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Signature of mitochondria of steroidal hormones-dependent normal and cancer cells: potential molecular targets for cancer therapy

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1. ABSTRACT

The cross-talk between the cell nucleus and mitochondria appears to control hormone-induced signaling involved in the apoptosis, proliferation, and differentiation of both normal and malignant cells. Evaluation of the defects in genetics and physiology of human endocrine diseases, such as cancer, may manifest as a result of mitochondrial physiologic and metabolic compensation of genetic defects. Steroidal agents control biogenesis and maintenance of mitochondria through the crosstalk between nuclear and mitochondrial genomes. The regulation of mitochondrial transcription by steroidal hormones, presumably occurring through pathways similar to those that take place in the nucleus, opens a new way to better understand steroid hormone and vitamin action at the cellular level. In addition to the steroid hormone receptors, estrogen generated mitochondrial oxidants together with an estrogen-driven increase in epithelial cell proliferation have been shown to participate in the initiation and promotion of the neoplastic lesions in estrogen-sensitive tissues. Mitochondria generation of ROS appears to transduce signals to the nucleus for the activation of transcription factors involved in the cell cycle progression of estrogen-dependent cancer cells. Therefore, an in-depth analysis of such redox regulatory mechanisms is pertinent to the development of novel drugs and gene therapy strategies for the treatment of steroid hormone-dependent diseases related to mitochondrial disorders including cancer.

2. INTRODUCTION

Physiological concentrations of estrogens are essential for the growth of hormone responsive organs, estrogen receptor-mediated cell signaling and several other biochemical and molecular activities. Estrogen is considered to elicit different growth responses in various tissues through binding to the estrogen receptor (ER) alpha and ER beta (1,2,3,4). Supra-physiological or pharmacological concentrations of both natural and synthetic estrogens are known to produce adverse effects, such as immunotoxicty, teratogenicity and carcinogenicity (4). Over the years, stilbene estrogen, diethylstilbestrol (DES) and 17β-estradiol (E2) have been shown to induce mammary, bladder, ovarian, testicular, lymphatic, uterine, and prostatic tumors in mice and rats; ovarian and mammary tumors in dogs; endometrial carcinomas in rabbits; and kidney, testicular, and uterine tumors in hamsters (5,6). In 1999, the International Agency for Research on Cancer (IARC) categorized combined oral contraceptives consisting of the steroid hormone estrogen in combination with a progestogen and postmenopausal estrogen therapy as human carcinogens (6). In 2002, the US National Toxicology Program (NTP) listed steroidal estrogens used in estrogen replacement therapy and oral contraceptives as human carcinogens (7,8). The Women's Health Initiative randomized trial of estrogen alone and estrogen plus progestin showed an increased risk of breast cancer among women taking these hormones (8). In 2005,

IARC reported that the combined estrogen-progestogen oral contraceptives and combined estrogen-progestogen menopausal therapy are carcinogenic to humans (9). The neoplastic transformation of human breast epithelial cells by E2 clearly suggests the role of estrogen in the initiation of breast cancer (10,11). The exact mechanisms of initiation and progression of estrogen-related cancers are not clear.

Increasing evidence indicate that both endogenous and exogenous estrogen-induced genetic instability are critical for the development of estrogen-dependent cancers. Although ER-mediated genomic signaling pathways play a role in the promotion of initiated cells and progression of tumor, however, these pathways cannot fully explain estrogen-induced genetic alterations observed in estrogen-dependent cancers. Estrogens after aromatic hydroxylation gets converted into catechol estrogens by cytochrome P450/peroxidase enzymes (12,13,14,15). Catalytic oxidation of catecholestrogens gives rise to estrogen-quinones, which react with DNA to form adducts (12,15). These adducts can either be stable DNA adducts that remain in DNA unless removed by repair or it can form depurinating adducts that are released from DNA by destabilization of the glycosyl bond (16). Quinone and semi-quinone forms of catechol estrogens-induced DNA adducts are found in various target tissues of cancer (16). LC-MS-MS analysis of mammary tissue extract from rat showed the formation of an alkylated depurinating guanine adduct induced by equine estrogen metabolite 4-hydroxyequilenin (17). Estrogen DNA-adducts have been detected in human breast tumor tissue and healthy tissue by combined nano LC-nano ES tandem mass spectrometry (18). Recently, 4-hydroxy catechol estrogen conjugates with glutathione or its hydrolytic products (cysteine and N-acetylcysteine) were detected in picomol amounts in both tumors and hyperplastic mammary tissues from ERKO/Wnt-1 mice demonstrating the formation of CE-3,4-quinones (19). 2-OHE2 quinone-derived DNA adducts have been shown to be mutagenic generating primarily G --> T and A --> T mutations in simian kidney (COS-7) cells (20). E2-3,4-quinone induced rapidly-depurinating 4-hydroxy estradiol (4-OHE2)-1-N3Ade adduct and abundant A to G mutations in H-ras DNA was observed in SENCAR mouse skin treated with estradiol-3,4-quinone (E2-3,4-Q) (21). These studies indicate that estrogen metabolites react with DNA to form adducts in both humans and experimental models, and these adducts generate mutations and may contribute to tumor initiation. However, it is unlikely that these base modifications occur immediately after E2 exposure, as E2 would have to be hydroxylated and oxidized to a reactive intermediate in the endoplasmic reticulum prior to its transport to the nucleus. Although this type of adduction may play a role in the generation of mutations, it appears to be a later event.

Recently Felty *et al*, 2005 (22) have reported that physiological concentrations of E2 stimulate a rapid production of intracellular reactive oxygen species (ROS), and ROS formation in epithelial cells depends on cell adhesion, the cytoskeleton, and integrins. In our studies of E2-induced ROS generation in MCF7 and other cells, we were not able to find any hydroxylated estrogen metabolites or their adducts immediately after addition of E2, and this rules out the possibility of ROS generation by redox cycling of hydroxylated estrogens. These events occur earlier than ER-mediated genomic actions. E2-stimulated ROS production does not depend on the presence of the ER in breast cancer cells as the ER negative cell line MDA-MB 468 produced ROS equal to that of ER-positive cell lines MCF7, T47D, and ZR75. ROS formation upon E2 exposure can explain oxidative damage to hormone-dependent tumors and subsequent genetic alterations as reported earlier by Malins and others (23,24,25,26). E2-induced ROS generation also provides mechanistic support to the generation of mutations by physiological concentrations of estrogens (27,28).

Besides apoptosis, respiration, and oxidative phosphorylation; mitochondria also control ion homeostasis and the synthesis of heme, lipids, amino acids, and nucleotides. Steroidogenesis is also controlled by mitochondria. The recent resurgence of research interests implicating mitochondria in the regulation of developmental processes, cell proliferation and differentiation, and redox signaling to nucleus are timely and strategically important. The key role of mitochondria in cellular physiology is evident from the recent discoveries of a wide variety of human diseases associated with mitochondrial alterations such as diabetes, Alzheimer's disease, Parkinson's disease, and several types of cancer (29,30,31,32,4,31,33,34,35,36,37,38). In the last decade, the list of human diseases related to mitochondrial dysfunction has been steadily growing. The mitochondrial alterations include genetic defects in mt DNA or nuclear DNA (n DNA) that affect the functioning of respiratory electron transport chain as well as ADP phosphorylation (39,40,41,42). The expression of various mitochondrial disorder-related diseases can be ascribed to a number of qualititative as well as quantitative changes in mitochondria. During cell division, mt DNAs are randomly distributed into the newly formed mitochondria and into the daughter cells. In normal cells there is only one type of mitochondria DNA sequences (homoplasmy). In abnormal cells, mitochondria contain *two or more* types of mt DNA sequences (heteroplasmy). Cells with and without mitochondrial abnormalities because of mtDNA alterations may harbor a mixture of mutant and wild-type mtDNA within each cell (heteroplasmy) (43). Heteroplasmic as well as homoplasmic mutations of mtDNA can lead to the development of a number of disorders that express the phenotypes of diseases (36,37,38,39) . However, the proportion of mutant mtDNA must exceed a critical threshold level before a cell expresses an abnormality of the mitochondrial signaling (the threshold effect) because each cell contains more than a dozen mitochondria. A quantitative decrease in mtDNA copy number has also been linked to pathogenesis of various diseases (44). It has been recognized for a long time that malignant cells have a reduced respiratory rate coupled with an increased rate of aerobic glycolysis. However, recent studies indicate that this metabolic pattern is neither unique to malignant tumors nor an essential characteristic of all varieties of hormonal

cancer(45). Most of the benign and malignant endocrine tumors have lower respiratory capacities that may arise either due to a decrease in mitochondrial population in the cell or due to deficiencies in the electron transport activities.

