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Metabolomics and Transcriptional Responses in Estrogen Receptor Positive Breast Cancer Cells

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1. Introduction

Estrogen exposure is well recognized as a high risk factor of breast cancer, despite the fact that the hormone transcriptionally regulates the expression of tumor suppressor genes, like BRCA1 (Hockings *et al.*, 2008). Estrogen acts through the estrogen receptor α (ERα) in target cells (Ali and Coombes, 2000). ERα overexpression occurs in about 70% of breast cancers, referred as "ER positive" (ER+) (Dickson and Lippman, 1988). ERα is a primary target for chemoprevention as well as other therapeutic interventions for breast cancer with the ER+ phenotype. Clinically, in pathological lesions such as women with benign breast tumors, who underwent surgery, presented with a high proportion of ER positivity than the control group. In such cases, logistic regression yielded an adjusted odds ratio of 6.5 for risk of development of breast cancer compared to 0.3 odds ratio for PR (progesterone receptor); there was an increased proportion of cells expressing ERα by immunohistochemical staining, thereby demonstrating the importance of ERα as a breast cancer risk marker (Khan *et al.*, 1994). Estrogen or E2 mostly exerts its mitogenic effects by the modulation of metabolic responses and through the transcriptional induction of genes regulating crucial cellular processes like the cell cycle (Prall *et al.*, 1998). Microarray and RNA interference studies suggest that the transcriptional regulation of about 60% of estrogen responsive genes (induced or repressed) is dependent upon ERα and Sp1/3 transcription factors (Bazley and Gullick, 2005). The basal expression of ERα, in turn, is regulated by Sp1 in ER+ breast cancer cells (deGraffenried *et al.*, 2002). Sp1 and Sp3 are ubiquitously expressed in mammalian cells and are abnormally expressed in various cancers, including breast (Li and Davie, 2010). Sp1 and Sp3 bind to the same DNA sequence defined as Sp1/3 sites, with similar affinity. Sp1 and Sp3 play important roles in regulating genes critical to the initiation and progression of breast cancers (Hirokawa, 1984; Lu and Archer, 2010). Targeting these proteins is a promising cancer therapeutic approach (Jia *et al.*, 2010).

In most cases, ER+ breast cancers present a better clinical prognosis than the ER- breast cancers. Moreover, it is an encouraging fact that the leading antiestrogenic drugs such as tamoxifen have effectively improved the overall survival of pre and postmenopausal women by the reduction of cancer incidence and formation of new tumors (Ferguson and Davidson, 1997; Muss, 2001). However, a major obstacle to breast cancer treatment is the development of drug resistance. Resistance is commonly associated with an increased expression of Erb

proteins including EGFR (epidermal growth factor receptor); in addition, there is evidence of non-hormonal role of ERα in hormone sensitive MCF-7 cells to maintain basal proliferation (Salazar *et al.*, 2011). Interestingly, there was no expression of Erb2 and 3, but ERα supported a population of basal fraction of actively dividing S phase cells which were unaffected by hormone depletion or hydroxy tamoxifen treatment (Salazar *et al.*, 2011). Like breast tumors, breast cancer cell lines can also yield clonal populations of tamoxifen resistant cells that have variable ER dependence (Coser *et al.*, 2009). This *in vitro* evidence supports the fact that the basal population of hormone sensitive cells may be a prerequisite condition for eventual occurrence of hormone insensitive tumors where current therapies such as tamoxifen are no more effective (Salazar *et al.*, 2011). Development of resistance to tamoxifen or aromatase inhibitors (AIs) is a result of adaptive changes which lead to the activation of alternate signaling pathways. ER+ tumors unresponsive to front line therapy drugs such as tamoxifen or AIs will eventually become resistant; as seen in xenograft or cell line models, there is an eventual constitutive activation of the RAS-MAPK (RAS-mitogen activated protein kinase) signaling pathway and dependency on growth factor receptor (EGFR/HER1 and HER2/neu) signaling. Constitutive RAS-MAPK activation by EGFR or HER2 overexpression in ER- breast cancers results in the downstream activation of mitogen and stress activated kinase 1 and 2 (MSK1 and MSK2) as well as several MSK substrates that are implicated in oncogenic programming. Given these detrimental adaptive cellular changes, there is a lot of attention given to breast cancers during the ER+ stage, such that alternative strategies can be used to circumvent the problem of drug resistance. One such approach is the use of synthetic peptides with sequences of natural biological molecules, such as ERα. A peptide analog, ERα17p with a 17 amino acid motif (PLMIKRSKKNSLALSLT; P295-T311 of ERα) was synthesized which contained the third nuclear localization signal of ERα, a proteolysis site, as well as a binding site for calmodulin. This 17 amino acid motif of ERα is responsible for a number of post-transcriptional modifications as well as of the recruitment of co-regulators. It was reported to elicit (pseudo)-estrogenic responses in ER+ MCF-7 cells (Gallo *et al.*, 2007). Further studies showed that the ERα17p peptide induced apoptosis in the ER+ MCF-7 and T47D cells and also in the ER- MDA-MB-231 and SKBr-3 cells through ERα independent mechanisms. This peptide also caused regression of ER- breast cancer tumor xenografts without apparent toxicity. This suggested a potentially new attractive tool for the development of promising therapeutic approaches, and also provided an insight to the cellular fate of ERα (Pelekanou *et al.*, 2011).

The differences of the ER+ or ER- breast cancer not only relate to their morphology, but are also largely due to the differential metabolomics and differences in their transcriptional responses, which overall is a reflection of the selectivity of gene usage. Differences between these two wide phenotypes are reflected in the metabolism of sugars, fatty acids, proteins or even drugs that accelerate or decelerate proliferation. In the ER+ breast cancer cells, E2 is pivotal in controlling diverse energy metabolic pathways such as glucose transport, glycolysis, TCA (tricarboxylic acid) cycle, mitochondrial respiratory chain, adenosine nucleotide translocator and fatty acid β-oxidation and synthesis respectively. Estrogen has positive effects on carbohydrate and lipid metabolism. Absence of ERα causes hyperplasia and hypertrophy of the adipose tissue, insulin resistance, and reduced energy expenditure respectively [(Chen *et al.*, 2009) and references therein]. Hence disturbances in the E2/ER metabolic pathways are likely to cause metabolic diseases such as heart disease, obesity and also cancer. Thus breast cancer progression and unresponsiveness to therapy are interrelated.

MCF-7 is a model cell line of the ER+ phenotype and the highly metastatic MDA-MB-231 is a model cell line of ER- phenotype. Both these cell lines have been widely used for *in vitro* cell culture and *in vivo* xenograft studies. We used microarray expression data obtained from public repositories for MCF-7 (non-stimulated) and MDA-MB-231 to compare interrelated factors that regulate the transition of breast cancer towards the more aggressive phenotype (Mandal *et al.*, 2007b; Mandal and Davie, 2007). In this chapter, we review, discuss, and compare some salient features and experimental findings of major energy metabolic pathways, the importance of pathways that are constitutively activated and transcriptional responses with particular emphasis on ER+ breast cancers.

2. Glucose metabolism

The binding of estrogen to its receptor induces crucial metabolic changes through the activation of various estrogen responsive genes. Estrogen stimulates glucose metabolism, including glycolysis and glycogen synthesis, in target organs such as the uterus, which is a classic estrogen target organ (Shinkarenko *et al.*, 1994; Smith and Gorski, 1968). It has been shown in many studies that estrogen stimulates the incorporation of labeled glucose (^{14}C-labeled glucose) into lipid, RNA, and protein (Bitman *et al.*, 1965; Nicolette and Gorski, 1964; Swigart *et al.*, 1961). The influence of estrogen in glucose metabolism is also well studied in another estrogen target tissue, the breast. Several studies in breast cancer cell lines have that the incorporation of the glucose analogue, [^{18}F]-fluoro-2-deoxy-D-glucose (^{18}F-FDG) varies with the ERα status, the acquisition of drug resistance, or alterations in the expression of ERα. Clinically, ^{18}F-FDG is used for non-invasive detection of breast cancer and staging of axillary lymph node metastases by PET (positron emission tomography) scan (Utech *et al.*, 1996). PET technology utilizes the metabolic characteristic of enhanced glucose utilization by tumor cells (Moreno-Sanchez *et al.*, 2007). Hence this advanced technology can be effectively used to study metabolic characteristics in drug resistance or hormone related metabolic changes.

Tumors refractory to chemotherapy are prone to the acquisition of drug resistance. One of the mechanisms of acquired resistance is through alterations of glucose metabolism and the differential expression of GLUT (glucose transporter) expression. The higher gene and protein expression of GLUTs in tumors is well documented (Macheda *et al.*, 2005; Medina and Owen, 2002; Wood and Trayhurn, 2003). This phenomenon provides an advantage to tumors to harness increased glucose utilization through the glycolysis pathway. Glucose transporters such as GLUT12, initially identified in MCF-7 cells, have been shown to have deregulated and increased expression in breast tumors (Rogers *et al.*, 2003). Facilitated transport of polar glucose molecules across the plasma membrane is dependent on GLUT proteins. GLUT proteins 1-5 have been identified in mammalian cells. These transporter proteins impart unique metabolic properties to cells (Rogers *et al.*, 2003). There is evidence that in both the ER+ MCF-7 cells and the ER- MDA-MB-231 cells, there is an increased expression of GLUT1 under hypoxic conditions (Rivenzon-Segal *et al.*, 2003). MCF-7 cells rendered resistant to the drug 5-fluorouracil by long term exposure (the drug-sensitive parental cell line in medium with increasing concentrations for a period of two years), had a decreased ^{18}F-FDG incorporation. Microarray data showed that the expression of GLUTs 8 and 10 was decreased in resistant cells, while GLUT1 was only increased in cells resistant to the lowest concentration of 5-fluorouracil. There were no alterations in the hexokinase activity, but there was a increase in glucose transport (Smith *et al.*, 2007). On the other hand, estrogen produces an positive

increase in the ^{18}F-FDG uptake which has been demonstrated in ER+ cells. A comparative study between the ER+ T47D cells and the ER- cell lines, MDA-MB-231 and MDA-MB-468 demonstrated the dependence of estrogen in mediating the glucose uptake. In the ER- breast cancer cells, the ^{18}F-FDG uptake was totally absent; this correlated positively with the fact that these cells are totally unresponsive to the estrogen dependent effect on glucose uptake. To further demonstrate this estrogen dependence, it was seen that when the ER+ T47D cells were pretreated with the pure antiestrogen ICI182,780 (fulvestrant), the glucose uptake was totally abrogated. Unlike fulvestrant, the partial antiestrogen tamoxifen was incapable of blocking this estrogen response (Ko *et al.*, 2010).

