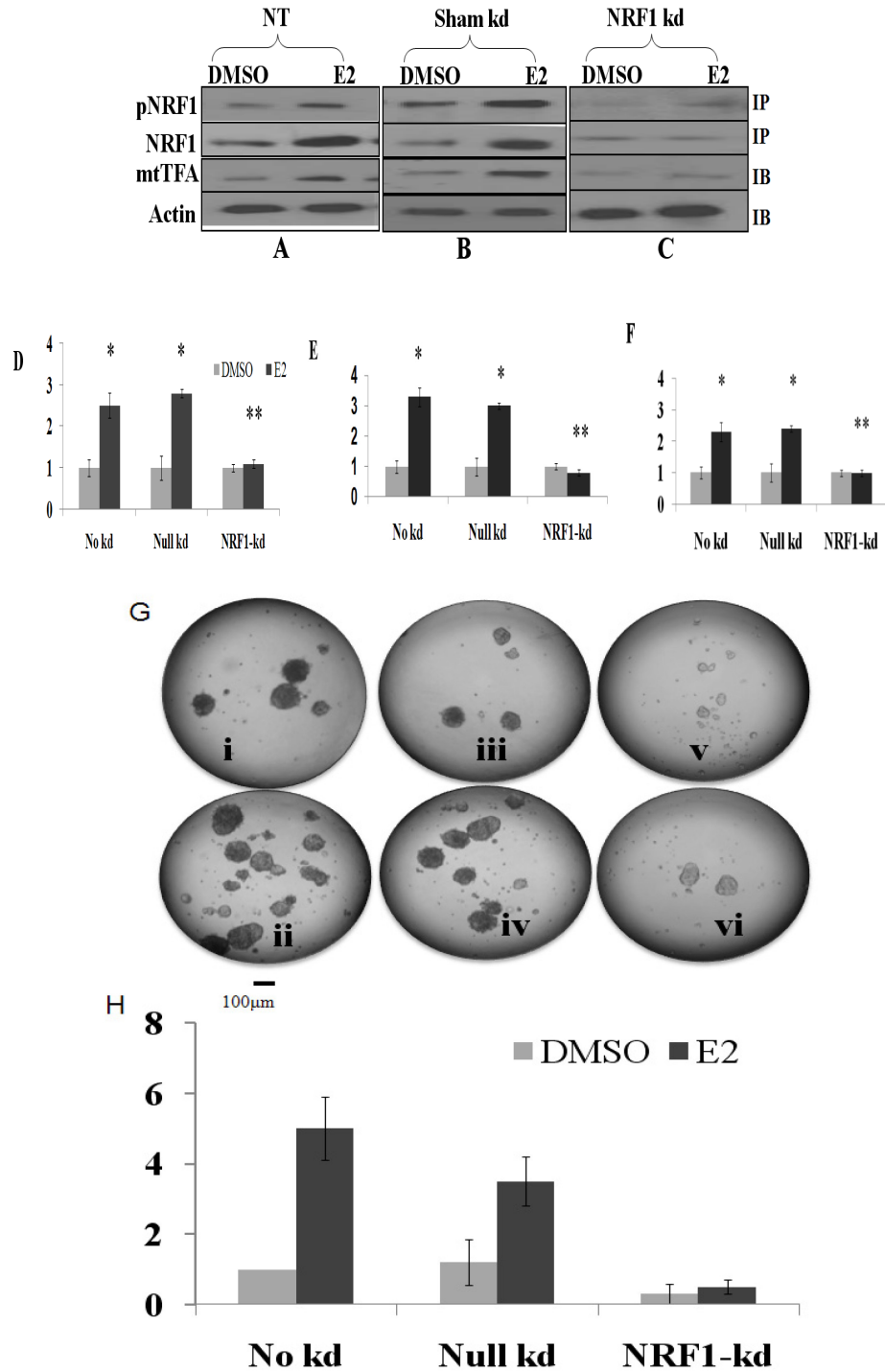
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Fig. 7. Silencing of NRF1 attenuates estrogen induced in vitro growth of MCF-7.
MCF-7 cells were transfected with shRNA vector or NRF1 shRNA plasmid for 96 hrs.
Post transfection, cells were seeded for colony assays or treated with DMSO or 100 pg
E2 for 30mins, then harvested and lysed with RIPA buffer. 500 ug total proteins were co-
immunoprecipitated with anti NRF1 antibody, and blots probed with either anti NRF1,

anti phosphoserine and anti mtTFA antibodies. (Panel A) Non transfected cells (NT) probed with anti serine, anti NRF1, anti mtTFA and anti actin respectively. (Panel B) Vector transfected cells probed with anti serine, anti NRF1, anti mtTFA and anti actin respectively. (Panel C) NRF1 kd cells probed with anti serine, anti NRF1, anti mtTFA and anti actin. (D-F) Densitometry of NRF1 knocked on (D) phosphorylated NRF1 compared to total NRF1, (E) total NRF1 compared to actin. (F) mtTFA expression compared to actin. (G) Representative pictures of colony assay of MCF-7 cells with NRF1 knockdown + estrogens treatment; (i) NT-DMSO, (ii) NT-E2, (iii) vector-DMSO, (iv)vector-E2, (v) NRF1-kd-DMSO, (vi) NRF1-kd-E2. (H) Fold change of colonies compared to NT-DMSO. (*) Indicates that E2 treatment group is significantly different from control (DMSO) groups while (**) indicates that other treatments groups are significantly different from E2 treatment groups. Data is expressed as mean of 3 experiments, OD ± SE, (P<0.05)
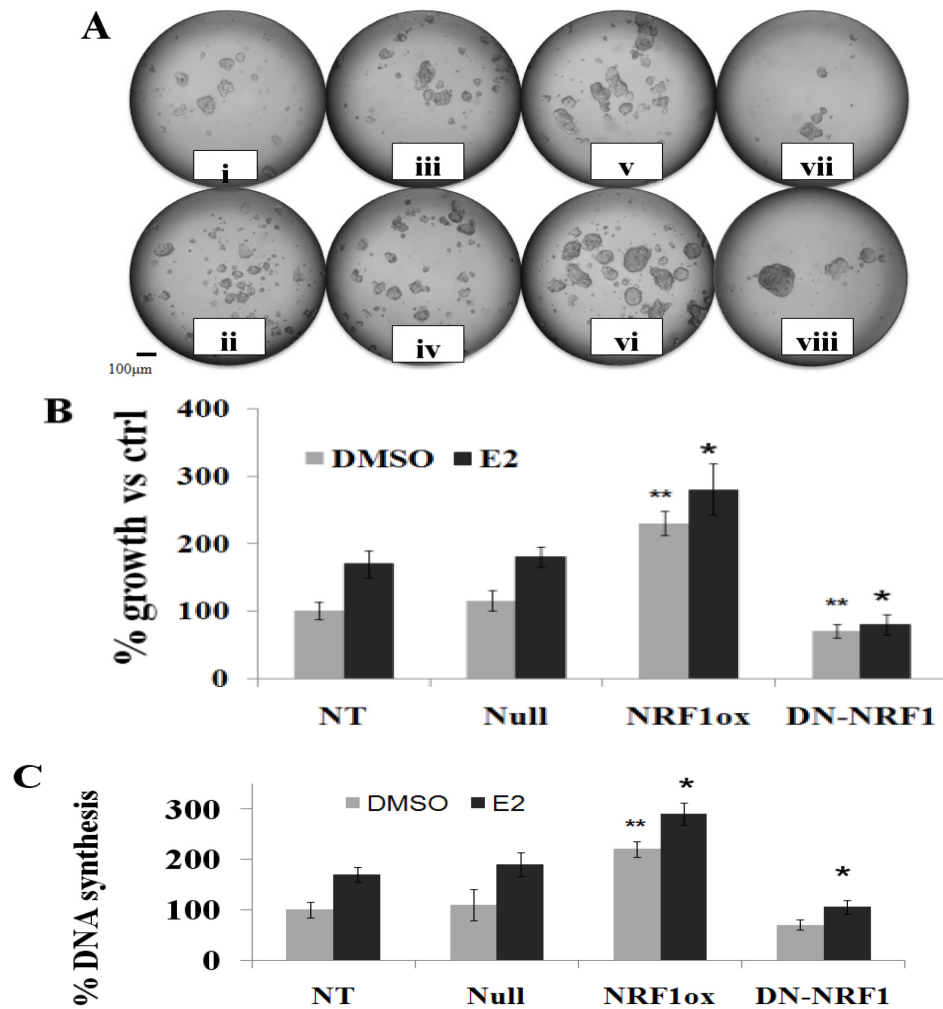
Fig. 8. NRF1 expressions modulates estrogen induced proliferation and growth of MCF-7 cells. MCF-7 cells were transfected with plasmid either over-expressing or under-expressing NRF1 (NRF1-ox and NRF1-kd for 48hrs. Transfected cells were subsequently used for colony assay, with or without 100 pg/ml E2 treatments for 21 days. (A) Colonies formed by MCF-7 cells transfected with NRF1 modulating plasmid with vector control. (i) NT-DMSO, (ii) NT-E2, (iii) Vector-DMSO, (iv) vector-E2, (v) NRF1-ox-DMSO, (vi) NRF1-ox-E2, (vii) NRF1-kd-DMSO, (viii) NRF1-kd-E2. (B). Enumerated colony count were expressed as percentage change from NT treatment group +/- SD of three experiments. (*) indicates treatment significantly different from E2. (**) indicates treatment significantly different from control. (P<0.05)
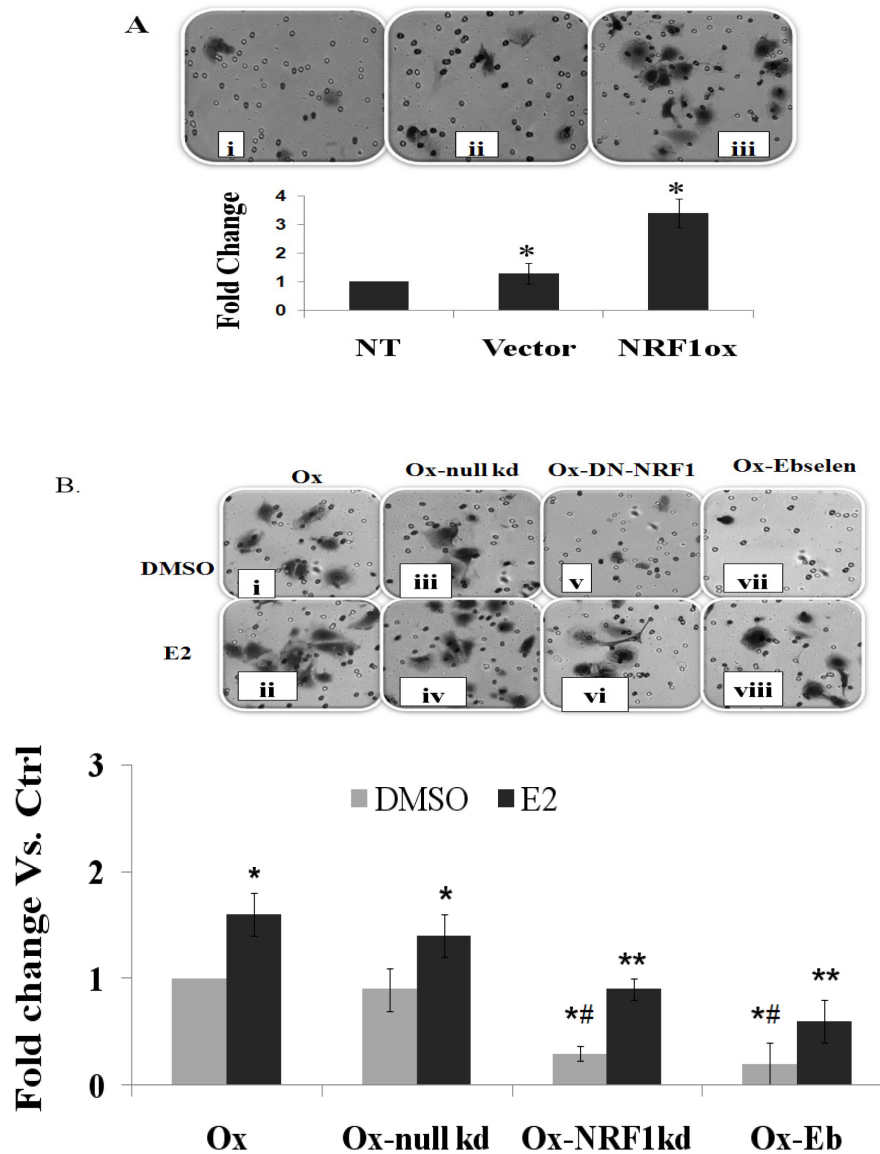
Fig. 9. NRF1 over-expression modulates invasion of MCF-7 cells. MCF-7 cells were transfected with either Null vector (Vector) or NRF1 over-expressing vector (Ox). In a separate experiment, cells were also co-transfected with Ox, Ox+ vector, Ox+NRF1 knockdown plasmids (Ox+Kd). 1.0 x 10^4 cells were seeded on matrigel invasion chamber with 8.0 μM pore size and 10% FBS media with or without 100 pg/ml estrogen were added to the bottom of wells as chemoattractant. Cells were cultured for 72 hrs in CO2 incubator. Invaded cells at bottom of wells were stained and images were acquires with Nikon microscope over 20x objective, and metamorph software. Data represents invaded cells over 5 fields, +/- SD. (A)Invasion of NRF1ox expressing cells; i) DMSO, ii) vector, iii) NRF1ox. (B) Invasion of Co-transfected NRF1ox plasmid with or without estrogen as chemoattractant; i) Ox+DMSO, ii) Ox+E2, iii) Ox+vector+DMSO, iv) Ox+vector+E2, v) Ox+ NRF1 kd+ DMSO, vi) Ox+NRF1+E2. (*) indicates treatment significantly different from Ox/DMSO. (**) indicates treatment significantly different from Ox/Null/E2. (*#) indicates treatment significantly different from Ox/Null/DMSO. ($P<0.05$)