The mitochondrion is an integral component of steroidogenesis. The presence of steroid hormone response elements and recent localization of hormonal receptors in the mitochondria, particularly receptors for estrogen, thyroid, and glucocorticoid (46,47,48,49) has provided new insight into the regulation of mitochondrial genome transcription and mitochondrial biogenesis. These and other studies show that several steroid hormones regulate mitochondrial biogenesis and its activity, leading to a search for the physiological importance of the mitochondria in steroid hormone actions and its involvement in steroid hormone-related diseases. Therefore, a better understanding of mitochondrial deficiencies associated with human degenerative diseases and the imbalance in steroid hormones is of utmost importance. The elucidation of genetic and bioenergetics markers of mitochondria that influence the pharmacology of steroidal hormones is timely. For example, recent studies on the mutagenic action of estrogenic compounds have revealed that mitochondria are important targets of these steroidal agents (50,2,51,52,53). There is about 15% of cellular protein is in the mitochondria.

The recognition of steroid hormone interactions with mitochondria as primary or secondary targets will lead us to understand the mechanisms underlying steroidal hormone-related diseases and therefore help in developing new drugs to eliminate or minimize these effects. In this article, we have critically evaluated the role of mitochondria in the growth of estrogen-dependent cancer and non-cancer cells, and current understanding of defects in genetics and physiology of mitochondria, particularly in steroidal hormone-related endocrine organs and its association with human diseases. Through crosstalk between nuclear and mitochondrial genomes, the steroid hormones control biogenesis and maintenance of mitochondria. The understanding of the regulation of mitochondrial biogenesis by estrogenic compounds would open a new way to better understand steroidal and non-steroidal estrogen action at the cellular level. A detailed analysis of such regulatory mechanisms is therefore relevant for the development of novel drugs and gene therapy strategies for the treatment of mitochondrial disorder-related diseases.

3. INFLUENCES OF STEROIDAL HORMONES, PARTICULARLY, ESTROGEN ON PHENO- AND GENO-TYPES OF MITOCHONDRIA IN CELLS

The number of mitochondria per cell varies from a few dozen to several thousand. Mitochondria contain their own DNA and have their own transcription and replication machinery. The mitochondrial DNA (mtDNA) is maternally inherited, and the entire genome is about 16 Kbp. The mitochondrial genome (mt genome) encodes for 12s and 18s rRNAs, for 19s tRNAs, and for mRNAs of 13 proteins. NADH dehyrogenases (ND1-ND6 and ND4L) encode seven subunits of mitochondrial respiratory chain complex I. In the complex III subunit, cytchrome b is the only mtDNA-encoded protein. Cytochrome c oxidase I to III encode for three of the mitochondrial complex IV subunits, while the ATPase 6 and ATPase 8 genes encode for two subunits of mitochondrial respiratory chain complex V. Ribosomal and transfer RNA genes are interspaced between the protein-encoding genes. These sequences provide the necessary RNA components for intra-mitochondrial protein synthesis. The mitochondrial proteins encoded in the Mendelian-inherited nuclear genes, other than the thirteen proteins encoded by the mt genome, are synthesized in the cytoplasm and imported into mitochondria. It has been estimated that there are approximately 1,000 different polypeptides in the mitochondrion (54).

Several hormones such as thyroxine, estrogen, glucocorticoids, and vitamin D3 have been reported to exert their influences on mitochondria. The mitochondrial activity is regulated by steroid hormones, e.g., thyroxine, estrogen, glucocorticoids, and vitamin D3 (55,56) . Estrogen is considered to act via interactions with specific receptors located at the plasma membrane as well as the mitochondrial membrane (57,58). An increasing body of evidence has shown that mitochondrial transcription is enhanced by estrogen treatment. For instance, a 16-fold increase in cytochrome oxidase II (CO II) mRNA is reported in the GH4C1 rat pituitary tumor cell line when treated for 6 days with E2 (0.5nM) (59). The mitochondrial gene for subunit III of cytochrome oxidase (CO III) is induced as early as 3h following a single dose of E2 in the hippocampus of ovariectomized female rats (60). Other mitochondrial transcripts have also been reported to increase in the human hepatoma cell line, HepG2, and rat hepatocytes when exposed to ethinyl estradiol (EE). A 40 h exposure to an EE concentration ranging from 0.5 to 10 μM resulted in a 2- to 3- fold induction of CO I, CO II, and NADPH dehydrogenase subunit 1 (NADPH-DH1) mRNA (61). E2 (20 μM), although less potent than EE, showed a similar effect of induction from 1.5- to 1.8-fold in mitochondrial transcripts CO I, CO II, and NADPH-DH1 when treated for 12h. The E2 catechol metabolite 4-OH-E2 caused a greater response in CO I and CO II transcript levels as compared to E2 after 24 h of treatement with a dose of 10 μM. The mitochondrial gene for ATP synthase subunit 6 (ATPase 6) was also elevated in female rat liver tissue exposed to EE (5 μg/day) for 42 days. An increase in the transcript level of COX7RP (cytochrome c oxidase subunit IV-related protein) was reported after a 6h E2 (100nM) treatment in MCF7 cells (62). It is not known whether the COX7RP transcript is translated to a functional protein in the mitochondria, but the study proposed that COX7RP may represent a regulatory subunit of cytochrome c oxidase that modulates a high state of energy production in estrogen sensitive target tissues. More recently, a 12h E2 (0.3μM) treatment of MCF7 cells was demonstrated to enhance the mitochondrial transcript levels of CO I approximately fourfold and CO II~2.5-fold (63).

The mechanism of estrogen-induced mitochondrial gene transcription is not clearly understood. The involvement of estrogen responsive elements (EREs)

and/or the ER may be a possible mechanism in the increase of these mitochondrial transcripts. Sequences with partial similarity to the ERE consensus sequence, (AGGTCANNNTGACCT), have been reported in the mouse mitochondrial genome (47). These partial EREs were detected in genes CO I and CO II which may account for the observed increases in these two transcripts in rat GH4C1 pituitary cells and rat hepatocytes (59,61). Other genes in which the various EREs were detected include 12S rRNA, 16S rRNA , tRNA-gln, cytochrome oxidase b, unidentified reading frame (URF) 4, URF5, and the D-loop region (47). In the human mitochondrial genome, we identified partial or ERE ½ sites in the D-loop region, CO II, tRNA-met, 12S rRNA, 7S rRNA, URF1, and URF5 (2,3). The presence of these partial EREs in the mitochondrial genome may lend support to a novel ER signal transduction pathway. A mechanism of ER translocation into the mitochondria and ER binding to mitochondrial EREs remains unclear. Using electrophoresis mobility shift assay (EMSA) and plasmon resonance analysis, the recombinant human ER alpha- and ER beta-containing mitochondrial proteins were demonstrated to specifically bind putative EREs in the mtDNA D-Loop, and this ER binding was enhanced by E2 treatment and inhibited by ICI 182780 (63). Based on this evidence, it is biologically plausible that ER mediates mitochondrial transcription in the same manner as the glucocorticoid receptor (GR) which is translocated into the mitochondria and binds glucocorticoid response elements (GRE) after treatment with glucocorticoid (64,65). Whether estrogen-induced mitochondrial transcription participates in the development and growth of estrogen dependent breast cancer is not known. Long-term stilbene estrogen (diethylstilbestrol = DES) treatment of Syrian hamsters produced tumors in the kidney with a 5- to10- fold higher transcript level of CO III than age-matched control kidneys (66). These studies indicate that the steroid hormone interactions with mitochondria are primary events.

4. DEFECTS IN MITOCHONDRIA OF STEROIDAL HORMONE-DEPENDENT CANCERS

The differences in the structure and function of mitochondria between normal and cancer cells, including differences in metabolic activity, molecular composition, and mtDNA sequence have been recently reviewed (2,67). For example, increased mutations in 61% to 74% of primary breast tumors have been reported recently (68,69). The mtDNA content is significantly reduced in the human breast tumors relative to their corresponding normal (70). The changes in mtDNA levels do not correlate with tumor grade and metastasis, which suggests that alterations may occur in the early stages of malignancy (70). The greater incidence of breast abnormality in women taking hormone replacement therapy (HRT) compared to lower incidence in pathology among women not taking HRT appears to be associated with differences in mitochondrial content and activity (71). It has also been shown that MCF7 and MDA-MB-468 breast cancer cells have a defective repair system for 8-hydroxyguanine in mitochondria (72). Mitochondrial DNA appears more susceptible to formation of stilbene estrogen adducts than nuclear DNA. Obstruction of replication and/or transcription of the mitochondrial genes by covalent modifications of the mitochondrial DNA by estrogen may produce mitochondrial genomic instability *in vivo* and may also provide an explanation for the carcinogenic effects of estrogen (47,48,49). In tumors induced by mutagenic carcinogens or viruses, alterations in the size and structure as well as deletions and insertions of the mitochondrial genome have been observed (74). To date, the major emphasis of the mitochondrial involvement in breast oncogenesis has been limited to their role in apoptotic processes. Mitochondrial abnormalities in tumor tissues generally have been considered to be a consequence, rather than the cause of, tumorigenesis. However, recent reports argue against this concept and provide support to the idea that mitochondria may control the growth of cancer tissues. For example, genetic and sporadic cases of brain tumors (paraganglioma and pheochromocytoma) are caused by mutation of a mitochondrial-specific protein, succinate dehydrogenase, a Krebs cycle enzyme (73,74). Recently, mutations in another mitochondrial Krebs cycle protein, fumarase, have been associated with the development of uterine fibroids, skin leiomyomata and renal cell cancer (75). Mutations in these proteins appear to be involved in familial predisposition to benign and malignant tumours, such as malignant phaeochromocytomas and renal cell carcinomas.

