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Genes Related to Suppression of Malignant Phenotype Induced by Maitake D-Fraction in Breast Cancer Cells

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ABSTRACT It is already known that the Maitake (D-Fraction) mushroom is involved in stimulating the immune system and activating certain cells that attack cancer, including macrophages, T-cells, and natural killer cells. According to the U.S. National Cancer Institute, polysaccharide complexes present in Maitake mushrooms appear to have significant anticancer activity. However, the exact molecular mechanism of the Maitake antitumoral effect is still unclear. Previously, we have reported that Maitake (D-Fraction) induces apoptosis in breast cancer cells by activation of BCL2-antagonist/killer 1 (*BAK1*) gene expression. At the present work, we are identifying which genes are responsible for the suppression of the tumoral phenotype mechanism induced by Maitake (D-Fraction) in breast cancer cells. Human breast cancer MCF-7 cells were treated with and without increased concentrations of Maitake D-Fraction (36, 91, 183, 367 μg/mL) for 24 h. Total RNA were isolated and cDNA microarrays were hybridized containing 25,000 human genes. Employing the cDNA microarray analysis, we found that Maitake D-Fraction modified the expression of 4068 genes (2420 were upmodulated and 1648 were downmodulated) in MCF-7 breast cancer cells in a dose-dependent manner during 24 h of treatment. The present data shows that Maitake D-Fraction suppresses the breast tumoral phenotype through a putative molecular mechanism modifying the expression of certain genes (such as *IGFBP-7*, *ITGA2*, *ICAM3*, *SOD2*, *CAV-1*, *Cul-3*, *NRF2*, *Cycline E*, *ST7*, and *SPARC*) that are involved in apoptosis stimulation, inhibition of cell growth and proliferation, cell cycle arrest, blocking migration and metastasis of tumoral cells, and inducing multidrug sensitivity. Altogether, these results suggest that Maitake D-Fraction could be a potential new target for breast cancer chemoprevention and treatment.

KEY WORDS: • ***breast cancer cells*** • ***gene expression*** • ***Maitake D-Fraction*** • ***malignant phenotype*** • ***microarrays***

INTRODUCTION

NUMEROUS STUDIES HAVE CONFIRMED that Maitake has prominent beneficial effects on immune function.[1–9] It promotes the action of not only macrophages, but also a variety of other immune-related cells, such as natural killer (NK) cells and cytotoxic T-cells that can attack tumor cells. Maitake also increases the immune-related efficiency of these cells by increasing interleukin-1, interleukin-2, and lymphokines.[1–9]

The specific proteoglucan in the Maitake mushroom extract for fighting cancer tumors is called D-Fraction. Maitake D-Fraction has been reported to exert its antitumor effect in tumor-bearing mice by enhancing the immune system through activation of macrophages, T cells, and NK cells. The proteoglucan shows anticarcinogenic activity, prevents oncogenesis, and prevents metastasis. However, the exact molecular mechanisms and gene expression profiles generated by Maitake D-Fraction in the anticarcinogenesis process are still unclear. In our previous work, we have demonstrated that D-Fraction of Maitake mushroom was able to effectively induce apoptosis in MCF-7 breast cancer cells.[10] These findings were corroborated by a reduction in cell viability upon treatment with different concentrations of this fraction. Several genes promoting apoptosis were upregulated as assessed by cDNA microarray analysis. We have reported that D-Fraction Standard (Mushrooom Wisdom, Inc.) induces apoptosis in breast cancer cells by activation of *BAK1* gene expression; we also found that cytochrome C1 was released to the cytoplasm in a dose-dependent manner.[10] In the present work, we are trying to clarify the genomic expression induced by Maitake (D-Fraction) in MCF-7 breast cancer cells. We also want to demonstrate which genes are involved in the mechanism of suppression of the tumoral breast phenotype. We performed a new set of human cDNA microarray assays, including 25,000 genes employing MCF-7 cells treated with and without (control) increased doses of Maitake (D-Fraction) (36, 91, 183, and 367 μg/mL), during 24 h. The gene expressions are corroborating by real-time reverse transcription (RT)–polymerase chain reaction (PCR) assay employing commercial reagents and custom primers designed by Applied Biosystems, Inc.

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MATERIALS AND METHODS

Bioactive Maitake D-Fraction

The bioactive D-Fraction was obtained as a commercially available bottled liquid, product developed by Mushroom Wisdom, Inc. Basically, Maitake D-Fraction was ethanol extracted from *Grifola frondosa* mushroom, corresponding to the protein-bound polysaccharide compound, and was prepared by a standardized procedure developed by Maitake Products, Inc.

Cell culture

The human breast cancer MCF-7 cell line was obtained from the American Type Culture Collection (ATCC). MCF-7 cells were routinely cultured in the DMEM containing 10% inactivated FBS and 1% penicillin/streptomycin. Cell culture media, fetal bovine serum, and penicillin/streptomycin were purchased from Invitrogen Life Technologies. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere.

MCF-7 cells Maitake D-Fraction treatment

MCF-7 cells were treated with and without (control) increased concentrations of Maitake D-Fraction for 24 h, such as 36, 91, 187, or 367 μg/mL.

Total RNA isolation

The RNA was isolated by duplicate using Trizol (Invitrogen) following the classic phenol purification method.[11] The concentration and the quality of total isolated RNA were measured in the Nanodrop (Nanodrop Technologies) and in the Bioanalyzer (Agilent Technologies).

Labeling and cDNA human microarray hybridization

We employed direct labeling of probes with amine-modified random primers using 5 μg of RNA followed the protocol indicated previously.[10] Probes were purified, before hybridization, Cy3- and Cy5-labeled products were combined and 30 μL of water was added. The purified probes were pipetted onto microarrays, coverslips were applied, and the slides were placed in a hybridization chamber (Corning). Arrays were incubated at 42°C water bath for 16 h, and subsequently washed with 0.5× saline–sodium citrate buffer (SSC), 0.01% (w/v) SDS, followed by 0.06× SSC, at room temperature for 10 min each. Slides were spun for 5 min at 800 rpm (130 *g*) at room temperature.

Array scanning and data analysis

Arrays were read with a Affymetrix 428 fluorescent scanner (MWG Technologies) at a 10-μm resolution and variable photomultiplier tube voltage settings to obtain the maximal signal intensities with <1% probe saturation. The resulting images were analyzed and normalized by the Lowess Method employing the Nexus Expression Software (Biodiscovery). Hierarchical clustering was done by using the neighbor joining method applied to the Pearson correlation distance matrix. In addition, other hierarchical clustering methods and other distance-generating functions (such as Euclidean) were used. Only marginal deviations between the methods and the distance matrices used were observed. We found the most stable results for the neighbor-joining method applied to the Pearson correlation distance matrix.

Gene validation by RT-PCR amplification assay

To corroborated the gene expression induced by Maitake D-Fraction in MCF-7 cells, we validated by real-time RT-PCR assay, three upregulated genes (*ITGA2*, *SOD2*, and *Cul3*) and one downmodulated gene (intercellular adhesion molecule 3 [*ICAM3*]) using nucleotide sequences that we found by the gene accession number obtained from cDNA glass microarray and searching the National Cancer Institute website. TaqMan primer and probe sequences are listed below. The sense and antisense sequence primers were designed using Primer3 software and are the following. *ITAG2* (sense primer: TCT CAT CTG GAT TTT TGG TCA TC, antisense primer: AAC CTG ATG AGA AAG CCG AAG), *SOD2* (sense primer: TGC CTC CAG TCA ACA AGA TG, antisense primer: CGT TAG TGG TTT GCA CAA GG), *Cul3* (sense primer: GAC CCT AAA ACT GAG CAT CAA A, antisense primer: AGA CGT TAA GAA TGG CAG ATA AA), *ICAM3* (sense primer: GTA ACT GCC GCT CCG TTG, antisense primer: ACT TTG TCC CCG TCT TCG T). A β-actin primer was included as a control for gene expression. Primers were labeled with SyBro Green dye (Applied Biosystems). All RT-PCR reactions were performed on the ABI Prism 7000 Sequence Detection System.

