

# Inhibition of Dimethylbenz[a]anthracene-Induced Breast Tumors in Rats by Soy Protein is Mediated by Downregulation of MAPK/AP-1 Signaling

Shyamali Mukherjee <sup>2</sup>, Sutapa Mukhopadhyay <sup>1</sup> and Salil K. Das <sup>1\*</sup>

<sup>1</sup>Department of Biochemistry, Cancer Biology, Neuroscience & Pharmacology, Meharry Medical College, Nashville, TN 37208, USA.

<sup>2</sup>Department of Professional Education, Meharry Medical College, Nashville, TN 37208, USA.

\*Corresponding Author: Salil K. Das, Sc.D., D.Sc., Professor of Biochemistry Cancer Biology, Neuroscience & Pharmacology, Meharry Medical College, Nashville, TN 37208, USA. Tel: 615 327-6988; Fax: 615 327-6442.

Received date: **May 24, 2022**; Accepted date: **June 01, 2022**; Published date: **June 30, 2022**

**Citation:** Shyamali Mukherjee, Sutapa Mukhopadhyay and Salil K. Das. (2022). Inhibition of Dimethylbenz[a]anthracene-induced Breast Tumors in Rats by Soy Protein is Mediated by Downregulation of MAPK/AP-1 Signaling. *J Cancer Research and Cellular Therapeutics*, 6(4); DOI: 10.31579/2640-1053/122

**Copyright:** © 2022, Salil K. Das. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

## Abstract

We reported earlier that replacement of casein by soy protein in the diets of rats exposed to the carcinogen, dimethylbenz[a]anthracene (DMBA), not only delayed the initiation of breast tumor but also had a protective effect against the development of aggressive tumor. The aim of this study was to elucidate the molecular mechanism by which soy protein offers these beneficial effects. Tumor was developed by gavage administration of single dose of 80 mg/kg of DMBA into 50-day old female rats, maintained on a standard AIN-76A diet containing either casein or soy protein. After ~120 days of DMBA administration, we evaluated the role of MAPK phosphorylation and subsequent AP-1 activation on the differential effects of soy protein and casein on the development of aggressiveness and progression of DMBA-induced breast tumor and determined if soy protein controls MMP-9 and uPAR expression by modifying AP-1 activity. The present study demonstrates that the beneficial effect of soy protein in breast tumor development is mediated by control of MAPK/AP-1 signaling. It is concluded that deactivation of MAPK pathway lead to down-regulation of the AP-1 activation which in turn down regulates the target gene and may be responsible for controlling breast tumor aggressiveness. Thus, it is suggested that MAPKs (ERK, p38 and JNK), MMP-9 and uPAR may be a potential target for anticancer therapy inhibiting tumor vasculature and invasion stimulated by tumor-associated stroma, and regulating the target gene and may be responsible for the beneficial effects of soy protein.

**Keywords:** soy protein; casein; casein plus soy protein; breast cancer; MAPK and AP-1 signaling; proliferation; PCNA; Cyclin D1; uPAP; MMP-9

## Introduction

Breast cancer is the second most common cancer globally and one of the leading cancers in women in the world and its incidence gradually increases year by year according to the world Health Organization [1, 2]. Etiology of breast cancer is composed of many factors including age, genetic, reproductive, hormonal, environmental factors and lifestyle such as cigarette smoking, alcohol and diet [3-6]. Several recent reports indicate that soy proteins either as intact soy protein or functional or bioactive peptides derived from soybean processing have a variety of physiological functions such as immunomodulatory effects, hypolipidemic, anti-inflammatory, antioxidant, anti-hypertensive, antiangiogenesis, anti-cancer properties [7-12]. Observational studies show that among Asian women higher soy consumption is associated with an approximately 30% reduction in risk of the developing breast cancer. Furthermore, Clinical trials and epidemiologic studies involving over 11,000 women from USA and China show that post-diagnosis soy intake statistically significantly reduces recurrence and improves survival

[13-15]. Recent research also suggested that women who are at increased risk of breast cancer due to polymorphisms in genes associated with disease may especially benefit from high soy isoflavones intakes [16]. Furthermore, soy foods have long been recommended in the diets for breast cancer survivors and women at high risk for breast cancers [17]. Recent epidemiology evidence on the association of soy intake with breast cancer risk is still inconsistent due to different soy intake levels and or type of soy food such as whole soy, soy isoflavones, soy protein isolate [18-22].

Our previous study indicated that replacement of casein by soy protein in the diets of rats exposed to the carcinogen, dimethylbenz[a]anthracene (DMBA), not only delays the initiation of breast tumor development but also has a protective effect on the development of aggressive tumor [23]. While 100% of the breast tumor induced in rats fed soy protein was of grade I (non-aggressive) type, casein consumption produced more aggressive tumors (20% grade I, 60% grade II and 20% grade III). The mechanism by which soy protein

regulates the development of aggressiveness and progression of DMBA-induced breast tumors remains to be elucidated. However, our subsequent study also indicated that replacement of casein by soy protein in diet suppresses the expression of a tumor-promoting gene, translocator protein (TSPO) in breast tumors in rats [24].

The mitogen-activated protein kinase (MAPK) cascades are multi-functional signaling pathways that transduce signals from the cell membrane to the nucleus in response to variety of stimuli and participates in various intracellular signaling pathways that control a wide spectrum of cellular processes, including proliferation, growth, differentiation, development, inflammatory response and stress responses, and is known to have a key role in cancer progression. It is a critical pathway for human cancer cell survival, dissemination, and resistance to drug therapy [25]. Three MAP kinase (MAPKs) cascades, such as extracellular signal-regulating kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAP kinases have been characterized [26, 27]. MAPKs are proline-directed serine/threonine kinases that are activated by dual phosphorylation on threonine and serine residues in response to a wide variety of extracellular stimuli [28, 29]. They mediate signal transduction from the cell surface to the nucleus. The ERKs family consist mainly of a kinase domain (ERK1 and ERK2) and is activated by mitogenic stimuli such as growth factors, cytokines, and phorbol esters and plays a major role in regulating cell growth, survival, and differentiation. The JNK and p38 MAPK are weakly activated by growth factors but respond strongly to stress signals including environmental stress, tumor necrosis factor (TNF), interleukin-1, ionizing and ultraviolet radiation, hyperosmotic stress, and chemotherapeutic drugs and activation of this pathway is strongly associated with inflammation, apoptosis, cell differentiation and cell regulation. A number of studies have reported that the JNK signaling pathway acting via c-Jun or the related transcription factor ATF-2 is involved in the modulation of DNA repair and/or cell survival in response to various forms of genotoxic damage [30-32]. MAPKs regulate AP-1 both at the transcriptional and post-translational levels, affecting its transactivation potential, DNA binding capacity and stability. Several studies reported that MAPK signaling pathways were key upstream of activator protein-1 (AP-1) and activation of MAPK regulates the expression level of AP-1 through the substrate phosphorylation and by influence on the abundance of individual AP-1 components in cells [33]. AP-1 is an important regulatory protein involved in various physiological and pathological cellular processes including cell proliferation, growth, migration, differentiation, transformation, and apoptosis [34]. The AP-1 complex consists of homo- or hetero- dimers of Fos families (c-Fos, Fos B, Fra-1 and Fra-2), activating transcription factor families (ATF-1, ATF-2, B-ATF, JDP1 and JDP2) Jun subfamilies (c-Jun, Jun B and Jun D), and MAF (c-Maf, MafB, MafA and MafG/F/K) protein families that are characterized by highly conserved dimeric basic-region leucine-zipper (bZIP) DNA-binding sequences called 12-O-tetradecanoylphorbol-13 acetate (TPA) responsive elements (TRE, 5'-TGAG/CTCA-3' or cAMP response elements, CRE, 5'-TGACGTCA-3') [35]. The activity of this transcription-factor complex is modulated by growth factors, cytokines and tumor promoters [36]. The activated AP-1 dimer binds to specific DNA sequences in the regulatory regions of mitogen-responsive genes, several of which are involved in cellular processes such as proliferation or tumor invasion [37].

In breast cancer cells, the AP-1 proteins have been identified as important regulators of growth and invasion. As a member of the AP-1 transcription factor complex, c-Fos is an essential modulator of cell proliferation, differentiation, and transformation [38]. Depending on the cell type and environment, c-Fos can function either as a transcriptional activator or as a transcriptional repressor. According to Bland et al [39] increased c-Fos expression in breast cancer was associated with poor prognosis. Gee et. al [40] have observed a significant association between elevated Fos protein expression and increased proliferation. Furthermore,

Fra-1, Fos family member and an AP-1 transcription factor component is known to induce promoter activity of several target genes, such as MMP-1 and MMP-9 and involved in cell proliferation and invasiveness of breast cancer cells [41, 42].

Substantial experimental studies indicated that many dietary natural products could affect the development and progression of breast cancer, such as soy, pomegranate, mangosteen, citrus fruits, apple, grape, mango, cruciferous vegetables, ginger, garlic, black cumin, edible macrofungi, and cereals [43]. In general, fruits and vegetables contain various dietary substances such as vitamin, fiber, phytochemical and phenolic compounds like flavonoids and vanilloids, which act as anti-cancer agents [43]. Their anti-breast cancer effects involve various mechanisms of action, such as downregulating ER- $\alpha$  expression and activity, inhibiting proliferation, migration, metastasis and angiogenesis of breast tumor cells, inducing apoptosis and cell cycle arrest, and sensitizing breast tumor cells to radiotherapy and chemotherapy [43, 44].

Soybean is an excellent source of dietary phenolic substances in the Asian diet [45, 46]. The two major isoflavones in soybean are genistein and daidzein. It has been reported that growth inhibitory effect of genistein may be linked to suppression of angiogenesis [47, 48] and cell cycle progression as well as stimulation of apoptosis [49]. Genistein has been shown to attenuate the transcription of c-Fos mRNA in breast cancer cells [50, 51].

Extracellular matrix metalloproteases such as MMP-1, MMP-3, MMP-7, and MMP-9 and the fibrinolytic system such as uPA (urokinase-type plasminogen activator) are important protease systems interacting with each other in charge of remodeling and recycling of tissues and they play important role in tumor invasion and metastasis [39, 51, 52]. A recent study has confirmed the association between MMPs and tumor growth, invasion, metastasis and prognosis of breast cancer [53]. Urokinase plasminogen activator receptor (uPAR), also designated CD87, is a glycoprotein surface receptor specific for urokinase plasminogen activator (uPA). Active uPA is extracellular matrix-degrading protease that promotes tumor progression and metastasis. It has been reported that uPAR in serum and in urine of breast cancer patients was significantly higher than in healthy control [54, 55]. An association between a high expression of MMP-2 and reduced survival in breast cancer patients as well as an association of the tumor grade with increased levels of MMP-9 in breast cancer tissue was reported [56]. However, despite increasing knowledge about the physiological functions of AP-1, how upstream signaling pathways, mainly MAPKs, regulate the transcriptional activity and how the proteins of the Fos and Jun families give rise to AP-1 signaling pathway in DMBA-induced rat breast tumors and how differential effects are achieved between soy protein treated group and casein treated group on MAPK/AP-1 signaling pathway in DMBA-induced rat breast tumors are not fully elucidated. To our knowledge, currently there are no data available on the effect of casein and soy proteins on the level of PCNA, cyclin D1, MMP-9 and uPAR in DMBA-induced rat breast tumors. Therefore, our specific aim was to evaluate the role of MAPK phosphorylation and subsequent AP-1 activation on the differential effects of soy protein and casein on the development of aggressiveness and progression of DMBA-induced breast tumor and whether soy protein control MMP-9 and uPAR expression by modifying AP-1 activity in DMBA-induced breast tumor.