Dehydroepiandrosterone (DHEA), a steroid of the adrenal gland and a precursor in the biosynthesis of potent estrogens and androgens, induces liver cancer in rats (76). In congenital lipoid adrenal hyperplasia – the most severe gene-based disorder of steroid hormone synthesis – affected people are almost completely unable to make steroid hormones because they cannot convert cholesterol to pregnenolone, the first step in the process of steroid hormone synthesis. This disorder results from a mutation in a mitochondrial phosphoprotein, designated steroidogenic acute regulatory protein (StAR) that blocks the use of cholesterol to synthesize steroid hormones (76). The resulting accumulation of cholesterol esters and related substances therefore damage the cells. The targeted disruption of StAR showed severe defects in adrenal steroids with a loss of negative feedback regulation at hypothalamic-pituitary levels whereas hormones constituting the gonadal axis did not differ significantly from levels in wild-type litter mates (77). The adrenal cortex of StAR knockout mice contained florid lipid deposits, lesser deposits in the steroidogenic compartment of the testis, and none in the ovary. The knock out of StAR differentially impacts adrenocortical and gonadal steroidogenesis. Patients with deletions in their mitochondrial DNA had various and often multiple endocrine abnormalities that may be associated with mitochondrial myopathy (78). Hepatocellular carcinomas do not arise from liver areas with the strongest peroxisome proliferation, but from focal lesions with abundant mitochondria (79). A profile of energy metabolizing enzymes in the mitochondria-rich preneoplastic lesions and biochemical studies of DHEA-treated liver show a characteristic pattern of metabolic alterations indicating a thyroid hormone-like effect of DHEA on the liver of both genders.

GH/TSH-secreting pituitary adenoma shows a morphological feature of mitochondrial gigantism (80). Thyroid oncocytoma is characterized by the presence of oncocytes containing abnormally large numbers of mitochondria (81). Thyroid oncocytic tumors showed a mean 5-fold increase in mitochondrial DNA when compared to corresponding controls [(82). In parathyroids with hyperplasia and oxyphil areas, defects of cytochrome c oxidase occurred significantly more often and tended to be larger than in adenomas (83). Mutational analysis of mtDNA in thyroid neoplasia, which is characterized by increased numbers of mitochondria, showed three different somatic mutations (23%) in papillary thyroid carcinomas and significant differential distributions of mtDNA sequence variants between thyroid carcinomas and controls (84). These variants were more frequent in the genes that encode complex I of the mitochondrial electron transport chain as compared to normal population controls. Recently, a significant decrease in the copy number of total mtDNA and the activity of a nuclear-encoded mitochondrial enzyme citrate synthase has been reported in estrogen-induced hamster kidney tumors compared to age-matched controls (85). In testicular tumors of King-Holtzman hybrid rats with testicular feminization, Leydig cells contain pleomorphic mitochondria with numerous cristae. Mitochondria were numerous and varied in size and shape (86). Mitochondrial protein, manganese super oxide dismutase (MnSOD), is considered to be a tumor suppressor in human breast cancer. Increased expression of this protein has been found to suppress the malignant phenotype of human breast cancer cells (87). Kuhl and his associates have reported that a mitochondrial protein, peripheral benzodiapene receptor, controls aggressive growth of breast cancer cells (88). Prostate cancer LNCaPs cells with reduced levels of prohibitin showed an increase in the percentage of population in cell cycle, while cells with increased prohibitin levels showed a reduction in the percentage entering cell cycle following dihydrotestosterone stimulation, when compared to controls (89). Thus, it appears that the regulation of the mitochondrial protein prohibitin is critical for the cellular growth response to androgen stimulation in LNCaPs. These data support the concept that the growth of prostate cancer cells is correlated with the mass of mitochondria. Earlier studies have indicated that Ca^{2+} ions and mitochondrial function are involved in the regulation of a certain segment of mitosis beyond metaphase (90). The G1-S transition of normal and cancer cells is impaired by specific inhibitors of the electron flow through the respiratory chain, although respiratory ATP can be replaced by glycolytic ATP. Adenine is equally effective in removing the block produced by specific inhibitors of the electron flow, pointing out a purine metabolism restricting cell recruitment into the S phase (91). The antiproliferative action of 2-methoxyestradiol (2-ME), an endogenous estrogen metabolite, in pancreatic cancer cells is mediated either by G2-M arrest (PANC-1) with Bcl-x(L) phosphorylation or by the accumulation of tetraploid cells in G1-S region (MIA PaCa-2) without Bcl-2/ Bcl-x(L) phosphorylation (92). Mitochondria control NADPH oxidase 1 (Nox1) redox signaling. Nox1 is highly expressed in breast (86%) and ovarian (71%) tumors (93). The loss of control of this signaling is considered to contribute to breast and ovarian tumorigenesis (93). From the above studies it appears that there are significant differences in the structure and function of mitochondria between normal and cancer cells. Interactions of steroidal hormones with mitochondria molecules and defects in the in the structure and function of mitochondria between normal and cancer cells merit furture investigations, which may shed new light on the role of mitochondria in growth of cancer cells.

5. THE ROLE OF MITOCHONDRIA IN THE DEVELOPMENT OF STEROIDAL HORMONE-DEPENDENT CANCERS

Though the exact mechanisms by which mitochondria may participate in the development of steroidal estrogen-related cancers are not clear, however, the increasing evidences indicate that the dysregulation of mitochondrial signaling is an important mechanism of steroidal hormone-related carcinogenesis. The ability of steroidal hormones to produce structural and functional alterations to mitochondria indicate that steroidal hormones through dysregulation of the cross-talk between the cell nucleus and mitochondria may be involved in the development of preneoplastic lesions and the progression of these cancers (Figure 1). In addition to hormone receptor mediated signaling, steroidal hormones may support the growth of tumor cells by alternative pathways. Below we have discussed the highly novel concept that exposure of steroidal hormones, particularly estrogen, through directly acting on the mitochondria, leading to the production of calcium and ROS is involved in the induction of instability in the genome and in the growth of cancer cells (Figure 1). It was envisioned that this is an important mechanism that drives the carcinogenesis process, but that it occurs in the context of other processes such as hormone receptor-mediated signaling and estrogen reactive metabolite-associated genotoxicity that may also contribute to the process. We have discussed below four alternative pathways that may be influenced upon exposure to steroidal hormones, which in turn, may participate in the developmental of hormonal cancers.

5.1. Steroid Hormones Control of Mitochondrial Biogenesis and Crosstalk between Nuclear and Mitochondrial Genomes

The mitochondrial biosynthetic capacity correlates with the rate of organelle biogenesis rather than the steady-state concentration of a marker enzyme. The copy number of mitochondrial DNA does not seem to play a major role in determining either mitochondrial transcript levels or functional mass. Differentiated cells contain a large excess of mtDNA molecules that are not transcribed at a given time. It appears that the rate of transcription from a subpopulation of mitochondrial DNA seems to determine the amount of mitochondrially encoded mRNAs and consequently mitochondrial proteins, whereas the copy number of mtDNA does not seem to be of major importance (94,95,96). Though the coordination between expression of mtDNA encoded proteins and that of the hundreds of nuclear genes encoding mitochondrial proteins