Statistical analysis

Normalization and statistical analysis of the expression data were carried out using Linear Models for Microarray Data.[12–14] For detecting the differential expression of genes that might not necessarily be highly expressed, background correction using the normexp method in Linear Models for Microarray Data was done for adjusting the local median background estimates, a correction strategy that avoids problems with background estimates that are greater than foreground values and ensures that there were no missing or negative corrected intensities. An offset of 100 was used for both channels to further dampen down the variability of log ratios for low-intensity spots. The resulting log ratios were normalized by using the print-tip group Lowess method with a span of 0.4, as recommended by Smyth.[14] Moderated t statistic was used as the basic statistic for significance analysis; it was computed for each probe and for each contrast.[14] The false discovery rate was controlled using the BH adjustment of Benjamini and Hochberg.[15,16] All genes with *P* value below a threshold of .05 were selected as differentially expressed, maintaining the proportion of false discoveries in the selected group below the threshold value, in this case 5%.[17]

RESULTS

cDNA microarray analysis

Employing the cDNA microarray analysis, we demonstrated that Maitake D-Fraction modified the expression of

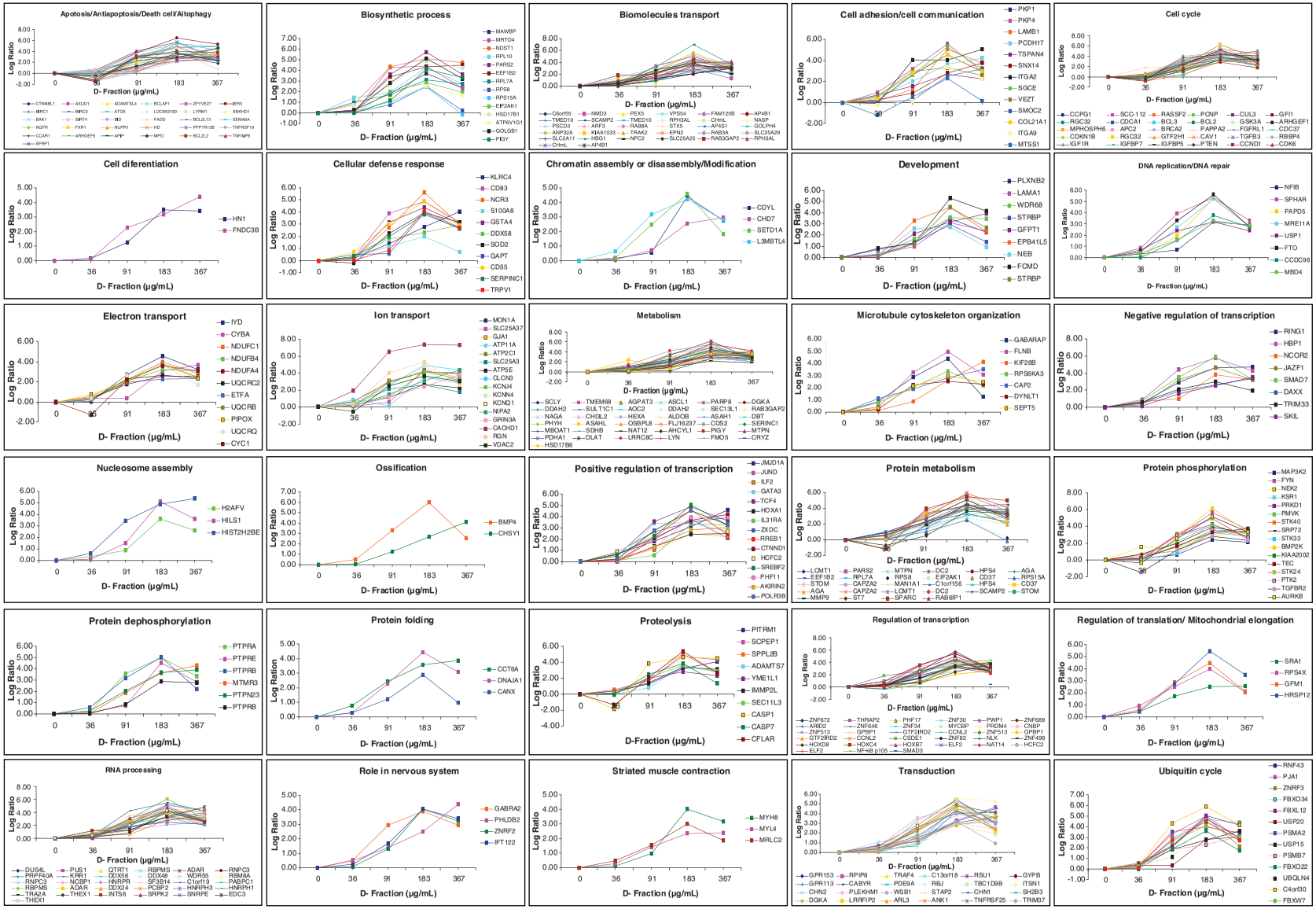

FIG. 1. Gene expression profile of upmodulated genes induced by Maitake D-Fraction in MCF-7 cells. Human breast cancer MCF-7 cells were treated with and without (control) increased concentrations of Maitake D-Fraction for 24 h, namely, 36, 91, 187, or 367 μg/mL. Genomic analysis using complementary DNA (cDNA) microarrays was used to monitor expression levels of 25,000 human known genes. The resulting array images were analyzed and normalized employing the Nexus Expression Software (Biodiscovery). Color images available online at www.liebertpub.com/jmf

4068 genes (2420 were upmodulated and 1648 downmodulated) in MCF-7 breast cancer cells in a dose-dependent manner compared to control (untreated cells) during 24 h of treatment. Under more stringent conditions, we found that 505 genes modified their expression, 430 genes of them were found upregulated, and 75 genes were downregulated at 367 μg/mL of Maitake D-Fraction after 24 h of treatment with a log2 ratio ≥ 2.0 for the upmodulated and a log2 ratio < -2.0 for the downmodulated genes, respectively. Those genes are related to several biological functions as the following (Fig. 1 and Supplementary Table S1; Supplementary Data are available at www.liebertpub.com/jmf): 31 genes related to apoptosis/antiapoptosis/cell death/autophagy, 14 related to biosynthetic process, 59 related to transport, 13 related to cell adhesion/cell communication, 30 genes related to cell cycle, 2 related to cell differentiation, 11 related to cellular defense response, 4 genes related to chromatin assembly/disassembly or modification, 9 genes related to development, 8 related to DNA replication/DNA repair, 65 related to metabolism, 7 related to microtubule cytoskeleton organization, and 56 to regulation of transcription. Three genes are related to nucleosome assembly, 2 related to ossification, 22 to protein phosphorylation/dephosphorylation, 3 genes related to protein folding, and 10 genes related to proteolysis. Four genes are related to regulation of translation/mitochondrial translational elongation, 32 related to RNA processing, 4 genes with roles in nervous system, 3 to striated muscle contraction, 24 to transduction, and 14 genes related to ubiquitin cycle (Fig. 1 and Supplementary Table S1). Also, we detected that 75 genes were downmodulated in a dose-dependent manner in MCF-7 cells. After Maitake D- Fraction treatment, we found that 91 μg/mL of Maitake (D-Fraction) during 24 h induces the maximum downmodulation in those genes (Fig. 2 and Supplementary Table S2) that were related to several biological functions as the following: 1 genes related to apoptosis, 2 related to biosynthetic process, 5 genes related to cell adhesion/cell communication, 5 related to cell cycle, 1 related to chromatin assembly or disassembly, and 7 genes related to transport. Eight genes related to metabolism, 1 related to microtubule cytoskeleton organization, 1 to muscle contraction, and 6 related to protein phosphorylation/dephosphorylation. Four genes related to proteolysis, 9 related to regulation of transcription, 1 to regulation of translation, 1 to RNA processing, 8 to transcription, and 15 genes related to signal transduction (Fig. 2 and Supplementary Table S2).

Validation of differential expression

To confirm the differential expression results, a subset of four genes, three of them upregulated (*ITGA2*, *SOD2*, and Cullin 3 [*CUL3*]) and one downregulated (*ICAM3*), were selected and their mRNA expression were corroborated by real-time quantitative PCR assay employing reagents acquired from Applied Biosystems.

DISCUSSION

Out of 505 genes that showed statistically significant differential expression in MCF-7 cells after treatment with Maitake (D-Fraction) in a dose-dependent manner compared to control, we investigate in the public literature the relationship between those genes and neoplasic disease, specifically in breast cancer. In this manner, we identify genes that are responsible for the suppression of the tumoral phenotype mechanism induced by Maitake (D-Fraction) in breast cancer cells. Thus, we can elucidate the molecular mechanism induced by Maitake in the anti-tumoral action. In the following section, we will describe certain genes that were considered more important in the suppression of the tumoral phenotype mechanism.

Maitake D-Fraction induces apoptosis in MCF-7 cells through activation of BAK1, BCLAF1, RASSF2, FADD, SPARC, *and* BCL2L13 *genes and by downregulated PI3K-AKT signaling*

Evidence demonstrates that *BAK1* promotes cell death and counteracts the protection from apoptosis provided by *BCL2*.[18,19] Chittenden *et al.*[18] found that enforced expression of *BAK1* induced rapid and extensive apoptosis of serum-deprived fibroblasts, suggesting that *BAK1* may be directly involved in activating cell death machinery. Accordingly, Kiefer *et al.*[19] reported that the *BAK1* gene product primarily enhances apoptotic cell death after an appropriate stimulus. Previously, we have published that Maitake (D-Fraction) induces apoptosis in breast cancer cells by activation of *BAK1* gene expression.[10] Activated BAK1 proteins oligomerize at the mitochondrial membrane and cause the release of several mitochondrial factors, such as cytochrome C, which in combination with *APAF-1* and procaspase 9 form an apoptosome. Activated caspase 9 probably then activates caspase 7 and caspase 1, allowing apoptosis to proceed.[10] In concordance with that, in the present work, we found that Maitake D-Fraction induces the overexpression of the proapoptotic *BAK1* gene (2.18-fold) at 183 μg/mL in MCF-7 cells (Fig. 3). However, more research must be done to determine the exact apoptotic mechanism and its regulation. Also, here we have shown that Maitake D-Fraction induces the expression of *BCL2*-associated transcription factor 1 (*BCLAF1*; 5.43-fold), another proapoptotic gene. BCLAF1 is a nuclear protein that was originally identified in a screening of proteins that interact with *BCL2*. It has been shown that *BCLAF1*-deficient cells did not experience cell death after exposure to various apoptotic stimuli.[20] Over expression of *BCLAF1* was shown to induce apoptosis as well as transcriptional repression, properties that were reversible in the presence of *BCL2* or *BCL-XL*.[21] Taken together, these studies potentially support a role for *BCLAF1* in apoptosis through events that control transcription.[22] In addition to the overexpression of *BCLAF1*, we also found upmodulation of the antiapoptotic *BCL2* gene. This could be due probably in response to controlled or regulated cell death mechanisms already triggered by Maitake D-Fraction through activation of several apoptotic genes in MCF-7 tumoral cells. We reiterate that after 24 h of treatment with this compound, we observed $>85\%$ of the MCF-7 cells died because of apoptosis;[10] the surviving $\sim 15\%$ could be