## Materials and Methods

### Development of breast tumor in female rats

Adult female Sprague Dawley rats were purchased at 22 days of age from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). They were housed individually in polycarbonate cages. The method was described in detail previously [24]. Briefly, animals were divided into three groups. Each group contained 10 animals. Animals in groups 1 and 2 were fed a

standard AIN-76A diet containing 20% casein as protein source and those in group 3 were fed with same diet replacing casein with 20% soy protein as protein source. The diets were prepared by Harlan Teklad (Madison, WI) [23, 24].

The rats were placed on the either casein (group 2) or soy protein (group 3) at 25 days of age and remained on the same diet for the rest of the study. Rats were allowed to feed and drink *ad libitum*. Mammary tumors were induced in rats of groups 2 and 3 by a single intragastric administration 80 mg/kg b.wt. of dimethylbenz[a]anthracene (DMBA; Sigma-Aldrich Chemical Co, St. Louise) in sesame oil at 50 days of age. Control animals (group 1) received the vehicle only by gavage. This group served as a control for group 2 and 3, which received DMBA. Since our previous study indicated that replacement of casein by soy protein had no effect on either morphology or biochemical characteristic in breast tissue of rats, which did not receive DMBA [23, 24]. Hence, we used casein protein in control group.

Animals were weighed and also palpated triweekly to detect tumors beginning four weeks after the administration of carcinogen. At almost 120 days post-administration of DMBA, animals were sacrificed by CO<sub>2</sub> asphyxiation. All tumors were weighed and measured for volume, and a section of the tumor was fixed in buffered formalin pH 7.5. Sections of the paraffin-embedded tumors were stained with hematoxylin (H) and eosin (E) for morphological analysis. The remaining tissues were stored at -80°C for biochemical studies. For biochemical studies, we took tissues from three different tumors (in case of casein group Grade I, II and III) and mixed them and designated them as one sample (n=5)

#### **Histological grading of mammary gland adenocarcinoma**

The slides were examined and scored as described earlier [23]. The final grade was determined by the total scored based on the tubule formation within the neoplasm, nuclear pleomorphism and mitotic count per 10 high power field (hpf). The tumor was scored 1-3 depending on the number of mitotic figures per 10hpf. Using these parameters mammary gland adenocarcinomas were graded I, II and III. Grade I was less aggressive and had the best prognosis and grade III was the most aggressive and had the worst prognosis [23].

#### **Immunohistochemical staining of Ki-67 for assessment of mitotic activity**

Formalin (10%) fixed sections were incubated with 0.3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity. The sections were washed and incubated with normal rabbit serum for 30 min to block non-specific binding. Afterwards, the sections were incubated with a polyclonal antibody for Ki-67 (diluted 1/50 in PBS, DAKO). The sections were washed and then incubated with biotinylated secondary antibody for 1 h. The sections were washed and incubated with HRP-Streptavidin for 1 h at room temperature and reacted with diaminobenzamide until reddish brown color developed. Sections were then counterstained with Gill's hematoxylin solution, and cover slipped. Ki-67 reactivity was evaluated by counting the number of positive and negative epithelial cell nuclei in six randomly selected fields in each section (n=5) [23].

#### **Assay of MAPK (Total/Phospho) multispecies assay by enzyme-linked immunosorbent assay (ELISA)**

The breast tissues were homogenized with cell extraction buffer (Biosource International, Inc., Camarillo, CA) and centrifuged for 10 minutes at 10,000 rpm. Protein concentration of supernatant was measured by Lowry method [57] and stored at -80°C for further analysis. The MAPK activity in the breast tissues was measured by ELISA kits (Biosource International, Inc., Camarillo, CA) following the protocol provided by manufacturer. The absorbency at 450 nm was performed by

a Benchmark Plus Microplate Spectrophotometer (Bio-Rad, Hercules, CA).

#### **AP-1 activation assay**

The activation of DNA binding activity of individual family member of AP-1 was quantified by ELISA using the AP-1 transcription factor family assay kit (Active Motif, Carlsbad, CA), according to the manufacturer's protocol. Briefly, the breast tissues were homogenized in a 5 volume of ice-cold tissue lysis buffer (Biosource International, Inc, Camarillo, CA) containing protease inhibitor cocktail (0.01%, Sigma – Aldrich Chemicals, St. Louis, MO) with 0.1 mM PMSF and incubated in 96-well plates to which oligonucleotide containing a TPA responsive element (TRE) was immobilized. AP-1 binding to the target oligonucleotide was detected by incubation with primary antibodies specific for c-Fos, Fos-B, Fra-1, Fra-2, p-c-Jun, JunB, JunD and followed by subsequent incubation with secondary anti-IgG horseradish peroxidase conjugate and visualized with developing solution and quantified at 450 nm with a reference wavelength of 655 nm by a Benchmark Plus Microplate Spectrophotometer (Bio-Rad, Hercules, CA) [58].

#### **Western blot Analysis**

Breast tissues were homogenized in a 5 volume of ice cold 210 mM mannitol, 70mM sucrose, 10 mM Tris-HCl, 1mM EGTA, 2mM CaCl<sub>2</sub> buffer, pH 7.2 containing protease inhibitor cocktail (0.01%, Sigma –Aldrich Chemicals, St. Louis, MO) by using a Brinkman Polytron (setting 6-7, 30 sec). Proteins were measured by Lowry method [57] and 50µg protein was separated by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred electrophoretically onto PVDF membranes Immobilon-P (Millipore, Bedford, MA). The membrane was immunoblotted with primary antibodies of c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, JunD, ATF-2, L-ZIP, cyclin D1 and proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology Inc., CA) primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibody. Binding of antibodies to the blots was detected with an ECL-detection system (Perkin Elmer, Boston, MA) following manufacturer's instructions. Stripped blots were re-probed with β-actin specific polyclonal antibodies (Santa Cruz Biotechnology Inc., CA) to enable normalization of signals between samples. Band intensities were analyzed using Bio-Rad Gel Doc (Bio-Rad, Hercules, CA). For MMP-9 and uPA analysis, we used secondary antibody with fluorescent using manufacture's protocol. Band detection and intensities were analyzed using an Odyssey (LI-COR Biosciences, NE) infrared fluorescent at 680 and 780nm [59].

#### **Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from 100 mg of breast tissue using a Qiagen RNAEASY kit (Qiagen, CA) according to manufacture protocol. The concentration and purity of RNA were analyzed using a UV spectrophotometer [59].

RT-PCR of c-Fos and c-Jun and GAPDH was performed using 5µg of total RNA from control, casein and soy sample, using one-step RT-PCR kit (Invitrogen, MD). The primer sequences were: 5'-GCCAGTGAGGAATATCTGGA-3' (forward) and 5'-ATCGCAGATGAAGCTCTGGT-3' (reverse) for c-Fos; 5'-TCGCAGATGAAGCTCTGGT-3' (forward) and 5'-GGCCATCTCTTGCTCGAAGTC-3' (reverse) for c-Jun and 5'-GGTATCGTGGAAGGACTCATGACC-3 (Forward) and 5'-TCGCTGTTGAAGTCACAGGACACA -3' (Reverse) for GAPDH). RT-PCR was performed in a thermal cycler (Biometra, TRIO gradient) as follows: 1 cycle for 30 min at 45°C, 1 cycle at 94°C for 2 min, 39 cycles for 1 min at 94°C, 1 min at 50°C, 2 min at 72°C and 1 cycle for 10 min at 72°C. The RT-PCR products were electrophoresed on a 1% agarose gel and band intensities were quantified using Bio-Rad Gel Doc (Bio-Rad,

Hercules, CA). Then bands are purified using QIA quick PCR purification kit (Qiagen, CA).

**Statistical analysis**

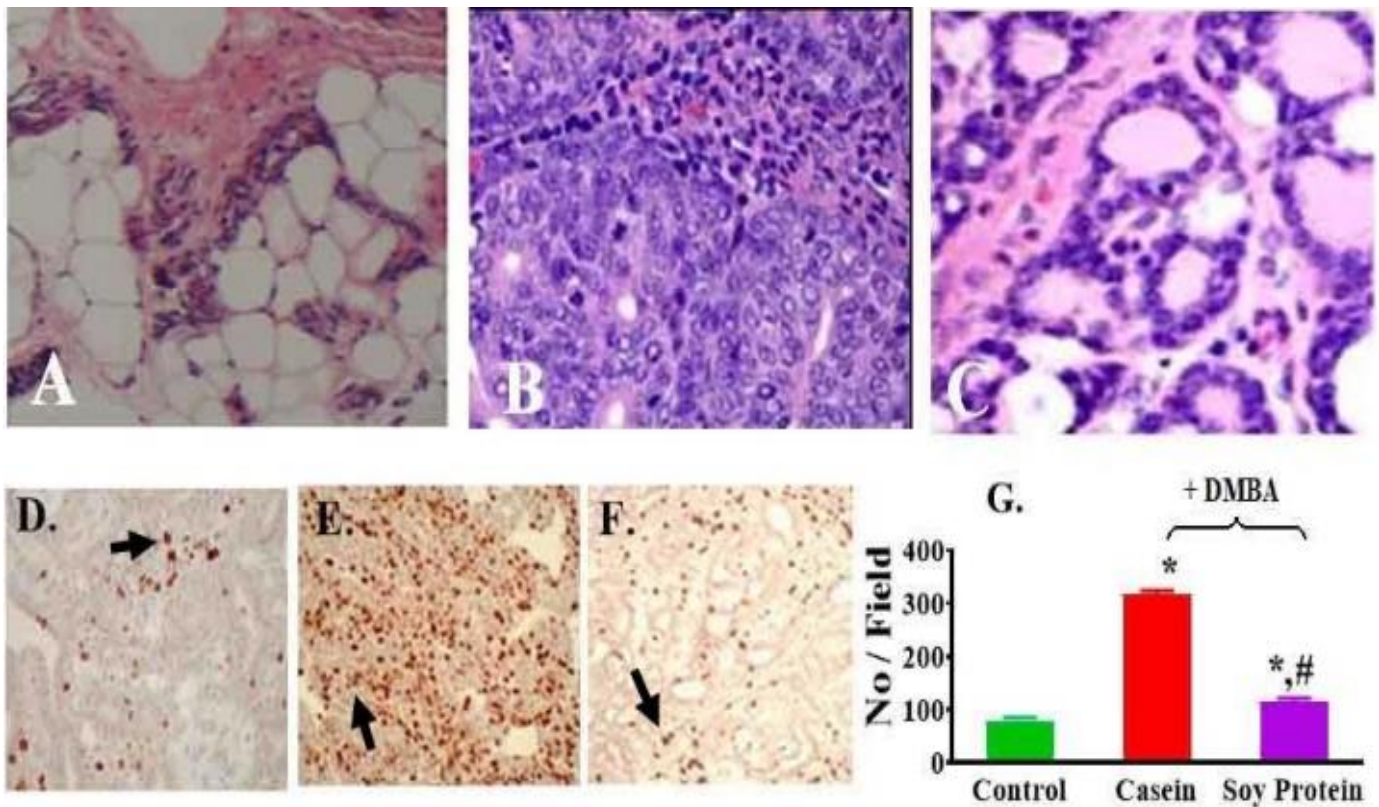
Data were expressed as means ± standard error of mean (S.E.M.). Statistical significance was determined by the student’s t-test or Dunnett’s test after one-way analysis of variance (ANOVA), using GraphPad prism Version 9.0 (GraphPad software, SanDiego, CA). Results were considered statistically significant at  $p < 0.05$ .