CHAPTER VI

THE ACTIVATION OF NRF1 BY ESTROGEN IS NECESSARY FOR BREAST CELL
SUSCEPTIBILITY TO DEVELOP A MALIGNANT PHENOTYPE AND FOR
INVASIVE GROWTH OF BREAST TUMORS

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ABSTRACT

Understanding the contribution of NRF1 activation to the susceptibility for the
development of malignant phenotype cells by estrogen is the focus of this study.
Consistent with the redox-sensitivity of NRF1, 4-OH-E2-induced NRF1 expression and
its phosphorylation and acetylation were inhibited by the glutathione peroxidase mimic,
ebselen and by overexpression of ROS modifying genes, catalase and MnSOD. NRF1
was bound to the promoters of the cell cycle genes pcna, cyclin b1, and cdc25c, and prc1,
confirming the prediction that these promoters contain NRF1 elements. The exposure of
MCF10 A cells to a carcinogenic dose of 4-OHE2 increased the binding of NRF1 to the
promoters of these genes and that 4-OHE2-induced binding could be inhibited by
cotreatment with ebselen (antioxidant), or over expression of catalase (H2O2 scavenger).
These results suggest that 4-OH-E2-generated oxidants, particularly H2O2, may mediate
NRF1 binding to these gene promoters. Decreasing NRF1 protein levels via specific
siRNA Knock down of NRF1, PCAF and AKT inhibited 4-OH-E2 induced generation of
malignant phenotype. Together, these data suggest that ROS accelerate cell-cycle
progression and anchorage-independent growth in 4-OH-E2-transformed cells by

controlling the expression of cell cycle genes in an NRF1-dependent manner. In summary, estrogen-mediated activation of a redox sensitive signaling pathway regulates NRF1 phosphorylation and acetylation events. These two events enhance its activating function by increasing its localization close to nuclear targets and its affinity for promoters of target genes. This, in turn, contributes to the susceptibility of normal breast epithelial cells to develop malignant phenotype.

INTRODUCTION

NRF1/α-PAL (nuclear respiratory factor-1/α-palindrome-binding protein) is a transcription factor (1-3). Initially, this transcription factor was mostly famous for controlling nuclear-mitochondrial interactions that coordinate regulation of nuclear and mitochondrial genes during organelle biogensis. A genome-wide analysis has revealed that NRF1 binding elements are present in genes involved in DNA replication, mitosis, and cytokinesis, suggesting that NRF1 plays an important role in cell cycle regulation (4,5). Interestingly, the NRF1 recognition site is one of the seven transcription factor binding sites which are most frequently found in the proximal promoters of ubiquitous genes, indicating a broader spectrum of target genes for NRF1. NRF1 overexpression has been observed in hepatoma, neuroblastoma and thyroid oncocytoma (6-8). In B cell chronic lymphocytic leukemia (CLL) patients, the mRNA expression of the mitochondrial biogenesis factors NRF1 and TFAM in the majority of leukemic cells is higher than normal lymphocytes (9). While there are more than 20 Oncomine studies with NRF1 over expression, the (actual) role NRF1 plays in breast cancer remains the least studied of all transcription factors. We have recently meta-analyzed 18 published

breast cancer microarray data (10). From our study, we found that: 1) NRF1 was significantly overexpressed in ER+ tumors compared to ER- tumors; and 2) NRF1 was significantly overexpressed as breast tumor's grade increased. Investigation of the role of NRF1 in breast carcinogenesis is the central focus of this study.

Elevated lifetime estrogen exposure is a well-known major risk factor for breast cancer, however the underlying mechanisms of breast susceptibility to estrogen's carcinogenic effect remain elusive. In human breast cancer cells, the expression of almost 15% of the genes significantly affected by E2 contains the NRF1 binding element, and the NRF1 binding signature is significantly enriched in the promoters of genes induced by estrogen treatment (11). Recently, Klinge's group reported that the NRF1 promoter contains a half imperfect ERE, and E2 stimulates NRF1 expression (12) through the ER pathway. Motifs bound by ELK1, E2F, NRF1 and NFY positively correlate with malignant progression of breast cancer (13). From these studies, it appears that NRF1 may contribute to estrogen-induced malignant transformation of breast epithelial cells. Therefore, we first investigated in this study whether NRF1 influences the susceptibility of the breast epithelial cells to develop tumors in response to exposure to a well known major breast cancer risk factor, estrogen. 4-OH-E2 is the most prevalent E2 metabolite in human breast tumor tissues, with tumor tissue concentrations exceeding normal tissue concentrations by several folds (14-16). Moreover, 4-OH-E2 is strongly carcinogenic, while E2 is weakly carcinogenic and the other E2 metabolite, 2-OH-E2, is non-carcinogenic. Based on these data, 4-OH-E2 was used as the main model compound in

this study to produce malignant phenotype in breast epithelial cells differentially expressing NRF1.