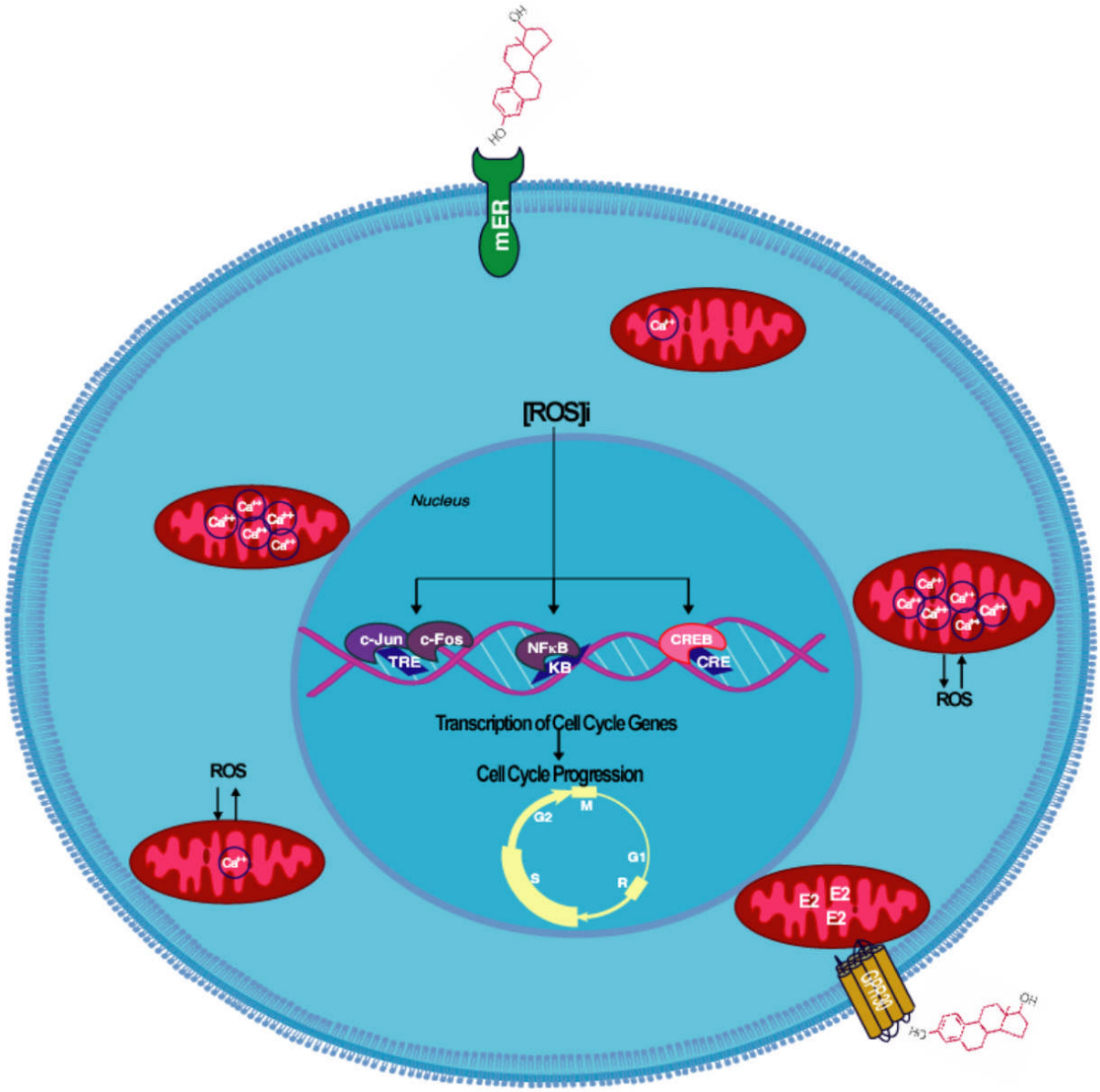

Figure 1. A scheme outlining 17 beta- estradiol (E2)-induced signaling pathways from mitochondria. (*i*) E2 binding to a plasma membrane protein(s) generates ROS which leads to redox sensitive kinase activation. (*ii*) E2-induced rise in ROS leads to increased mitochondrial calcium. This leads to the activation of calcium regulated kinases. Increased redox signaling results in the activation of transcription factors responsible for cell cycle progression.

is not fully understood, a positive correlation between cytochrome oxidase activity and levels of the nuclear-encoded mRNAs for cytochrome oxidase subunit VIc and Va indicates that transcription regulates the number of nuclear-encoded OXPHOS subunits and that both genomes are expressed in a coordinated fashion in the steady-state. Mitochondrial biogenesis stimulated by various physiological challenges such as the endurance training of skeletal muscle, hyperthyroidism in liver, and skeletal muscle or hyperglucocorticoidism in colon epithelium lead to increased tissue levels of mRNAs for both nuclear and mitochondrially encoded OXPHOS subunits, thus supporting the important role of transcription regulation (97) During cold adaptation in brown adipose tissue and heart hypertrophy induced by thyroid hormone, the increase in functional mitochondrial mass is produced by both elevated levels of such transcripts and an additional, specific stimulation of mitochondrial translational capacity. This also suggests that up-regulation of mtDNA copy number is of minor importance for adaptive stimulation of mitochondrial biogenesis.

Mitochondrial biogenesis in mammalian cells has requirements for gene products from two physically separated genomes: one contained within the mitochondria and the other within the nucleus. The biogenesis and

function of mitochondria in mammalian cells relies upon the regulated expression of the nuclear genome's origin transcriptional activators and coactivators (98). Mitochondrial transcription factor A, mtTFA, is a key regulator of mtDNA copy number and mitochondrial transcription in mammals and therefore it is essential for mitochondrial biogenesis and embryonic development. MtTFA translocates to mitochondria and initiates the transcription and replication of the mt genome. Mitochondrial translation initiation factor 2, MTIF2 is yet another nuclear-encoded protein that functions in mitochondria to initiate the translation of proteins encoded by the mitochondrial genome. The region surrounding the transcription start point of the MTIF2 gene contains consensus binding sites for transcription factors, Sp1, nuclear respiratory factor 2, NRF-2, and estrogen receptor (99). Steroid hormones, androgens, and thyroxine induce changes in mRNA and rRNA derived from the mt genome through increasing mtTFA mRNA expression from the nuclear genome (100). The tissue specific coactivators of steroid receptors including ER such as peroxisome proliferators-activated receptor coactivator-1 (PGC-1) and PGC-1 related proteins PRC and PERC, coactivate the expression of mtTFA (reviewed in 98). Recent evidence points to both transcriptional activators (NRF1/2) and coactivators (PGC-1, PRC) as important mediators of mt maintenance and proliferation (95). These activators and coactivators also control mitochondrial biogenesis through the induction of mTFA. Several studies show an increase in the number of mitochondria upon expression of retroviral-mediated PGC-1. We have recently shown that mitochondria can modulate the expression of nuclear cell cycle genes in breast cancer cells (2,3). Genetic and biochemical evidence suggest that when these factors act together they can bring about mt biogenesis and increase oxidative phosphorylation.

5.2. Imprinting of phenotypes of mitochondria from blastocysts to adult cells: influence of steroid hormones during development

The regulation of mt DNA expression is crucial for mitochondrial biogenesis during development and differentiation. The mitochondrial genome is transcribed in early mouse embryos. Also, the protein synthesis is essential for the normal growth of mouse embryos (101). In normal implanting blastocysts, both oxygen consumption and the intensity of cytochrome oxidase histochemical staining increased rapidly upon estrogen stimulation (102). Within the first hour of extrauterine life, the pre-existing fetal mitochondria are rapidly transformed into energy producing mitochondria and new mitochondria are synthesized parallel with the surge in testosterone levels (103,104,105). In most cases, mtDNA levels correlate with the number of copies of mitochondria per cell (66,67). The rapid induction of mitochondrial biogenesis occurs in the liver and other organs after postnatal onset (66,67).

At birth, mitochondrial DNA sequences are usually all identical (homoplasmy). Mitochondrial biogenesis during postnatal development results in both (i) an increase in the number of mitochondria per cell, a continuous process that spans most of the two-thirds of the suckling period, and (ii) an increase in functional capabilities of already pre-existing mitochondria that occurs during the first postnatal hour (66,67). These increases in the number of mitochondria and their functional capabilities parallels a postnatal rise in testosterone levels observed within only a few hours after birth in several male mammals (106). A postnatal stilbene estrogen exposure that either inhibits the development of the new generation of Leydig cells or blunts the steroidogenic capacity of the existing Leydig cells may prevent a postnatal surge of testosterone production that in turn may prevent mitochondria biogenesis (27,107). The neonatal exposure of GnRH antagonist to monkeys lowers the mitochondrial DNA content in Leydig cells (108). On the other hand, androgen exposure to prostate cells increases mitochondria contents by 80% (109). Chronic exposure to hCG stimulates testosterone production and thus increases the volume and surface area of mitochondria (110).

The number of mitochondria per cell is double in mature cells from the pubertal animals as compared to the number of immature cells from developing young animals (66,67). Similarly, cells from young animals have twice the number of mitochondria per cell than the cells of the neonatal tissues (66,67). During liver development, although the relative amount of nuclear encoded mt mRNAs are several fold higher in the fetus than in the adult, the concentrations and activities of the corresponding proteins are very low or even undetectable. This indicates that although nuclear encoded mt mRNAs are by some means accumulated, they are translationally repressed during fetal liver development, becoming accessible to the translational machinery just 1 hr after birth (66,67). Rapid postnatal differentiation and proliferation of mitochondria is controlled both at the post-translational and post–transcriptional levels (66,67).