**Results**

Representative photograph of light microscopy from breast tissue of control animals regardless of whether they were fed casein protein is shown in Figure 1A-1C. It reveals scattered normal lobuloalveolar units lined by a layer of cuboidal epithelium and a discontinuous layer of flattened myoepithelial cells and terminal end buds in an adipose tissue stroma (H & E x 40). No tumor was noticed in any control animal. Figure 1B shows grade III modified Scarff-Bloom-Richardson (SBR) mammary duct carcinoma in casein group. This tumor is composed of sheets of infiltrating tumor without tubular elements comprising more than 75% of the tumor cells arranged in solid structures. The nuclei are crowded, enlarged, pleomorphic, with dense finely to coarsely granular chromatin, and visible nucleoli. More than 20 mitoses per 10 hpf at x 40 are present in this animal. Areas of tumor necrosis with polymorphonuclear neutrophil, lymphocyte, and plasma cell infiltrates are present.

Representative photograph of light microscopy from breast tumors found in the DMBA/soy protein group are shown in Figure 1C (H & E x 40). It reveals the tumors to be composed of cells arranged in isolated small round to oval elongated tubules lined by columnar epithelial cells with one or two layers of basal nuclei. The lining epithelial cells exhibit enlarged, crowded, uniform, hyperchromatic nuclei with evenly disbursed finely granular chromatin and indistinct nucleoli. The basal nuclei of the tubular epithelium show an absence of myoepithelial cells, indicating infiltrating neoplastic cells. The tubules vary in size and shape, are mostly isolated, widely spaced, and surrounded by a copious amount of fibrous connective tissue. Occasional tubules show little intervening stroma. The solid areas displayed in the lesion are less than 25% of the lesion. This lesion is comparable to a low-grade tumor, grade I modified Scarff-Bloom-Richardson mammary duct carcinoma.

The mitotic activity assessed in grading the tumors was verified by immunohistochemical reactions for Ki-67 antibody (Figure 1D -1F). Tumors from animals in the DMBA/casein group (Figure 1E) had more 3.0-fold of the cells staining positive with Ki-67 antibody than control (Figure 1D). However, tumors from the animals of the DMBA/soy protein group showed 1.4-fold increase of the cells staining with Ki-67 antibody (Figure 1F) than control group. Thus, proliferative index was significantly lower (2.8 fold) in the soy protein group than that in the casein group (Figure 1G).



**Figure 1:** Histological section (H and E staining; A-C) and Ki 67 labelling index (D-F) from breast of rats (A, D) fed either casein or soy without treatment with DMBA (B, E) fed casein and (C, F) fed soy and treated with DMBA and (G) the percentage of positively stained tumor cells among the total number of malignant cells assessed in 20 randomly selected fields in each section (N= 6 section/group; N=5 animals). X 40.

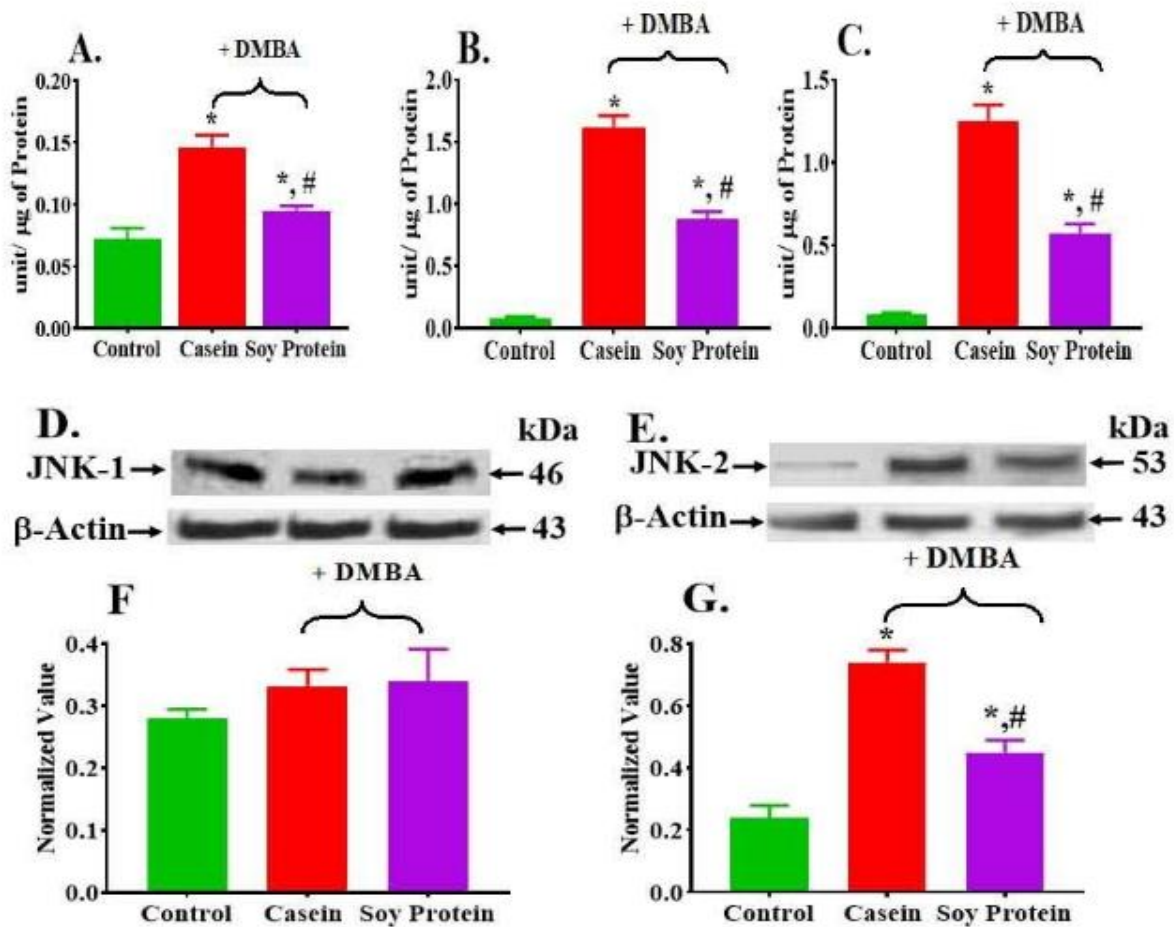
Tumor had higher activity of ERK1/2, p38 and JNK than control (Figure 2A-2C). However, tumors in the animals fed soy protein had significantly lower total levels and activities for all MAPKs than animals fed casein (Figure 2A-2C, Table 1).

Tumor Group	Normalized with respect to			
	ERK 1/2		P38	
	Ratio (Phospho/Total)	% Change	Ratio (Phospho/Total)	% Change
Casein	0.72	52.78↓	7.53	34.5↓
Soy protein	0.34		4.93	

**Table 1:** Effects of casein and soy protein on mitogenic activated kinase (MAPK) activity in breast with or without DMBA treatment

The activity of ERK1/2 increased 1.4-fold in casein than control, whereas it decreased 1.7-fold in soy group than casein (Figure 2A). Furthermore, the ratio of PhosphoERK1/2 vs total decreased 2.1-fold in tumor from soy group than casein group (Table 1). Figure 2B shows the total of p38 MAPK activity. Both total p38MAPK activity and ratio of phospho p38MAPK vs total p38 MAPK increased 1.9-fold and 7.5-fold in casein group than control, whereas it decreased 1.8-fold and 1.5-fold in soy group than casein respectively (Figure 2B, Table 1). The total JNK1/2 activity increased 14.7-fold and 6.7-fold in both casein and soy group than control (Figure 2C, Table 1) whereas both total and ratio of phospho JNK1/2 decreased in soy protein group 2.2-fold and 1.6-fold than casein respectively (Figure 2C, Table 1). Tumors of both casein

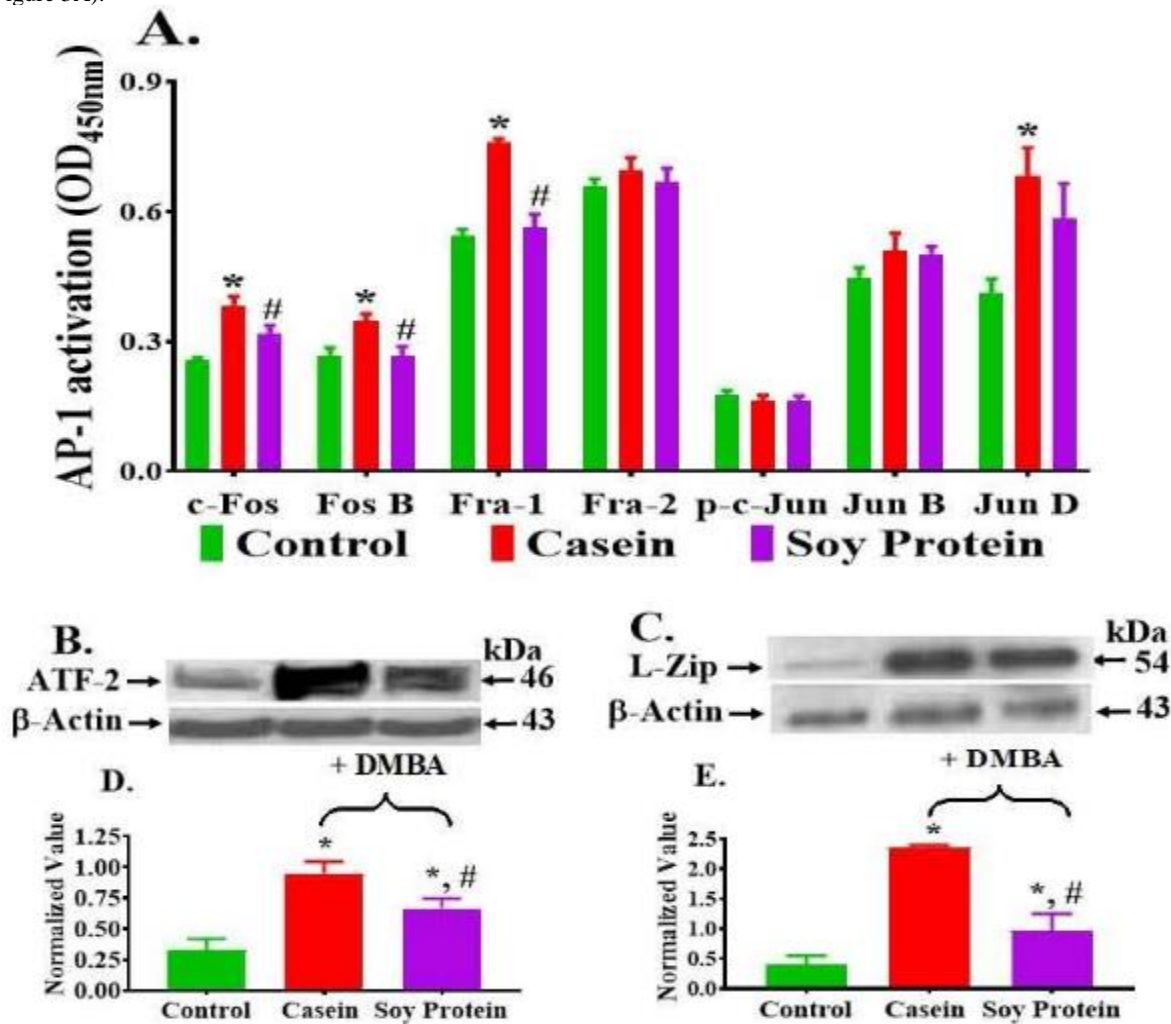
and soy groups had higher phospho ERK 1/2, phospho p38 and phospho JNK1/2 activity than corresponding control (Table 1). However, tumors in animals fed soy protein had significantly lower level of activities for all phospho MAPKs than animals fed casein (Table 1). Since we observed a significant increase in the activity of the JNK family in tumors, we measured the protein level of individual members of the JNK family (JNK-1 (D, F) and JNK-2 (E, G) in Figure 2). While no significant change in the protein level of JNK-1 (Figure 2F) was observed, a significant increase in JNK-2 (Figure 2G) was observed in the tumors of animals fed either casein (3.1 fold) or soy protein (1.9 fold) than control (Figure 2G). But this level was much elevated in the casein group than soy protein group (1.7 fold decreased than casein, Figure 2D -2G).