NRF1 is highly redox sensitive protein (17,18-22). Similar to the other transcription factors, the activation of NRF1 is essential for its optimal binding to the promoters of target genes. NRF1 can bind to DNA in the absence of its phosphorylation. However, both phosphorylation and acetylation increase NRF1 DNA binding to the promoters and transcriptional activation (23,24). We and others have shown that estrogen exposure to breast cells produce reactive oxygen species (ROS) (18,25,26,27). We have shown that NRF1 binding to its promoter is inhibited by mitochondrial inhibitors as well as by antioxidants (18). Thus, estrogen-generated ROS may also regulate NRF1 expression. Therefore, we also examined whether a carcinogenic dose of 4-OH-E2 increases NRF1 phosphorylation and acetylation in MCF-10A cells and that these changes are inhibited by antioxidants or overexpression of catalase. Our findings revealed that activation of NRF1 by phosphorylation and acetylation depended on estrogen-induced ROS formation. This is essential for NRF1 to contribute to the susceptibility of breast epithelial cells to estrogen's carcinogenic effect. We further determined whether NRF1 over-expression converts non-aggressive breast tumors to aggressive and invasive tumors, and a dominant negative suppressor of NRF1 is sufficient to prevent aggressive and invasive tumor formation in vivo. We confirmed our in vitro findings of the role of NRF1 in breast cancer invasion by in vivo.

MATERIALS AND METHODS

Reagents

17β-Estradiol (E2), 2-hydroxyestradiol (2-OH-E2), 4-hydroxyestradiol (4-OH-E2), Ebselen, N-acetyl-cysteine (NAC), Dimethylsulfoxide (DMSO) were all purchased from Sigma (St Louis, MO, USA). All antibodies; PI3K (p110), phospho PI3K (p85), phospho-Akt (ser 473) and total Akt antibodies were purchased from Cell Signaling Technology Inc. (Boston, MA). All tissue cultures reagents were purchased from Invitrogen Corporation (CA) unless otherwise specified.

Culture of cells and MCcells and Adenovirus Gene Transfer

Human mammary epithelial cells (MCF-10A) and breast cancer MCF-7 and MDA-MB 231 cells were obtained from American Type Culture Collection (ATCC). MCF-10A cells were routinely cultured in phenol red-free DMEM-F12 media (1:1) supplemented with 5% horse serum, hydrocortisone (0.5 μg/ml), insulin (10 μg/ml), epidermal growth factor (20 ng/ml), 100 ng/ml cholera toxin and penicillin-streptomycin (100 μg/ml each) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Breast cancer cells were cultured in DMEM-F12 Media with 10% fetal calf serum. The cell culture media, serum, antibiotics, and growth supplements except cholera toxin (Calbiochem, La Jolla, CA) were purchased from Invitrogen Corp CA). For experimental purposes, culture media were changed to starvation media (serum free media + antibiotics) and allowed to incubate for 48 hrs prior to commencement of most experiments, unless otherwise indicated. Serum deprivation synchronizes cells in the G0/G1 phase of the cell cycle.

The Adenovirus-CMV (empty vector), Adenovirus-MnSOD (AdCMVMnSOD), and Adenovirus-Catalase (AdCMVCat) constructs were purchased from ViraQuest, Inc. (North Liberty, IA, USA). The adenovirus constructs used were replication-defective, E1- and E3-deleted recombinant adenovirus. Inserted into the E1 region of the adenovirus genome was either the human MnSOD or catalase gene, both of which are driven by a cytomegalovirus promoter. Cells were seeded in plates at 15%-70% confluence. The following day, cells were infected with Adenoviruses over-expressing MnSOD or catalase or vector at 100 MOI in serum free media. Control cells were treated with 100 MOI of the adenovirus-CMV construct. This viral load were determined to achieve greater than 50% growth arrests of MCF-10A cells without significant cell death for duration of experiment. Infected cells were cultured for 48 hrs after which cells were used for experiments.

Akt1 and NRF1 RNAi transfections

Pre-designed and verified human shRNA for Akt1 or NRF1 and corresponding null vector controls were purchased from OriGene (OriGene Technologies, Inc. Rockville, MD). Transfections of cells were carried out in sub-confluent cell population using FuGENE 6 (Roche) transfection reagents according to the manufacturer's protocol. Briefly, MCF10A cells were seeded in 6 well plates with growth factor supplemented media (SM) overnight. Post seeding, cells were transfected with 2 μl of Fugene-6 (Roche) preincubated for 20 min at room temperature with 0.5 μg plasmid RNAi or its null controls (sham). Forty eight hrs post transfection, media were changed to serum-free (starvation media) media and incubated for additional 48 hrs, after which cells were used

for various experiments. Transfection efficiencies ranged between 60-80% as quantified by decreased protein expressions levels.

Cell viability assay

CellTiter-Fluor™ Cell Viability kit was purchased from promega corporation and used according to manufacturer's instructions. Briefly, cells were seeded in 96 well plate at a density of $1.0x10^4$ cells/well, serum starved for 48 hrs and treated with estrogens or ROS modifiers. At the end of treatments procedure, substrate reagent (GF-AFC) were mixed with substrate buffer and dispensed into well. This assay measures protease activities in live cells as opposed to MTT or MTS assay kits that measure formations of formazon crystals by mitochondrial enzymes. Plates were read on a fluorescence plate reader at 380–400 nm excitation and 505 nm emission and data is expressed as mean of three experiments +/- SD.

Cell transformation

The cell transformation was carried out by a modified protocol of Russo's group (18). Briefly, MCF-10A cells were seeded at 30% density in a 10 cm dish. After 24 hrs of seeding, media were replaced with stavations media and allowed to culture for 48 hrs, and then cells were subjected to two treatment cycles with E2 or its catechol metabolites. A treatment cycle includes 48 hrs starvation period, 48 hrs treatment period (100 ng/ml of either E2, 2-OHE2, and 4-OHE2), and 48 hrs recovery period in growth media containing 10% horse serum (HS) and no growth suppliments. At the end of two treatments cycles, cells that would be used for immunoprecipitation and Western blot analysis were treated

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for additional 30 mins with estrogens, lysed with RIPA buffer, immunoprecipitated and processed for western analysis. For anchorage independent growth assay 5000 cells/well were used for colony formation assays in soft agar.

Anchorage independent Growth

Anchorage-independent growth, an indicator of neoplastic transformations of cells, was assessed soft agar assay. Briefly, base support agar were made fresh by diluting 1.0% molten agarose mixed with 1:1 2x culture media (2x DMEM/F12 media, 20% HS, 2x penstrep antibiotics and 200 pg/ml estrogens) to a final 0.5%. Molten agar was left at 42 oC in a water bath until dispensed at 200 ul/well in 48 well plates, then allowed to solidify for 4 hrs at room temperature. Top agarose overlay were made fresh by mixing 0.7% molten agarose with 2x culture media containing appropriate 5000 cells/well, then gently overlaid over base agar. Cells were incubated for a minimum of 21 days in 37°C incubator with 5% CO_2. Cultures were fed every week with top agar layer and colony formation was assumed when cell masses are 100 micron or greater as measured on a Nikon TE2000U inverted microscope (Nikon Corp., USA) with Metamorph software (Universal Imaging, USA), Images were acquired by using an Olympus C-5060 digital camera attached to the Nikon TE2000U inverted microscope with 4x objective. Four wells were enumerated for each groups and data expressed as mean of five wells +/- SD.

Invasion Assay

In order to determine invasiveness of cells NRF1 overexpressing, cells (5.0 x 103 cells/ml) were seeded over 8 μm pore transwell filter insert (Transwell, Coastar Cambridge, MA) precoated with Matrigel (Collaborative Research, Bedford, MA).

Chemoattractants used were reduced growth factor supplemented media or media with 10% FBS media positive control cells MDA MB 231. Matrix invasion were allowed for 16 hrs at 37 °C in a CO2 incubator. The non-invaded cells inside chambers were wiped off with a cotton swab, and the filters were fixed, stained by Diff Quick (Sigma, St. Louis, MO), cut out and mounted onto glass slides. The total number of cells that crossed the membrane were counted under a light microscope, enumerated and expressed as fold increase compared to parent cell line. The experiments were repeated five times and results are expressed as the mean±S.E.

Chemical antioxidant treatments

The treatment procedure for Ebselen (a glutathione peroxidase mimetic which also removes both H2O2 and peroxynitrite) or NAC (a precursor of glutathione and scavenger of ROS) (31, 32) varies according to the experiments design. For all experiments, 40 μM ebselen and 1.0 mM NAC were used for cell treatments. For example, in DCF assays, antioxidants were pre-loaded onto cells for 2-4 hrs before ROS measurement commences. For BrdU assays, cells were cultured with the chemical antioxidants throughout the experimental procedure. For transformation regimen, antioxidants were applied to cells each time cells were treated with estrogens. For anchorage independent growth assays, antioxidants were added to soft agar matrix media and during weekly feeding of colonies.