Comparison of metabolic and morphological differences between normal breast and ER+ breast cancer cells gave insights into the adaptive responses adaptive responses which enable cancer cells to better utilize cellular energy resources. Glucose metabolism was studied in the finite lifespan normal human mammary epithelial cell line (HMECs) as well as the classical ER+ the classical ER+ MCF-7 cancer cell line. Cells were induced to grow rapidly under routine tissue culture conditions in nutrient rich media containing ^{13}C-labeled glucose and the isotopic enrichment of cellular metabolites was quantified to calculate metabolic fluxes in key pathways. Cells grown in culture dishes of various sizes (with different surface areas) exhibited very different metabolic and morphological profiles. MCF-7 cells have about an 80% smaller exposed surface area and contain 26% less protein per cell than the HMECs. The per-cell glucose consumption, lactate production, and glutamine consumption rate was 225-250% higher per cell relative to the cancer cells. However, the calculated flux per the exposed area for glucose, lactate, and glutamine was much higher in MCF-7 cells; MCF-7 cells also consumed amino acids at rates much higher than that required for protein synthesis demonstrating a greater efficacy of transport mechanisms (Meadows *et al.*, 2008). In addition, the energy efficiency was much higher in MCF-7 cells which also had a higher dependence on the TCA cycle. These observations form the basis of rational drug design for metabolic drugs (Meadows *et al.*, 2008).

2.1 Glucose metabolism – A comparative analysis in ER+ and ER- breast cancer cells

Glycolysis is the metabolic pathway where glucose is oxidized to pyruvate under aerobic conditions to yield ATP; however, under anaerobic conditions, pyruvate is converted to lactic acid. This biochemical pathway is regulated by the enzymes hexokinase, phosphofructokinase, and pyruvate kinase respectively, which are also potential sites of control and in most organisms these steps are irreversible. The opposite action of glycolysis is gluconeogenesis, which occurs when the blood sugar level falls, thus aiding in the replenishment of blood sugar levels. The gene expression in glycolysis and gluconeogenesis pathways is depicted in Fig. 1A in the ER+ MCF-7 cells and the ER- MDA-MB-231 cells. Some of the key patterns of gene expression are seen between the ER+ and ER- phenotypes:

a. In both cases, the expression of genes and proteins coding for facilitated transmembrane GLUTs (SLCs, solute carrier family of proteins) is either absent or down-regulated. Interestingly, the protein encoded by the gene, *GPI* (phosphoglucose isomerase) catalyzing the irreversible isomerization of G-6-PO_4 (glucose-6-phosphate) to F-6-PO_4 (fructose-6-phosphate), is key in energy pathways and is down-regulated in MDA-MB-231 cells whereas, it is up-regulated in MCF-7 cells. This agrees with our previous studies where we had shown that the more aggressive ER+ cells have a reduced utilization of genes involved in the energy production and expenditure pathways similar to the ER- breast cancer phenotype (Mandal and Davie, 2007).

b. Of the three isozymes of PFK (phosphofructokinase) which catalyze the conversion of fructose 6-phosphate (F-6-PO_4) to the 1, 6-diphosphate form, PFKM is highly expressed in MCF-7 cells and is down-regulated in MDA-MB-231 cells; PFKM is responsible for the phosphorylation of F 6-PO_4 to F 1, 6-bisPO_4. On the other hand, expression of PFKP is quite high in MDA-MB-231 cells and this isoform is responsible for the catalytic conversion of F 6-PO_4 to F 1, 6-bisPO_4 and PFKP is a key regulatory enzyme of glycolysis. Mutations in this key enzyme can cause glycogen storage disease.
c. A crucial difference is the down-regulated expression of the isozymes of lactate dehydrogenase (LDH) in MCF-7 (LDHA, LDHC) versus their up-regulation in MDA-MB-231 cells. The proteins encoded by both these genes catalyze the conversion of L-lactate and NAD to pyruvate and NADH as the final step of anaerobic glycolysis, a feature of the metastatic phenotype.
d. Genes encoding for glycolytic enzymes like phosphoglycerate kinases (*PGK1/2*) are either down-regulated (*PGK1* in MCF-7), or have low expression (*PGK1* in MDA-MB-231), or are absent (*PGK2,* in MCF7 and MDA-MB-231).
e. Aldolases catalyzing the reversible aldol cleavage of fructose 1, 6-biphosphate and fructose 1-phosphate to dihydroxyacetone phosphate and either glyceraldehyde-3-phosphate or glyceraldehyde respectively are either absent (*ALDOA, B*) or are highly expressed in MCF-7 cells, but down-regulated in MDA-MB-231 cells. The same pattern of opposite gene expression is also seen with *TPI1* (triosephosphate isomerase 1), which catalyzes the isomerization of glyceraldehydes 3-phosphate (G3P) and dihydroxy-acetone phosphate (DHAP) in glycolysis and gluconeogenesis.

Glycogenesis is the glycogen synthesis process, in which glucose molecules are added to chains of glycogen for storage. We compared the gene expression analysis between MCF-7 and MDA-MB-231 cells where in general, there was a down-regulation or absence of genes involved in critical steps [such as debranching (AGL), phosphate transfer to glucose molecules (*PGM1*)] within the pathway (Fig. 1B). Notably, mutations of most of the genes involved in the glycogenesis pathway are associated with glycogen storage disease. Interestingly, there was a selective up-regulation of genes *(PPP2R3A, PPP2CA, PPP2R5A, PPP2R5B, PPP2R2C)* in MDA-MB-231 cells encoding for protein phosphatases; the proteins of these genes are involved in the negative control of cell division and growth.

3. Fatty acid (FA) metabolism

The first step to the biosynthesis of fatty acids is the transport of citrate from the mitochondria to the cytoplasm. Lipogenesis is controlled by the enzyme fatty acid synthase or FASN, which is highly expressed in breast carcinomas and is thus an important cancer therapeutic target. The breakdown of fatty acids is regulated by carnitine palmitoyltransferase-1 or CPT-I. The preferential expression of FASN in cancer cells including breast cancer, in particular in ER+ breast cancer cells, is of importance because the production and levels of palmitate substantially differ in cancer cells. Blocking FASN has antiproliferative effects on breast cancer cells. A natural antibiotic, known as cerulenin derived from the fungus, *C. ceruleans*, caused apoptosis in cancer cells; C75 is a natural derivative of cerulenin. C75 has antitumor effects and its action is postulated to be on CPT-1 and production of high levels of malonyl CoA respectively. In a comparative study between C75 and EGCG (epigallocatechin-3-gallate), a main constituent of green tea, it was seen that EGCG caused induction of apoptosis uncoupled to the effects of CPT-1 in breast cancer cells through the targeted inhibition of FASN (Puig *et al.*, 2008). (Information source of individual genes: www.ncbi.nlm.nih.gov).