**Figure 2:** The effect of casein and soy protein on mitogen-activated protein kinase (MAPK) activity (A-C) and the level of JNK-1 (D) and JNK-2 (E) at Breast tissue. (A) ERK 1/2 activity, (B) p38 activity, (C) JNK 1/2 activity. Results are expressed as mean ± SEM (N= 5 /group). (F) Histograms summarizing western blot analysis data for JNK-1. (G) Histograms summarizing western blot analysis data for JNK-2. Results are expressed as Mean ± SEM; (N=5/group). \*p < 0.05 for DMBA exposed animals fed casein compared to normal and #p < 0.05 for DMBA-exposed animals fed soy protein vs DMBA-exposed animals fed casein.

According to Figure 3A, DNA binding activities of members of Fos family, such as c-Fos (1.5 fold), FosB (1.3fold), Fra-1 (1.4fold), Fra-2 (1.1 fold) and members of Jun family such as Jun B (1.2 fold) and JunD, (1.7fold) was significantly higher in tumors of animals fed casein than corresponding control (Figure 3A). However, this increased activation of c-Fos (1.2 fold), FosB (1.3 fold), Fra-1 (1.3 fold) and JunD (1.2 fold) in tumors was downregulated when casein was replaced by soy protein in diets, but c-Fos and JunD activity increased in tumor of soy group from the control (Figure 3A).

Since we observed higher activation of AP-1 transcription factor in casein group than soy protein group, we determined the protein levels of individual members of AP-1 family. We found a significant increase in ATF-2 (2.9 fold and 2.0-fold; Figure 3B, 3D) and L-ZIP (5.9 fold and 2.4-fold; Figure 3C, 3E) protein expression in the tumors of animals fed either casein or soy protein respectively. But this level was much elevated in tumors of casein group (1.4 fold and 2.5-fold) than soy protein group (Figure 3B-E).

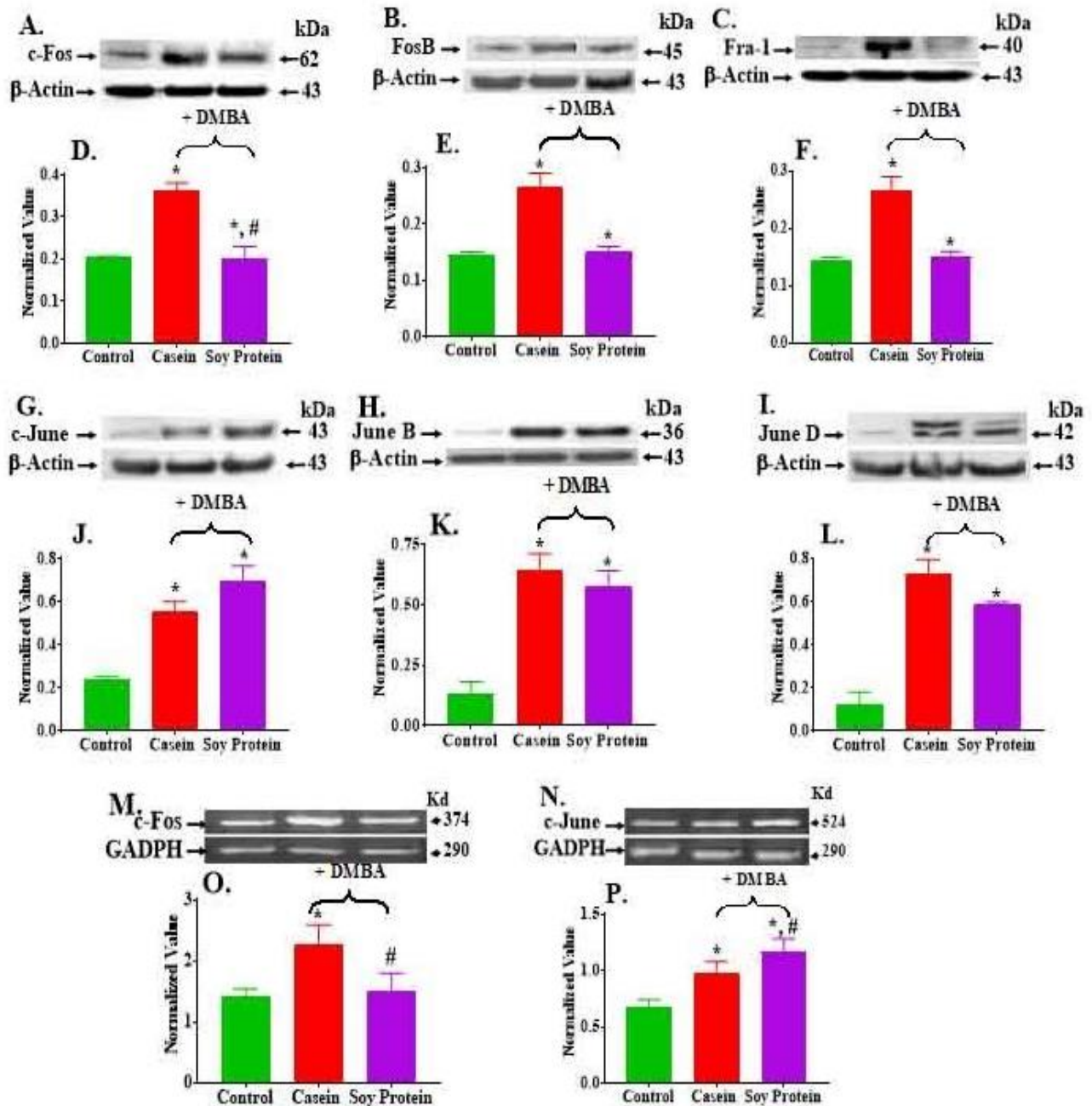


**Figure 3:** The effect of casein and soy protein on (A) AP-1 activation and level of (B) ATF-2, and (C) L-ZIP in control and DMBA-induced rat breast tumors (N = 5 in each group). (D) Histograms summarizing western blot analysis data for ATF-2 and. (E) Histograms summarizing western blot analysis data for LZIP. Results are expressed as Mean ± SEM. \*p < 0.05 for DMBA exposed animals fed casein compared to normal. (D) Histograms summarizing western blot analysis data for LZIP, \*p < 0.05 compared to normal, and #p < 0.05 for DMBA-exposed animals fed soy protein vs DMBA-exposed animals fed casein.

We also observed a significant increase in the protein levels of c-Fos (1.8 fold; Figure 4A, 4D), Fos-B (1.8 fold; Figure 4B, 4E) and Fra-1 (2.0 fold; Figure 4C, 4F) in tumors of casein group than control breast tissues. But this level was significantly decreased (1.8-fold, 1.7-fold and 2-fold respectively) when casein is replaced by soy protein (Figure 4A- 4F). No significant change was observed in Fra-2 protein expression between tumors and control breast tissues (data not shown). A significant increase in the protein levels of c-Jun (2.3 fold and 2.9-fold; Figure. 4G, 4J), Jun-B (4.9 fold and 4.4-fold; Figure. 4H, 4K) and Jun-D (5.9 fold and 4.9-fold; Figure 4I, 4L) was observed in both casein and soy group tumors respectively than control breast tissues (Figure 4G -4L).

However, no significant difference was observed between casein group and soy protein group (Figure 4G -4L).

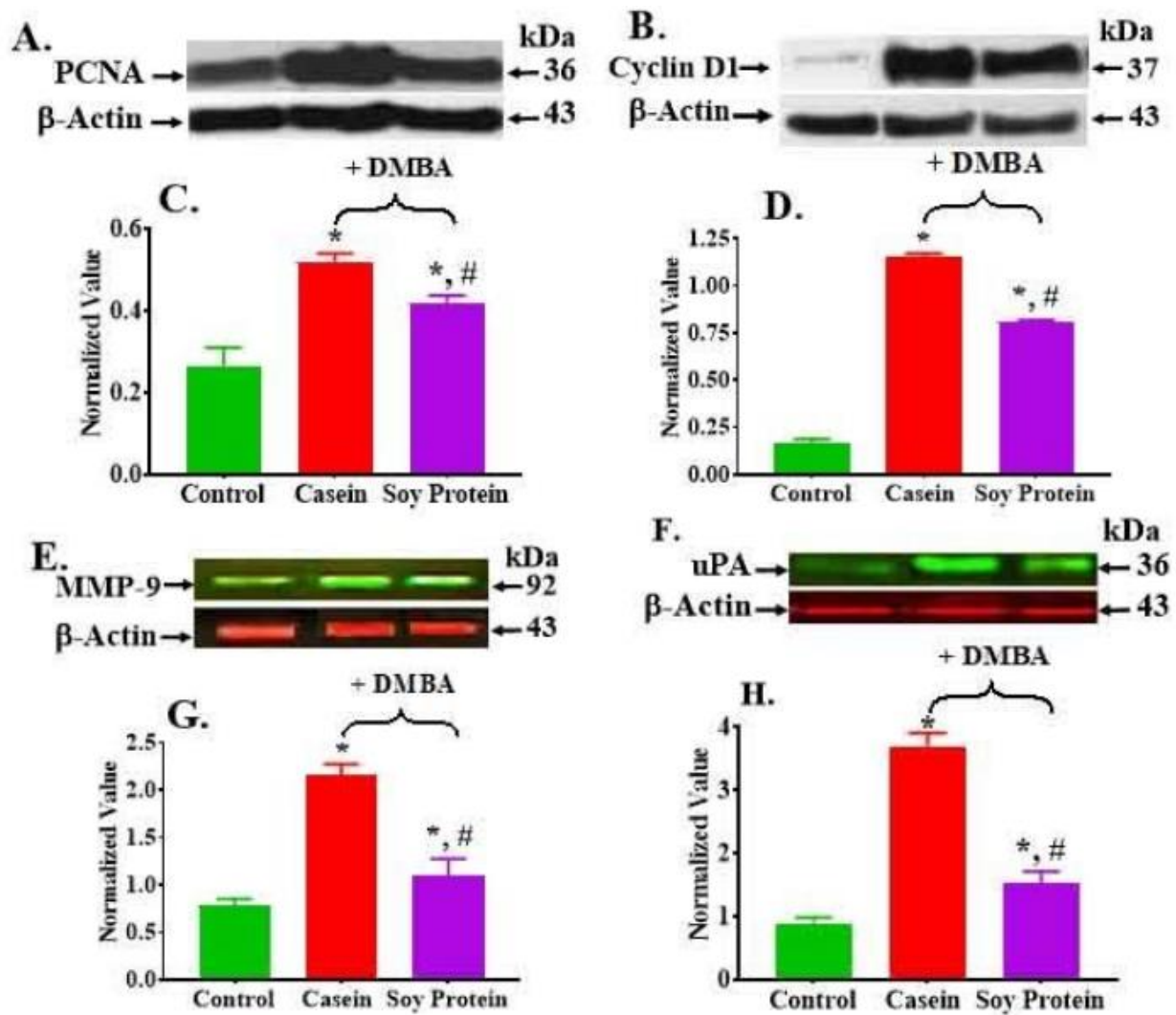
Since we observed significant variation of c-Fos and c-Jun level in tumor of both casein and soy protein groups, we measured the expression of both c-Fos and c-Jun in both tumors and control groups. The band densities obtained for c-Fos and c-Jun after normalization with that of GAPDH are shown in Figure 4M to 4P. We observed a significant increase (1.6 fold) increase in c-Fos gene expression in tumor of casein group than control (Figure 4M, 4N). c-Fos expression was decreased 1.5-fold in tumor when casein is replaced by soy protein (Figure 4M, 4O), whereas c-Jun expression was significantly higher in both casein (1.5 fold) and soy protein group (1.8 fold) respectively than control (Figure 4N, 4P).