The proteins involved in the mitochondrial oxidative phosphorylation (OXPHOS) process are tissue specific and are developmentally regulated (111). Adrenal steroid hormones regulate perinatal maturation of mitochondria in rat kidney and these hormones are involved in the fetal mitochondrial biogenesis in a tissue-specific manner (112). Adrenalectomy leads to a decrease in fetal kidney cytochrome-*c* oxidase messengers and mtDNA content while the administration of dexamethasone restores normal levels. Adrenalectomy leads to a decrease in mt DNA content in the fetal kidney of rats, but no change in mt DNA content of fetal liver and heart was observed (73). Treatment with cyclosporine inhibits the growth of Leydig cells (113). In normal and tumor Leydig cells, inhibition of expression of a mitochondrial protein, the peripheral benzodiapene receptor (PBR), decreases cholesterol transport into the mitochondrial matrix and subsequent steroid synthesis (114). Ultrastructual analysis by electron microscopy demonstrated the abnormalities in the morphology of mitochondria derived from phospholipid hydroperoxide glutathione peroxidase (PHGPx)-defective spermatozoa. The failure of the expression of mitochondrial PHGPx in spermatozoa is considered as one of the causes of oligoasthenozoospermia in infertile men (115). The age-

related decrease in female fertility is associated with a decrease in follicle numbers and oocyte quality. The meiotic division errors, mitochondrial DNA mutations, and ageing itself have been suggested to play important roles in the age-associated reduction in oocyte quality (116). Time-dependent accumulation of delta mtDNAs (spontaneous deletions of mtDNA) occurs in normal oocytes (117). Since delta mtDNAs are functionally inactive, an accumulation of such aberrant genomes may compromise ATP-dependent energy-utilization in these cells. Consequently, these deficiencies affect the functioning of the somatic follicular cells that surround and secrete important paracrine factors to the oocyte.

In females, exposures during the perinatal period and the period between age at menarche and age at first full-term pregnancy are thought to be extremely influential in breast, prostate, endometrial and testicular tumor development (29). The possibility of disruption during these time periods is supported by studies showing early exposures to some chemicals can have permanent effects on breast development and susceptibility to carcinogens (reviewed in 4, 29, 32). Several studies have shown that indicators, such as twin pregnancy, primigravid state and obesity, high birth weight, and neonatal jaundice, of high levels of endogenous pregnancy hormones, are associated with an increased risk of testicular and prostate cancer (4-6,29,30,32). A review of 30 cases of breast cancer in men reported worldwide between 1946 and 1972 showed that, among 30 cases, 16 men were treated with stilbene estrogen alone (dose varied from 200-44,200 mg) and the breast cancer appeared from 1-57 months after the start of treatment; 6 of the tumors were new primaries and 10 were metastasis (5). Above studies indicate that peri- and post-natal exposure to steroidal hormones imprint mitochondrial biogenesis of cells. Perinatal imprinting of the mitochondrial biogenesis in the interstitial cells of the testes by stilbene estrogen (diethylstilbestrol=DES) is involved in the etiopathology of testicular lesions (4). In immature rodents, exposure to natural and stilbene estrogen results in infertility and cancer; and in hamsters and mice neonates, testicular tumors develop from its exposure (4). Our studies into the underlying mechanisms of DES-induced carcinogenesis show that exposure of neonates to DES leads to hyperplasia and loss of germ cells in testis of adult animals. This was accompanied by hyperproduction of proinflammatory cytokines, TNF and IL-1 in testes after 60 days of DES exposure to neonates. Exposure of neonates to DES produced a 2-fold increase in 8-OH-dG levels and mutations in the testicular DNA compared with that in age-matched controls after 60 days. Administration of IL-1 antisense oligonucleotides or pentoxifylline, an inhibitor of cytokine synthesis, inhibited the effects of neonatal exposure to DES on the testes in the puberatal animals (4). Based on these findings, we propose that imprinting of mitochondrial pheno- and geno-types may be involved in the development of neoplastic lesions resulting from pre- or neo-natal steroidal estrogen exposure.

5.3. Bioenergetics of Mitochondria, Ca^{2+}, and Functions of Steroid Hormones

Mitochondria play very important roles in the cellular processes via regulation of intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. By sequestering Ca^{2+} rapidly through a uniporter, Ca^{2+} is then released more slowly to the cytoplasm. Mitochondrial Ca^{+2} uniporter are a highly selective ion channel with a very high affinity for Ca^{2+}. The Ca^{2+} is extruded from the mitochondria via the Na^+/ Ca^{2+} exchanger. The Na^+/H^+ antiporter that is located in the mitochondrial inner membrane exchanges 1 Na^+ for 1 H^+ whereas H^+/Ca^{2+} exchanger exchanges $2H^+$ for every Ca^{2+}. The Ca^{2+} transport across the mitochondria is therefore driven by the proton-motive force generated by the oxidative respiratory chain. Estrogen has been shown to rapidly increase intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, by initiating a signal through a G protein coupled membrane ER (118,119). 17β-estradiol- (E2-) induced mitochondrial (mt) reactive oxygen species (ROS) act as signal-transducing messengers that control the G_1/S transition of G_0-arrested estrogen dependent cells (120,22). Calcium is also a well established second messenger that has been shown to modulate the early G_1 phase and the G_1/S phase boundary of the cell cycle (121). A transient rise in Ca^{2+} within the perinuclear compartment of a dividing cell was followed by an elevation in the whole cell's $[Ca^{2+}]_i$, but ceased once cells progressed into the S phase. We previously showed a localization of ROS production in perinuclear mitochondria when cells were exposed to E2; therefore a potential relationship between perinuclear mtROS and mitochondrial Ca^{2+} (Ca^{2+}_{mito}) is intriguing because both factors are known to play a role in cell cycle progression.

Phytoestrogens have been shown to activate the mitochondrial Ca^{2+} uniporter (122). In addition, Ca^{2+} transport across the mitochondrial inner membrane is facilitated through the Ca^{2+}-induced mitochondrial permeability transition pore (PTP) (123) which has also been shown to be modulated by E2 (124). During cell activation, mitochondria play an important role in Ca^{2+} homoeostasis due to the presence of a fast and specific Ca^{2+} channel in its inner membrane, the mitochondrial Ca^{2+} uniporter. This channel allows mitochondria to buffer local cytosolic $[Ca^{2+}]$ changes and controls the $[Ca^{2+}]_{mito}$, thus modulating processes such as oxidative phosphorylation, cytosolic calcium signaling, and apoptosis (123). Previous studies, though few in number, have shown the modulation of mitochondrial calcium in brain cells by E2 exposure (125,126). More recently, E2 has been shown to modulate mitochondrial calcium uptake in cancer cells (127). However, this study was limited in terms of spatial resolution because it measured total Ca^{2+}_{mito} uptake of an E2 treated cell population rather than monitoring Ca^{2+}_{mito} uptake within individual cells. In addition, the reported effects occurred at toxicological concentrations (1 µM to 40 µM) of E2 which raises concerns about their physiological significance.

Distinct populations of mitochondria with different biochemical and respiratory properties exist in cells (27). Previous studies have failed to identify a differential mitochondrial response after E2 exposure because they did not investigate the spatial distribution of Ca^{2+}_{mito} within the cells. The temporal and spatial regulation of signaling molecules within the cell is critical

in the regulation of various cellular functions, and mitochondria play crucial roles in these processes. We have shown that Ca2+mito uptake within an individual cell can differ between two distinct populations of mitochondria with respect to their response to E2-induced Ca2+mito uptake. Mitochondria that had a [Ca2+]mito ≥0.8 μM were responsive to E2 treatment while mitochondria with a lower [Ca2+]mito showed no response (117). Our findings are corroborated by other studies that observed similar heterogeneous Ca2+mito uptake (128,129,130). Since the low Ca2+ capacity mitochondria did not show a response to E2 nor to treatment with mitochondrial Ca2+ specific blockers, these findings suggest that only high Ca2+ capacity mitochondria are involved in the response to estrogen. Our data suggests that high Ca2+ capacity mitochondria are recruited in response to the new metabolic requirements of E2 stimulation or, as in this case, to promote oxidative signaling. More interestingly, the suppression of E2-induced Ca2+mito uptake by the mitochondrial inhibitor rotenone, which is not known to bind the ER; supports the idea that these effects on Ca2+mito uptake and/or mtROS promote oxidative signaling without involving nuclear ER signaling. Mitochondria may coordinate signal transduction pathways that run parallel to nuclear ER signaling. Therefore, we postulate that a subpopulation of mitochondria act like E2 sensors, potentially allowing forces triggered by altered cytoarchitecture in response to E2 exposure to be transmitted to these organelles; which in turn increases the release of ROS signals to the cytosol.