Immunoprecipitation and Western Blot Analysis

After the respective treatments, cells were rinsed twice with ice cold phosphate buffered saline (PBS), harvested with lysis buffer (150 mM NaCl, 0.5% deoxycholate, 0.1%

Nonidet P-40, 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (Roche). Samples were diluted to 500 μg of protein in 1 ml of lysis buffer, and pre-cleared for 1 hr at 4 °C with 10 μl of 1:1 slurry of protein A-agarose beads (Invitrogen Corp) in lysis buffer. After a brief centrifugation to remove pre-cleared beads, 2 μg of desired capture antibodies were added to each supernatant and incubated on a rocking platform at 4 °C overnight and captured proteins were precipitated with 40 μl of protein A-agarose beads for 2 hr. The beads were washed five times with lysis buffer and resuspended in 40 μL sample loading buffer, subjected to electrophoresis and electro-blotted onto a PVDF nylon membrane. Primary antibodies used for Western blots were diluted 1:1000 in phosphate buffered saline Tween-20, PBST and horseradish peroxidase-conjugated secondary antibodies were diluted 1:50,000 in PBST. Blots were treated with ECL reagents (Amersham Biotech), and proteins were detected by autoradiography. Band intensity was quantified with Bio-Rad Gel Doc Imaging System.

Immunofluorescence Labeling

MCF-10A cells were seeded and treated in chamber slides as indicated in legends to the figures. Post treatments, cells were fixed with ice cold methanol for 15mins, and permeabilized with 0.5% Triton X-100 for 30. Cells were blocked with 1% normal goat sera for 1 hr after which they were probed with antibodies diluted 1:500 for Akt and 1:500 for phospho Akt. Alexa Fluor labeled secondary antibody directed against Akt antibody was diluted 1:1000. The confocal fluorescence images were scanned on a Nikon TE2000U inverted fluorescence microscope equipped with a Nikon D-Eclipse C1 laser scanning confocal microscope system (Nikon Corp., USA). The z-series scanning were

done at every 1 μm up to a z-depth of 10 μm by using a Nikon 40 x 1.30 NA DIC H/N2 Plan Fluor oil immersion objective. The built-in Nikon EZ-C1 software was used for confocal image acquisition and analyses.

RESULTS

Estrogen-induced ROS regulate NRF1 expression: Recently, Klinge's group reported that the NRF1 promoter contains a half imperfect ERE, and E2 stimulates NRF1 expression (12) through the ER pathway. ROS is an important mediator of mitochondrial biogenesis by controlling the expression of the nuclear respiratory factor-1 and mitochondrial transcription factor A. NRF1 expression is increased by menadione, lipopolysaccharide (LPS), tertiary butyl hydroperoxide (t-BH), and H2O2 (19,20). Using physiologically achievable E2 concentration of 1 nM, which corresponds to the estrogenic menstrual peak, we have shown that E2 induces rapid formation of ROS in MCF-7 cells (21,22). Therefore, we tested the effect of carcinogenic regimen of 4-OH-E2 on the NRF1 expression in presence of ROS modifiers. The expression of NRF1 was increased by several folds by 4-OH-E2 and this increase in the expression of NRF1 was inhibited by over-expression of catalse and MnSOD and co-treatment with Ebselen (Figures 1 and 2). This indicated that 4-OH-E2-induced NRF1 expression is redox sensitive.

The exposure of MCF-10A cells to carcinogenic dose regimen of 4-OH-E2 increased the phosphorylation and acetylation of NRF1 (Figures 3-5). Both phosphorylation and acetylation of NRF1 by two independent signaling molecules may control NRF1 activation. We assessed phosphorylation and acetylation of NRF1 by Western blotting

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and confocal microscopy after treating MCF-10A cells with 4-OH-E2 in serum-free medium in the same condition that produces malignant phenotype. Cell lysates were immunoprecipitated with NRF1 antibody, resolved on an gel, transferred to a membrane, and probed individually with anti-serine, anti-lysine, anti-AKT, anti-NRF1 and anti-PCAF antibodies. In addition to increased phosphorylation and acetylation of NRF1 by 4-OH-E2 treatment, we also detected AKT and PCAF in the NRF1 immunoprecipitates (Fig. 3 and 4). 4-OH-E2 exposure to MCF-10A cells induced serine phosphorylation of NRF1 and overexpression of catalase and MnSOD blocked 4-OH-E2-induced phosphorylation of serine residues on NRF1. NRF1 phosphorylation preceded P/CAF recruitment for acetylation, because silencing of AKT prevented acetylation of NRF1.

The exposure of MCF-10A cells to a carcinogenic dose of 4-OH-E2 increased NRF1 binding to the gene promoters for the cell cycle regulators cyclin B1, Cdc25c, and Pcna: MCF-10 A (5x105 cells /dish) were seeded, grown for 24 hrs, serum starved for another 24 hrs, and transfected with NRF1, PCAF, and AKT RNAi, as well as with AdMnSOD (MnSOD) and Adcatalase (Cat). They were then treated with ebselen using the cell transformation condition as described above, and then treated with 4-OH-E2. In a preliminary ChIP analysis, we found that NRF1 is bound to the promoters of the cell cycle genes Pcna, Cyclin B1, and Cdc25c, confirming the prediction that these promoters contain NRF1 elements (Fig. 6). We also found that the exposure of MCF-10A cells to a carcinogenic dose of 4-OH-E2 increased the binding of NRF1 to the promoters of these genes, and that 4-OH-E2-induced binding could be inhibited by cotreatment with ebselen (antioxidant), by an over expression of catalase (H2O2 scavenger) and MnSOD, and by

RNAi of NRF1, PCAF and AKT. These results suggest that 4-OH-E2-generated oxidants, particularly H2O2, may mediate NRF1 binding to these gene promoters.

Silencing of NRF1 and its interacting partners (PCAF or AKT) prevented 4-OH-E2-induced anchorage-independent growth of MCF-10A cells: 4-OH-E2 treatment was able to induce AIG positive colony formation (Figures 2-4 and 7); and in NRF1, AKT, or PCAF RNAi silenced cells, 4-OH-E2 was not able to produce any colony. The effects of 4-OH-E2 on colony formation in cells transfected with scrambled RNAi of NRF1, PCAF, and AKT were similar to WT cells. This indicates that 4-OH-E2-dependent anchorage-independent growth of MCF-10A cells is dependent on NRF1 signaling.

DISCUSSIONS

The novel finding emerged from this study are that the increased expression of NRF1 renders support to the growth of MCF-7 cells to form tumors in vivo. Alternatively, the knockdown of NRF1 blocks the ability of invasive ER-MD-MBA-231 breast cancer cells to form aggressive tumors or prevents these cells to metastasize to other organs. Phosphorylation and acetylation of NRF1, which are redox sensitive, enhanced its binding to promoter consensus sequences of cell cycle genes and subsequently, transcriptional activation. Therefore, it is plausible that phosphorylation and acetylation of NRF1 could contribute to the growth as well as invasiveness of the breast tumor.

Recent studies show several genes with the ability to transform normal cells to malignant cells that are direct downstream targets of NRF1 (HP1α, SMYD3, CAPNS1) (28,29,30).

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The overexpression of a NRF1 regulatable gene, heterochromatin protein 1 alpha (HP1α), involved in gene silencing produces genomic instability in cells (31-33), which is essential for cell transformation. Similarly, overexpression of another NRF1 transcription target gene, SMYD3, produces transformed phenotypes (34). Since these genes are overexpressed in human breast cancer tissues, we postulate that these NRF1 regulatable genes alone or in concert with others may contribute to the estrogen-induced malignant phenotype. Recently, Klinge's group reported that the NRF1 promoter contains a half imperfect ERE, and E2 stimulates NRF1 expression (12) through the ER pathway in MCF-7 cells. Even if a small amount of ERβ present in ERα negative MCF-10A cells is involved in an increased synthesis of NRF1, it would not explain how increased localization close to nuclear targets or enhanced ability to bind to the promoters of target genes will be achieved upon estrogen exposure. Furthermore, ER signaling may be under the influence of NRF1 through its target gene, SMYD3. SMYD3 functions as a co-activator of ERα and potentiates ERα activity in response to its ligand (35).

We have shown that NRF1 binding to its promoter is inhibited by mitochondrial inhibitors as well as by antioxidants (18). We and others have shown that estrogen exposure to breast cells produce reactive oxygen species (ROS) (18,25-27).Thus, estrogen-generated ROS may also regulate NRF1 expression. Both phosphorylation and acetylation of NRF1 protein control its activation (23,24). NRF1 has been shown to be phosphorylated by redox sensitive kinases p38MAPK, ERK, and AKT (19,20) and acetylated by P/CAF. The protein tyrosine phosphatases (PTPs) which control ERK and AKT phosphorylation and histone deacetylases which control histone acetyl transferase

(HAT) activity of P/CAF are highly sensitive to ROS (35-41). The oxidation of PTEN's active site by ROS makes them inactive, which, in turn, stop dephosphorylation of PI3K to occur and thus result in an active AKT (PKB) (42-46). In fact, a higher activation of stress-activated protein kinases has been reported in E2-treated MCF-7 cells, which is considered to contribute to the E2-proliferative effect (47). Thus, estrogen-generated ROS may inactivate PTEN leading to an active phosphorylated PI3K. Subsequently, the active PI3K may activate AKT which will directly phosphorylate NRF1. By a similar mechanism histone decetylases, such as, HDAC3 and hSirT1, are inactivated by ROS (42-46), which in turn can allow P/CAF to acetylate NRF1. Our studies showed that a carcinogenic dose of 4-OH-E2 increased NRF1 phosphorylation and acetylation in MCF-10A cells and that these changes were inhibited by antioxidants or overexpression of catalase. This suggests that phosphorylation and acetylation of NRF1, which are redox sensitive, enhance its binding to promoter consensus sequences and subsequently, transcriptional activation. Findings of this study further showed that NRF1 binding to the promoters of cell cycle genes, PCNA, cyclin B1 and Cdc25C was inhibited by knockdown of NRF1, PCAF and AKT by their shRNA. Therefore, it is plausible that phosphorylation and acetylation of NRF1 could contribute to the aggressiveness and invasiveness of the breast tumor.