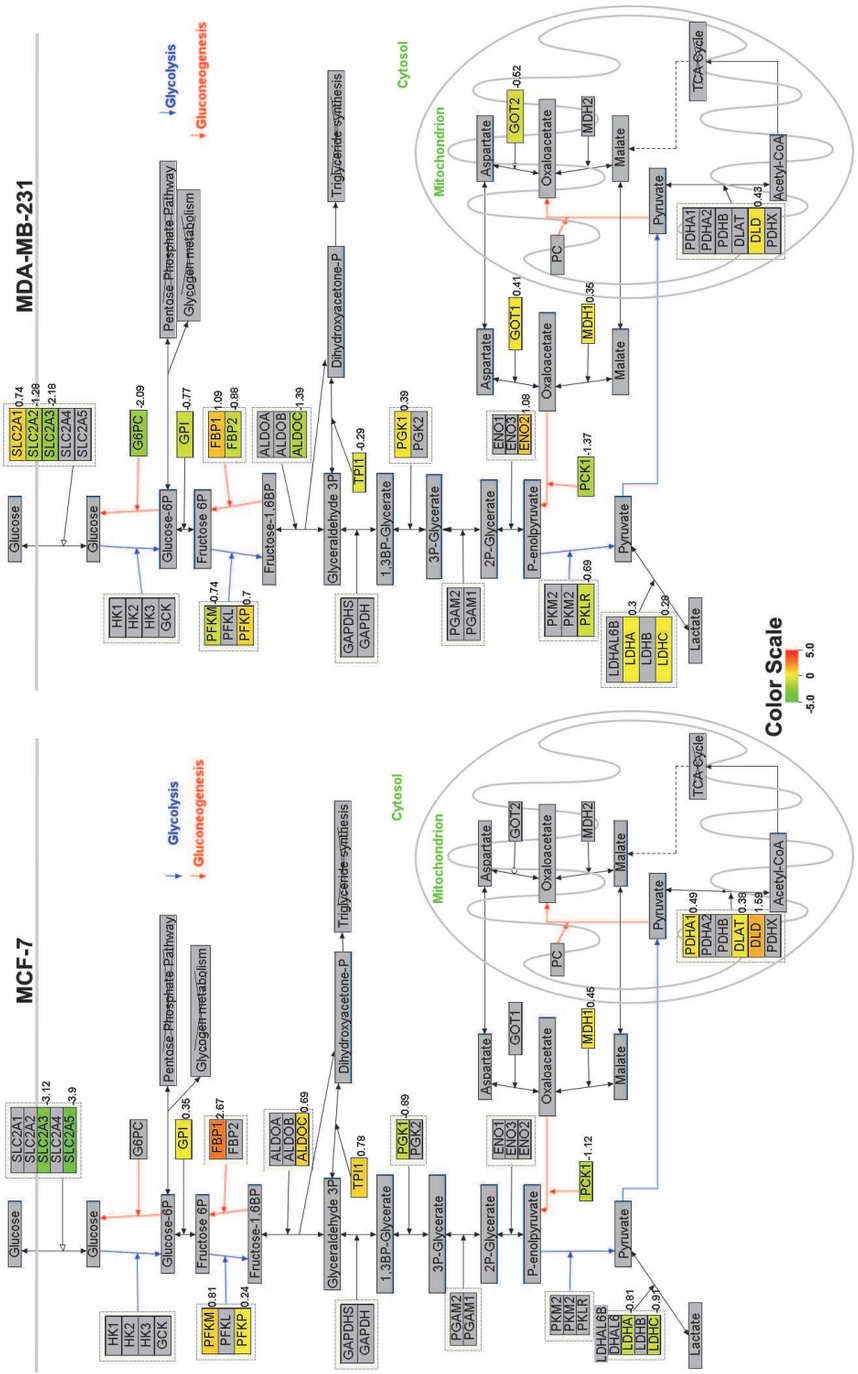


Fig. 1A. A comparative view of the glycolysis and gluconeogenesis pathways operating in the ER+ MCF-7 and ER- MDA-MB-231 cells is shown. The pathway maps were generated using the pathway visualization tool, PathVisio (Version 2). The software generated a color scale for the visualization of expression (red is up-regulation and green is down-regulation of gene expression). Expression values (SLR, signal log ratio) with a cutoff value of ≥ 0.2 or ≤- 0.2 was used and in case of duplicate SLR values, an average SLR was taken. **Note**: Only the controlled irreversible steps of each pathway are colored.

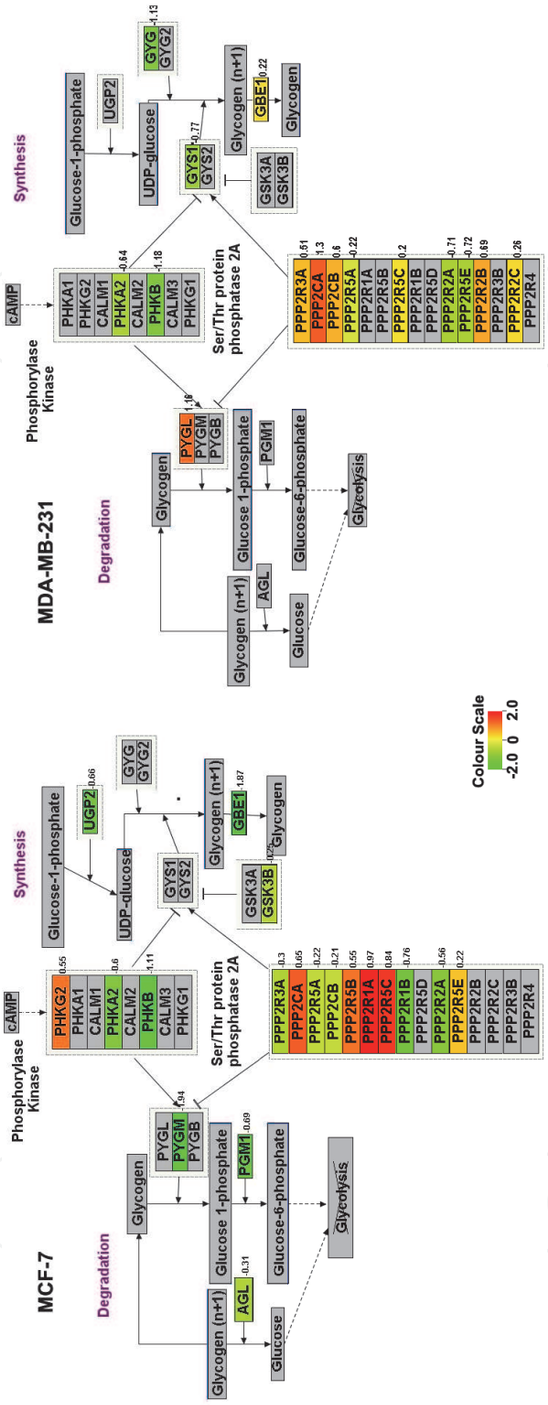


Fig. 1B. A comparative view of the glycogenesis pathway operating in MCF-7 and MDA-MB-231 cells is shown. The pathway maps were generated using the pathway visualization tool, PathVisio (Version 2). The software generated a color scale for the visualization of expression (red is up-regulation and green is down-regulation of gene expression). Expression values (SLR, signal log ratio) with a cutoff value of ≥ 0.2 or ≤- 0.2 was used and in case of duplicate SLR values, an average SLR was taken.

The n-3 PUFAs (n-3 polyunsaturated fatty acids) have beneficial effects as chemopreventive and chemotherapeutic agents in the treatment of cancer. It is of particular interest that in recent years several *in vitro* and *in vivo* xenograft studies and clinical trials have presented evidence that there are beneficial effects of n-3 PUFAs when administered with conventional antineoplastic drugs and radiotherapy against many different types of cancers including breast cancer. Combination of drugs such as doxorubicin/epirubicin/paclitaxel with the n-3 PUFA, DHA (docosahexaenoic acid) had antiproliferative effects such as apoptosis induction, inhibition of neo-angiogenesis, or inhibition of invasiveness in MCF-7 and MDA-MB-231 cells [(Calviello *et al.*, 2009) and references therein]. The cellular antiproliferative actions of n-3 PUFAs include modulation of metabolism, expression of cell cycle and apoptosis factors including caspases, and the ability of fatty acids to sensitize tumor cells to anticancer drugs by increasing membrane permeability. Such beneficial properties have been exploited to develop an interesting strategy of drug delivery, which is the design of liposomes for the targeted delivery of anticancer drugs. This system utilizes the fact that conventional drugs like doxorubicin and cisplatin in liposomal formulations are activated by secretory phospholipase A_2 or $sPLA_2$ which is overexpressed in inflammatory and tumor tissues (Andresen *et al.*, 2005; Laye and Gill, 2003). However, such an approach has not been successful in ER+ (MCF-7) or ER- (MDA-MB-231) cells (Rasmussen *et al.*, 2010). The metabolic modulatory effects of PUFAs leading to antiproliferation include alterations in arachidonic acid (AA) oxidative metabolism and metabolic conversion of n-3 PUFAs to bioactive derivatives. The cellular production of some AA-derived eicosanoids such as prostaglandin E_2 is high in cancer and their formation is inhibited by n-3 PUFAs. Moreover, n-3 PUFAs also competitively inhibit the COX2 enzyme (a very potent oncogene) to produce the less biologically effective n-3 PUFA derived eicosanoids (Chell *et al.*, 2006). A promising finding is the ability of n-3 PUFA to overcome resistance of breast cancer cells treated with tamoxifen. As the n-3 PUFAs are known to inhibit the Akt activity, it was found that EPA (eicosapentaenoic acid) cotreatment of breast cancer cells overexpressing Akt made cells more responsive to tamoxifen. Conjugated preparations of novel anticancer agent, propofol with n-3 PUFAs, DHA and EPA caused significant induction of apoptosis, induction of caspase 3 activity, inhibition of cell adhesion and migration in MDA-MB-231 cells. The drug alone or in combination with the n-3 PUFAs was minimally effective to cause these effects (Siddiqui *et al.*, 2005).

3.1 FA metabolism – A comparative analysis in ER+ and ER- breast cancer cells

In general, the ER+ MCF-7 cells show a greater number of up-regulated genes for proteins catalyzing the FA biosynthesis in comparison to the ER- MDA-MB-231 cells (Fig. 2A). The key features of gene expression are as follows:

a. Notably, the gene expressing the primary enzyme for FA biosynthesis is *FASN*, which is up-regulated in MCF-7 cells in contrast to a down-regulated expression in MDA-MB-231 cells. Genes encoding for enzymes catalyzing the production of FA acetyl Co-A esters from the TCA cycle intermediate, citrate and acetyl CoA (*ACYL, FASN*), or via its intermediate malonyl CoA (ACACB), were up-regulated in MCF-7 cells, but were down-regulated or absent in MDA-MB-231 cells (*ACYL, FASN, ACACA, ACACB*).
b. Interestingly, in both cell types, genes for the enzymes catalyzing the production of triglycerides from FA CoA (*ACSL1, 3, ACAS2*) were down-regulated. These ligases play a key role not only in biosynthesis, but also in FA degradation. Thus, while it is evident that MCF-7 cells differ from ER- cells in the production of palmitate, the synthesis of triglycerides seems to be restricted in both cell types and may be a predominant adaptive response of cancer cells (Fig. 2A).

Similar to FA biosynthesis, genes encoding for enzymes of FA degradation and cholesterol biosynthesis (Fig. 2B) are also mostly up-regulated in MCF-7 cells in contrast to MDA-MB-231 cells.

a. Genes regulating cholesterol biosynthesis may be favored in a hormone responsive environment, cholesterol being the building block of all steroid hormones, including estradiol. Notably among them, is *SQLE* (squalene epoxidase), the protein product of which is a key enzyme in cholesterol key enzyme of cholesterol biosynthesis. MCF-7 cells have an up-regulated expression of the gene, while it is down-regulated in MDA-MB-231 cells. Clinical studies have shown that higher expression of *SQLE* is inversely correlated with distant metastasis-free survival in ER+ stage 1/II breast cancer patients (Helms *et al.*, 2008).
b. Other notable differences include the up-regulated expression of genes of enzymes (*MVK, PMVK*) producing the phosphorylated forms of mevalonic acid, which is a key intermediate of steroid biosynthesis, in MCF-7 cells.
c. Gene of the enzyme catalyzing the dimerization of 2 farnesyl diphosphate molecules to form squalene, is down-regulated, but forms an important step in cholesterol synthesis. It may be assumed that this step of cholesterol biosynthesis, and hence the synthesis of squalene is tightly controlled with respect to the need of steroid hormone synthesis.
d. Notably, in both cell lines, the NAD(P) dependent steroid dehydrogenase-like (NSDHL) enzyme is slightly up-regulated; this may be indicative of an advantage of cancer cells to produce cholesterol depending on its cellular needs.
e. Two other genes whose proteins (*SC4MOL, CYP51A1*) are involved in the synthesis of the cholesterol intermediate, lathosterol are either absent (MDA-MB-231) or down-regulated (MCF-7). Notably, among these, the *CYP51A1* gene encodes for a member of the cytochrome P450 superfamily of monooxygenase enzymes which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. The absence or down-regulation of this enzyme allows cancer cells to metabolize therapeutic drugs aimed to arrest or cause death and thus aid in their overall survival.