E2-induced mtROS precedes Ca2+mito uptake. The mechanism of Ca2+mito uptake in response to E2 is not clear. Although E2 has been shown to increase mitochondrial retention of Ca2+ by inhibiting Na-dependent Ca2+ efflux (125), our data implies that a sufficient amount of E2 would need to cross the plasma membrane and bind to the mitochondrial membrane in 20 s for this mechanism to work; which is not likely to occur in this short amount of time. Under physiological conditions mitochondria only transiently take up Ca2+ released by proximate endoplasmic reticulum upon stimulation(131). ROS have been shown to increase the level of cytosolic Ca2+ through the mobilization of intracellular Ca2+ stores and/or through the influx of extracellular Ca2+ (132). Relatively low concentrations of hydroperoxides have also been shown to cause transient changes in [Ca2+]i (133,134). These studies in conjunction with our results suggest that a low level of mtROS triggered by E2 exposure stimulates the release of Ca2+ from the endoplasmic reticulum and this is taken up by mitochondria. The regulation of cell proliferation and the cell cycle by Ca2+ is well recognized. Deregulation of calcium homeostasis and signaling is associated with hormonal cancer. The estrogen stimulated high capacity mitochondria Ca2+ uptake plays a role in cell growth based on our data that showed inhibition of breast cancer cell growth by the calcium chelator BAPTA and mitochondrial calcium uniporter blocker Ruthenium Red (135,136). Our findings support a significant role for Ca2+ in the control of E2-induced MCF7 cell cycle progression.

5.4. E2-Induced Cell Signaling Through Mitochondrial ROS

Mitochondria are a predominant source of ROS in most cell types with unique characteristics that may allow it to participate in growth signal transduction. Mitochondria produce low levels of ROS that can be effectively scavenged by the cell's antioxidant defenses at resting conditions. The low basal level of ROS produced by the mitochondria at rest, which makes mitochondrial ROS ideal signaling molecules since its contribution to the intracellular level of ROS is not at so high a level to induce oxidative stress; instead, a low oxidant level provides a physiologically safe window for redox signaling which allows the cell to regulate mild to moderate oxidative changes and critically respond to them by activating cellular processes such as proliferation and differentiation rather than triggering cell death. Mitochondria are unique because they are a regulatable source of ROS in response to external stimuli. For example, cortical neurons exposed to N-methyl-D-aspartate (NMDA) were reported to couple a rise in intracellular calcium with mitochondrial $O_2^{\bullet -}$ production (137). Tumor necrosis factor alpha (TNF-α) is another example of stimulated mitochondrial generation of $O_2^{\bullet -}$ in L929 cells and this ROS generation is coupled to the cytokine by the TNF-α receptor (138,139). Few other examples exist of mitochondria producing ROS in response to external stimuli, but more recently integrins (cell surface receptors that interact with the extracellular matrix) were reported to modulate mitochondrial ROS production for signal transduction (140). Although signal pathways involved in triggering mitochondrial ROS remain largely unknown, it has been proposed that mitochondria participate in integrin signaling in a nonapoptotic manner, which leads to gene expression and cell differentiation.

Mitochondrial ROS can enter the cytosol as either H_2O_2 or $O_2^{\bullet -}$ where it can participate in redox signaling. Within the mitochondria, MnSOD can dismutate $O_2^{\bullet -}$ to H_2O_2 which is a highly diffusible signaling molecule that can exit the mitochondria. In addition to H_2O_2, $O_2^{\bullet -}$ was demonstrated to be released by mitochondria to the cytosol via the voltage-dependent anion channels (VDACs) (141). In regard to turning the mitochondria ROS signal off, cellular antioxidant defenses such as SOD, catalase, and glutathione peroxidase easily degrade ROS, which terminates the signal. Therefore, mitochondrial ROS fulfill the prerequisites of a 2nd messenger since they are short-lived (rapidly generated and degraded), produced in response to a stimulus, highly diffusible, and ubiquitously present in most cell types.

Steroidal Estrogen Exerts a Spatial and Temporal Influence on Mitochondrial Oxidant Stimuli Generation: Mitochondria are highly dynamic structures capable of changing their shape (by elongation, branching, swelling) and their location inside a living cell (142). It is becoming clear that the morphological, functional, and genetic differences (heteroplasmy) that exist within the mitochondria population may reflect a division of labor within the cell. Mitochondria have been reported to be morphologically heterogeneous and unconnected within individual cells (129). Pancreatic acinar cells were reported to contain distinct groups of mitochondria classified by their cellular location that included perinuclear, subplasmalemmal, and perigranular mitochondria (143). In light of this finding, the highly diffusible H_2O_2 generated

by mitochondria may become a specific signaling molecule as a function of location. For example, perinuclear mitochondria may generate H_2O_2 that only transduces signals to the nucleus or the subplasamlemmal mitochondria may only activate signal cascades of plasma membrane origin. Additionally, perinuclear, subplasmalemmal, and perigranular mitochondria were independently activated by intracellular calcium signals in their immediate environment, which supports distinct calcium functions for each type of mitochondria.

It is significant that mitochondria can create subcompartments or 'microzones' within the cytoplasm because signal transduction depends on the close proximity of substrates and effector molecules to be an efficient process. In addition, given the presence of other endogenous ROS sources besides the mitochondria such as NADPH oxidase, peroxisomes, cytochrome p450, xanthine oxidase, cyclooxygenase, lipooxygenase, and γ-glutamyl transpeptidase (144); and because ROS is involved in a variety of signal cascades, understanding how mitochondrial ROS is activated at the right place and at the right time is vital in understanding the organelle's role in signal transduction. Compartmentalization has already been reported to play a key role in redox signaling and we consider this attribute when describing the mitochondria as a signal transducer (145). In adult cells, mitochondrial clustering functions to create steep gradients of low molecular weight species such as O_2, ATP, and pH resulting in specialized microzones that may facilitate signal specificity (146). In the cytosol, the volume occupied by mitochondria in cells is highly variable and ranges from 15% to 50%. Based on volume, mitochondria compose a significant compartment within the cytosol that harbors signaling molecules. H_2O_2 produced within the mitochondria is highly diffusible in contrast to $O_2^{\bullet -}$, which cannot diffuse through membranes making it easily compartmentalized. Thus, mitochondrial generated $O_2^{\bullet -}$ may be kept separated from the cytosol until an appropriate stimulus releases it through VDACs. Another route for $O_2^{\bullet -}$ release may be through the mitochondrial permeability transition pore (MPTP) as low molecular weight compounds up to molecular weight 1500, can be exchanged between the mitochondrial matrix and the cytosol via this pore (147). Since the MPTP is reported to reversibly open/close naturally in intact cells without resulting in apoptosis, mitochondrial signaling molecules could be exchanged with the cytosol by the transient 'flickering' (open/closing) of the MPTP in response to certain stimuli (148).

In addition to location and compartmentalization, protein scaffolds mediate the selective activation of the mitogen activated protein kinase (MAPK) signaling pathway which raises the question of whether mitochondria may also act as a protein scaffold for signaling complexes (149). A-kinase anchoring protein (AKAP) is reported to tether protein kinase A (PKA) to the mouse mitochondrial outer membrane (150,151). What makes AKAPs unique is their ability to simultaneously bind multiple signaling enzymes such as other kinases and phosphatases (152). This multivalent scaffold has been described as a 'transduceosome' capable of integrating signals from multiple pathways (153). Whether these multivalent scaffolds exist on mitochondria is not clear at this time, but these signaling complexes could be a mediator of signals between the mitochondria and the nucleus during cell division. For example, AKAP84/121 has been demonstrated to concentrate on mitochondria in interphase and on mitotic spindles during metaphase transition alluding to its role in the cell cycle (154). In addition to AKAP, a mitochondrial signaling complex has been reported to activate MAPKs. The PKCε can form signaling complexes with ERKs, JNKs, and p38 MAPKs in the murine heart (155). Activated PKCε was shown to increase phosphorylation of mitochondrial ERK and p38 MAPKs. Whether the anchoring of PKC to mitochondria depends on AKAP is not known at this time.

We have very convincingly shown tha the rise of ROS in mitochondria precedes an increase in mitochondrial Ca^{2+} ,$[Ca^{2+}]_m$, and mtROS and $Ca^{2+}{}_{mito}$ followed a similar temporal pattern (135,136). The variation observed in both calcium uptake and ROS production among the cells depended on cell density. Cells with less cell-cell contact generated more ROS and calcium uptake compared to cells with more contacts. This suggests that sparse cells generate more ROS and calcium uptake in response to estrogen because they require it for growth where as confluent cells do not need it and consistently showed less ROS production and calcium uptake. Our observations our are corroborated by another study which showed that the arrest of growth induced by cell confluence ("contact inhibition") is due, at least in part, to a decrease in the steady-state levels of intracellular ROS and the consequent impairment of mitogenic redox signaling (156). The mechanism for these increases in ROS and $[Ca^{2+}]_m$ is not clear. There is some evidence which suggests allosteric inhibition of a respiratory complex may be a mechanism for hormone induced ROS formation. The inhibition of respiratory complex I is known to favor ROS generation. Rat brain mitochondria that respired on complex I substrates produced a substantial level of ROS when inhibited with rotenone concentrations as low as 20nM (157). Since estrogen is known to inhibit respiratory complex I, we speculate that complex I interactions with the hormone could favor ROS production in a manner similar to rotenone. The phytoestrogen genistein is another flavinoid besides quercetin that acts like a pro-oxidant at the level of mitochondria. Genistein (50μM) treatment of rat liver mitochondria was shown to increase ROS formation through interaction with respiratory complex III which resulted in the opening of the membrane transition pore (158). Besides hormone interactions with respiratory enzymes, post-translational modifications such as phosphorylation-/-dephosphorylation that affect the activity of mitochondrial proteins should also be considered in ROS generation. The cAMP-dependent protein kinase is reported to phosphorylate 6-, 18-, 29-, 42-kDa mitochondrial proteins in bovine heart and phosphorylate the human 18-kDa subunit which promotes the activity of complex I (159,160,161,162). Since estrogen is reported to stimulate cAMP-dependent protein kinase activity in hippocampal neurons, it raises the possibility for estrogen to induce

cAMP accumulation in mitochondria (163,164). If estrogen increased cAMP levels within mitochondria, then cAMP-dependent phosphorylation of mitochondrial respiratory complexes may modulate $\Delta\Psi_m$ and/or $[Ca^{2+}]_m$ in favor of ROS generation.