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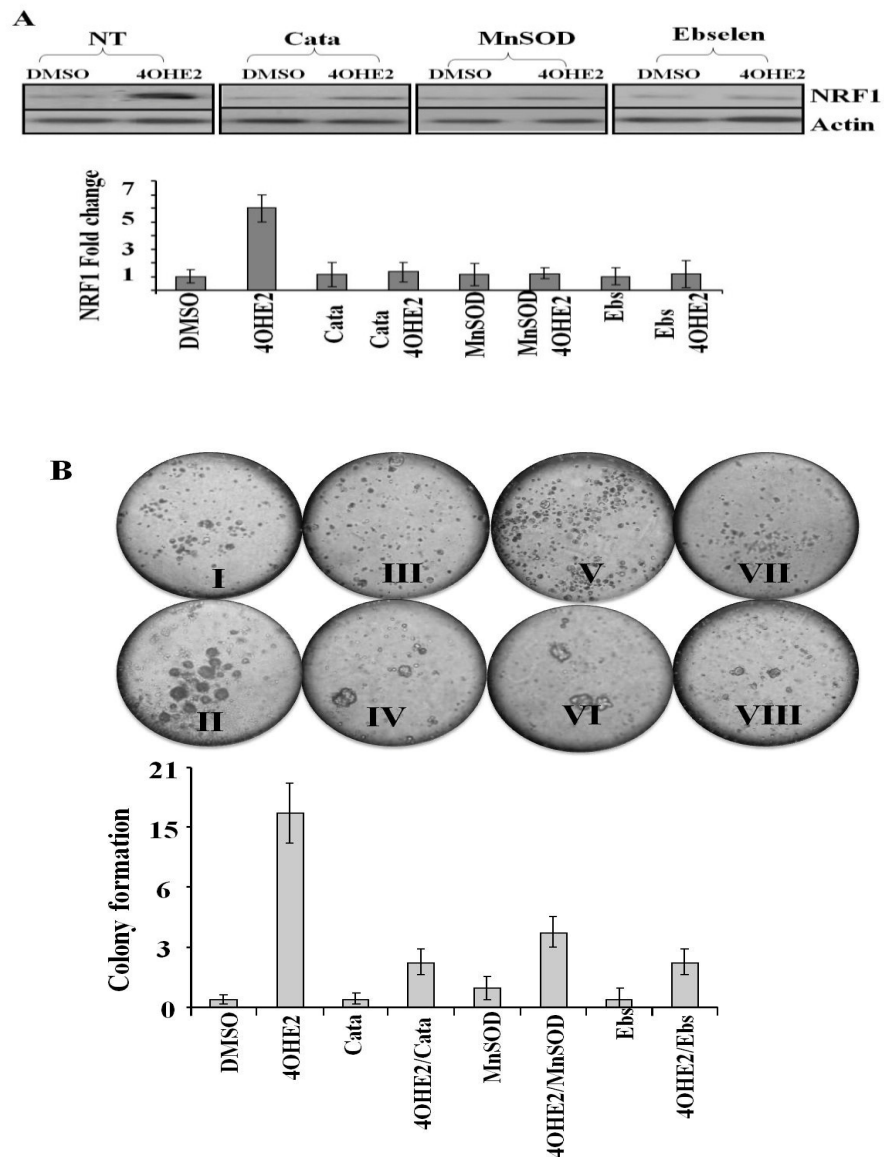47. Nishina, H. et al. Physiological roles of SAPK/JNK signaling pathway. J Biochem 136,123-6, 2004.

Fig. 1. Redox expression of NRF1 modulates 4OHE2 induced mammary transformation. MCF-10A cells were seeded for 24 hrs and infected with Adenovirus overexpressing catalase, MnSOD, or null construct at moi 100. 24 hrs post infection, cells were subjected to transformation regimen with or without 100 ng/ml estrogens. For groups treated with chemical antioxidant, these cells were co-treated with 40 μM ebselen and estrogen for the duration of transformation protocol except during recovery period. Post transformation, cells were either lysed with RIPA buffer, fractionated on 12% SDS-PAGE gel, then blot probed for NRF1 and beta actin expression, or seeded for anchorage independent growth on soft agar. A) Immunoblot of transformed cells normalized to Null/DMSO and band intensity quantified with Biorad geldoc 2000. B) Representative picture of colony count

per area in 24 well plate, normalized to Null/DMSO. Data expressed as mean of 3 experiments +/- SE, (P<0.05). (I) AdNull/DMSO, (II) AdNull/4OHE2, (III) AdCata/DMSO, (IV) AdCata + 4-OHE2, (V) AdMnSOD/DMSO, (VI) Ad/MnSOD+ 4-OHE2, (VII) Ebselen, (VIII) Ebselen + 4-OHE2.
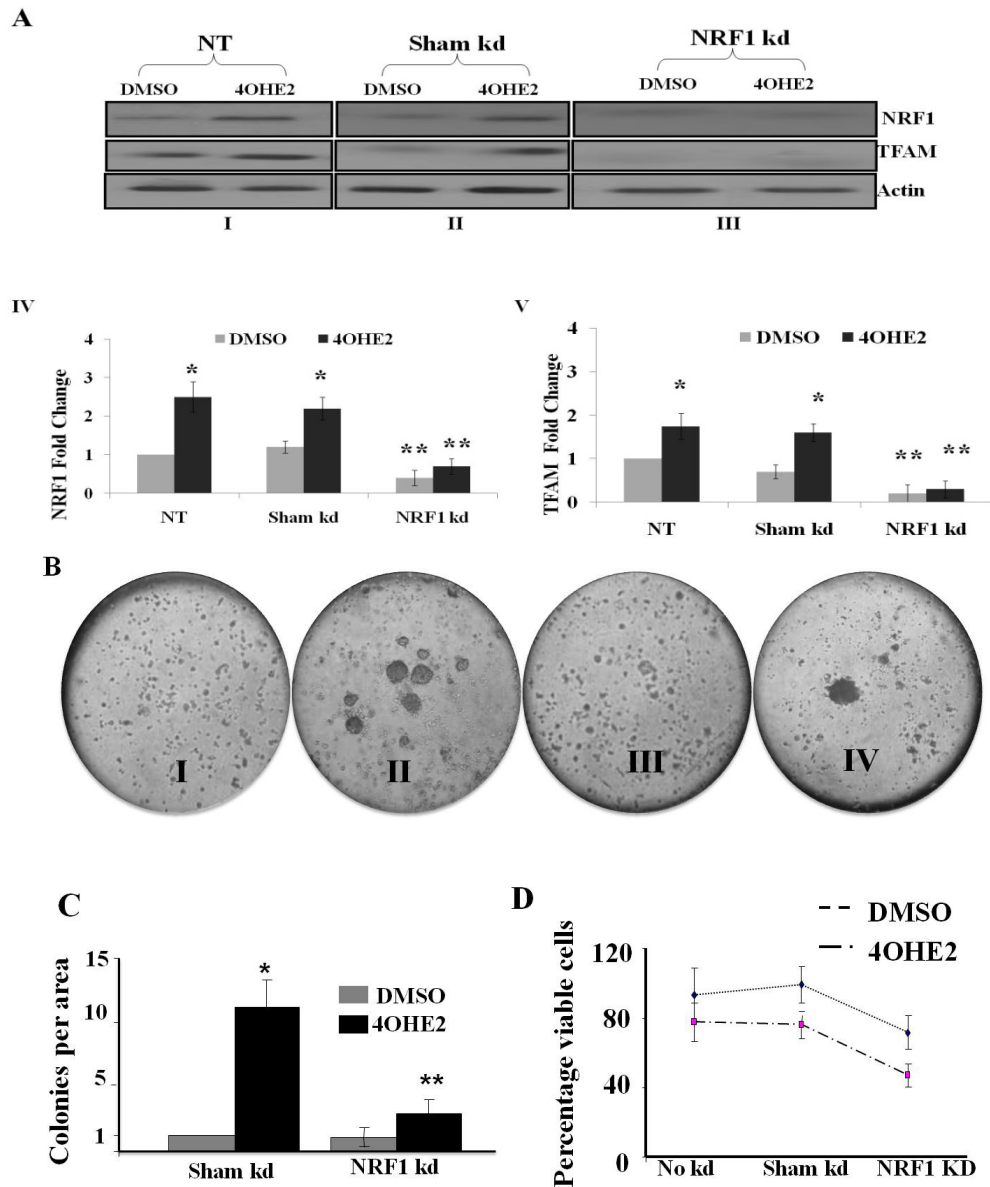

Fig. 2 NRF1 expression mediates 4OHE2 induced mammary tumorigenesis. MCF-10A cells were seeded at 20-30% confluency overnight and transfected with NRF1 shRNA or it corresponding null vector. Post transfection, cells were subjected to transformation regimen as previously described. Post transfection, cells were used for western blot analysis probed for NRF1, TFAM and actin expressions, or anchorage independent growth assay. A) Immunoblot of transformed cells normalized to non transfrected cells