**Figure 4:** The effect of casein and soy protein on the level of *c-Fos* (A), *Fos-B* (B) *Fra-1* (C), *c-Jun* (G), *Jun B* (H) and *JunD* (I) and PCR-analysis product of *c-Fos* (M) and *c-Jun* (N) in control and DMBA-induced rat breast tumors (N = 5 in each group). Histograms summarizing western blot analysis data for *c-Fos* (D); *Fos B* (E), *Fra-1*(F), *c-Jun* (J), *Jun B* (K), *Jun D* (L) and PCR analysis data of *c-Fos* (O) and *c-Jun* (P). Results are expressed as Mean ± SEM. \**p* < 0.05 for DMBA exposed animals fed casein compared to normal and #*p* < 0.05 for DMBA-exposed animals fed soy protein vs DMBA-exposed animals fed casein.

Emerging evidence suggests that PCNA is at the very heart of many essential cellular processes, such as DNA replication, repair of DNA damage, chromatin structure maintenance, chromosome segregation and cell-cycle progression. Cyclin D1 is an important regulator of the cell cycle progression and development of cancer. To further explore the involvement of AP-1 in cell proliferation and cell-cycle progression, we determined the protein levels of cell cycle proteins, PCNA (Figure 5A,

5C) and cyclin D1 (Figure 5B and 5D). The data obtained after normalization with β-actin reveal that the protein levels of both PCNA (2.0 fold) and cyclin D1 (7.0 fold) were significantly higher in casein tumors than control breast tissues (Figure 5A-5D). However, the levels PCNA (1.2 fold) and cyclin D1 (1.4 fold) were significantly lower in soy protein group than casein group (Figure 5A-5D).



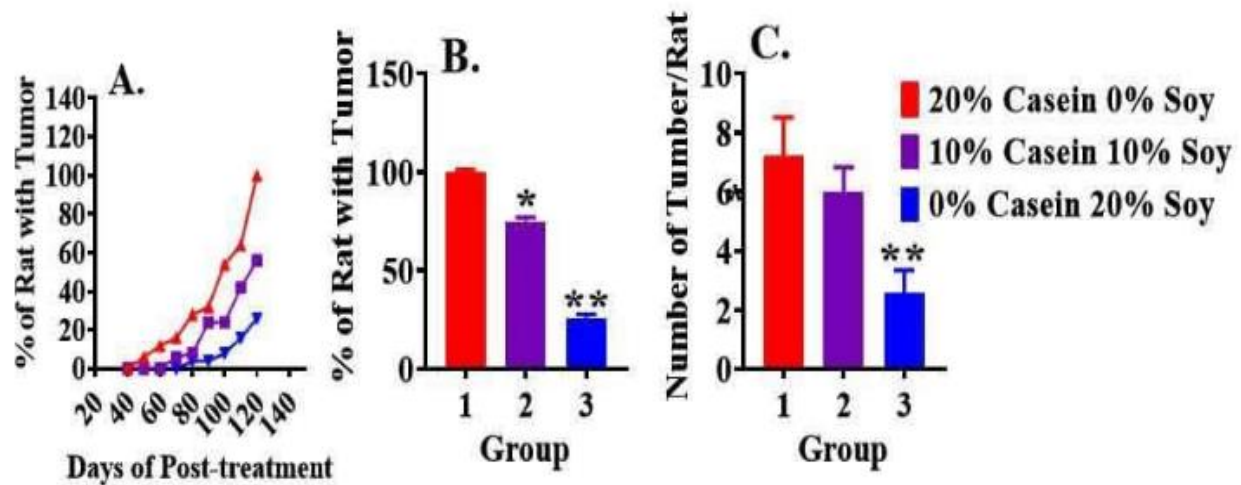
**Figure 5:** The level of PCNA (A), cyclin D1 (B), MMP-9 (E) and uPA (F) in control breast tissue and DMBA-induced breast tumors in rats fed either casein or soy as dietary protein ( $N = 5$  in each group). Histograms summarizing western blot analysis data for PCNA (C), cyclin D1 (D), MMP-9 (G) and uPA (H). Results are expressed as mean  $\pm$  SEM. \* $p < 0.05$  for DMBA exposed animals fed casein compared to normal and # $p < 0.05$  for DMBA-exposed animals fed soy protein vs DMBA-exposed animals fed casein.

Matrix metalloproteinases (MMPs) have been implicated in diverse roles in breast cancer development and progression. While many of the different MMPs expressed in breast cancer are produced by stromal cells MMP-9 is produced and AP-1 was the crucial transcriptional factor for expression of MMP-9 and uPA. Hence, we explore whether there is any differential effect between casein and soy protein on the level of MMP-9 (Figure 5E, 5G) and uPA (Figure 5F, 5H) in DMBA-induced breast tumor. Figure 5E-5H show that the level of MMP-9 and uPA were increased 2.7-fold and 4.2-fold respectively at casein group than control, but it decreased 2.0-fold and 2.4-fold respectively, when casein is replaced by soy protein (Figure 5E-5H).

Previously, we reported that total replacement of casein protein with soy protein causes not only causes delay initiation and progression but also decreased the number of breast tumor present in the rat [23]. To

further explore whether the combination of casein and soy protein has any effect on tumor incidence and total number of tumors in DMBA-induced rat breast. Our preliminary data indicated that 10% casein with 10% soy also causes gradual delay the time course of palpable tumor incidence (Figure 6A) than 20% casein only. Both number of rats with tumor (Figure 6B) and the number of tumors per rat (Figure 6C) decreased as soy protein replace casein. The initiation of tumor development was observed as follow 20% Casein (Group 1) <10% casein pulse 10% soy protein (Group 2) < 0% casein and 20% soy (Group 3, Figure 6A) after post-treatment with DMBA. After 120 days, we observed that 100% contain multiple tumors in group I and it decreased 25% when 10% casein replace with 10% soy in group 2 and 75% group 3 when we totally replace with soy protein (Figure 6B). Furthermore, we also observed significantly number of tumors per rat decreased 1.6-fold in group 2 and 6.3-fold in group 3 than group 1 (Figure 6C).





**Figure 6:** Effect of diet on breast A. time course of palpable tumor (initiation), B. number of rats with tumor and C. Total number of tumor/rats. Results are expressed as mean  $\pm$  SEM, N = 5. \*  $p < 0.05$ ; \*\*  $p < 0.001$ .

## Discussion

Breast cancer is the most common cancer among women in both developed and less-developed countries. According to GLOBOCAN, cancer is a major health problem worldwide. The number of cases increases every year and rates in countries with high consumption of soybeans and they are lower than those in the United States. Barak and Fridman [60] reported that the people who adhere to the Mediterranean Diet have lower incident of cancer. We previously reported that both soy protein and  $\alpha$ -lactalbumin, which is one of the active components of whey, has a beneficial effect on the initiation, progression and development of more aggressive breast cancer [23, 61]. The aim of this study was to not only validate our previous observation, but also elucidate the possible mechanism by which soy protein mediates its delay the initiation tumor and protective effect on the development of aggressive breast tumors. We observed that 100% of the tumors in DMBA/soy protein group was non-aggressive (Grade I) where as DMBA-casein group higher percentage of aggressive tumors (Grade I > Grade II > Grade III) of aggressive tumors as reported previously by us [23]. Based on recent a large cohort data analysis of a clinical cancer registry, it was demonstrated that Ki-67 is frequently determined in clinical study for the prediction of the risk of reoccurrence [62]. We observed that the number of Ki-67 positive staining intensities i.e., the proliferative activity of the tumor per field significantly increases in DMBA/casein group where as it was significantly reduced in DMBA-soy group than that in casein group (Figure 1G). Positive Ki-67 staining correlates with degree of differentiation, vascular invasion, lymph node metastasis and aggressiveness of the tumors as reported by us and others [23, 61-63]. Recent studies also indicated that the proliferation biomarker Ki-67 is also considered as a prognostic factor for breast cancer and level can be used as a valuable biomarker in breast cancer patients [64, 65]. Our present study also demonstrate that there was a strong correlation between grading (Figure 1A-1C) and Ki-67 (Figure 1D-1G) and validate our previous observation that the DMBA-soy protein group contained lower grade tumor than DMBA-casein group. This result indicates that when casein protein was replaced with soy protein not only significantly and physiologically delay development of mammary tumor in rats exposed to DMBA but also inhibits the aggressiveness of tumor progression.

To identify the signaling pathways that is involved in mediating this differential effect of casein and soy protein on mammary gland, we measured the signaling cascade of individual members of both total and

phosphoMAPK family i.e., ERK (Figure 2A), p38 (Figure 2B), and JNK (Figure 2C, Table 1) in the breast tissues. According to our results, a significant increase was observed both the activity of total and phosphoMAPKs (ERK1/2, p38 and JNK1/2) was higher in tumor of both DMBA-casein and DMBA-soy than control breast tissues. Soy protein showed a significant decrease in the activity of all MAPKs (Figure 2, Table 1). Western blot analysis revealed that it was JNK-1 (Figure 2D, 2F) and not JNK-2 (Figure 2E, 2G) was increased in both DMBA-Casein and DMBA-soy protein group tumors than control breast tissues (Figure 2D-2G), but soy protein decreased significantly the protein level of JNK-2 in comparison to the DMBA-casein group (Figure 2E, 2G). Chen et. al [66] reported that ERK plays a significant role in progression of tumor. Recent report indicates that rapidly accelerated fibrosarcoma/mitogen-activated protein kinase (MEK)/ERK signaling pathway is significantly correlated with the clinicopathological features and prognosis for patient's breast cancer having axillary lymph node metastasis [67]. Studies have shown that p38MAPK promotes breast carcinoma invasion and metastasis [67]. Multivariate analysis indicates that triple negative breast cancer (TNBC) patient with ERK-2 – overexpressing tumor had a lower overall survival rate than those with low ERK-2-expressing tumors. ERK-2 and pMAPK are valuable prognostic markers in TNBC. Further studies are justified to elucidate ERK's role in TNBC tumorigenicity and metastasis [68]. Current evidence clearly shows that MAPK pathways such as JNK and p38 play important role in the not only enhance the efficacy but also development resistance and toxicity of chemotherapeutic drugs [69]. P38MAPK signaling in tumor cells promotes breast carcinoma growth and metastasis by altering the tumor microenvironment (TME). Inactivation of p38MAPK signaling in breast carcinoma cells reduced growth and spontaneous metastasis of tumor xenografts [70]. The present study indicates that soy protein significantly inhibits all three MAPKs activity and can provide new therapeutic options for treatment of breast cancer including metastasis disease.