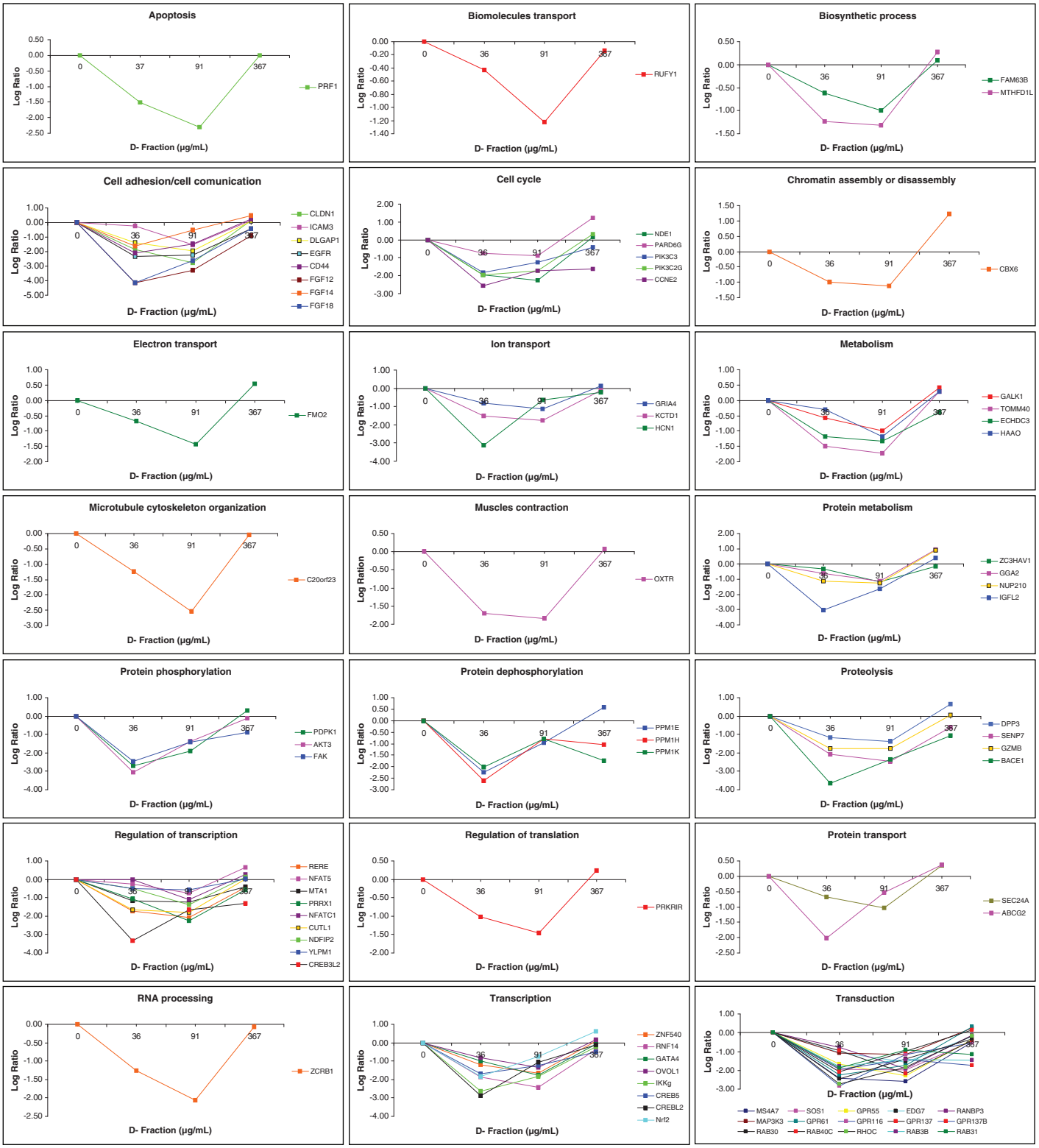

FIG. 2. Gene expression profile of downmodulated genes induced by Maitake D-Fraction in MCF-7 cells. Human breast cancer MCF-7 cells were treated with and without (control) increased concentrations of Maitake D-Fraction for 24 h, namely, 36, 91, 187, or 367 μg/mL. Genomic analysis using complementary DNA (cDNA) microarrays was used to monitor expression levels of 25,000 human known genes. The resulting array images were analyzed and normalized employing the Nexus Expression Software (Biodiscovery). Color images available online at www.liebertpub.com/jmf

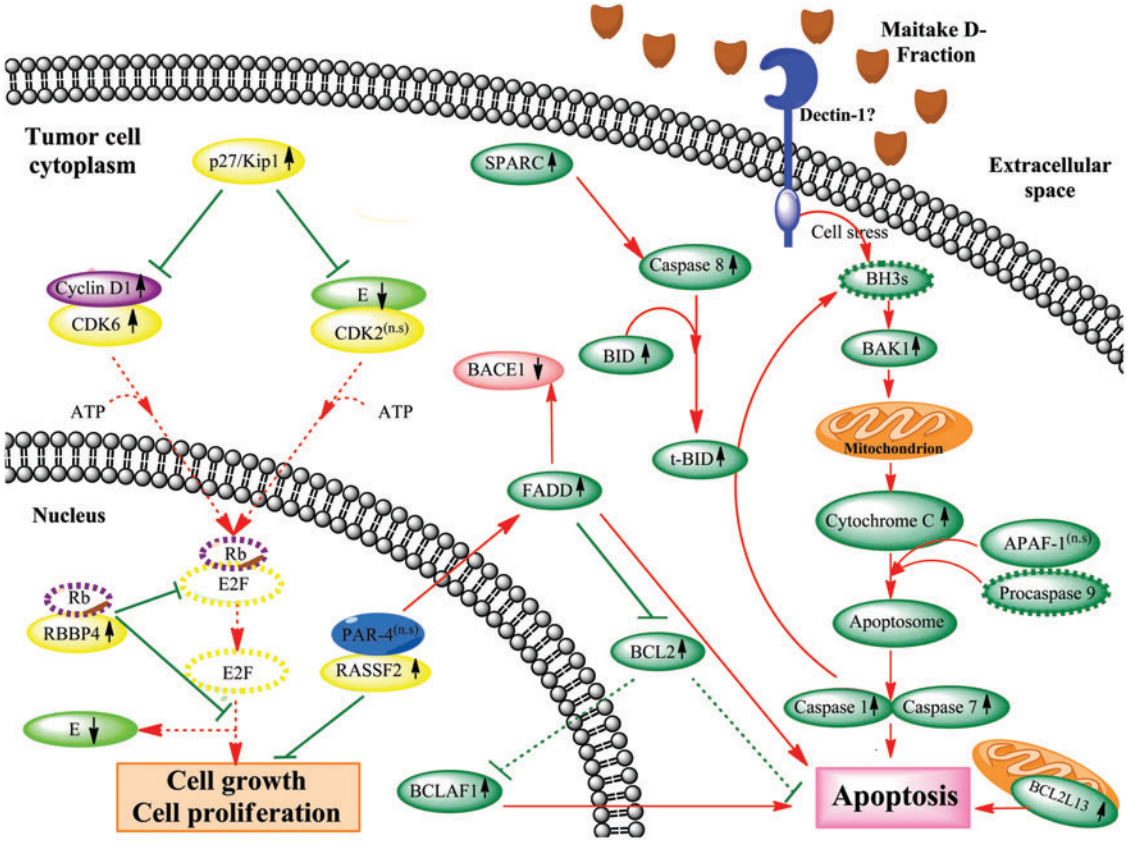

FIG. 3. Maitake D-Fraction induces apoptosis mediated by *BAK1* stimulation and blocks tumor cells growth and proliferation in MCF-7 cells through activation of *p27/Kip1* and *RASSF2* genes. The putative molecular mechanism of apoptosis and anti-tumoral cell proliferation induced by Maitake D-Fraction in breast cancer MCF7 cells is shown, with red lines indicating stimulated pathways, green lines indicating inhibitory pathways, and discontinuous gray lines representing the genes whose expression has not been changed after Maitake treatment. Color images available online at www.liebertpub.com/jmf

explained by the antiapoptotic signal induced by *BCL2* gene expression. In conclusion, it is well known that *BCLAF1* activation is related to apoptosis; however, its exact mechanism in these events remains unclear (Fig. 3).