4. Retinoic acid (RA) metabolism

The retinoids comprise of RA (vitamin A, all-*trans* retinoic acid*)* and related signaling molecules which are essential in the differentiation of various types of stem cells. A compromised retinoid signaling is often seen early in carcinogenesis including in breast carcinogenesis, where retinoids interact with the estrogen signaling pathways. These molecules are used for cancer therapeutics because they can induce differentiation and growth arrest. Efficacy of retinoid treatment is often challenged by the fact that these molecules are rapidly metabolized. Moreover, compromised responses, including resistance, to pharmacological doses of retinoids to cancer cells are also the result of epigenetic changes, such as the expression of various transcriptional coactivators and corepressors, the hypermethylation of CpGs in specific promoters in cancer cells, and the activities of other signaling pathways respectively. A combination therapy of retinoids with epigenetic drugs such as histone deacetylase or DNA methytransferase inhibitors or with other classical chemotherapeutic drugs may be a potential alternative route of treatment (Tang and Gudas, 2011). Moreover, another reason limiting the potent anticarcinogenic activities of RA is the fact that it exhibits a paradoxical behavior in that in some cancers it facilitates proliferation rather than inhibition of growth (Schug *et al.*, 2008). (Information source of individual genes: www.ncbi.nlm.nih.gov)

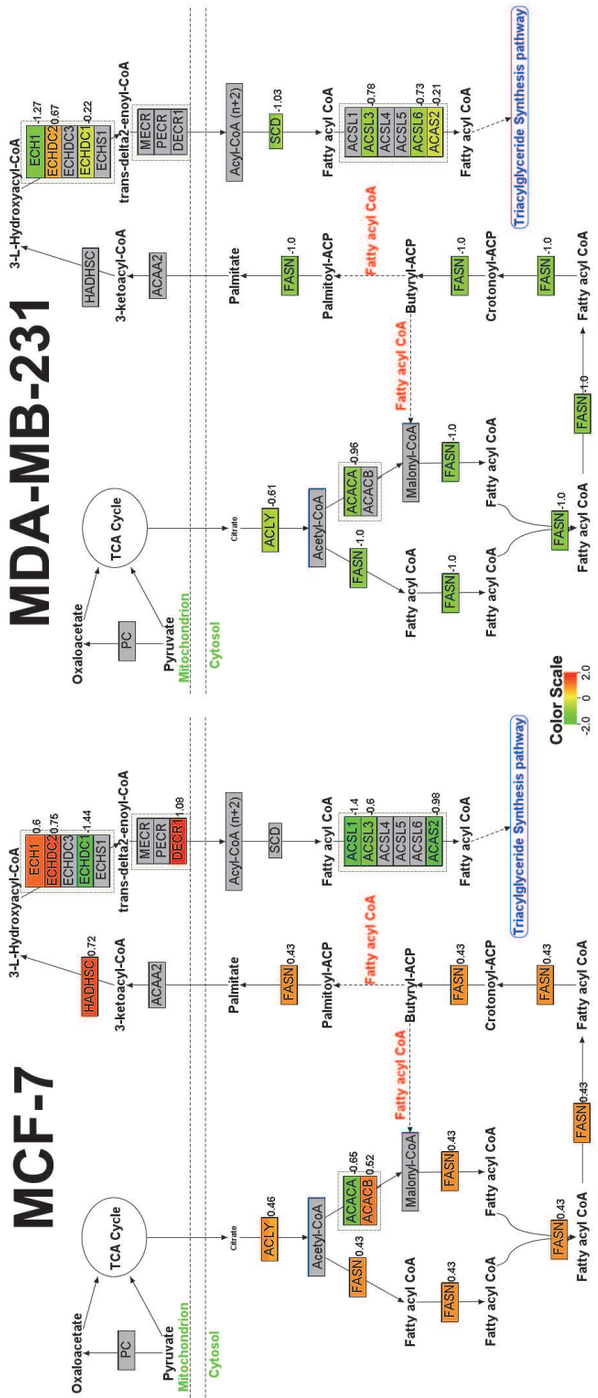


Fig. 2A. A comparative view of fatty acid biosynthesis pathways operating in MCF-7 and MDA-MB-231 cells is shown. The pathway maps were generated using the pathway visualization tool, PathVisio (Version 2). The software generated a color scale for the visualization of expression (red is up-regulation and green is down-regulation of gene expression). Expression values (SLR, signal log ratio) with a cutoff value of ≥ 0.2 or ≤- 0.2 was used and in case of duplicate SLR values, an average SLR was taken.

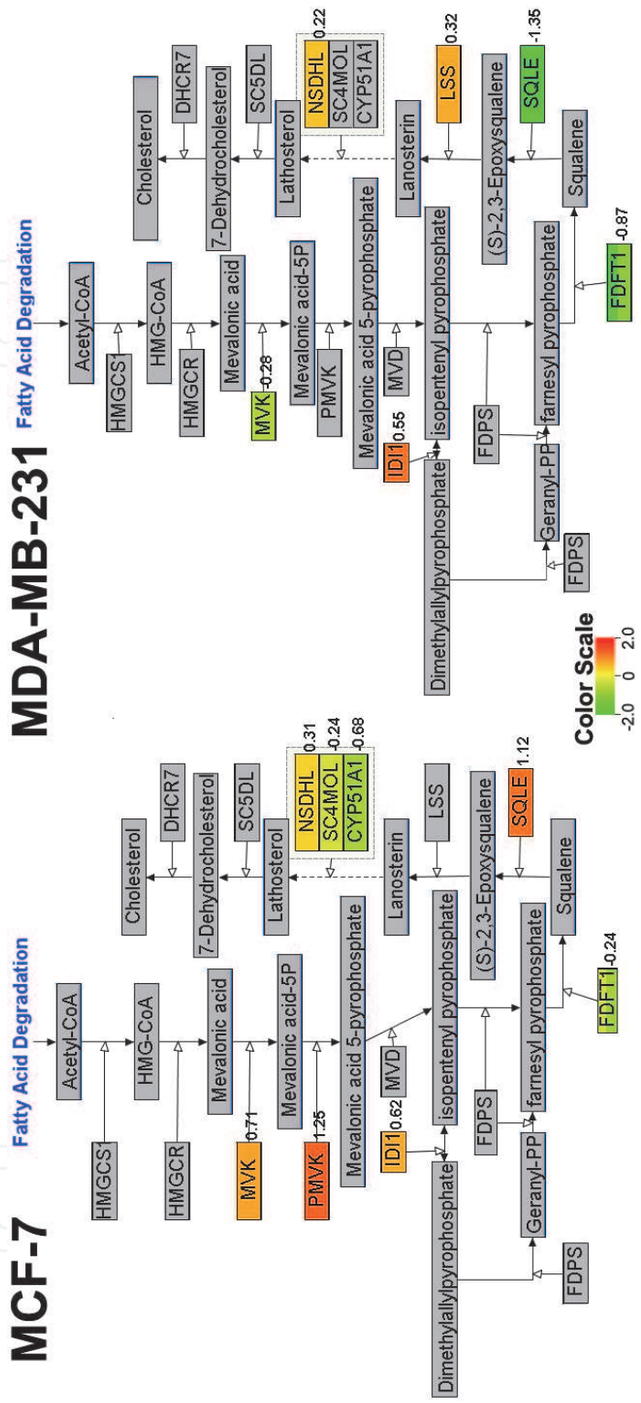


Fig. 2B. A comparative view of fatty acid degradation pathway operating in MCF-7 and MDA-MB-231 cells is shown. The pathway maps were generated using the pathway visualization tool, PathVisio (Version 2). The software generated a color scale for the visualization of expression (red is up-regulation and green is down-regulation of gene expression). Expression values (SLR, signal log ratio) with a cutoff value of ≥ 0.2 or ≤- 0.2 was used and in case of duplicate SLR values, an average SLR was taken.

The intracellular concentrations of various forms of retinoids are under the control of several metabolic enzymes (Duester, 2008). Within the RA generating tissue, retinol or vitamin A (retinol) is first converted to the aldehyde form (retinaldehyde) and then to the carboxylic acid (retinoic acid, RA). The expression of several alcohol and retinol dehydrogenases is widespread and overlapping in a variety of tissues. However, during mouse embryogenesis, the expression of the enzymes (retinaldehyde dehydrogenases) responsible for the second step which leads to RA generation, is tissue specific and non-overlapping (Mic *et al.*, 2002). Oxidation of RA leads to its degradation to more polar metabolites such as 4-oxo-RA (and other more polar RA metabolites); this oxidative degradation is catalyzed by three cytochrome P450 (CYP) enzymes, CYP26A1, B1, and C1 respectively (bu-Abed *et al.*, 1998). Enzymes controlling the synthesis of RA are expressed in very low levels in breast cancers relative to normal breast cells. The ability of normal HMECs from reduction mammoplasty has been shown to be competent in RA synthesis and the ability is still retained by immortal, non-tumorigenic breast epithelial cells, such as MCF10A. However, in the ER+ MCF-7 and T47D cells, the impaired RA activity appeared due to the impaired biosynthesis of RA; treatment with the CYP26 inhibitor, liarozole did not affect the low and undetectable levels of RA (Mira *et al.*, 2000). Likewise, a defect found in human primary breast tumors is the aberrantly high expression of the degradation enzyme, CYP26A1. The ducts and lobules of normal breast tissues demonstrated weak staining which was in contrast to distinct strong cytoplasmic staining in 46% of breast carcinomas (primary and metastatic breast carcinomas) examined within a tissue microarray. Kaplan-Meier analysis of these breast cancer cases suggested an association of high expression of CYP26A1 with a lower disease free and overall survival period. While overexpression of CYP26A1 in transfected AC2M2 (a breast metastatic ER-variant cell line) caused a decreased sensitivity to apoptosis, CYP26A1 siRNA silencing partially abrogated cells from the apoptosis sensitivity. In RA sensitive ER+ T47D cells, the induction of this enzyme was also more rapid than the ER- MDA-MB-231 cells. The growth inhibitory properties of RA were increased by the effect of treatment of an RA metabolism inhibitor, liarozole. Thus both *in vivo* and *in vitro* evidence suggested the potential oncogenic capacity (decreased apoptosis and increased cell survival) of RA degradation enzymes (Osanai *et al.*, 2010; Sonneveld *et al.*, 1998).