Distinct MAPK pathways are responsible for the phosphorylation and activation of AP-1 proteins [71]. The stress-responsive p38 and JNK MAPK pathways regulate cell cycle and apoptosis [72]. MAPKs have significant roles in mediating signals triggered by cytokines, growth factors, and environmental stress; and are involved in controlling cell proliferation, cell differentiation, and death [73, 74]. Recently, it has been reported that soy sauce induced mitochromesis and increased oxidative stress tolerance, which is mediated through p38 MAPK pathway [75]. Development and

progression of breast tumors involve a complex series of events, including oxidative stress, dysregulation of cellular differentiation, excessive proliferation and resistance to apoptosis [76]. Moreover, our laboratory reported that antioxidant liposome significantly counteracted the half mustard gas-induced oxidative stress and activation of AP-1 transcription factors by modulating Fos, Jun and ATF family as a result it protects from mustard gas induced lung injury [58]. Oxidative stress is a crucial factor for cancer progression and therapy. Several studies have shown that DMBA can be used to induce experimental breast carcinomas in rats and that this process involves disruption of tissue redox balance; in turn, this suggests that biochemical and pathophysiological disturbances may result from oxidative damage [77, 78]. Soy, phytochemicals and other antioxidant intake decreases oxidative stress as well as their ability to induce apoptosis in cancer cells [79, 80]. Our result suggests that soy protein may have imparted its beneficial effects by controlling the MAPK pathway. The precise nature of the effect of oxidative stress on cancer development and/or response to treatment with soy protein requires further exploration.

AP-1 is the downstream signal of MAPKs signaling pathways and the blocking of MAPKs leads to the inhibition of AP-1 transactivation and subsequent cell transformation [34]. AP-1 activity plays a critical role in the process of tumorigenesis [35]. AP-1 transcription factors are targeted by many signal transduction pathways and regulate a multiple of cellular processes, such as cell proliferation, survival, differentiation, invasion and carcinogenesis, depending on their dimer composition [80-83]. To elucidate the downstream signal of MAPK pathway, we monitored the protein levels of AP-1 family. Since AP-1 is a homo or hetero dimers of Fos family, Fos/activating transcription factor (ATF) and Jun subfamilies of basic-region leucine-zipper (L-ZIP) proteins, we measured the protein levels of individual components. For ATF, we studied only ATF-2 since it is the predominant form in vertebrate. The protein levels of Fos/activating transcription factor (ATF-2) and Jun subfamilies of basic-region leucine-zipper (L-ZIP) proteins were higher in tumor samples from casein group than corresponding controls. The protein level of both ATF-2 (Figure 3B, 3D) and L-ZIP (Figure 3C, 3E) were decreased if casein was replaced by soy protein. It has been reported that pATF2 has been shown to facilitate the transcription of MMP2, which increases migration in H-Ras-transformed MCF10A human breast epithelial cells indicating that ATF2 may play a role in breast cancer metastasis [84]. It has been also reported that Knocking ZIP expression down in breast cancer cells leads to dysregulated proliferation, indicating its potential role as a tumor suppressor [85]. However, whether ATF-2 and ZIP play important role in delay initiation and progression of breast tumor by soy protein requires further exploration.

We also measured the protein level of individual family members of both Fos (c-Fos, Fra-1, Fra-2 and Fos B) and Jun (c-Jun, Jun B and Jun D). The protein levels of certain family members were higher in tumor samples than corresponding control (Figure 4). Soy protein caused a decrease in the protein levels of c-Fos, Fra-1 and Fos-B (Figure 4A-4F) and also members of Jun family Jun B and Jun D (Figures 4H, 4K and 4I, 4L) except Jun C (Figure 4G, 4J). It has been reported that there was significant association between increased Jun B expression and reduced tumor size and tumor stage [82]. Jun and Fos proteins differ significantly in both their DNA binding and transactivation potential as well as their target gene regulation [86]. In vitro studies have shown that Jun/Fos heterodimers are more stable and have a stronger DNA binding activity than Jun homodimers [87, 88]. Although soy protein increased the expression of c-Jun (Figure 4N, 4P) but the c-Fos expression in the tumors of the soy protein group was at the basal level (Figure 4M, 4O). Since c-Fos and c-Jun dimerization is necessary to activate AP-1, we conclude that activation of AP-1 is higher in tumors from casein group than that in tumors from soy protein group. There is

no variation in c-Fos or c-Jun gene in tumor of both casein and soy group than normal. Sundqvist et al [89] reported that the AP-1 component Jun B was required for expression of many late invasion mediating genes, creating a feed-forward regulatory network that aggravates breast cancer invasion. Recent report also shown that the AP-1 member Fra-1 expression level is correlated with the expression of genes that have been implicated in cancer, influences cell proliferation, in vitro cell invasiveness and motility of breast cancer cells as a result it play a pivotal role in breast cancer progression and aggressiveness of TNBC [90, 91]. The present study indicates that replacement of casein protein by soy protein significantly decreased c-Fos, Fos B and Fra-1 (Figure 4A-4F) level in DMBA-induced rat breast tumor. Hence, this is the first report that consumption of soy protein rather than animal protein modulates both AP-1 and MAPK signaling and as a result it delays the initiation and aggressiveness DMBA-induced rat breast tumor.

Proliferating cell nuclear antigen (PCNA) is a cofactor of DNA polymerase delta and is required for DNA synthesis. The PCNA gene contains AP-1 sites in the promoter region and its expression is regulated by AP-1 activity [91]. Cyclin D1, the regulatory subunit of several cyclin-dependent kinases, is required for, and capable of shortening, the G1 phase of the cell cycle. AP-1 proteins bind the cyclin D1-954 region. Cyclin D1 promoter activity was stimulated by over expression of mitogen-activated protein kinase through the proximal 22 base pairs [92]. Several AP-1 proteins are shown to bind these sites and activate cyclin D1 expression [93]. Our result shows an up regulation of both PCNA and cyclin D1 in DMBA- exposed animal fed casein compared with control (Figure 5A-5D). But these increase of PCNA and Cyclin D1 are down regulated when casein is replaced by soy protein (Figure 5A-5D). Hence, our results show that replacing casein with soy protein down regulate AP-1 dependent cell cycle proteins as well as cell differentiation marker animal exposed to DMBA (Figure 5A-5D) as a result it inhibits tumor cells proliferation and differentiation.

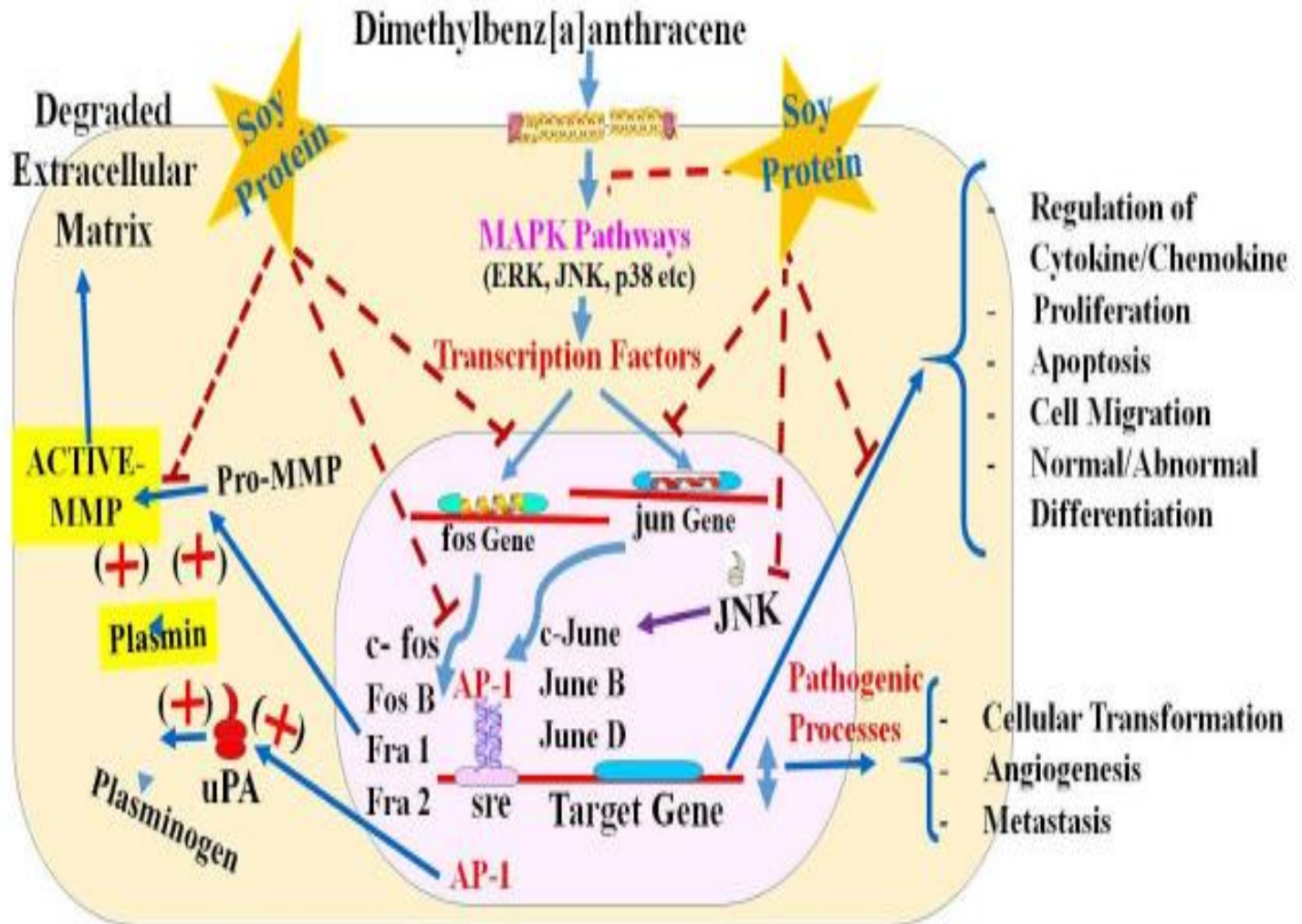
The progression, invasion and metastasis are augmented by proteolytic enzymes like metalloproteinase and serine protease, because they degrade the basement membrane enabling the tumor cells to invade the adjacent tissues [94]. In the present study we observed that casein significantly increased both MMP-9 and uPA level (Figure 5E-5H) than control, whereas replacing casein protein with soy protein significantly decreased the level of MMP-9 and uPA in DMBA-induced rat breast tumor. Recent meta-analysis demonstrated that overexpression of both MMP-2 and MMP-9 in tumor was associated with larger tumor size, lymph node metastasis and poor prognosis [54, 57]. It has been reported that the uPA activates many intracellular signaling and play important role in inflammation and tissue remodeling [95] and overexpression of uPA enhanced malignant potential in TNBC [96]. Moreover, simultaneous knockdown of both uPA and MMP-9 not only reduced breast cancer progression but also decreased the epithelial cells to mesenchymal transition (ENT) in the tumor micro-environment by altering the expression of ENT genes [97]. In the present study, we observed that soy protein significantly decreased the level of both MMP-9 and uPA in DMBA-induced rat tumor (Figure 5E-5H) and aggressiveness of tumor (Figure 1). However, the precise mechanism of action of soy protein in modulation of uPA and MMP-9 function on regulation of aggressiveness breast cancer and whether deactivation of MAPK pathway and/or down-regulation of AP-1 by soy protein requires further investigation.

We previously reported that replacing casein with soy protein not only delay tumor initiation and decreased tumor size and number, but also inhibited progression of higher clinical stage of tumors. In the present study, we observed that feeding 10% casein plus 10% soy protein delay tumor initiation in rat after DMBA post-treatment (Figure 6A) than 20% casein group but earlier than 20% soy protein group.