Likewise, it is well known that *RASSF2* (*Ras* association [*RalGDS/AF-6*] domain family member 2) is a novel proapoptotic effector of *K-Ras*. *RASSF2* is implicated as a tumor suppressor, whose inactivation facilitates transformation by disconnecting apoptotic responses from *Ras*. It has been demonstrated that *RASSF2* forms a direct and endogenous complex with the tumor suppressor prostate apoptosis response protein 4 (*PAR-4*).[23] This interaction is regulated by *K-Ras* and is essential for the full apoptotic effects of *PAR-4*, a proapoptotic protein capable of selectively inducing apoptosis in cancer cells. It has been shown that *PAR-4* sensitized the cells to diverse apoptotic stimuli and causing regression of tumors in animal models.[23] *PAR-4* induces apoptosis in certain cancer cells by activating *Fas TNFRSF6*-associated via death domain (*FADD*) prodeath pathway and coparallel inhibition of *NF-κB* transcriptional activity.[23] In the present work, we found that Maitake D-Fraction treatment stimulates the apoptosis mechanisms by increasing the expression (4.62-fold) of *RASSF2* gene and upmodulation of the *FADD* gene (3.87-fold) through interaction with *PAR-4*. It is well known that the FADD protein inhibits the expression of the antiapoptotic protein *BCL2* and maintains the stimulated apoptotic pathway in these tumor cells. However, we found *BCL2* gene expression upmodulated (3.47-fold) after Maitake treatment; based on previous reports, we can suggest that the apoptosis pathways in MCF-7 cells are positively balanced due to the BCL2 interaction with the FADD protein, which could affect the *BCL2* pre-mRNA splicing and processing to play a pivotal role in the regulation of apoptosis. By another hand, it has also been demonstrated that *FADD* may be directly involved in regulating the amyloid precursor protein (APP) cleavage activity of *BACE1*,[24] which was found downmodulated (−2.37-fold) in our experiments; thus, we can also suggest a potential role of Maitake D-Fraction in Alzheimer's disease, preventing APP accumulation. In conclusion, here we show that Maitake D-Fraction significantly increased the expression of *RASSF2*, a potential tumor suppressor, which may promote apoptosis in MCF-7 cells (Fig. 3). However, the exact molecular mechanism of *RASSF2* in apoptosis is not yet defined.

Also, researchers show that *SPARC* (secreted proteins, acidic, cysteine-rich) potentiates apoptosis in the presence of chemotherapy in colorectal cancer, by augmenting the

signaling cascade in a caspase-8-dependent manner. They found that this occurs independently of death receptor activation and leads to downstream involvement of *Bid* and subsequent apoptosis.[25] In this study, we found that Maitake D-Fraction induces the expression of the *SPARC* gene (5.45-fold) in MCF-7 cells. Thus, we can suggest that Maitake D-Fraction induces the extrinsic pathway of apoptosis by overexpression of the *SPARC* gene (Fig. 3).

BCL2L13, also called *BCL*-rambo, is a widely expressed *BCL-2* member that shows an overall structural homology containing sequence conservation clustered in *BCL-2* homology (BH) domains. In mammalian cells, *BCL*-rambo was localized to mitochondria, and its overexpression induces apoptosis in those cells.[26] Similar to *Bax* and *Bak*, overexpression of *BCL*-rambo causes the release of cytochrome C to the cytosol, indicating that mitochondrial damage occurs during *BCL*-rambo–induced cell death. Surprisingly, it has been found that overexpression of anti-apoptotic *BCL-XL* and *BCL-2* genes had little effect on *BCL*-rambo–mediated cell death. Thus, *BCL*-rambo appears to induce apoptosis, at least in part, independently of known mitochondrial signaling pathways.[26] In our experiments, we found that Maitake D-Fraction treatment increases the expression of *BCL2L13* (3.40-fold) in MCF-7 cells; thus, we suggest that this compound could be activated by the apoptotic pathway independently by the overexpression of the antiapoptotic *BCL-2* gene (Fig. 3).

Decades of study have demonstrated an important role of phosphoinositide-3(OH) kinase (*PI3K*)–*Akt* signaling in the formation and progression of a wide variety of tumors. There are many diverse mechanisms by which aberrant activation of *PI3K-AKT* signaling occur in cancer cells, but all lead to similar downstream signaling events: from cell growth and proliferation to survival and motility, which drive tumor progression.[27,28] Particularly, the mechanism by which *AKT* protects cells from death is likely to be multifactorial, because *AKT* directly phosphorylates several components of the cell death machinery.[28] In this work, we found that D-Fraction downregulated *PI3K-AKT* signaling at 91 μg/mL in MCF-7 cells by downmodulation of *PIK3C3* (−1.25-fold), *PIK3C2γ*, (−1.71-fold), *PDPK1* (−1.91-fold), and *AKT3* (−1.38-fold) genes. Thus, we can suggest that D-Fraction could decrease activation of the *AKT* anti-apoptotic pathway, which is frequently overactivated in cancer (Fig. 4). *ICAM3* and *PTEN* genes are also involved in the downregulation of that pathway.

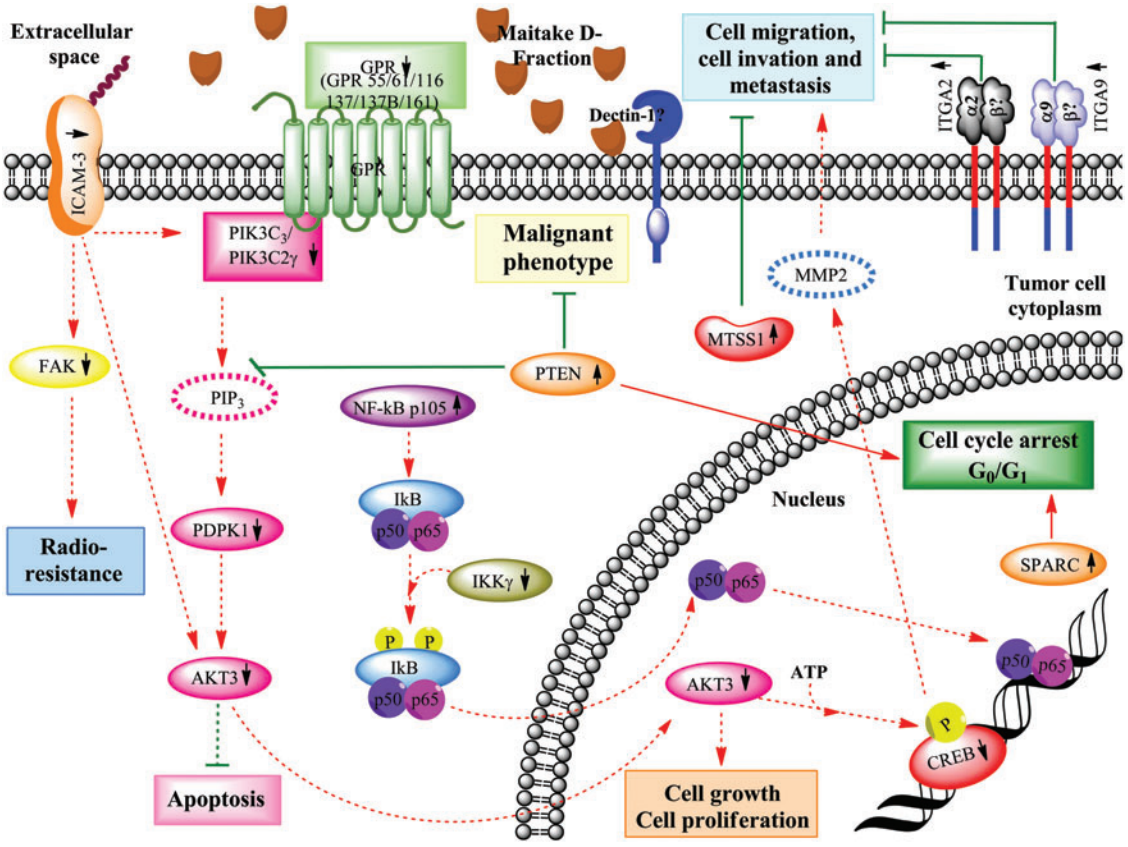

FIG. 4. Maitake D-Fraction blocks the malignant phenotype, cell invasion, and metastasis in MCF-7 cells through activation of *PTEN* and *MTSS1*. The putative molecular mechanism of cell cycle arrest and blocking of malignant phenotype induced by activation of *PTEN* gene expression after Maitake D-Fraction treatment is depicted. As shown, *MTSS1* blocks cell migration, invasion, and metastasis in MCF-7 cells. Stimulated (red) and inhibitory (green) pathways are indicated. Discontinuous gray lines represent the genes whose expression has not been changed after Maitake treatment. Color images available online at www.liebertpub.com/jmf

Maitake D-Fraction blocks tumor cell growth and proliferation

In our experiments, we found that Maitake D-Fraction increases (3.28-fold) the expression of cyclin-dependent kinase (*CDK*) inhibitor 1B (*p27/Kip1*) gene in MCF-7 cells. *p27* or *CDKN1B* is a *CDK* inhibitor directly involved in the cell cycle arrest, and it acts as a typical tumor suppressor that regulates GO to S phase transitions by binding to and regulating the activity of *CDK*s.[29] Recently, it has been reported that an increase in *p27* gene expression is associated with cell growth arrest, cell differentiation, and apoptotic pathways, whereas its downmodulation is related to cell proliferation stimulation and breast tumorigenesis.[30] Moreover, high levels of *p27* are expressed in normal human mammary epithelium, but loss of *p27* is frequent in breast cancer and has been demonstrated to have prognostic implications.[31] Also, in primary tumors, low levels of *p27* have been associated with reduced time to disease relapse and/or reduced overall patient survival.[32] It has been demonstrated[33] that only patients with tumors expressing high levels of *p27* benefited from a combined treatment with tamoxifen and goserelin. Based on these findings and our results, we can postulate that Maitake D-Fraction could be directly involved in the breast tumor cell growth arrest, acting as a tumor suppressor by stimulating the *p27/Kip1* gene expression. Also, we can hypothesize that Maitake D-Fraction could benefit patients who receive certain treatment by increasing the *p27/Kip1* gene expression (Fig. 3).