(NT)/DMSO and band intensity quantified with Biorad geldoc 2000. (I) immunoblot of non transfected (NT) cells; (II) immunoblot of null transfected cells (III), immunoblot of NRF1 shRNA transfected cells; (IV) Fold change of NRF1 expression normalized to NT/DMSO; (V) Fold change of TFAM expression normalized to NT/DMSO. (B,C) Colonies formed after 21 days in soft agar assay were enumerated and expressed as numbers colonies per area. Five areas from each well was averaged over 3 plates and expressed as mean, +/- SE. (P<0.05). (*) indicate significant difference from non transfected (NT/DMSO) group. (**) indicate significant difference from 4OHE2 treatment group. (D) Trypan blue cell viability assessment of single cell suspension from colony assay at the end of 21 days culture per 1000 cells.
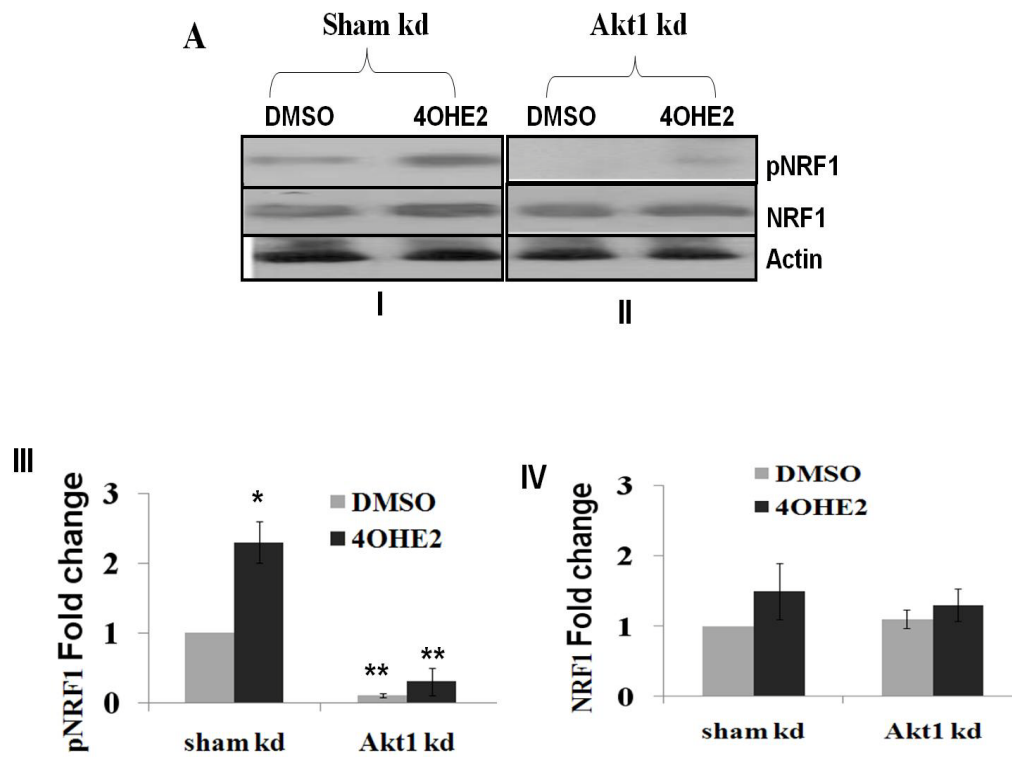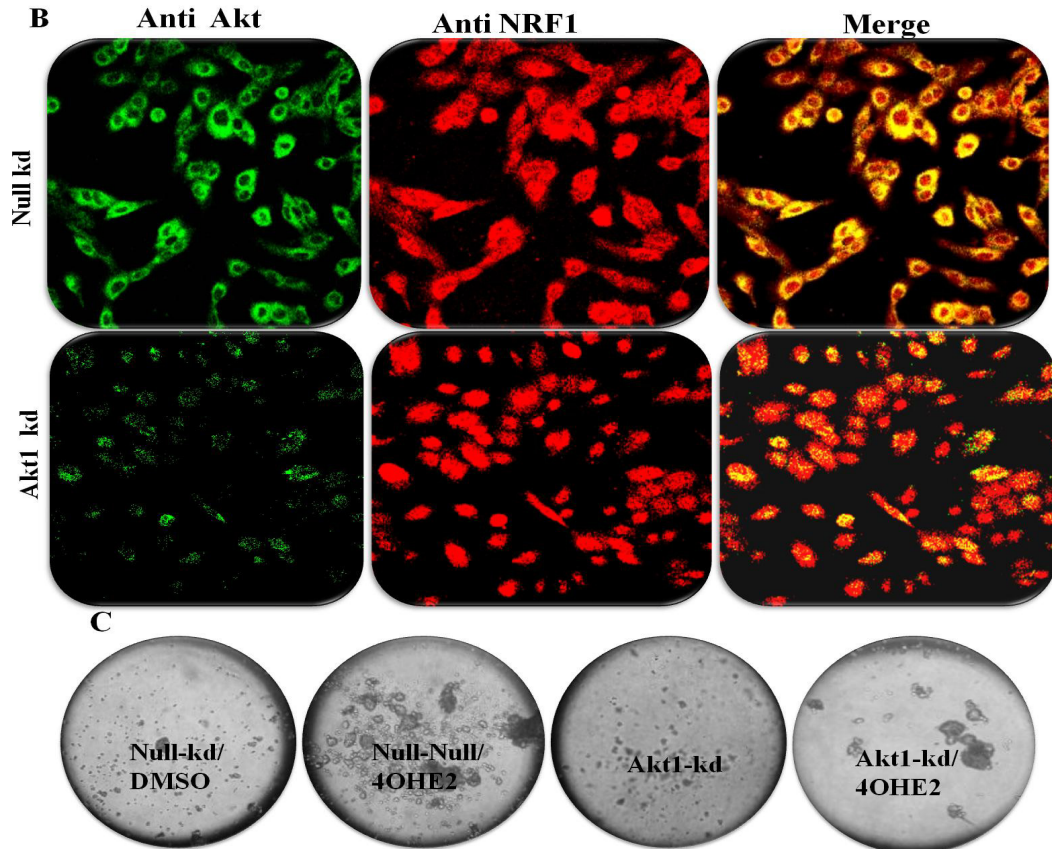
Fig. 3. Activation of Akt phosphorylate NRF1 during mammary tumorigenesis. Cells were transfected with Akt shRNA or null constructs, and then subjected to transformation regimen as previously described. Post transformation, cells were either treated with estrogen for additional 30 mins and used for co-immunoprecipitation western blot analysis, or for used for colony assay. A) Western blot analysis of NRF1 immunoprecipitated proteins probed with anti phosphoserine (pNRF1) or anti NRF1 antibodies respectively. (I) Immunoblot of null transfected cells (sham); (II) immunoblot of Akt1 knockdown cells (Akt1-kd); (III) phosphorylated NRF1 fold change compared to sham/DMSO treated group; (IV) NRF1 expression fold change compared to NRF1 expression from sham/DMSO. Data expressed as mean of three experiments, +/- SE. (P<0.05). (*) indicate significant difference from null (sham/DMSO) group. (**) indicate significant difference from 4OHE2 treatment group. B) Immunoflorescence label of sham or Akt1-kd transformed cells probed for Akt (green) or NRF1 (red) expression. Yellow is co-localization of Akt and NRF1 expression. C) Anchorage independent growth assay of transformed cells transfected with sham or Akt shRNA.
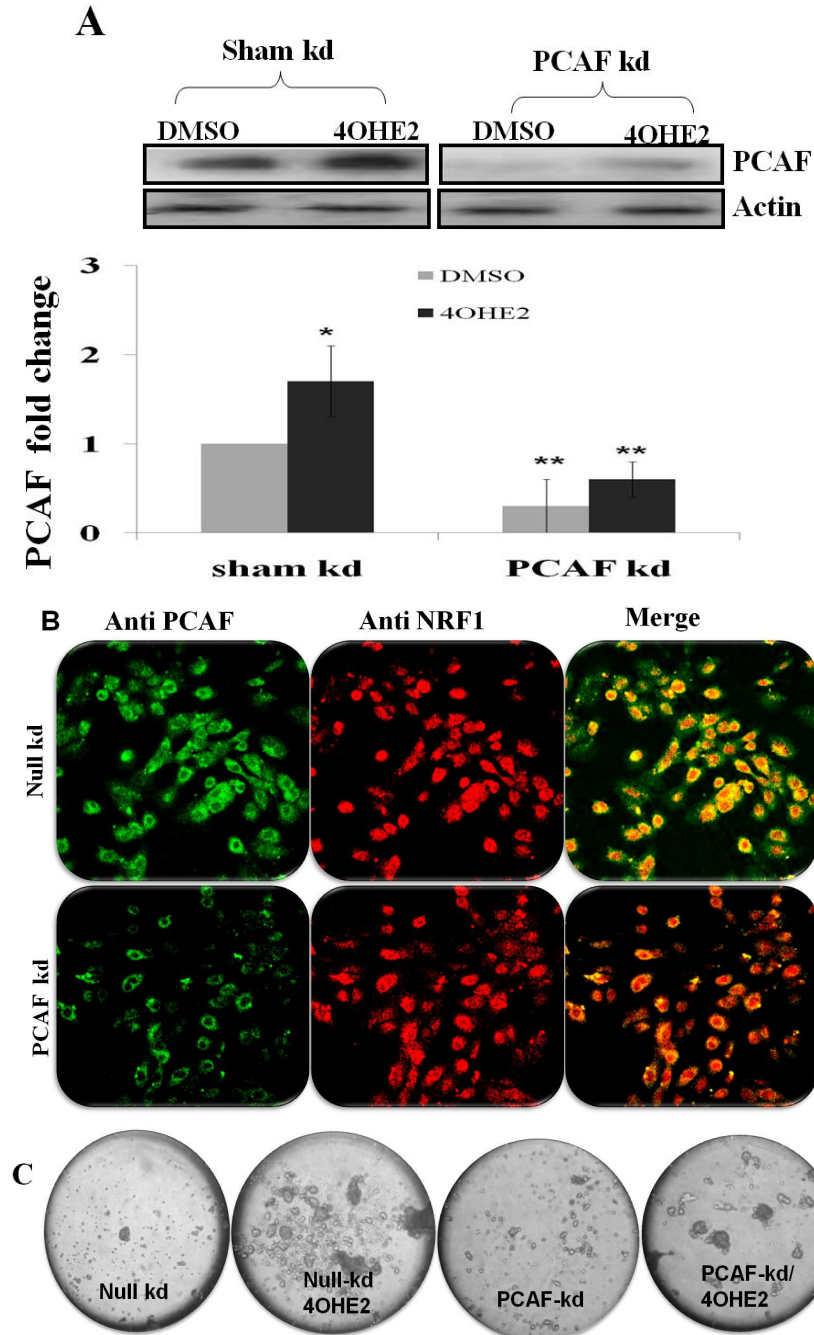
Fig. 4. PCAF expression participates in 4OHE2 induced mammary transformation. Cells were transfected with PCAF shRNA plasmid and cells were subjected to transformation regimen. Post transformation, cells were either treated with estrogen for additional 30 mins and used for western blot analysis, or seeded for colony assay formation and confocal immunoflorescence analysis. A) Immunoblot of sham or PCAF RNAi transfected cells and the fold change of PCAF expression compared to sham/DMSO treated group. Data expressed as mean of three experiments, +/- SE, (P<0.05). (*) indicate significant difference from null (sham/DMSO) group while (**) indicate