Furthermore, we also observed that both numbers of rat with tumor and number of tumor present in each rat varies as follow 20% casein (group 1) > 10% casein plus 10% soy protein (group 2) > 20% soy (group 3) (Figure 6B and 6C). Furthermore, we also observed that both tumor size and clinical grading of rat tumor decreased with gradual replacing casein with soy protein as follow 20% casein (group 1) < 10% casein plus 10% soy < 20% soy only (group 3) respectively (data not shown). Hence, our preliminary data indicates that total replacement of casein by soy protein is required for delay initiation, progression and aggressiveness of DMBA-induced rat breast tumor, but needs further validation.

## Conclusion

The present study demonstrates that the beneficial effect of soy protein in breast cancer prevention may be mediated by control of MAPK/AP-1 pathway. We conclude that deactivation of MAPK pathway lead to down-regulation of the AP-1 activation which in turn down regulates the target gene and may responsible for the beneficial effects of soy protein. Possible mechanism of action by which soy protein modulate MAPK/AP-1 signaling in DMBA-induced breast cancer are shown in Figure 7.



**Figure 7:** Schematic presentation of possible mechanism of action of Soy protein. (modified from reference 35).

Furthermore, our findings suggest that 38MAPK, MMP-9 and uPA may be a potential target for anticancer therapy inhibiting tumor vasculature and invasion stimulated by tumor-associated stroma.

Soy protein diets have been associated with lower risk of various diseases, including type 2 diabetes, cardiovascular disease, and other cardiometabolic risk factors. However, the association between soy protein diet quality and breast cancer remains unclear. This study provides evidence that adherence to a healthful soy protein diet may reduce the risk of breast cancer, especially those that are more likely to be aggressive tumors. Moreover, total replacement of animal protein by soy/plant protein may provide a new therapeutic option for treatment/management of breast cancer, including metastatic disease.

## Acknowledgements

### Funding Information

This study was supported by the US Army Grant DAMD 17-03-1-0352, FAMRI grant 02416, NIH grant 5U54MD007953 and RCMI5U54MD007586-34.

### Authors' contributions

Salil K. Das and Shyamali Mukherjee designed the experiments. Sutapa Mukhopadhyay and Shyamali Mukherjee conducted the experiments. Shyamali Mukherjee and Salil K. Das wrote the manuscript, and all authors participated in the review and approval of the final manuscript.

## Conflict of interests

The authors declare no conflicts of interest.

## Data availability statement

All data generated or analyzed during the present study are included in these articles.

## ORCID

Salil K. Das <https://orcid.org/0000-0002-4907-5252>  
Shyamali Mukherjee <https://orcid.org/0000-0003-3357-7079>

## Ethics approval and consent to participate

Ethical approval was obtained from the Institutional Review Board (IRB) and Institutional Animal Care and Use Committee (IACUC) prior to commencing this study.

## References

- Sung H, Ferlay J, Siegel RL, et al. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Cancer J Clin*. 71:209-249.
- World Health Organization Breast Cancer Fact sheets, access on March 22, 2022.
- World Cancer Report 2020; World Health Organization, International Agency for Research Cancer, WHO Press: Geneva, Switzerland, 2020.
- Kawaguchi H, Masuda N, Nakayama T, et al. (2018). Factors associated with prolonged time to treatment failure with fulvestrant 500 mg in patients with post-menopausal estrogen receptor-positive advanced breast cancer: a sub-group analysis of the JBCRG-C06 Safari study. *Curr Med Res Opin*. 34(1):49-54.
- Vineis P, Wild CP. (2014). Global cancer patterns: Causes and prevention. *Lancet*. 383:549–557.
- Ahmad A, Azim S, Zubair H, et al. (2017). Epigenetic basis of cancer health disparities: looking beyond genetic differences. *Biochim Biophys Acta Rev Cancer*. 1868(1):16–28.
- Brennan SF, Cantwell, MM, Cardwell CR, et al. (2010). Dietary patterns and breast cancer risk: A systematic review and meta-analysis. *Am J Clin Nutr*. 91:1294-1302.
- Buck K, Vrieling A, Flesch-Janys D, et al. (2011). Dietary patterns and the risk of postmenopausal breast cancer in a German case-control study. *Cancer Causes Control*. 22:273–282.
- Chatterjee C, Gleddie S, Xiao CW. (2018). Soybean Bioactive Peptides and Their Functional Properties Nutrient. 10 (9):1211-1227.
- Varinska L, Gal P, Mojzisova G, et al. (2015). Soy and breast cancer: focus on angiogenesis. *Int J Mol Sci*. 16(5):11728-11749.
- Chen M, Rao Y, Zheng Y, et al. (2014). Association between soy isoflavone intake and breast cancer risk for pre- and post-menopausal women: a meta-analysis of epidemiological studies. *PLoS One*. 9(2):e89288.
- Nachvak SM, Moradi S, Anjom-Shoae J, et al. (2019). Soy, Soy isoflavones, and protein intake in relation to mortality from all causes, cancers and cardiovascular diseases: A systematic review and dose-response meta-analysis of prospective cohort studies. *J Acad Nutr Diet*. 119(9):1483-1500.
- Messina M. (2016). Impact of soy foods on the development of breast cancer and the prognosis of breast cancer patients. *Forsch Komplementmed*. 23(2):75-80.
- Messina M. (2016). Soy and health update: Evaluation of the clinical and epidemiologic literature. *Nutrients* 8(12):754.
- Fan Y, Wang M, Li Z, et al. (2022). Intake of soy, soy isoflavones and soy protein and risk of cancer incidence and mortality. *Front Nutr*. 9:847421.
- Magee PJ, Rowland I. (2012). Soy products in the management of breast cancer. *Curr Opin Clin Nutr Metab Care*. 15(6):586-591.
- Gertraud Maskarinec. (2005). Soy foods for breast cancer survivors and women at high risk for breast cancer? *J. Am Diet Assoc*. 105(10):1524-1528.
- Wei Y, Lv J, Guo Y, et al. (2020). Soy intake and breast cancer risk: a prospective study of 300,000 Chinese women and a dose-response meta-analysis. *Eur J Epidemiol*. 35(6):567-578.
- Zhong X, Zhang C. (2012). Soy food intake and breast cancer risk: a meta-analysis. *Wei Sheng Yan Jiu*. 41(4):670-676.
- Kazemi A, Barati-Boldaji R, Soltani S, et al. (2021). Intake of various food groups and risk of breast cancer: A systematic review and dose-response meta-analysis of prospective studies. *Adv Nutr*. 12(3):809-849.
- Papadimitriou N, Dimou N, Gill D, et al. (2021). Genetically predicted circulating concentrations of micronutrients and risk of breast cancer: A Mendelian randomization study. *Int J Cancer*. 148(3):646-653.
- McTiernan A. (2021). Diet and prognosis in women with breast cancer. *Cancer Epidemiol. Biomarkers Prev*. 30(2):252-254.
- Mukhopadhyay S, Ballard BR, Mukherjee S, et al. (2006). Beneficial effects of soy protein in the initiation and progression against dimethylbenz[a]anthracene-induced breast tumors in female rats. *Mol Cell Biochem*. 290(1-2):169-176.
- Mukhopadhyay S, Rajaratnam V, Mukherjee S, et al. (2008). Control of peripheral benzodiazepine receptor-mediated breast cancer in rats by soy protein. *Mol Carcinog*. 47(4):310-319.
- Morrison DK. (2012). MAP kinase pathways. *Cold Spring Harb Perspect Biol*. 4(11):a011254.
- De Luca A, Maiello MR, D'Alessio A, et al. (2012). The RAS/RAF/MEK/ERK and the PI3K/AKT signaling pathways: role in cancer pathogenesis and implications for therapeutic approaches. *Expert Opin Ther Targets*. 16(Suppl 2):S17–27.
- Shigeru Shiizaki S, Naguro I, Ichijo H. (2012). Activation mechanisms of ASK1 in response to various stresses and its significance in intracellular signaling. *Adv Biol Regul*. 53(1):135-144.
- Pearson G, Robinson F, Gibson TB, et al. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev*. 22:153-183.
- Munshi A, Ramesh R. (2013). Mitogen-activated protein kinases and their role in radiation response. *Genes Cancer*. 4(9-10):401-408.
- Cuadrado A, Nebreda AR. (2010). Mechanisms and functions of p38 MAPK signalling. *Biochem J*. 429(3):403-417.
- Insua-Rodríguez J, Pein M, Hongu T, (2018). Stress signaling in breast cancer cells induces matrix components that promote chemoresistant metastasis. *EMBO Mol Med*. 10(10):e9008.
- Lee S, Rauch J, Kolch W. (2020). Targeting MAPK signaling in cancer: Mechanisms of drug resistance and sensitivity. *Int J Mol Sci*. 21(3):1102.
- Plotnikov A, Zehorai E, Procaccia S, et al. (2011). The MAPK cascades: Signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochim Biophys Acta Mol Cell Res* 2011; 1813(9):1619-1633.
- Ibrahim SAEI-F, Abudu A, Jonhson E, Aftab N, Conrad S, Fluck M. (2018). The role of AP-1 in self-sufficient proliferation and migration of cancer cells and its potential impact on an autocrine/paracrine loop. *Oncotarget*. 2018; 9(76):34259-34278.