Studies show that two critical steps in RA metabolism responsible for synthesis and degradation of RA are sometimes impaired in breast cancers. Thus, defects in RA metabolism influences its effectiveness as a cancer antiproliferative drug in the ER+/ER- breast cells. RA inhibited cell growth and induced apoptosis in ER+ breast cancer cell lines, MCF-7 and T47D, but not in ER- MDA-MB-231 and MDA-MB-453 cells. The differences were due to the ability of ER+ cells to uptake RA rapidly (by hour 2) and by hour 24 there was a disappearance of RA uptake from the medium and cells leaving oxidation products in the culture medium. In sharp contrast, ER- cells showed lower accumulation without any sharp increase and subsequent steep decline; the result was the presence of more RA in these cells and the culture medium (Okamoto *et al.*, 2000). Interestingly, the ER- cell line MDA-MB-468 cells still retain the ability to synthesize RA (Mira *et al.*, 2000). Though a triple negative ER- cell line with EGFR amplification, the MDA-MB-468 cells are responsive to novel drugs along with the nuclear localization of a pseudo wild-type p53 and delocalization of mutant p53 (Mandal *et al.*, 2007a).

4.1 Comparative analysis of RA metabolism between ER+ and ER- cells

RA acts through its binding to the RA receptor (RAR), which in turn is bound to DNA as a heterodimer with the retinoid X receptor (RXR) in the RA response elements (RAREs).

Binding of RA ligand to RAR alters the conformation of the RAR resulting in the transcriptional induction or repression of RA target genes. As discussed in the previous paragraphs, the genes of proteins catalyzing the metabolism of RA has profound effects on breast cancer and hence also on its antiproliferative actions. Here we discuss the pattern of expression of genes encoding for proteins involved in RA metabolism in the ER+ and ER- breast cancer cells (Fig. 3). Essentially, there is a generalized down-regulation of genes encoding proteins involved in the production of various RA metabolites. Major differences are seen in RAR, RA binding proteins, and sulfotransferase enzymes (SULT) respectively. The key differences and similarities are discussed below:

a. The protein encoded by SCARB1 is a plasma membrane receptor for HDL (high density lipoprotein) cholesterol. SCARB1 protein mediates cholesterol transfer to and from HDL. This gene is down-regulated in both cell lines. Among the metabolic genes, there is retinol saturase, (*RETSAT*); the protein of this gene catalyzes the conversion of all-trans retinol to all-trans-13, 14-dihydroretinol, an intermediate of 13, 14-dehydroRA. All-trans-13, 14-dihydroretinol binds to the RARs in the nucleus and it is selectively up-regulated in MCF-7, but is absent in MDA-MB-231 cells. Also, in the ER+ MCF-7 we see a selective up-regulation of CYP2E1, a RA degradation enzyme, which is highly up-regulated in aggressive breast tumors as discussed above. The first step of RAR activation is mediated by the delivery of RA from the cytosol to the receptor in the nucleus, a step which involves the cellular retinoic acid-binding protein 2 (*CRABP2*); this gene is highly up-regulated in MCF-7 cells (Schug *et al.*, 2008).
b. In MCF-7 cells the expression of genes encoding for SULT enzymes, *SULT2A1* and *B1*, are highly up-regulated. These enzymes catalyze the sulfate conjugation of many hormones, neurotransmitters, drugs, and xenobiotic compounds. In particular, the enzyme SULT2B1 sulfates dehydroepiandrosterone, but not 4-nitrophenol, a typical substrate for the phenol and estrogen sulfotransferase subfamilies. MDA-MB-231 cells did not show expression of these genes. In support of this, we found that the GO term molecular functions are associated with sulfotransferase activity within the RA network in E2 stimulated MCF-7 cells; genes of the RA network are associated with biological processes such as the cell cycle and its regulation, protein metabolic processes amino acid methylation and alkylation and sulphur compound biosynthetic process respectively.
c. *RARa/RXRa*-these RARs are selectively up-regulated in MCF-7, but are low in expression and absent in MDA-MB-231 cells. This selective expression can be correlated to the fact that RARa protein is involved with functions such as apoptosis and differentiation and together as hetero/homodimers mediate the biological effects by RA-mediated gene activation.
d. *RARb/RXRb-RARb* binds to RA and mediates cellular signaling in embryonic morphogenesis, cell growth and differentiation. This protein has antiproliferative effects in many cell types. This gene is down-regulated in both cell types; however, its heterodimeric counterpart, RXRb gene lies within the MHC class II region on chromosome 6a, is selectively up-regulated in MDA-MB-231 cells. The significance of this discrepancy is unknown, but may be a feature of de-regulated immune responses present in ER- breast cancer cells (Mandal *et al.*, 2007b).
   RARg/RXRG-these nuclear hormone receptors are ligand-dependent transcriptional regulators. RXRg, the counterpart of RARg, mediates the antiproliferative effects of RA. RXRg is expressed at significantly lower levels in non-small cell lung cancer cells. This receptor pair is down-regulated in MDA-MB-231 cells, but RARg is selectively up-regulated in MCF-7 cells. (Information source of individual genes: www.ncbi.nlm.nih.gov)

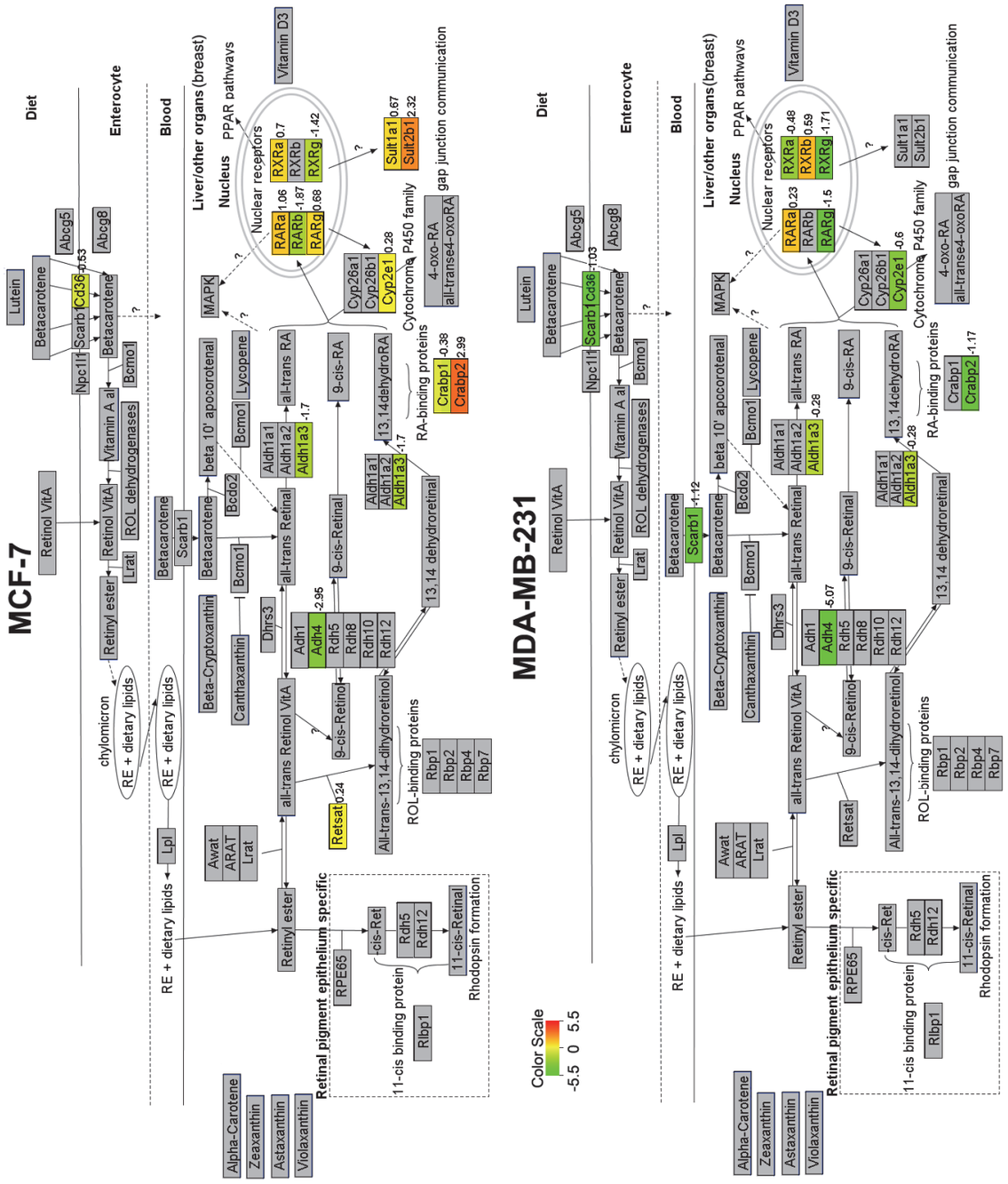


Fig. 3. A comparative view of retinoic acid metabolic pathways operating in MCF-7 and MDA-MB-231 cells is shown. The pathway maps were generated using the pathway visualization tool, PathVisio (Version 2). The software generated a color scale for the visualization of expression (red is up-regulation and green is down-regulation of gene expression). Expression values (SLR, signal log ratio) with a cutoff value of ≥ 0.2 or ≤- 0.2 was used and in case of duplicate SLR values, an average SLR was taken. Abbreviations used: ROL=retinol; RAL=retinaldehyde; RA=retinoic acid; RE=retinyl ester; RPE=retinal pigment epithelium.