It is well known that the retinoblastoma binding protein 4 gene (*RBBP4*) encodes an integral component of transcriptional silencing corepressor complexes. It was found that *RBBP4*, among several cellular proteins, binds directly to the retinoblastoma protein to regulate cell proliferation, and also seems to be involved in transcriptional repression of *E2F*-responsive genes.[34] In our experiments, we found that Maitake D-Fraction (183 μg/mL) increases the expression (4.70-fold) of the *RBBP4* gene in MCF-7 cells after 24 h of treatment. We can suggest that Maitake D-Fraction inhibits tumor cell growth and proliferation pathways by overexpression of the *RBBP4* gene in breast cancer cells by blocking the *RB-E2F* protein complex formation (Fig. 3).

It has been demonstrated that *ICAM-3* expression can contribute to cancer progression by inducing cancer cell proliferation through the *PI3K/AKT* pathway.[35] In this study, we found that Maitake D-Fraction induces downmodulation of the *ICAM-3* gene (−1.53-fold) and downregulated *PI3K-AKT* signaling (as described earlier) in MCF-7 cells. Thus, we can suggest that Maitake D-Fraction could decrease activation of the AKT proliferative pathway by repression of *ICAM-3* gene expression (Fig. 4). However, the exact ICAM-3 molecular mechanisms remain unclear.

Many studies have demonstrated that *RASSF2* may function as a tumor suppressor gene in *in vitro* cell migration, cell cycle progression, and colony formation assays. Recently, it has been reported that *RASSF2* (isoform A) can function as a tumor suppressor gene *in vitro* inhibiting the growth of breast cancer cell lines in colony formation and soft agar growth assays, and *in vivo* inhibiting tumor formation when cells expressing *RASSF2A* were subcutaneously injected into SCID mice.[36] All together and in agreement with the literature, we found that Maitake D-Fraction significantly increased the expression of the *RASSF2* gene (4.62-fold) in MCF-7, and thus may be implicated in the suppression of tumor cell growth (Fig. 3).

Transforming growth factor-β (*TGF-β*) is probably the best-characterized antigrowth pathway. Acquired resistance to growth inhibition induced by TGF-β signaling is a characteristic of the early stages of many solid tumors.[37] SMAD proteins function as a transcriptional modulator activated by *TGF-β* and are thought to play a role in the regulation of carcinogenesis.[38] SMAD3 target genes in epithelial cells include *CDK* inhibitors that generate a cytostatic response.[39] Chen *et al.*[39] defined how SMAD3 can mediate transcriptional repression of the growth-promoting gene *c-Myc*. Although it has been suggested that SMAD7 blocks downstream signaling of *EGFR*, the role of SMAD7 in the *EGF* signaling pathway has not been fully elucidated. In our experiments, we found that Maitake treatment resulted in significant overexpression of the TGF-β Receptor II (2.80-fold) and its ligand *TGF-β3* (3.98-fold) in MCF-7 cells. Also, SMAD7 are upmodulated (5.92-fold) in these cells after Maitake treatment. We did not find SMAD3 significantly upmodulated after treatment. It is well known that the heterodimer SMAD3/SMAD7 is responsible for inducing the expression of the *CDK* inhibitor (*p27/KIP1*) directly involved in the arrest of cell cycle, which was detected after Maitake D-Fraction treatment. Also, these heterodimers are able to exert inhibition of *c-Myc* oncogene expression, a nuclear phosphoprotein that plays a role in cell cycle progression, suggesting a negative regulation of *TGF-β* in the cell proliferation. Recent work demonstrates that a phosphatase-dependent mechanism operates in the export of dephosphorylated SMAD2 out of the nucleus during termination of *TGF-β* signaling.[40] PPM1A has been identified as a SMAD2 phosphatase, which can promote nuclear export of SMAD2,[41] but the exact mechanism has not been elucidated. In our experiments, we found that the phosphatase proteins PPM1E, PPM1H, and PPM1K are downmodulated (−0.95-, −0.79-, and −0.80-fold, respectively), by Maitake D-Fraction treatment in MCF-7 cells; likewise, we observed downmodulation of *RanBP3* gene expression (−1.92-fold), a key component that mediates *Smad3/7* nuclear export, suggesting that the downstream signaling of *TGF-β* is not terminated under this stimulus, maintaining thus the tumor cell-growth and proliferation inhibited (Fig. 3). Moreover, other authors reported that *RanBP3* directly recognizes dephosphorylated *Smad2/3*, and mediates nuclear export of *Smad2/3* in a *Ran*-dependent manner.[42] Also, it has been reported that increased expression of *RanBP3* inhibits *TGF-β* signaling in mammalian cells and Xenopus embryos, but depletion of *RanBP3* expression or dominant negative inhibition of *RanBP3* enhances *TGF-β*–induced antiproliferative and transcriptional responses.[42] Altogether, and in agreement with the literature, we can conclude that Maitake D-Fraction significantly inhibits the tumor cell

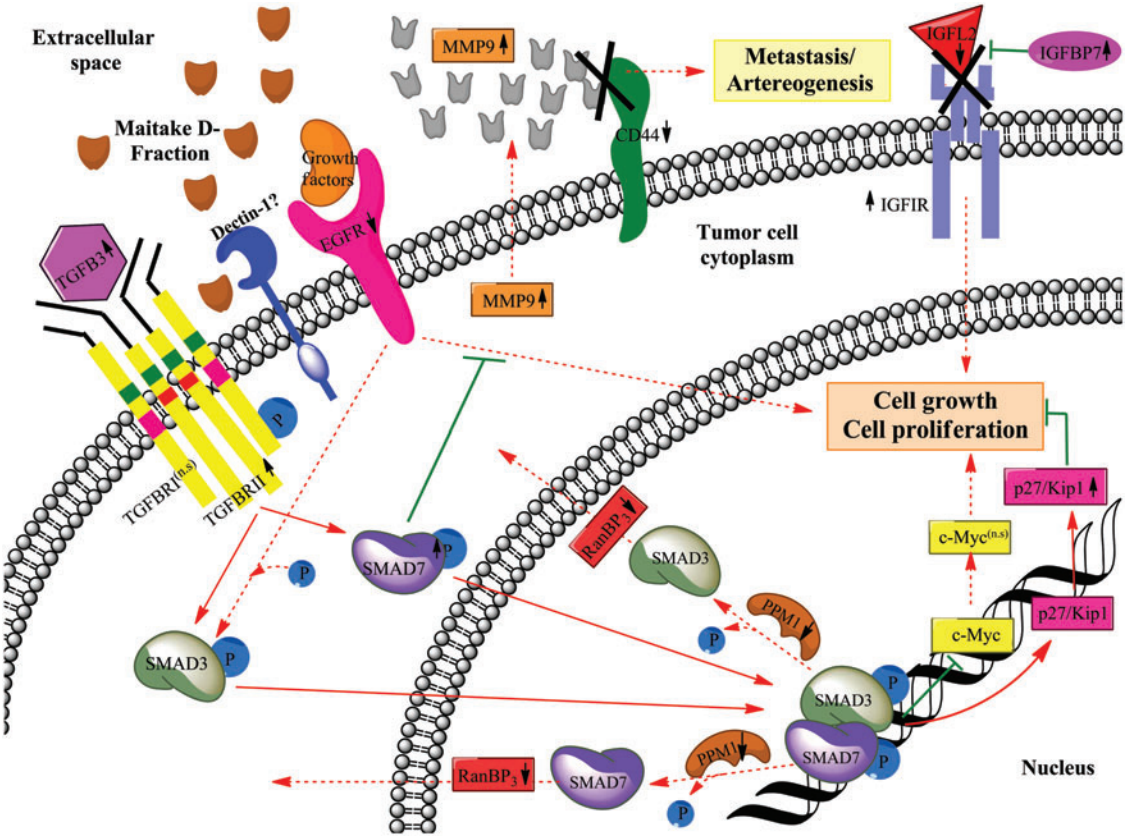

FIG. 5. Maitake D-Fraction blocks tumor cells growth and proliferation in MCF-7 cells through activation of *p27/Kip1* gene expression. The putative molecular mechanism of cell growth and proliferation inhibition by activation of *p27/Kip1* gene expression is shown; this process is stimulated by SMAD3 and SMAD7 proteins, which are activated after *TGFBRII* interaction. Stimulated (red) and inhibitory (green) pathways are indicated. Discontinuous gray lines represent the genes whose expression has not been changed after Maitake treatment. Color images available online at www.liebertpub.com/jmf

growth and proliferation through activation of *TGF-β* signaling (Fig. 5).