The rapid stimulation of intracellular ROS by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and nerve growth factor (NGF) suggests that this underlying mechanism of cell growth may be shared with other growth factors including estrogen (165). Exogenous addition of low concentrations of H_2O_2 and/or $O_2^{\bullet}$ has been demonstrated to stimulate cell growth in a variety of cell types including muscle cells, fibroblasts, amnion cells, prostate cancer cells, and aortic endothelial cells (144). The molecular signaling mechanism that initiates ROS production by mitochondria is not clear, however, other cell processes besides apoptosis may be coupled to this signaling event. Tumor necrosis factor alpha (TNF-α) induces gene expression via mitochondrial respiratory chain dependent activation of NF-κB, AP-1, JNK, and MAPKK (140). The proliferative response of endothelial cells to hypoxia was demonstrated to be initiated upstream by mitochondrial ROS which activated the MEK/ERK pathway (166). Although other endogenous ROS sources besides mitochondria such as NAD(P)H oxidase exist, mitochondria will be the focus of this paper for the following reasons: (*i*) mitochondria are the principal source of intracellular ROS in epithelial cells. (*ii*) the growth of adenocarcinomas occur in tissue of epithelial cell origin.

A characteristic of rapidly dividing cancer cells is their capacity to produce significant amounts of intracellular ROS, which has been implicated in the promotion of accelerated cell cycle activity in neoplastic cells. Mitochondria have long been suspected to play a role in the development and progression of cancers. The ROS molecules H_2O_2 and NO have been demonstrated to stimulate mitochondrial biogenesis, a process that depends on the flow of molecules into and out of the organelle (167,168). Since mitochondrial proteins are encoded in two separate genomes (mitochondria and nuclear genome), biogenesis is a coordinated effort in which mitochondria transmit signals to the nucleus and vice versa. The question of how mitochondria transmit these signals in the process of cell proliferation has risen from reports of its involvement in cell growth. Cerebral granular cells isolated from newborn rats with high mtNOS activity were reported to exhibit maximal proliferation rates which depended on NO and H_2O_2 levels. In addition, MnSOD displayed an increased pattern of activity similar to mtNOS (169). NO has been proposed to inhibit cytochrome c oxidase in favor of $O_2^{\bullet-}$ production and therefore MnSOD may dismutate $O_2^{\bullet-}$ generated by NO-dependent inhibition into the signaling molecule H_2O_2. Ethinyl estradiol, E2, and estrogen catechol metabolites at a dose of 0.25 to 5μM are reported to increase mitochondrial $O_2^{\bullet-}$ in cultured rat hepatocytes and HepG2 cells (170). Although the biological significance of the estrogen-induced mitochondrial $O_2^{\bullet-}$ is not known at this time, ROS has been demonstrated to modulate ER protein expression in various cell lines. Treatment of human breast cancer cells MCF7 and T-47D with H_2O_2 (2.5μM) increased the protein level of ERβ (171). In addition, PMA (100 ng/ml) treatment increased the expression of ERβ in the macrophage cell line J774A.1.

Oxidative stress has been shown to affect mitochondrial proteins of chronically estrogenized Syrian hamster kidney. A decrease in thiol/sulfhydryl groups was reported in the mitochondrial fraction at a preneoplastic stage of carcinogenesis (172). Estrogen-induced oxidative stress may be responsible for these post-translational modifications in mitochondrial proteins. This finding is significant in the context of cell signaling because redox reactions involving cysteine thiol groups transduce signals by breaking or forming protein dithiol/disulfide bridges (173). Since estrogen can induce mitochondrial ROS, we infer that the oxidation of thiols in response to estrogen converts the oxidative stress to a change in protein function involved in cell growth. Oxidative stress modifies mitochondrial matrix protein thiols (174). Similarly, thiols on protein subunits 51-kDa and 75-kDa of NADH dehydrogenase (complex I) have been reported to form mixed disulfides with glutathione (glutathionylation) in response to mitochondrial oxidative stress. This post-translational modification was reversible and correlated with an increase in mitochondrial $O_2^{\bullet-}$ production (175). Evidence in support of a ROS signal transduction pathway originating from complex I comes from a study which reported that the mitochondrial complex I inhibitor, rotenone, blocked ROS mediated signaling (42).

Role of steroidal estrogen-generated mitochondrial oxidants in redox signaling and cell proliferation: Evidence for the involvement of redox signaling with estrogen-induced cell proliferation has been demonstrated in several studies. Liposomes containing SOD or catalase inhibited *in vitro* estrogen-induced proliferation of Syrian hamster renal proximal tubular cells (176). The cytokines IL-1β and TNF-α are known to cause the release of $O_2^{\bullet-}$ from human fibroblast cells. Co-treatment with an inhibitor of IL-1β and TNF-α synthesis, pentoxifylline, inhibited stilbene estrogen-induced increase in myeloperoxidase activities, 8-hydroxydeoxyguanosine (8-OHdG) formation, mutations in the testicular genome, and prevented estrogen-induced testicular preneoplastic lesions (4). Recently, we have shown that estrogen-induced stimulation of macrophage cells and MCF7 cells in part occurs through ROS (177,178). We have also observed inhibition of estrogen-induced MCF7 cell growth by ROS scavengers such as N-acetylcysteine, ebselen, and catalase (19,77). It is interesting to note that E2 can stimulate the phosphorylation of a-raf and cell cycle progression in MCF7 cells (179). Whether the estrogen induced phosphorylation of a-raf depends on ROS is not known. Mitochondrial PKCδ and PKCε could also activate the raf/MEK/ERK pathway or directly activate MAPKs, respectively (180,181). Rapid effects of estrogen have been demonstrated to mediate the DNA binding activity and phosphorylation of transcription factors. E2 treatment of rat adipocytes doubled AP-1 DNA binding and phosphorylated CREB protein within 15 min (182). The redox sensitive

protein Akt is known to phosphorylate an upstream kinase, IKKα, which stimulates the degradation of Iκ-B (183). Estrogen-induced mitochondrial ROS may stimulate Akt leading to the degradation of Iκ-B and activation of the transcription factor NF-κB. Whether estrogen treatment can activate Akt via mitochondrial derived ROS is not clear, however, phosphorylation and translocation of Akt to the mitochondria was demonstrated when cells are treated with estrogen (184). Given that E2 can stimulate mitochondrial ROS generation; ER, src, a-raf, Akt, and PKC are targets of oxidative stimuli localized at the mitochondria; and the transcription factors AP-1, NF-κB, and CREB are stimulated by oxidants (170,165,185); it is possible that estrogen specific effects at the level of mitochondria can activate these transcription factors. Based on these studies we postulate that estrogen-induced mitochondrial ROS stimulates oxidant sensors a-raf, Akt, or PKC, which in turn activate transcription factors such as NF-κB, CREB, or AP-1 via the MEK/ERK pathway resulting in the transcription of cell cycle genes containing DNA responsive elements for NF-κB, CREB, or AP-1 and ultimately estrogen-induced cell proliferation. ROS can modulate effector molecules such as PKC, p53, extracellular regulated kinase (ERK), nuclear factor-κB (NF-κB), and the c-fos/c-jun heterodimer (AP-1); and these effector molecules are known to participate in growth signal transduction [Felty and Roy, 2004]. Recently we have demonstrated evidence for the involvement of redox signaling with estrogen-induced cell proliferation (77). Physiological concentrations of E2 stimulate a rapid production of intracellular ROS which lead to the phosphorylation of c-jun and CREB; and increased activity of redox sensitive transcription factors Nrf-1, c-jun and CREB known to be involved in the regulation of cell cycle genes. Although direct ER transcription factor effects are required to promote DNA synthesis, our recent data showed that MCF7 cell growth and cyclin D1 expression are suppressed by antioxidants and mitochondrial blockers which supports our novel finding that nongenomic estrogen-induced mitochondrial ROS modulate the early stage of cell cycle progression (77).