5. The role of mitogen and stress activated kinase (MSK) in breast cancer

Mitogen and stress activated kinases 1 and 2 (MSK1/RPS6KA5, MSK2/RPS6KA4) are serine-threonine kinases that are activated by the p38 or RAS-MAPK signaling pathway. In breast cancer, there is overexpression of the growth factor receptors EGFR/HER1 and HER2/neu; in 25-30% of breast tumors HER2 is overexpressed and 45% of breast tumors test positive for EGFR (Klijn *et al.*, 1992; Slamon *et al.*, 1989). The overexpression of these receptors leads to the constitutive activation of the RAS-MAPK signaling pathway and the subsequent phosphorylation and activation of MSK1 and MSK2. Once activated, MSK1 and 2 can act on several substrates that are relevant to cancer signaling pathways. Importantly, MSKs can phosphorylate the serine residues S10 and S28 on the N-terminal tail of the histone protein H3 (Soloaga *et al.*, 2003). MSK1/2 mediated H3 modification is an event that can lead to chromatin remodeling and the expression of immediate-early (IE) genes such as *FOS*, *COX2*, and *JUN*, which are often up-regulated during oncogenesis (Drobic *et al.*, 2010; Dunn *et al.*, 2005). MSK proteins can also phosphorylate and activate various transcription factors such as CREB, ATF1, ER81, and the p65 subunit of NFκB, which activate the transcription of genes involved in tumorigenic and metastatic progression (Arthur and Cohen, 2000; Vermeulen *et al.*, 2003; Wiggin *et al.*, 2002).

The role of MSKs in cancer progression spans from their contribution to the process of anchorage-independent growth to a potential role in epithelial mesenchymal transition (EMT). MSK has been implied in the anchorage independent growth of mouse epidermal cells, *H-ras* transformed mouse fibroblasts and v-Src transformed mouse Balb3T3 cells (Kim *et al.*, 2008; Perez-Cadahia *et al.*, 2011; Tange *et al.*, 2009). Inhibition of MSK by the small molecule inhibitor H89 implicates MSK in the EMT process in *H-RAS* overexpressing Caco-H human colon cancer cells (Pelaez *et al.*, 2010). In the ER+ breast cancer cell line MCF-7, stimulation with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) causes the activation of the RAS-MAPK pathway and hence H3S10 phosphorylation and the induction of the E2 response gene, *TFF1* (trefoil factor 1), which has been implicated in breast cancer progression (Espino *et al.*, 2006). Interestingly, MSK activation by TPA in MCF-7 cells induces H3S10 phosphorylation but not H3S28 phosphorylation by an unknown mechanism (Espino *et al.*, 2006). However, a recent study has called into question the value of using *TFF1* as a prognostic marker in breast cancer, as it demonstrates that knockdown of *TFF1* in MCF-7 cells leads to enhanced colony formation in soft agar, and that *TFF1* knock-out mice have a higher tumor incidence in mammary glands (Buache *et al.*, 2011).

In the ER- MDA-MB-231 and SKBR-3 cells, MSK is implicated in the expression of the pro-inflammatory cytokine IL-6, whereby IL-6 is constitutively expressed and MSK is recruited to the IL-6 promoter following TNF-α signaling. The MSK-mediated expression of IL-6 involves MSK-mediated phosphorylation of NFκBp65^{S276}, recruitment of MSK and phosphorylation of H3S10 on the IL-6 promoter, followed by subsequent chromatin remodeling of the promoter via the recruitment of the BRG1 subunit of the SWI/SNF chromatin remodeling complex (Ndlovu *et al.*, 2009). Similarly, MSK mediated H3S10 phosphorylation can induce the chromatin remodeling of the mouse mammary tumor virus (MMTV) promoter in MTVL breast cancer cells that have a stably integrated luciferase reporter gene driven by the MMTV promoter (Vicent *et al.*, 2008). In this system, progestin treatment leads to the formation of a ternary complex comprised of the PR and phosphorylated ERK (pERK) and MSK1 (pMSK1). The PR/pERK/pMSK1 complex is recruited to the MMTV promoter followed by phosphorylation of H3S10 by pMSK, leading

to the displacement of the HP1γ repressor complex and the subsequent activation of gene transcription in these cells (Vicent *et al*., 2008; Vicent *et al*., 2009). In the ER- cells (eg., MDA-MB-231) MSK has been implicated in the migration and invasion of cells to lymphatic endothelial cells *in vitro* as well as in *in vivo* mouse xenograft model through the possible NFκB binding to the chemokine receptor CCR7 (Pan *et al*., 2009). MSK has also been implicated in cancer-related inflammation through *COX2* expression. It has been observed that MSK-mediated *COX2* expression in mouse epidermal cells induces neoplastic transformation (Yan *et al*., 2006). MSK recruitment and H3S10 and H3S28 phosphorylation occur at the upstream regulatory promoter region of *COX2* in mouse fibroblasts in addition to the recruitment of chromatin remodeling factors and transcriptional activation histone marks at these sites (Drobic *et al*., 2010). In MCF-7, TPA selectively induces *COX2* expression (Degner *et al*., 2006). From these data it can be speculated that MSK may be involved in *COX2* expression/regulation in breast cancer cells, although this has yet to be experimentally reported. MSKs contribute to breast cancer progression through a variety of mechanisms including the induction of genes involved in transformation or inflammation, and priming gene promoters for chromatin remodeling and activation.

5.1 A comparative analysis of the MAPK pathway in ER+ and ER- breast cancer

As discussed in the previous paragraphs, genes of the MAPK pathway have profound effects on the progression of breast cancer. An overall comparison between MCF-7 and MDA-MB-231 shows that all the caspases are either absent or down-regulated in both cell lines, *EGFR* is up-regulated in the MDA-MB-231. cells, and the early responsive genes, *FOS* and *JUN* are oppositely expressed, and the p50/p105 (NFκB1) subunit of NFκB is up-regulated. *BDNF* (brain-derived neurotrophic factor) is highly expressed in MDA-MB-231 cells, but is totally absent in MCF-7 cells. The high expression of *BDNF* is of speculative importance because the gene encoding for this protein of the nerve growth factor family is responsible for the survival of striatal neurons and also responds to stress. Different members of MAP kinases are differntially up/down-regulated in the two cell lines, which may be a reflection of aggressiveness. The key differences and similarities are discussed below (Fig. 4):

a. *DUSP*- dual specificity protein phosphatase enzymes play important roles in regulating cellular response to environmental stress are also negative regulators of cell proliferation. They negatively regulate members of the mitogen-activated protein (MAP) kinase superfamily, which are associated with cellular proliferation and differentiation. Different members of this family of phosphatases have distinct substrate specificities for various MAP kinases, different tissue distribution and subcellular localization, and different modes of inducibility of their expression by extracellular stimuli. Interestingly, genes encoding for these phosphatases are down-regulated in MCF-7 and are up-regulated in MDA-MB-231 cells.
b. *PRKC (B, Z, H, G, D)*-Protein kinase C (PKC) is a family of serine- and threonine-specific protein kinases that can be activated by calcium and second messenger DAG (diacylglycerol). PKC family members phosphorylate a wide variety of protein targets and participate in diverse cellular signaling pathways. PKCs are major receptors for phorbol esters; in the comparative gene analysis, we find that most of the PKCs are down-regulated or absent with the exception of a few, but we have shown that the ER+ MCF-7 cells activate the MAPK pathway in response to TPA (Espino *et al*., 2006). (Information source of individual genes: www.ncbi.nlm.nih.gov)

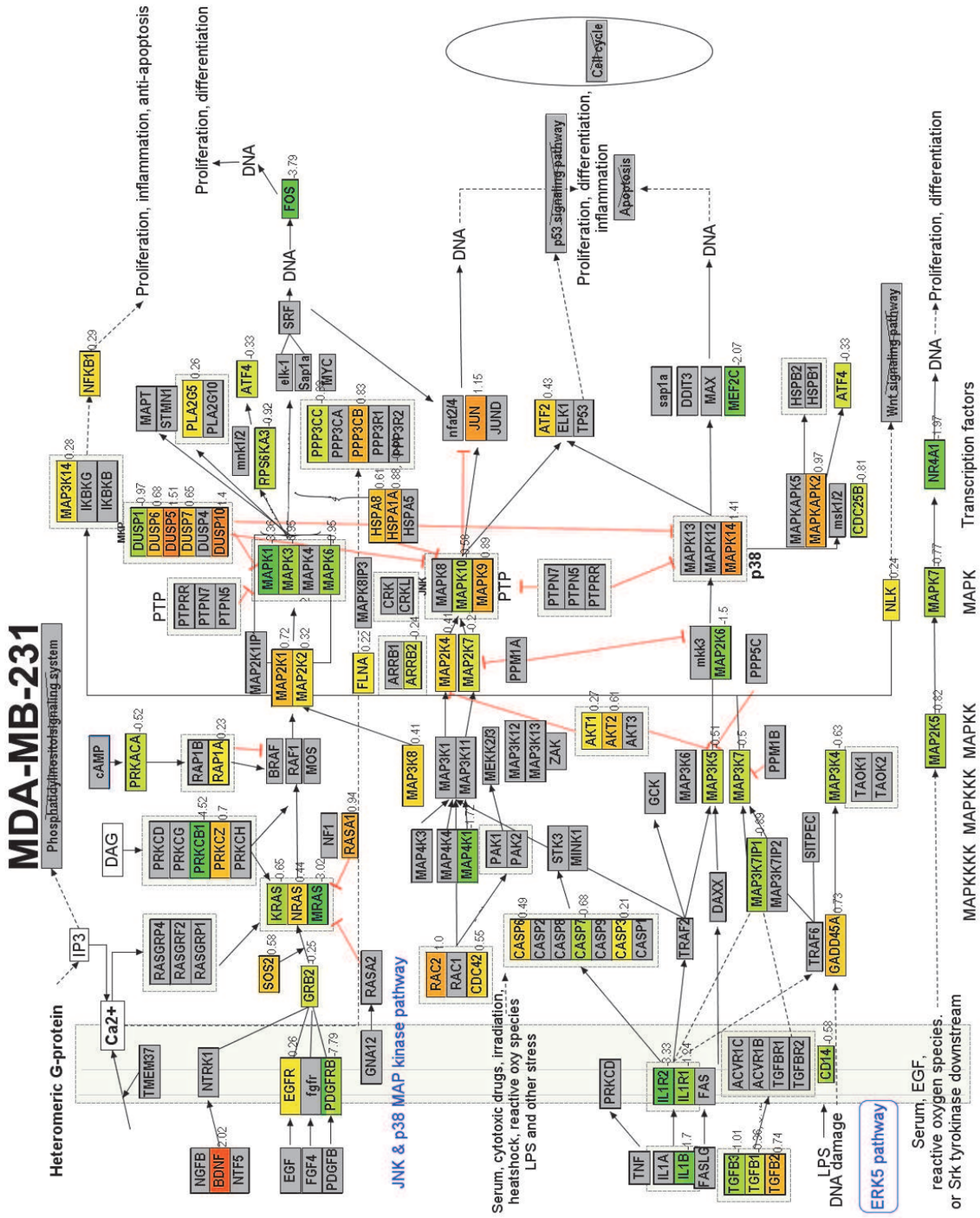


Fig. 4. (Continued)