significant difference from 4OHE2 treatment group. B) Immunoflorescence label of sham or PCAF-kd transformed cells probed for PCAF (green) or NRF1 (red) expression. Yellow is co-localization of PCAF and NRF1 expression. C) Anchorage independent growth assay of transformed cells transfected with sham or PCAF shRNA.
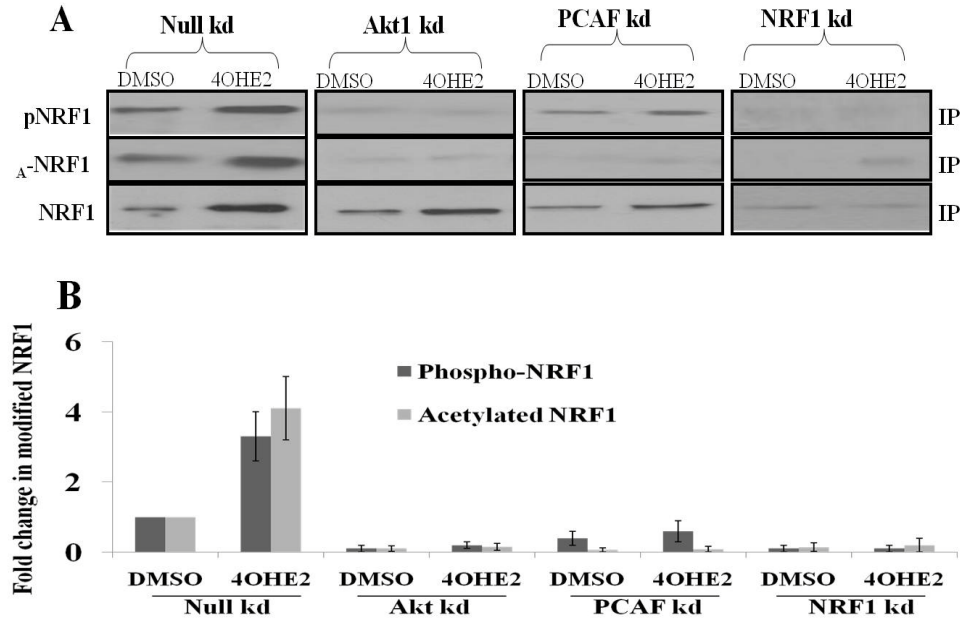

Fig. 5. Akt Phosphorylation of NRF1 induces its Acetylation during mammary transformation. MCF-10A cells were transfected with NRF1, Akt1, PCAF or null shRNA plasmids and the cells were subjected to transformation regimen as previously described. Post transformation, cells were treated with estrogen for additional 30 mins and lysed with RIPA buffer. 750 µg of whole cell lysate (WCL) were immunoprecipitated with NRF1 antibody and eluent fractionated on 12% SDS-PAGE gel. Blots were probed with anti serine (pNRF1), anti acetyl lysine ($_A$NRF1), anti NRF1(NRF1) antibodies respectively. A) Immoblot depicting the effects various RNAi on NRF1's phosphorylation and acetylation during MCF-10A transformation with 4OHE2. B) Densitometry of phosphorylated and acetylated NRF1 upon Akt1, PCAF and NRF1 knockdown. Data expressed as mean of three experiments, +/- SE, (P<0.05).
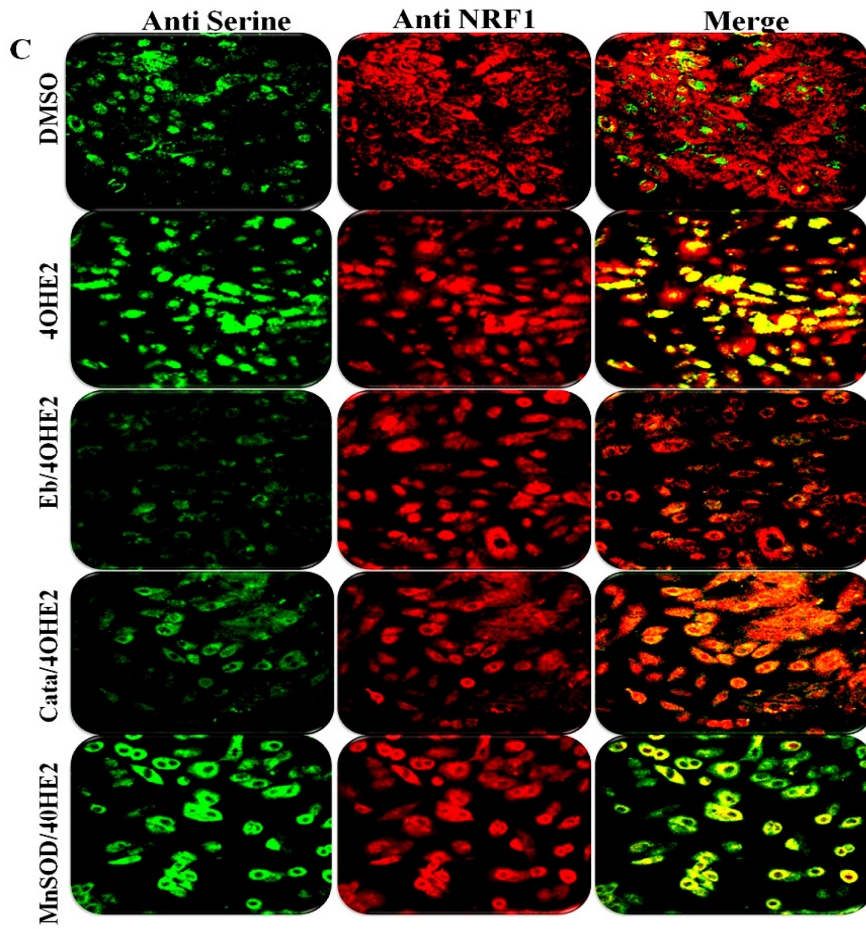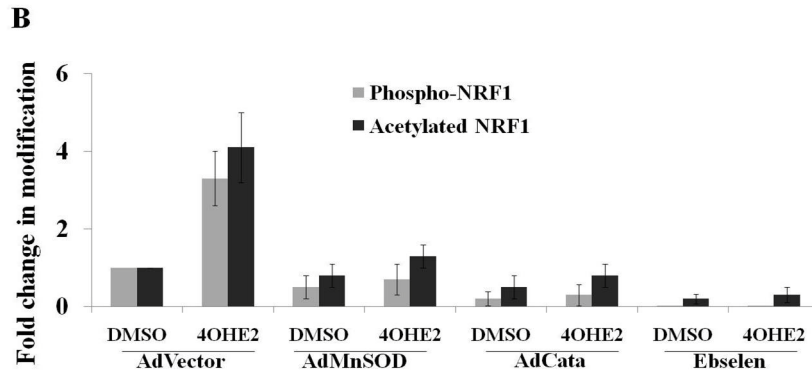
A

Vector   MnSOD   Cata   Ebs

DMSO 4OHE2 DMSO 4OHE2 DMSO 4OHE2 DMSO 4OHE2

pNRF1 — IP
A-NRF1 — IP
NRF1 — IP
Actin — IB

B

Fold change in modification

6

4

2

0

Phospho-NRF1
Acetylated NRF1

DMSO 4OHE2   DMSO 4OHE2   DMSO 4OHE2   DMSO 4OHE2
AdVector   AdMnSOD   AdCata   Ebselen