These studies suggest that in addition to ovarian estrogens, mitogenic signals may also come from E2 directly acting on mitochondria of epithelial or immune cells through TNF-α - and IL-1β-generated superoxide and hydrogen peroxide. This, in turn, would help to fix the genotoxic effect of the estrogen and/or inflammation; and the production of mutational changes in the genome. In the absence of mitogenic stimuli, DNA damaged viable cells might undergo senescence or apoptosis. Therefore, G1 arrested cells waiting for the repair of DNA damage may receive pressure from the mitogenic signals produced by estrogens and/or TNF-α and IL-1β generated low concentrations of superoxide and H_2O_2, which may force cells to exit out of G1 arrest. This mitogenic pressure may allow cells to enter into the S phase and proceed through G1/S checkpoint in order to complete cell division. This would result in an increased rate of fixation of DNA damage leading to mutation. Since estrogen-induced ROS exert mitogenic effects in the cells, this may also contribute to the potential for fixation of damage to bases of DNA leading to a probability of higher mutational frequency. These studies suggest that the estrogen generated oxidants together with an estrogen-driven increase in epithelial cell proliferation may initiate and promote neoplastic lesions in estrogen-sensitive tissues (Figure 1).

6. ANTIESTROGEN, GLUCOCORTICOID, VITAMIN D AND OTHER PHARMACOLOGICAL AGENTS: PRIMARY OR SECONDARY ACTIONS AT MITOCHONDRIA

We have described here some pharmacological agents such as tamoxifen, glucocorticoid, retenoic acid, vitamin D; some antibiotics, such as tetracycline, chloramphenicol; inhibitors of the mitochondrial protein synthesis, and other pharmacological agents, which exert their effects by influencing mitochondria. For example, doxycycline, an inhibitor of mitochondrial protein synthesis, reduces tumor burden in a mouse model of breast cancer-derived osteolytic bone metastasis and is under clinical trial in Canada for early combination therapy for breast cancer and prostate cancer patients (186). Cisplatin (DDP)-resistant human ovarian carcinoma cells show significant changes in their mitochondrial and plasma membrane potentials as well as in their mitochondrial morphology (187). Mitochondria appear to play an important role in the cellular pharmacology of DDP. These DDP-resistant 2008 cells have an elevated plasma membrane potential and alterations in their mitochondria as indicated by their membrane potential, morphology, and sensitivity to mitochondrial poisons.

Dexamethasone treatment specifically affects mitochondrial energy metabolism through the increase of basal proton conductance of mitochondria in the liver (188,189). The antiapoptotic effect of melatonin on glucocorticoid-treated thymocytes appears to be a consequence of an inhibition of the mitochondrial pathway (190). In differentiated T37i cells, aldosterone modulates endogenous mineralocorticoid receptors and glucocorticoid receptors, thus preventing isoproterenol or retinoic acid-induced increased expression of the uncoupling proteins and changes in the mitochondrial membrane potential (191).

Retinoic acid up-regulates mitochondrial gene expression while vitamin A, the precursor of naturally occurring retinoids, influences mitochondrial activity in rat liver and heart (192,193). It has been shown that peroxisome proliferators such as clofibrate that function via the peroxisome proliferator activated receptor also influence mitochondrial activity (194).

The E2-mediated effect on the transcription of mitochondrial encoded genes is inhibited by the pure ER antagonist, ICI182780 (195). The estrogen receptor ligands tamoxifen, nafoxidine and clomiphene alter membrane potential in a cyclosporin A-like manner of SH-SY5Y mitochondria. Thus, it indicates that estrogen receptor ligands affect mitochondria in a cyclosporin A-like manner in human neuroblastoma cells (196). In addition to estrogen receptor (ER)-positive cells, the antiestrogen tamoxifen

(TAM) inhibits the growth of estrogen receptor (ER)-negative cells. Based on the recent reports indicating multiple effects of TAM on mitochondrial bioenergetic functions – including depolarization of the membrane potential, uncoupling of the mitochondrial respiration, and decreased oxidative phosphorylation efficiency – it appears that TAM may produce inhibition of ER-independent cells through influencing mitochondrial activity.

7. CONCLUSION

The number of mitochondria per cell and amount of mt DNA per cell vary widely in mammalian cells. The number of mitochondria per cell ranges from more than a dozen to 2000 or more mitochondria in various cell types. However, mt DNA/mitochondrion is constant in all mammalian cell types. A quantitative decrease in mtDNA copy number has also been linked to pathogenesis of various diseases. These findings helped to emerge the concept that the proportion of abnormal mt must exceed a critical threshold level before a cell expresses an abnormality of the mitochondrial signaling (the threshold effect). However, this threshold concept may not hold true because of the recent knowledge of structural and functional heterogeneity of mitochondria within the cell. Highly energized mitochondria are found close to plasma membrane or endoplasmic reticulum, and therefore these mitochondria could accumulate more Ca^{2+} and produce more ROS than their perinuclear counterpart. Energized mitochondria are presumably found in those local domains of the cells where functional or signaling stimulus arises upon physiological challenges by exogenous or endogenous signals. The temporal range for the functioning of ROS and Ca^{2+} as primary signaling molecules span microsecond and milliseconds, such as in release of neurotransmitter at the synaptic junctions in neurons, cardiac contraction, to min and hours, such as in cell division and gene expression. The live cell is not homogenous with respect to ionic concentrations and ROS generation.. The changes in the spatial heterogeneity, such as, Ca^{2+}, pH, ROS would also determine the recruitment of sub-population of mitochondria in response to external or internal stimuli. The structural heterogeneity of mitochondria leads to functional heterogeneity that could give rise to heterogeneous ROS and Ca^{2+} accumulation, membrane potential, mitochondrial transition pore opening, or mt mRNA transcription or translation. Cells with and without mitochondrial abnormalities due to mt DNA, mt mRNA or mt encoded proteins alterations may harbor a mixture of abnormal and wild-type mt. Heteroplasmic as well as homoplasmic mt DNA, mt mRNA or mt encoded protein alterations can lead to the development of a number of disorders that express the phenotypes of diseases. Lack of correlation between mtDNA copy number which reflect the total mass or entire population of mitochondria within the cell and mitochondrial biogenesis in fetal, neonatal and adult cells also suggests that not the entire population of mitochondria, but a subpopulation of mitochondrial DNA upon various physiological challenges, such as, hyperthyroidism, hyperestrogenia, or hyperglucocorticoidism, may be transcribed to mitochondrially encoded mRNAs and, consequently, mitochondrial proteins or may be involved in signaling for adaptive stimulation of mitochondrial biogenesis. The stimulation of a subpopulation of mitochondria in the cell presumably depends upon the local domains in the cell that receive the physiological, biochemical or molecular signal (s) in response to external or internal stimuli . Our recent data showing increased ROS and Ca^{2+} concentration in a high calcium capacity mitochondrial population, but not in a low calcium capacity mitochondrial populations, upon exposure to E2 suggests that a sub-population of mitochondria is recruited to respond to the new metabolic requirements required by estrogen triggers or, as it is in this case, to promote oxidative signaling. Mitochondria appear to participate in signal transduction by capturing Ca^{+2} and/or producing RO/NS. Thus, the mitochondrial phenotype of the cell may be affected by molecules that influences redox state. More interestingly, mitochondria may coordinate parallel signal transduction pathways triggered by the altered cytoarchitecture that takes place during changes in cell upon exposure to estrogen or other steroids and RO/NS.

Steroid hormones influence at mitochondrial level. The presence of receptors of the steroid hormones in mitochondria, transport of ligands to the mitochondria, sequences of hormone response elements in the mitochondrial genome, and modulation of mitochondrial encoded genes by steroids supports the concept of a direct action of steroid hormones on mitochondrial gene transcription. Parallel to the primary actions of the steroid hormones on nuclear gene transcription, the hormones in mitochondria, act as a mechanism to coordinate regulation of mitochondrial biogenesis. The cross-talk between the cell nucleus and the mitochondria appears to control steroid hormone-induced signaling involved in the apoptosis, proliferation, and differentiation of both normal and malignant cells. The steroidal agents control biogenesis and maintenance of mitochondria through the crosstalk between nuclear and mitochondrial genomes. These interactions between estrogen and mitochondria merit furture investigations, which may shed new light on the role of mitochondria in cell growth. Several variants of human mitochondrial diseases specifically in steroids hormone-related endocrine organs manifest as a result of mitochondrial physiologic and metabolic compensation of genetic defects. The regulation of mitochondrial transcription by steroidal hormones presumably occurs through pathways similar to those that take place in the nucleus thus opening a new way to better understand steroid hormone and vitamin action at the cellular level. These findings may also provide the basis for the development of novel cancer therapy that targets mitochondria for the treatment of hormone dependent cancers.

8. ACKNOWLEDGEMENTS.

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