Insulin-like growth factor (*IGF*)–binding proteins (IGFBPs) are soluble proteins that bind *IGF*s with high affinity. Their principal functions are to regulate *IGF* availability in body fluids and tissues and to modulate IGF binding to its receptors. The *IGFBP* gene family plays a key role in regulating proliferation, differentiation, and apoptosis in different organ systems, including the human mammary gland. The principal mechanism of action of *IGFBP*s is believed to involve binding to and influencing the actions of *IGF*s, although *IGFBP*s may also affect cellular response through an *IGF*-independent pathway.[43] *IGFBP*s may prevent interaction between *IGF* and *IGF-IR* or, when anchored to the extracellular matrix or the cell surface, may act as a reservoir where they may localize and release *IGF*s to cell-surface receptors, thereby enhancing *IGF* action.[43] In agreement with that, in this study we found that the ligand *IGFL2* was downmodulated (−1.61-fold) after D-Fraction treatment in MCF-7 cells. Although IGFIR was upmodulated (4.31-fold), we have also found that *IGFBP-7* was upmodulated (3.67-fold), suggesting that the *IGFR* signaling pathway cannot be triggered after treatment, thus blocking cell growth and proliferation in these tumoral cells (Fig. 5). A study supports the role of *IGFBP-1*, *-3*, and *-7* as potential tumor suppressor genes in human breast cancer.[44] More recently, it has been demonstrated that *IGFBP-7* expression is inversely correlated with disease progression in breast cancer and is associated with poor outcome.[45] They showed that ectopic overexpression of *IGFBP-7* significantly reduced the growth of the *IGFBP-7*–transfected MDA-MB-468 cells compared to the parental MDA-MB-468 cells. The role of *IGFBP-7* on cell migration, a key determinant of malignant progression and metastasis was also checked.[45] These results suggest that the growth of breast cancer could be prevented by the forced expression of the IGFBP-7 protein.[45] Based upon these evidences and our results, we can suggest that Maitake D-Fraction induces the expression of the putative tumor suppressor gene *IGFBP-7* in breast cancer cells to reduce tumor cell proliferation and malignancy (Fig. 5). Also, in this work, we found that Maitake D-Fraction induces the overexpression of the *IGFBP-5* gene (5.21-fold) in MCF-7 cells (Fig. 6). Butt and colleagues have shown that *IGFBP-5* prevents the *in vitro* and *in vivo* growth of human breast cancer cells.[46] In their study, stable expression of *IGFBP-5* inhibited the growth of tumors derived from MDA-MB-231 cells by elevated *BAX* and decreased *BCL-2* at the mRNA level. Thus, we can suggest that

Maitake D-Fraction may be involved in tumor cell growth inhibition.

Caveolin 1 (*CAV1*) is greatly reduced in most oncogenically transformed and human cancer cells. It has been shown that the level of *CAV1* is correlated with mammary cancer progression *in vitro* and the overexpression of *CAV1* results in substantial growth inhibition of mammary tumor cells, which normally do not express endogenous *CAV1*.[47] More recently, it has been demonstrated that *CAV1* expression decreases the MCF-7 cell proliferation rate and markedly reduces their capacity to form colonies in soft agar.[48] In our experiments, we found that Maitake D-Fraction induces the expression of *CAV1* (2.91-fold) in MCF-7 cells after 24 h of treatment. Thus, and in concordance with the literature, we can suggest that D-Fraction inhibits cell growth and cell proliferation in breast cancer cells (Fig. 6).

Maitake D-Fraction downregulates pathway induced by NF-κB *(p105)*

NF-κB is a pleiotropic transcription factor that is present in almost all cell types. *NF-κB* regulates the expression of genes that play a key role in the development and progression of cancer, such as proliferation, migration, and apoptosis. It is well known that aberrant or constitutive *NF-κB* activation is present in many human malignancies.[49] p50 and p52 are DNA-binding members of the *NF-κB* family. They are synthesized as precursors of p105 and p100, respectively, which are proteolytically processed to generate active DNA-binding p50 and p52 peptides.[50] Also, it is well known that *NF-κB* complexes are held in the cytoplasm as inactive state complexes with members of the *NF-κB* inhibitor (*IκB*) family.[51] In a conventional activation pathway, *IκB* is phosphorylated by *IκB kinases* (*IKK*s) in response to different activators, subsequently degraded, thus liberating the active *NF-κB* complex, which translocates to the nucleus.[51] In the cytoplasm, *NF-κB* is inhibited by *IκB*. The upstream activating signal may cause phosphorylation of *IκB* by *IKK*. These trigger the degradation of *IκB* through the ubiquitin system, where the target molecule is masked by a chain of ubiquitins for degradation by the 26S proteasome. The free *NF-κB* can then translocate to the nucleus and activate transcription. In our experiment, we found that the expression of the *NF-κB* p105 subunit is induced (4.08-fold) by Maitake D-Fraction in MCF-7 cells; however, we did not find p50 or p52 subunits activated after treatment.

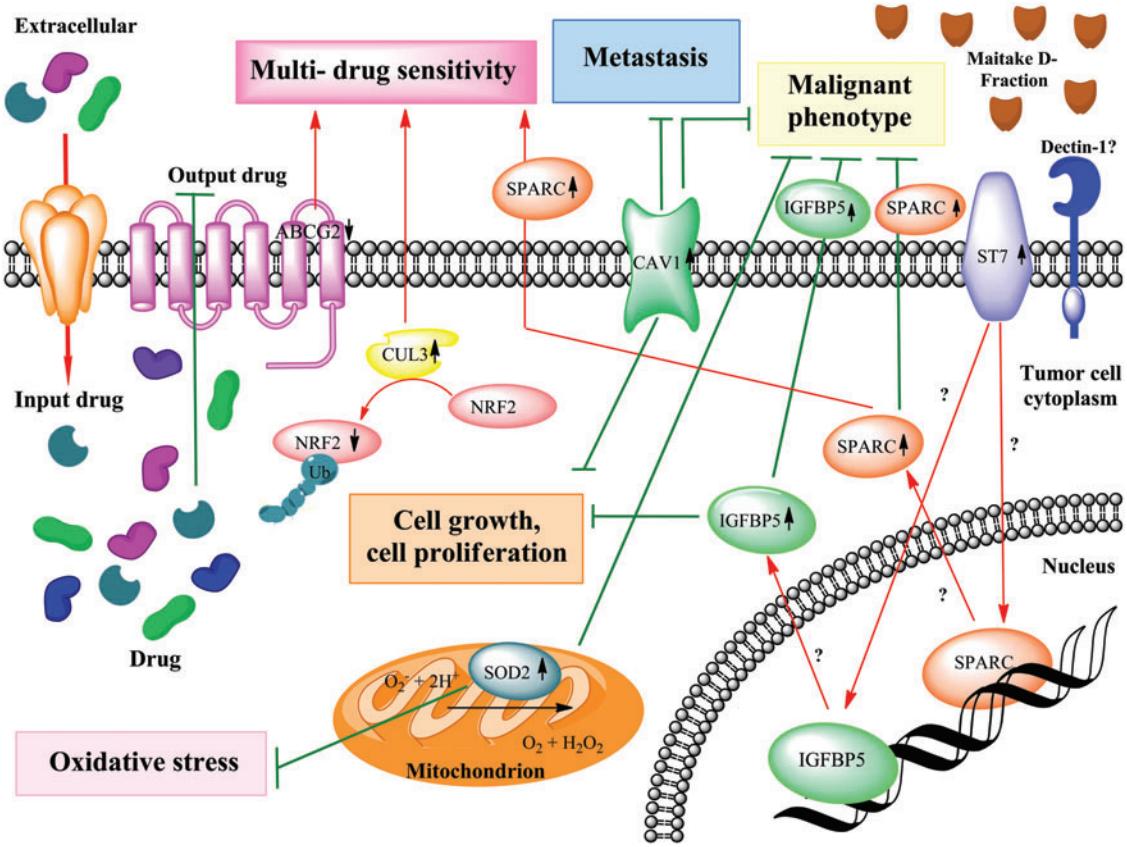

FIG. 6. Maitake D-fraction blocks malignant phenotype through activation of *SPARC* gene expression, and in collaboration with *CUL3*, they promote multi-drug sensitivity. The putative molecular mechanism of inhibition of malignant phenotype through activation of *SPARC* gene expression in MCF-7 cells is depicted. Also shown are the means by which *SOD2* blocks the oxidative stress in tumoral cells and *CUL3* participates in multi-drug sensitivity, blocking the output drug in those cells. Stimulated (red) and inhibitory (green) pathways are indicated. Discontinuous gray lines represent the genes whose expression has not been changed after Maitake treatment. Color images available online at www.liebertpub.com/jmf

Also, we found that Maitake D-Fraction significantly downmodulated (−1.82-fold) *IKKγ* gene expression, thus suggesting that active *NF-κB* DNA-binding peptides are not generated after treatment, thus avoiding *NF-κB* nuclear translocation, DNA binding, and transcription of factors involved in the regulation of a wide variety of biological responses (Fig. 4).

Maitake D-Fraction induces cell cycle arrest in MCF-7 cells

It has been reported that *SPARC* causes cell cycle arrest at G1 phase in endothelial cells[52,53] and in human colorectal cancer (MIP101 cells).[54] In the present study, we found that Maitake D-Fraction significantly induces expression of the *SPARC* gene (5.45-fold) in MCF-7 cells, probably involved in arresting the cell cycle of breast cancer cells (Fig. 4). However, the exact *SPARC* molecular mechanisms remain unclear. In addition, other studies show that the expression of *PTEN* induces a marked decrease of cell proliferation due to cell cycle arrest in the G1 phase, which is attributed to a *p27/Kip1* increase and *cyclin D1* nuclear localization.[55] In this work, we have observed that *PTEN* gene expression is upmodulated (3.27-fold) by Maitake D-Fraction treatment and thus, we can suggest that this compound induces cell cycle arrest through *p27/Kip1* activation (Fig. 4).