C

Anti Serine   Anti NRF1   Merge
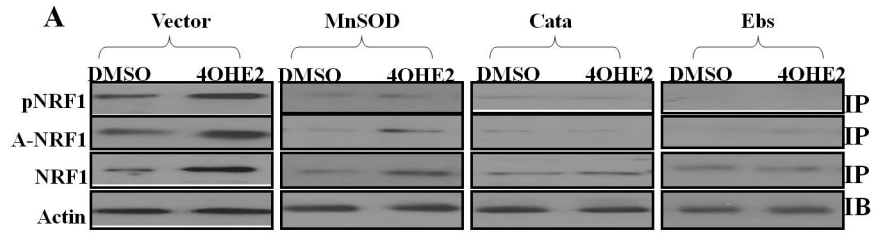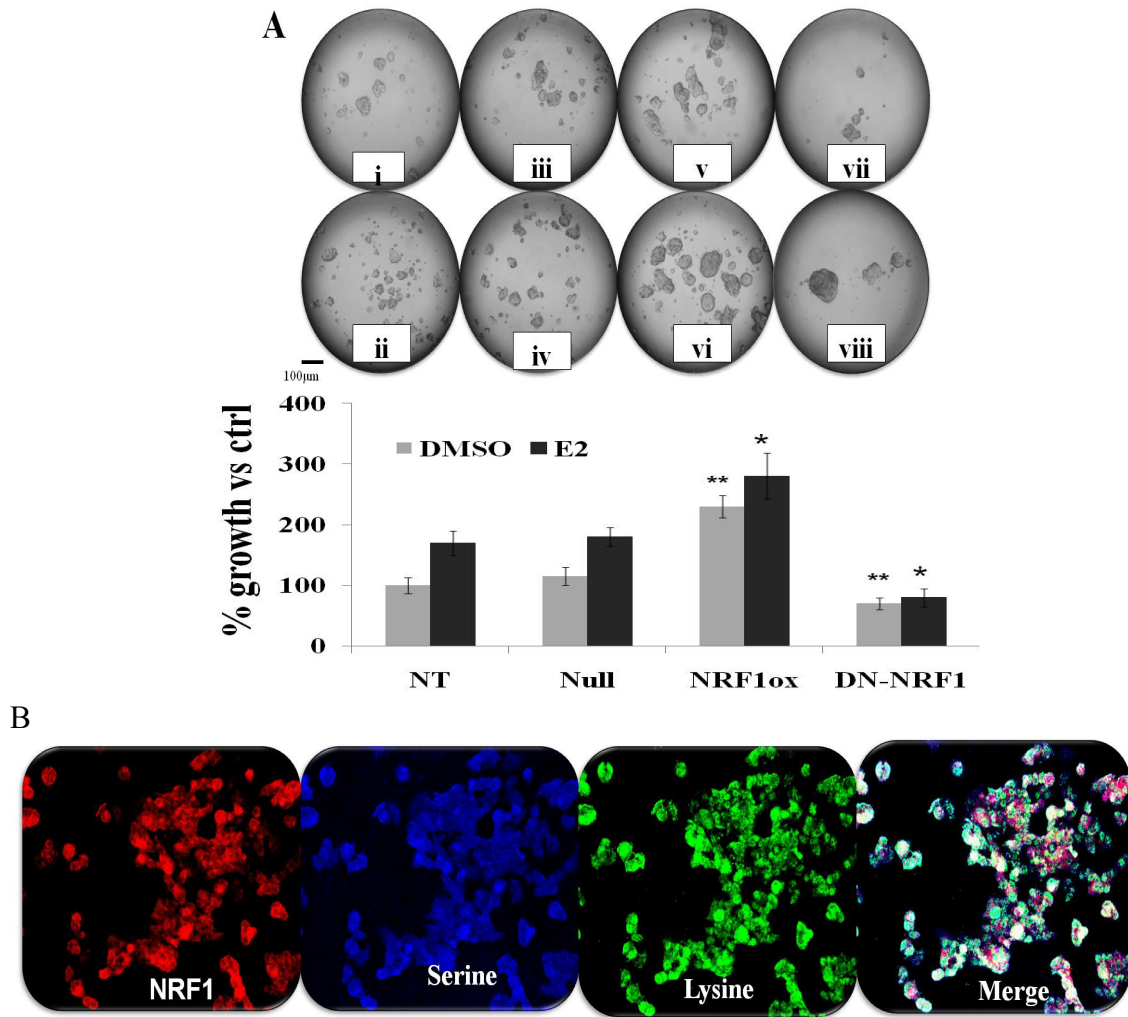
DMSO

4OHE2

Eb/4OHE2

Cata/4OHE2

MnSOD/4OHE2

Fig. 6. Transactivation of NRF1 during mammary tranformation is redox dependent. MCF-10A cells were co-treated with estrogens and ROS modulators as previously described. Post transfection, cells were either seeded on chamber slide for confocal microscopy analysis, or treated for additional 30 mims, then harvested with RIPA buffer. 750 µg of WCL were immunoprecipitated with NRF1 antibody and eluent were fractionated on 12% SDS-PAGE gel. Blots were then probed with anti serine (pNRF1), anti acetyl lysine ($_A$NRF1), anti NRF1(NRF1) antibodies respectively. 50 µg of WCL was also fractionated on SDS-PAGE gel and blot probed with beta actin anti body. A) Immoblot depicting the effects various RNAi on NRF1's phosphorylation and acetylation during MCF-10A transformation with 4OHE2. B) Normalized band intensity of phosphorylated and acetylated NRF1 upon Akt1, PCAF and NRF1 knockdown respectively. Data normalized to vector/DMSO and expressed as mean fold change of three experiments, +/- SE, ($p < 0.05$). C) Immunoflorescence label of cells co-treated with biological and chemical ROS modifiers, then probed for serine (green) or NRF1 (red) co-localization (yellow).
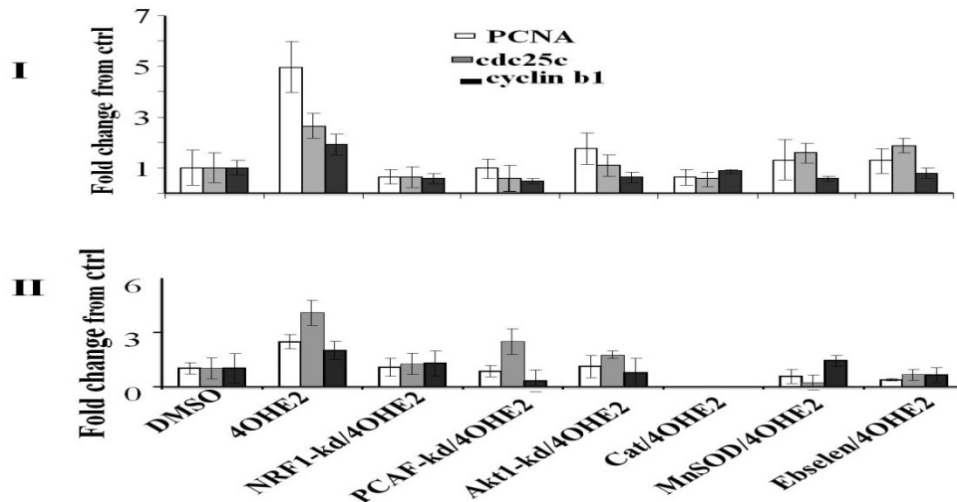

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C

Fig. 7. NRF1 expression is involved in invitro growth of breast cancer cells. A) Colonies formed by MCF-7 cells transfected with NRF1 modulating plasmids, with null vector control. (i) NT-DMSO, (ii) NT-E2, (iii) Vector-DMSO, (iv) vector-E2, (v) NRF1-ox-DMSO, (vi) NRF1-ox-E2, (vii) NRF1-kd-DMSO, (viii) NRF1-kd-E2. (B). Enumerated colony count were expressed as percentage change from NT treatment group +/- SD of three experiments. (*) indicates treatment significantly different from E2. (**) indicates treatment significantly different from control.
($p < 0.05$). B) Immunoflorescence labeling of MCF7 cells treated for 45 mins with 10 nM estrogens, then probed for NRF1, serine, acetyl lysine antibodies respectively. NRF1 (red), serine (blue), acetyl lysine (green), merge of NRF1/serine/acelty lysine (white). C) MCF-10A cells were seeded for transformation as described in methods. At end of transformation period, cells were treated for additional 18 hours with vehicles or estrogens after which they were washed with cold PBS with protease inhibitors. Cells were detached with trypsin and 2.0×10^6 cells were counted and used for either ChIP assay or qRT-PCR analysis. PCR reactions with DNA or cDNA primers for cell cycle regulatory genes were assembled in 25ul final volume and PCR reactions on ABI Real time PCR machine. Data represents mean of 3 different experiments +/-SE, ($p < 0.05$). (I) CHiP analysis for NRF1 promoter occupancy of selected cell cycle regulatory gene. (II) Quantitative RT-PCR (qRT-PCR) for transcriptional expression of selected cell cycle regulatory gene.

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PUBLICATIONS
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ABSTRACTS AND MEETINGS
Deodutta Roy, Quentin Felty, Victor Okoh, and Nana-Aisha Garba.
Inhibition of Estrogen-Induced Growth of Breast Cancer Cells by Modulating In Situ Oxidant Levels. Poster Presentation at DoD Era of Hope Breast Cancer Research Program, 2008.

Deodutta Roy, Quentin Felty, Joyce Slingerland, Nana-Aisha Garba, Rosalind Penny, and Victor Okoh. Reversible Inactivation of CDC25A by Estrogen and Antiestrogen-Induced Reactive Oxygen Species may be Involved in the Phosphorylation of P27. Poster Presentation at DoD Era of Hope Breast Cancer Research Program, 2008.

HONORS
Phi Kappa Phi Honor society.
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Several Dean's List