35. Trop-Steinberg S, Azar Y. (2017). AP-1 Expression and its Clinical Relevance in Immune Disorders and Cancer. *Am J Med Sci* 2017; 353(5):474-483.
36. Kharman-Biz A, Gao H, Ghiasvand R, Zhao C, Zendehele K, Dahlgren-Wright K. (2013). Expression of activator protein-1 (AP-1) family members in breast cancer. *BMC Cancer* 2013; 13:441-451.
37. Ye N, Ding Y, Wild C, Shen Q, Zhou J. (2014). Small molecule inhibitors targeting activator protein 1 (AP-1). *J Med Chem.* 2014; 57(16):6930-6948.
38. Langer S, Singer CF, Hudelist G, Dampier B, Kaserer K, Vinatzer U, Pehamberger H, Zielinski C, Kubista E, Schreiber M. (2006). Jun and Fos family protein expression in human breast cancer: correlation of protein expression and clinicopathological parameters. *Eur J Gynaecol Oncol.* 2006; 27(4):345-352.
39. Bland KI, Konstadoulakis MM, Vazeridis MP, et al. (1995). Oncogene protein co-expression. Value of Ha-ras, c-myc, c-fos, and p53 as prognostic discriminants for breast carcinoma. *Ann Surg.* 221:706-720.
40. Gee JM, Ellis IO, Robertson JF, et al. (1995). Immunocytochemical localization of Fos protein in human breast cancers and its relationship to a series of prognostic markers and response to endocrine therapy. *Int J Cancer* 64:269-273.
41. García-Cortés D, de Anda-Jáuregui G, Cristóbal Fresno C, et al. (2020). Gene co-expression is distance-dependent in breast cancer. *Front Oncol.* 10:1232.
42. Rattanasinchai C, Llewellyn BJ, Conrad SE, et al. (2017). MLK3 regulates FRA-1 and MMPs to drive invasion and transendothelial migration in triple-negative breast cancer cells. *Oncogenesis.* 6(6):e345-e356.
43. Murthy NS, Mukherjee S, Ray G, et al. (2009). Dietary Factors and cancer chemoprevention: an overview of obesity-related malignancies. *Jr Postgraduate Med.* 55(1):45-54.
44. Li Y, Li S, Meng X, et al. (2017). Dietary natural products for prevention and treatment of breast cancer. *Nutrients.* 9:728-766.
45. Kapinova A, Kubatka P, Golubnitschaja O, et al. (2018). Dietary phytochemicals in breast cancer research: anticancer effects and potential utility for effective chemoprevention. *Environ Health Prev Med.* 23(1):36-54.
46. Chabot GG, Touil YS, Pham MH, et al. (2010). Flavonoids in Cancer Prevention and Therapy: Chemistry, Pharmacology, Mechanisms of Action, and Perspectives for Cancer Drug Discovery. *Alternative and Complementary Therapies for Cancer.* Chapter 23:583-612.
47. Varinska L, Gal P, Mojzisova G, et al. (2015). Soy and breast cancer: focus on angiogenesis. *Int J Mol Sci.* 16(5):11728-11749.
48. Li Y, Chen H, Hardy TM, et al. (2013). Epigenetic regulation of multiple tumor-related genes leads to suppression of breast tumorigenesis by dietary genistein. *PLoS One.* 8(1): e54369.
49. Kim IS. (2021). Current perspectives on the beneficial effects of soybean isoflavones and their metabolites for humans. *Antioxidants (Basel).* 10(7):1064.
50. Sohel M, Biswas P, Amin MA, et al. (2022). Genistein, a potential phytochemical against breast cancer treatment-Insight into the Molecular Mechanisms. *Processes.* 10(2):415.
51. Radisky ES, Radisky DC. (2015). Matrix metalloproteinases as breast cancer drivers and therapeutic targets. *Front Biosci (Landmark Ed).* 20:1144-1163.
52. Merdad A, Karim S, Schulten H-J, et al. (2014). Expression of matrix metalloproteinases (MMPs) in primary human breast cancer: MMP-9 as a potential biomarker for cancer invasion and metastasis. *Anticancer Research.* 34:1355-1366.
53. Li H, Qiu Z, Li F, et al. (2017). The relationship between MMP-2 and MMP-9 expression levels with breast cancer incidence and prognosis. *Oncology Letters.* 14:5865-5870.
54. Noh H, Hong S, Huang S. (2013). Role of urokinase receptor in tumor progression and development. *Theranostics.* 3(7):487-495.
55. Soyuncu HO, Duranyildiz D, Guney N, et al. (2012). Utility of Serum and Urine uPAR Levels for Diagnosis of Breast Cancer. *Asian Pac J Cancer Prev.* 13(6):2887-2889.
56. Jiang H and Li H. (2021). Prognostic values of tumoral MMP2 and MMP9 overexpression in breast cancer: a systematic review and meta-analysis. *BMC Cancer.* 21:149:149.
57. Lowry OH, Rosebrough NJ, Farr L, et al. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem.* 193:265-275.
58. Mukhopadhyay S, Mukherjee S, Stone WL, et al. (2009). Role of MAPK/AP-1 signaling pathway in the protection of CEES-induced lung injury by antioxidant liposome. *Toxicology.* 261:143-151.
59. Mukhopadhyay S, Rajaratnam V, Mukherjee S, et al. (2006). Modulation of the expression of superoxide dismutase gene in lung injury by 2-chloroethyl ethyl sulfide, a mustard analog. *J Biochem Mol Toxicol.* 20:142-149.
60. Barak Y, Fridman D. (2017). Impact of mediterranean diet on cancer: Focused Literature Review. *Cancer Genomics Proteomics.* 14(6):403-408.
61. Sinha Roy S, Mukherjee S, Ballard BR, et al. (2016). Protection against dimethylbenz[a]anthracene-induced breast cancer in female rats by  $\alpha$ -Lactalbumin. *Int J Cancer Oncol.* 3(1):1-6.
62. Milde-Langosch K, Karn T, Müller V, et al. (2013). Validity of the proliferation markers Ki67, TOP2A, and RacGAP1 in molecular subgroups of breast cancer. *Breast Cancer Res Treat.* 137(1):57-67.
63. Inwald EC, Klinkhammer-Schalke M, Hofstädter F, et al. (2013). Ki-67 is a prognostic parameter in breast cancer patients: results of a large population-based cohort of a cancer registry. *Breast Cancer Res Treat.* 139 (2):539-552.
64. Soliman NA, Yussif SM. (2016). Ki-67 as a prognostic marker according to breast cancer molecular subtype. *Cancer Biol Med.* 13(4):496-504.
65. Hashmi AA, Hashmi KA, Irfan M, et al. (2019). Ki67 index in intrinsic breast cancer subtypes and its association with prognostic parameters. *BMC Res Notes* 12(1):605.
66. Chen L, Shi Y, Jiang CY, et al. (2011). Expression and prognostic role of pan-Ras, Raf-1, pMEK1 and pERK1/2 in patients with hepatocellular carcinoma. *Eur J Surg Oncol.* 37(6):513-520.
67. Shao GL, Wang MC, Fan XL, et al. (2018). Correlation Between Raf/MEK/ERK Signaling Pathway and Clinicopathological Features and Prognosis for Patients with Breast Cancer Having Axillary Lymph Node Metastasis. *Technol Cancer Res Treat.* 17:1533034617754024.
68. Bartholomeusz C, Gonzalez-Angulo AM, Liu P, et al. (2012). High ERK protein expression levels correlate with shorter survival in triple-negative breast cancer patients. *Oncologist.* 17(6):766-774.
69. Lee S, Rauch J, Kolch W. (2020). Targeting MAPK signaling in cancer: Mechanisms of drug resistance and sensitivity. *Int J Mol Sci.* 21-(3):1102.
70. Wu X, Zhang W, Font-Burgada J, et al. (2014). Ubiquitin-conjugating enzyme Ubc13 controls breast cancer metastasis through a TAK1-p38 MAP kinase cascade. *Proceedings of the National Academy of Sciences of the United States of America.* 111:13870-13875.
71. Limoge M, Safina A, Truskinovsky AM, et al. (2017). Tumor p38MAPK signaling enhances breast carcinoma vascularization and growth by promoting expression and deposition of pro-tumorigenic factors. *Oncotarget.* 8(37):61969-61981.

72. Whitmarsh AJ, Davis RJ. (1996). Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med.* 74:589-607.
73. Angel P, Karin M. (1991). The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. *Biochim Biophys Acta.* 1072:129-157.
74. Kharman-Biz A, Gao H, Ghiasvand R, et al. (2013). Expression of activator protein-1 (AP-1) family members in breast cancer. *BMC Cancer.* 13:441-451.
75. Sugawara T, Saraprug D, Sakamoto K. (2019). Soy sauce increased the oxidative stress tolerance of nematode via p38 MAPK pathway. *Biosci Biotechnol Biochem.* 83(4):709-716.
76. Hecht F, Pessoa CF, Gentile LB, et al. (2016). The role of oxidative stress on breast cancer development and therapy. *Tumour Biol.* 37(4):4281-4291.
77. Giri U, Sharma SD, Abdulla M, et al. (1995). Evidence that in situ generated reactive oxygen species act as a potent stage I tumor promoter in mouse skin. *Biochem Biophys Res Commun.* 209:698-705.
78. Al-Malki AL, Moselhy SS, Rafai MY. (2011). Synergic effect of lycopene and tocopherol against oxidative stress and mammary tumorigenesis induced by 7,12-dimethylbenz[a]anthracene (DMBA) in female rat. *Toxicol and Industrial Health.* 28(6):542-548.
79. Batcioglu K, A Uyumlu AB, Satilmis B, et al. (2012). Oxidative stress in the in vivo DMBA rat model of breast cancer: suppression by a voltage-gated sodium channel inhibitor (RS100642). *Basic Clin Pharmacol Toxicol.* 111 (2):137-144.
80. Saha SK, Lee SB, Won J, et al. (2017). Correlation between Oxidative Stress, Nutrition, and Cancer Initiation. *Int J Mol Sci.* 18 (7):1544-1574.
81. van Dam H., Castellazzi M. (2001). Distinct roles of Jun: Fos and Jun: ATF dimers in oncogenesis. *Oncogene.* 20:2453-2464.
82. Ozanne BW, Spence HJ, McGarry LC, et al. (2007). Transcription factors control invasion: AP-1 the first among equals. *Oncogene.* 26:1-10.
83. Shaulian E, Karin M (2002) AP-1 as a regulator of cell life and death. *Nat Cell Biol.* 4:E131-E136.
84. Rudraraju B, Droog M, Abdel-Fatah TM, et al. (2014). Phosphorylation of activating transcription factor-2 (ATF-2) within the activation domain is a key determinant of sensitivity to tamoxifen in breast cancer. *Breast Cancer Res Treat.* 147(2):295-300.
85. Zhu N, Zhang J, Du Y, et al. (2020). Loss of ZIP facilitates JAK2-STAT3 activation in tamoxifen-resistant breast cancer. *Proc Natl Acad Sci.* 117(26):15047-15054.
86. Gustems M, Woellmer A, Rothbauer U, et al. (2014). c-Jun/c-Fos heterodimers regulate cellular genes via a newly identified class of methylated DNA sequence motifs. *Nucleic Acids Res.* 42(5):3059-3072.
87. Ryseck RP, Bravo R. (1991). c-Jun, JunB, JunD differ in their binding affinities to AP-1 and CRE consensus sequences: effects of fos proteins. *Oncogene.* 6:533-542.
88. Zhao C, Qiao Y, Jonsson P, et al. (2014). Genome-wide profiling of AP-1-regulated transcription provides insights into the invasiveness of triple-negative breast cancer. *Cancer Res.* 74(14):3983-3994.
89. Sundqvist A, Morikawa M, Ren J, et al. (2018). JunB governs a feed-forward network of TGFβ signaling that aggravates breast cancer invasion. *Nucleic Acids Res.* 46(3):1180-1195.
90. Luo YP, Zhou H, Krueger J, et al. (2010). The role of proto-oncogene Fra-1 in remodeling the tumor microenvironment in support of breast tumor cell invasion and progression. *Oncogene.* 29(5):662-673.
91. Shen Q, Uray IP, Li Y, et al. (2008). The AP-1 transcription factor regulates breast cancer cell growth via cyclins and E2F factors. *Oncogene.* 27:366-377.
92. Brown JR, Nigh E, Lee RJ, et al. (1998). Fos family members induce cell cycle entry by activating cyclin D1. *Mol Cell Biol.* 18:5609-5619.
93. Vartanian R, Masri J, Martin J, et al. (2011). AP-1 regulates cyclin D1 and c-MYC transcription in an AKT-dependent manner in response to mTOR inhibition: Role of AIP4/Ich-mediated JUNB degradation. *Cancer Res.* 9(1):115-130.
94. Zhan R, Li X, Zang L, et al. (2020). An Au-Se nanoprobe for the evaluation of the invasive potential of breast cancer cells via imaging the sequential activation of uPA and MMP-2. *Analyst.* 145(3):1008-1013.
95. Smith HW, Marshall CJ. (2010). Regulation of cell signaling by uPAR. *Nat Rev Mol Cell Biol.* 11(1):23-36.
96. Huber MC, Mall R, Braselmann H, et al. (2016). uPAR enhances malignant potential of triple-negative breast cancer by directly interacting with uPA and IGF1R. *BMC Cancer.* 16:615-627.
97. Moirangthem A, Bondhopadhyay B, Mukherjee M, (2016). Simultaneous knockdown of uPA and MMP9 can reduce breast cancer progression by increasing cell-cell adhesion and modulating EMT genes. *Tumour Biol.* 36(2):1261-1271.



This work is licensed under Creative Commons Attribution 4.0 License

To Submit Your Article, Click Here:

[Submit Manuscript](#)

DOI: [10.31579/2640-1053/122](https://doi.org/10.31579/2640-1053/122)

#### Ready to submit your research? Choose Auctores and benefit from:

- fast, convenient online submission
- rigorous peer review by experienced research in your field
- rapid publication on acceptance
- authors retain copyrights
- unique DOI for all articles
- immediate, unrestricted online access

At Auctores, research is always in progress.

Learn more <https://www.auctoresonline.org/journals/cancer-research-and-cellular-therapeutics->