Maitake D-Fraction blocks tumor cell migration, cell invasion, metastasis, and arteriogenesis

In our work, we found that 187 μg/mL of Maitake D-Fraction increases (4.05-fold) the expression of the α_2 integrin (*ITGA2*) gene in tumoral MCF-7 cells after 24 h of treatment. It has been found that α_2 integrin was significantly reduced in breast cancer compared to normal breast tissue in the Richardson cohort ($P = .038$).[56] Not only was the loss of *ITGA2* gene expression highly associated with malignant disease, the decrement in α_2 integrin expression consistently correlated with disease progression in two different public data sets.[57,58] We can postulate that the over expression of α_2 integrin may serve as an important biomarker with antimetastasic potential and patient survival (Fig. 4). We also found that *ITGA9* was upmodulated (3.35-fold) after 183 μg/mL Maitake treatment. It was shown that *ITGA9* is expressed in normal human breast tissue,[59] while in breast cancer, *ITGA9* expression was down regulated or lost in 44% of tumors. Even more, it has been found by real-time PCR that the downregulation of tumor-suppressor genes *RASSF1A* and *ITGA9* was associated significantly with lung adenocarcinoma progression.[60] Based on these findings, we can hypothesize that overexpression of *ITGA9* and *ITGA2* could be involved in the antitumoral effect of Maitake D-Fraction in MCF-7 breast cancer cells. However, the exact mechanisms of its action are not yet elucidated (Fig. 4).

Likewise, it has been previously demonstrated that *MTSS1* plays an important role in cancer metastasis.[61] It has been reported that the overexpression of *MTSS1* significantly suppresses ($P < .01$) the invasive, migratory growth and adherence properties of a human breast cancer cell line.[61] In this work, we observed *MTSS1* gene expression upmodulated (2.28-fold) after Maitake D-Fraction treatment, suggesting its role as a metastasic suppressor in MCF-7 breast cancer cells. Based on these findings, *MTSS1* could be a prognostic indicator of disease-free survival in breast cancer patients. However, the biochemical mechanisms by which *MTSS1* functions in cells and the physiological role of the MTSS1 protein in humans remain largely unknown (Fig. 4).

The ICAM proteins appear to have a dual role in cancer. Two works found that *ICAM* molecules (particularly *ICAM-1*) may target and block tumor progression by stimulation of an immune response. Conversely, other investigations have shown that *ICAM* molecules are involved in cancer malignancy and their overexpressions are associated with poor diagnosis, lower survival rates, and metastasis in some cancers, including melanoma, breast cancer, and leukemia.[62] It has been demonstrated that overexpression of *ICAM-3* increased *AKT* phosphorylation, which caused an increase in cellular migration/invasion and *MMP* activities. Even more, it has been shown that *CREB* is involved in *ICAM-3*–induced cell migration and invasion-intermediating signaling from *Akt* to *MMP* proteins.[62] In this study, our results suggest that D-Fraction induces downmodulation of *ICAM-3* (−1.53-fold) at 91 μg/mL in MCF-7 cells and thus reinforces the decreased activation of the *AKT* pathway. Also, we found that *CREB* was downmodulated (Fig. 4). Thus, we suggest that D-Fraction blocks cancer cell migration/invasion via the *ICAM-3/AKT/CREB/MMP* pathway.

Another gene related to metastasis is *CD44*. It has been demonstrated that *CD44* promotes tumor growth and metastasis by mechanisms that remain poorly understood.[63] *CD44* was associated with a proteolytic form of the matrix metalloproteinase-9 (MMP-9) on the surface of mouse mammary carcinoma and human melanoma cells, promoting cell-mediated collagen IV degradation *in vitro* and mediating tumor cell invasion of G8 myoblast monolayers.[63] Also, it has been demonstrated that the disruption of *CD44*/*MMP-9* cluster formation, by overexpression of soluble or truncated cell surface *CD44*, inhibits tumor invasiveness *in vivo*.[64] In agreement with this, in our work, we observed that *CD44* gene expression is downmodulated (−1.47-fold) by Maitake D-Fraction treatment, suggesting that the MMP-9, which was found upmodulated (4.47-fold), cannot bind the *CD44* receptor in the cell surface. Based upon this, we can postulate that because of *CD44* downmodulation after Maitake treatment, the complex *CD44*/*MMP-9* cannot occur, thus blocking tumor cell survival, metastasis, and arteriogenesis in tumoral MCF-7 cells. Moreover, other authors reported severely impaired arteriogenesis in *CD44−/−* knockout mice, accompanied by reduced leukocyte trafficking to sites of collateral artery growth and reduced expression of *FGF2* (Fig. 5).[65]

Finally, in our experiments, we found that Maitake D-Fraction induces the expression of *CAV1* (2.91-fold) in MCF-7 cells after 24 h of treatment. Many *in vitro* studies have demonstrated that *CAV1* expression in cancer

epithelial cells controls tumor cell behavior and indicate that *CAV1* downregulation drives aggressive tumor growth. Also, exogenous *CAV1* expression in a highly metastatic breast cancer cell line suppresses primary tumor growth and spontaneous metastasis formation in an orthotopic mouse model.[66] In contrast, it has been reported that many solid tumors upregulate *CAV1* in epithelial cells, which were correlated with poor clinical outcome.[67] Also, it has been shown that *CAV1* expression is significantly associated with a basal-like phenotype and decreased progression-free and overall survival.[68] Conversely, an association between *CAV1* overexpression and a favorable clinical outcome has been reported by fewer studies. It has been shown as clinical and mechanistic evidence that *CAV1* is a critical target for suppression by Stat3 in driving invasion and metastasis of breast cancer cells.[69] In our experiments, we found that Maitake D-Fraction induces the expression of *CAV1* (2.91-fold) in MCF-7 cells after 24 h of treatment (Fig. 6). However, more research must be done to reconcile these discrepancies between the extensive evidence showing the tumor-suppressor functions of *CAV1* and the clinical data showing high *CAV1* levels in human tumors.

Maitake D-Fraction induces multidrug sensitivity in MCF-7 cells

Multidrug resistance is a major obstacle to success in cancer treatment. One mechanism by which cells can become resistant to chemotherapy is the expression of *ABC* transporters that use the energy of ATP hydrolysis to transport a wide variety of substrates across the cell membrane.[70] The ATP-binding cassette transporter breast cancer resistance protein (ABCG2 or BCRP) is apparently a major determinant of multidrug resistance phenotype of tumors by extruding chemically diverse cytostatic drugs out of tumor cells. Thus, *ABCG2* is a xenobiotic transporter that appears to play a major role in the multidrug resistance phenotype of a specific human breast cancer.[71] In our experiments, we found that Maitake D-Fraction treatment reduces the expression of *ABCG2* (−0.52-fold) in MCF-7 cells, suggesting that this compound could be a potential agent for multidrug sensitivity in breast cancer cells. In conclusion, cancer cells that do not express multidrug transporters are sensitive to chemotherapy and are eliminated in the course of chemotherapy (Fig. 6).

It has been demonstrated that low expression of *SPARC* was involved in colorectal cancer resistance to chemotherapy.[72] Moreover, treatment of mice with *SPARC* conferred increased sensitivity to chemotherapy. These authors concluded that *SPARC*-based gene or protein therapy may ameliorate the emergence of resistant clones and eradicate existing refractory clones and offers a novel approach to treating cancer.[72] Recently, it has been identified that an *NT*-domain of *SPARC* and its 51-aa peptide are highly efficient in modulating and enhancing apoptosis, thereby conferring greater chemosensitivity to resistant tumors.[73] Also, it has been found that *SPARC* overexpression is a functionally important feature of a subset of triple-negative breast cancer patients. This study suggests that nab-paclitaxel may serve as a therapeutic agent for the subset of triple-negative patients that overexpress *SPARC*.[74] In the present study, we found that Maitake D-Fraction significantly induces the overexpression of the *SPARC* (5.45-fold) gene in MCF-7 cells to probably increase the sensitivity to chemotherapy in these breast cancer cells (Fig. 6).

Also, overexpression of *ICAM-3* was reported to increase the resistance of cancer cells to radiation by the activation of focal adhesion kinase (*FAK*).[75] In this study, we found that Maitake D-Fraction induces downmodulation of *ICAM-3* (−1.53-fold) and *FAK* (−1.40-fold) genes at 91 μg/mL in MCF-7 cells. Thus, we can suggest that these could be the mechanism by which D-Fraction decreases resistance to radiation in MCF-7 cancer cells (Fig. 4).