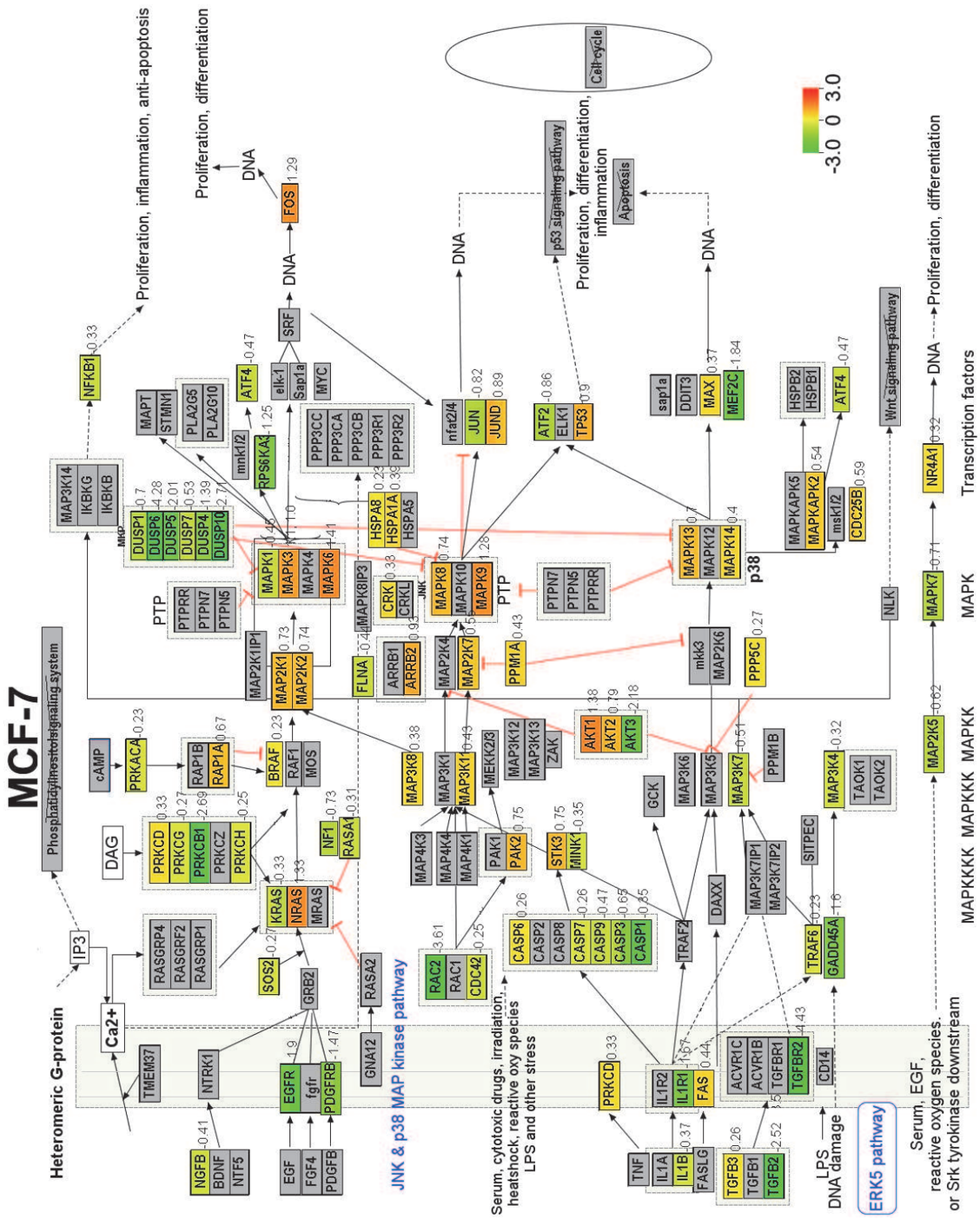


Fig. 4. A comparative view of the MAPK pathway operating in MCF-7 and MDA-MB-231 cells is shown. The pathway maps were generated using the pathway visualization tool, PathVisio (Version 2). The software generated a color scale for the visualization of expression (red is up-regulation and green is down-regulation of gene expression). Expression values (SLR, signal log ratio) with a cutoff value of ≥ 0.2 or ≤- 0.2 was used and in case of duplicate values, an average SLR was taken.

6. Role of transcription factors, Sp1 and Sp3 in breast cancer

Sp1 and Sp3 proteins are overexpressed in breast tumors and contribute to the proliferative and angiogenic phenotype, characteristics which correlate to poor prognosis (Li *et al.*, 2004; Mertens-Talcott *et al.*, 2007). There are an estimated 12000 Sp1 and Sp3 binding sites in human genome (Cawley *et al.*, 2004). Inhibition of Sp1 binding to DNA by mithramycin A or knocking down Sp1 to the normal expression level, decreased tumor formation, growth and metastasis (Lou *et al.*, 2005; Yuan *et al.*, 2007). Likewise, antiproliferative agents such as, betulinic acid and curcumin reduced the expression of Sp1 and Sp3 and their target genes (*EGFR, CCND1, VEGF, SREBF2, CD151*), which have roles in metastasis (Chadalapaka *et al.*, 2010; Kang and Chen, 2009). Knocking out Sp1/Sp3 was lethal and caused the death of knockout mice at different developmental stages. Compounded Sp1 and Sp3 knockout mice were not viable suggesting that both these transcription factors are required to maintain appropriate gene expression programs (Bouwman *et al.*, 2000; Kruger *et al.*, 2007; Marin *et al.*, 1997; Van Loo *et al.*, 2003). This agrees with the fact that Sp1 and Sp3 are autoregulated genes with Sp1 and Sp3 binding sites in their proximal promoter regions (Nicolas *et al.*, 2001).

In the estrogen and breast cancer context, it is known that many estrogen responsive genes have Sp1/3(s) sites in the estrogen response elements of their promoters (Castro-Rivera *et al.*, 2001; Higgins *et al.*, 2006; Mandal and Davie, 2010; Sun *et al.*, 2002; Sun *et al.*, 2005). *TFF1* is a highly expressed estrogen responsive gene in malignant breast epithelial cells but not normal mammary cells (Rio *et al.*, 1987). In our studies, we predicted and validated a Sp1/3 site 6 bp upstream of the imperfect ERE site and the dynamic association of ERα, Sp protein, KATs and HDACs on the *TFF1* proximal promoter in response to E2 using TFSEARCH, ChIP (chromatin immunoprecipitation) assay and promoter analysis. We found Sp3, but not Sp1, to be the major Sp protein activating the *TFF1* upstream promoter region in response to E2 (Sun *et al.*, 2005). This was supported by other studies where it was shown that Sp3 plays a major role in ERα/Sp-mediated gene expression in MCF-7 cells (Khan *et al.*, 2007). In general, Sp1 usually activates transcription and Sp3 represses or most weakly activates transcription (Higgins *et al.*, 2006; Jaiswal *et al.*, 2006; Noe *et al.*, 2001). Though in some studies it was shown that Sp1 and Sp3 synergistically enhanced gene promoter function, we have shown that Sp1 and Sp3 did not exist in the same protein complexes in the *TFF1* promoter in MCF-7 cells (Hantusch *et al.*, 2007; He *et al.*, 2005; Lee *et al.*, 2009; Li and Davie, 2008).

6.1 Regulation of transcriptional responses of Sp1 and Sp3

Sp1 and Sp3 have multiple isoforms, of which Sp1 has two and Sp3 has four isoforms (Li and Davie, 2010). Four Sp3 isoforms have been reported. The two long forms of Sp3 and Sp1 contain two trans-activation domains A and B, one serine /threonine rich domain for post-translational modifications (PTMs) and the DNA binding domain C. Sp1 has domain D at the C-terminus which is critical for its synergistic activation. As the Sp family signature, the DNA binding domain C features the three Cys2His2 zinc 'fingers' required for sequence-specific DNA association. The consensus Sp1 and Sp3 binding DNA sequence is GGGGCGGGG. Although recognizing the same DNA binding sequence, Sp1 and Sp3 differ structurally in the location of their inhibitory domains; it is at the N-terminus for Sp1 and for Sp3 this domain lies immediately in front of the DNA binding domain (Suske, 1999).