It has been demonstrated that simultaneous loss of *CUL3* and *p53* in hepatic progenitors turned these cells into highly malignant tumor-initiating cells, which formed largely undifferentiated tumors in nude mice. In addition, loss of *CUL3* and *p53* led to the formation of primary hepatocellular carcinomas.[76] Importantly, loss of *CUL3* expression was also detected in a large series of human liver cancers and correlated directly with tumor dedifferentiation. The overexpression of *CUL3* during hepatic differentiation therefore safeguards against transformation to progenitory cells.[76] Here we found that Maitake D-Fraction significantly induces the overexpression (4.56-fold) of the *CUL3* gene in MCF-7 cells after 24 h of treatment. It has been previously demonstrated that *Keap1* and the *CUL3* E3 ligase are responsible to maintain *NRF2* (*GA* binding protein transcription factor, alpha subunit 60 kDa) at very low concentrations by proteasomal degradation, a key transcription factor for cytoprotective gene programs.[77] Therefore, high levels of *CUL3*/low *NRF2* signature may be the key to cellular sensitivity to both chemical carcinogenic stimuli as well as to cytotoxicity of commonly used chemotherapeutic drugs in established breast cancers.[77] Moreover, knocking down *CUL3* constitutively upregulates the *NRF2* as well as multiple carcinogen-detoxifying genes.[77] Altogether, our data show that Maitake D-Fraction induces the overexpression of the *CUL3* gene and downmodulates the *NRF2* (−0.76-fold) gene, presenting a potential new target for breast cancer chemoprevention and treatment (Fig. 6).[78]

Maitake D-Fraction reverts the malignant phenotype MCF-7 cells

It has been demonstrated that the suppression of tumorigenicity 7 (*ST7*) gene functions as a tumor suppressor in both prostate and breast cancer cells.[79] A role for *ST7* in breast carcinogenesis is further supported by the finding that it is downregulated in a large proportion of primary breast cancers. These data, together with the observation that LOH at 7q31 is common in many tumor types, support the proposal that *ST7* may behave as a multitissue tumor suppressor.[79] Expression profiling of *PC-3* cells revealed that *ST7* predominantly induces changes in genes involved in remodeling the extracellular matrix such as *SPARC*, *IGFBP5*, and several matrix metalloproteinase.[79] In this study, we found that ST7 was upmodulated (3.62-fold) after D-Fraction treatment in

MCF-7 cells. The present data indicate that Maitake D-Fraction may mediate tumor suppression through *ST7* gene expression (Fig. 6). However, the specific mechanism through which *ST7* mediates tumor suppression remains unclear. Further studies of the function of *ST7* should be done in the tumor suppression pathway. In relation with *ST7*, in this work, we found that Maitake D-Fraction induces upmodulation of *IGFBP5* and *SPARC* genes in MCF-7 cells. *IGFBP*s are traditionally known as carrier proteins that regulate the activity of *IGF*s by prolonging their half-life and their circulation turnover. Importantly, there is increasing evidence that *IGFBP-2*, *IGFBP-3*, and *IGFBP-5* are important players in the phenotypes of various cancers.[80] Studies have supported the notion that *IGFBP-5* overexpression contributes to metastasis and poor prognosis of cancer.[81] However, recent studies have demonstrated that *IGFBP-5* may regulate the metastatic capacity through protein–protein interactions in downstream signal transduction pathways that are activated in breast cancer: *MAPK* and *PI3K/AKT* pathways.[80] In this work, we found that Maitake D-Fraction induces the overexpression of the *IGFBP5* gene (5.21-fold) in MCF-7 cells, which may be involved in the control of malignant phenotypes (Fig. 6). However, the exact role of this gene is not yet fully understood.

Likewise, it has been reported that in ovarian cancer, lower *SPARC* expression is noted in advanced tumors,[82] and studies using *SPARC–/–* animals reveal that loss of *SPARC* enhances growth of tumor xenografts of pancreatic and lung cancers.[83,84] The implication that loss of *SPARC* expression promotes tumor growth is supported by observations that it is downregulated following malignant transformation in ovarian cancers[82] and that upregulation of *SPARC* in these cells results in the development of nontumorigenic clones, while downregulation of *SPARC* results in more aggressive phenotypes.[85] In the present study, we found that Maitake D-Fraction significantly induces the expression of the *SPARC* gene (5.45-fold) in MCF-7 cells to probably revert the malignant phenotype (Fig. 6).

Also, in our experiments, we found that Maitake D-Fraction significantly increases (3.94-fold) the expression of the *SOD2* gene in MCF-7 cells after 24 h of treatment. It has been found that overexpression of *SOD2* (Mn superoxide dismutase) in pancreatic tumoral cells can significantly attenuate the malignant phenotype, by decreasing cell growth, plating efficiency, and growth in soft agar.[86] Moreover, it has been found that overexpression of *SOD2* in melanoma cells can alter the phenotype in culture; the cells lose the ability to form colonies, a trait characteristic of malignant cells.[87] Recently, it has been demonstrated that the expression of *SOD2* in neoplasm tissues, independent of the clinicopathologic characters, plays a critical role in breast cancer biology.[88] Thus, we can suggest that Maitake D-Fraction could be suppressing the malignant phenotype of MCF-7 cells by overexpression of *SOD2* (Fig. 6).

Finally, in this work, we have observed that *PTEN* gene expression is upmodulated (3.27-fold) by Maitake D-Fraction treatment. *PTEN* is a tumor suppressor gene and was found mutated in multiple cancers. It has been published that PTEN protein functions as a negative regulator of the *PI3K/Akt* oncogenic pathway. Reduced *PTEN* expression and the deregulated *PI3K/Akt* pathway were associated with aggressive breast cancer phenotypes and poor outcome of the disease.[89] Furthermore, tumors with reduced *PTEN* protein expression have been shown to carry a particular gene expression signature that predicts worse outcome and metastasis in breast cancer as well as in prostate and bladder carcinomas.[90] In conclusion, here we can hypothesize that Maitake D-Fraction is able to reduce the aggressive breast cancer phenotype by activating the *PTEN* gene in MCF-7 cells (Fig. 4).

Maitake D-Fraction reduces the oxidative stress in MCF-7 cells

Antioxidant enzymes maintain cellular redox homeostasis. *SOD2*, an enzyme located in mitochondria, is the key enzyme that protects the energy-generating mitochondria from oxidative damage. It has been found that levels of *SOD2* are reduced in many diseases, including cancer, neurodegenerative diseases, and psoriasis.[87] Moreover, it has been demonstrated that *SOD* expression played a critical role in free radical detoxification and it is directly correlated with the cell cycle, defining one of the most important characteristics of tumor cells, namely, cell growth and proliferation.[91] Recently, Sotgia *et al.*[92] postulated a possible mechanism by which mitochondrial oxidative stress contributes to tumor initiation and progression: ''[M]itochondrial oxidative stress in epithelial cancer cells leads to ROS production and ensuing DNA damage, resulting in an increased mutation rate and tumor evolution, via the positive selection of tumor cell mutations that confer a growth advantage.''[92] In support of this notion, in our experiments, we found that Maitake D-Fraction significantly increases (3.94-fold) the expression of the *SOD2* gene in MCF-7 cells after 24 h of treatment. These findings are in agreement with the hypothesis that *SOD2* could play a role as a putative tumor suppressor gene and negative regulation of oxidative stress in these cells (Fig. 6).

Maitake D-Fraction reduces general pain through inhibition of HCN gene in MCF-7 cells

The nonselective cation channel *HCN1* is a novel molecular target for the relief of pain, and has a relatively unexplored pharmacology. Development of a high-throughput compatible functional assay designed to detect antagonists of *HCN1* has been demonstrated.[93] As such, *HCN* channels may represent valid targets for novel analgesic agents. In the present work, we found that the *HCN1* gene was downmodulated (−0.65-fold) after Maitake D-Fraction treatment in breast cancer cells. The exact molecular mechanisms of *HCN1* in pain relief are not yet elucidated. However, screening studies for novel *HCN* channel blockers may be useful for the treatment of chronic pain in cancer patients. In agreement with that, we also observed in our *in vivo* experiments that 5 mg/kg/day of Maitake D-Fraction significantly reduces general pain in BALBc mice with mammary tumors after 15 days of i.p.

injection (data non shown). To demonstrate if the down-modulation of the *HCN1* gene is involved in the oncologic pain reduction in mice after Maitake treatment, we will measure the expression of *HCN1* by RT-PCR assay.

In conclusion, it is widely agreed that the tumoral phenotype is characterized to uncontrolled cell proliferation. The growth ability of tumor cells could be related not only to an abnormal signaling proliferative, but also to the inability of cancer cells to activate apoptosis. Here we showed that Maitake D-Fraction treatment blocks tumor cell growth and proliferation pathways in MCF-7 cells (Supplementary Fig. S1). Moreover, the present study showed that Maitake treatment increases proapoptotic gene activation, which will lead to cell death, while suppressing activation of genes that block apoptotic pathways in breast cancer MCF-7 cells. Also, tumor cells are characterized by invasion to surrounding tissues and metastatic spread to distant sites. Our results show that Maitake D-Fraction treatment plays a role in governing the metastatic nature on breast cancer MCF-7 cells by inducing the overexpression of *ITGA9*, *ITGA2*, *MTSS1*, *CAV1*, and blocking the *ICAM-3/AKT/CREB/MMP* pathway (Supplementary Fig. S1). The resistance of cancer cells to radiotherapy and chemotherapy is a major problem of these standard protocols; given that a select cell population may carry advantageous mutations, the standard treatments may block tumorigenesis only temporarily, ultimately making tumors no longer responsive to treatment. Our results show that Maitake D-Fraction could be a potential agent for multidrug sensitivity in breast cancer cells, promoting the elimination of the tumor cells during chemotherapy (Supplementary Fig. S1).

Our data support the concept that Maitake D-Fraction can influence the switching on and off of genes expressed in human breast cancer MCF-7 cells, and thus could be able to control the breast cancer phenotype and even could be able to revert the aggressive/malignant phenotype by the molecular mechanisms postulated. Here we have shown how a compound derived from an edible mushroom has the potential to activate expression of certain genes that could affect the malignant cell phenotype, avoiding their aggressiveness and invasion, affecting their sensitivity to chemotherapy, inducing their apoptosis, and probably involved in the reversion of the malignant phenotype (Supplementary Fig. S1). This work leaves a wide-open door to begin investigating each of the pathways shown here one by one and demonstrates how a nutrigenomic agent can be transformed into a therapeutic agent for breast cancer disease.

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AUTHOR DISCLOSURE STATEMENT

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