PTMs such as sumoylation and acetylation of Sp1 and Sp3 regulate their functional roles (Li *et al.*, 2004; Li and Davie, 2010). Sumoylated Sp1 was deficient in proteolytic processing; it

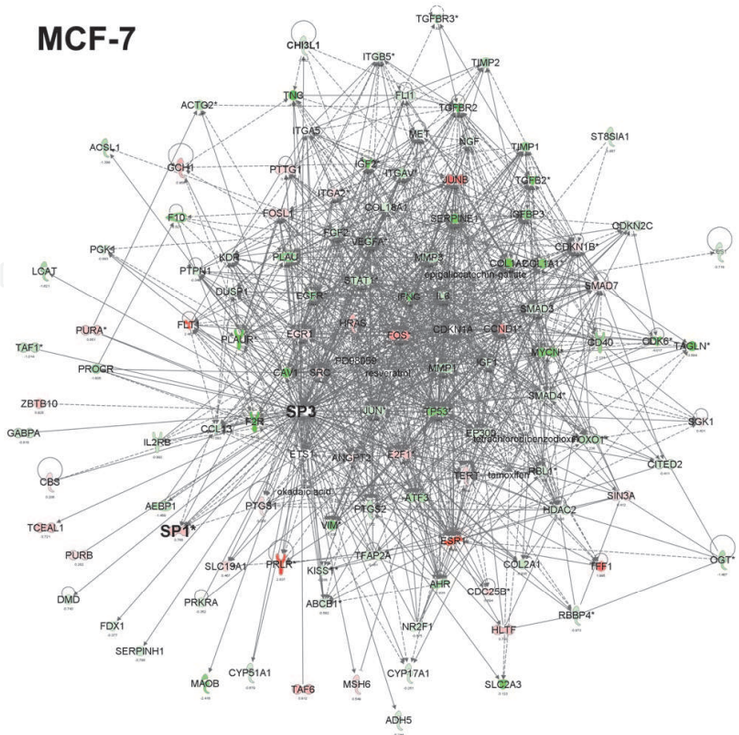
was proposed that Sp1 sumoylation preserves the integrity of a negative regulatory domain and inhibits the Sp1-dependent transcription (Spengler and Brattain, 2006). In another study however, sumoylation was found to aid in Sp1 interaction with the proteosome (Wang *et al.*, 2008). The major sumoylation of Sp3 long and short forms occurs at K551, which is a single lysine in its inhibitory domain and a minor sumoylation site (K120) is in the long form. The K551 modification silenced or significantly decreased Sp3 activity and mutation of this site converted the short Sp3 isoforms into potent transactivators of the SRC 1A gene promoter, but did not affect the transcriptional properties of Sp3 long forms (Ellis *et al.*, 2006; Ross *et al.*, 2002; Sapetschnig *et al.*, 2002; Spengler *et al.*, 2008). Interestingly, acetylation of K551 by KAT p300 converts Sp3 to a transcriptional activator; thus the activity of Sp3 can be repressing or activating depending on PTM of K551 (Ammanamanchi *et al.*, 2003). Sp1 can also be acetylated by p300, but the exact role of acetylation is unclear (Song *et al.*, 2003). Another PTM is methylation within or around Sp1/3 site(s), which regulates Sp1 and Sp3 association with DNA to regulate gene expression (Mudduluru and Allgayer, 2008). Whereas methylation within the consensus Sp1/3 site did not influence binding of Sp1/Sp3 to the $p21^{Waf1/Cip1}$ promoter, this modification outside the GC boxes reduced Sp1/Sp3 binding to $p21^{Waf1/Cip1}$ and mouse *Abcc6* promoter with repression of gene expression (Zhu *et al.*, 2003). Thus these Sp1/Sp3 PTMs likely influence the functional roles of these transcription factors for gene activation and repression.

6.2 A comparative analysis of Sp1-Sp3 interactions in ER+ and ER- breast cancer – The Sp1 and Sp3 target genes

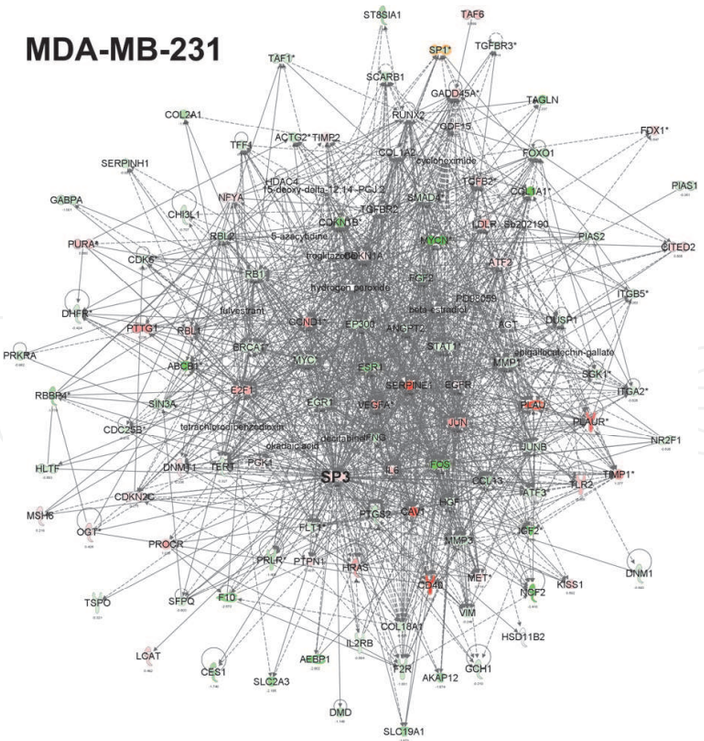
Sp1 and Sp3 participate in the regulation of tissue-specific, viral, and inducible genes (Davie *et al.*, 2008; Lu and Archer, 2010). Genes include regulators of cell cycle progression and arrest (eg. cyclins), pro- and anti-angiogenic factors involved in invasion and metastasis, pro- and anti-apoptotic factors involved in genomic stability, proto-oncogenes (eg. *MYC*), tumor suppressors (eg. *p53*), enhancers of cell proliferation and oncogenesis (Abdelrahim *et al.*, 2002; DesJardins and Hay, 1993; Feng *et al.*, 2000; Kavurma *et al.*, 2001; Lagger *et al.*, 2003; Olofsson *et al.*, 2007; Yuan *et al.*, 2007). Analysis by Ingenuity Pathway Analysis (IPA) tool showed that in MCF-7 and MDA-MB-231 cells, Sp1 and Sp3 share a network of interconnected genes inclusive of all categories discussed above (Fig. 5A, 5B). Fig. 5C is a snapshot cluster view of the Sp1-Sp3 gene network. Comparison showed that in the ER+ MCF-7 cells Sp1 was up-regulated and Sp3 was expressed at lower levels, while Sp3 was highly expressed in the MDA-MB-231 cells. In case of the $p21^{Waf1/Cip1}$ promoter, we also observed a lower Sp3 association in response to estrogen stimulation in MCF-7 cells (Mandal and Davie, 2007). We demonstrated that both Sp1 and Sp3 associated with *TFF1*, but Sp3, not Sp1, plays a major role in the estrogen activated ERα/Sp-mediated gene expression in MCF-7 cells (Khan *et al.*, 2007; Li and Davie, 2008). Therefore, in the context of estrogen stimulation, Sp1 and Sp3 play dual roles in regulating gene expression.

7. Conclusions

The evidence presented here show that metabolic patterns, transcriptional responses and gene expression in pathways in ER+ cells considerably differ from the ER- breast cancer counterpart. Generally, gene expression of energy metabolic pathways is low to absent for the more metastatic ER- cancer cells. Estrogen is a major mitogen which drives metabolic processes related to glucose and fatty acid metabolism and the hormone is also responsible



5A



5B

Fig. 5. (Continued)

5C

Fig. 5. A network of genes connected to the transcription factors Sp1 and Sp3 in (A) in the ER+ MCF-7 and the (B) ER- MDA-MB-231 cells. This network was generated using IPA (Ingenuity Pathway Analysis) tool. Red indicates positive and green indicates negative expression. (C) A cluster view of the genes in the network (Cluster Tree view; red is positive, green is negative, grey is total absence of expression); cluster analysis was done as described previously (Eisen *et al.*, 1998). Expression values (SLR, signal log ratio) with a cutoff value of ≥ 0.2 or ≤- 0.2 was used and in case of duplicate values, an average SLR was taken.

for the Sp1 and Sp3 transcriptionally driven expression of many estrogen responsive genes. The functional role of Sp1 and Sp3 in estrogen stimulated breast cancer cells is determined by the type of PTM, protein interaction, promoter context, collectively all of which contribute to the progression of the disease. The contribution of MSK to breast cancer progression is manifold. Importantly, MSK proteins can be inhibited by small molecules (e.g. H89) which reverse the epigenetic effects of MSK on gene transcription, and thereby make MSK an attractive target for cancer therapy. Moreover, it has been shown that treatment with MEK1/2 inhibitors such as PD98059, can cause radio-sensitization of MCF-7 cells in clonogenic survival assay. The MAPK pathway can also be proapoptotic in breast cancer cells. In the ER+ T47D cells, p38 kinase inhibitors (SB202190, SB203580, PD169316) abolished activin-mediated growth arrest, suggesting that this pathway can be utilized by a growth factor for growth inhibitory effects (Cocolakis *et al.*, 2001). Further studies are required to determine the mechanisms by which MEK1/2 inhibitors commit cells to killing via apoptotic and non-apoptotic mechanisms (Qiao *et al.*, 2002). On the other hand, targeting these major transcription factors is also a promising therapeutic approach. Most of the current studies have focused on Sp1 mRNA/protein expression and more studies on Sp3 isoforms are required to develop better Sp protein targeting cancer therapies. Several approaches to the development and delivery of metabolic drugs also hold promise to the treatment of hormone responsive tumors.

8. Acknowledgments

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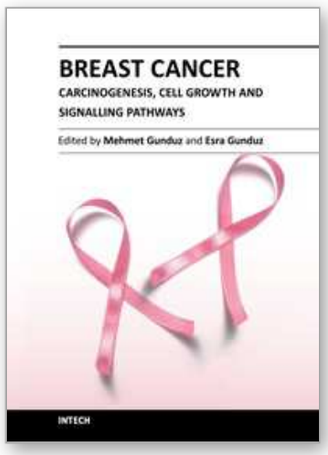

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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various aspects of breast cancer carcinogenesis from clinics to its hormone-based as well as genetic-based etiologies for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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