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# Farnesol, a Fungal Quorum-Sensing Molecule Triggers Apoptosis in Human Oral Squamous Carcinoma Cells[1]

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## Abstract

Farnesol is a catabolite within the isoprenoid/cholesterol pathway that has exhibited significant antitumor activity. Farnesol was recently identified as a quorum-sensing molecule produced by the fungal pathogen *Candida albicans*. In this study, we hypothesize that synthetic and *Candida*-produced farnesol can induce apoptosis *in vitro* in oral squamous cell carcinoma (OSCC) lines. Cell proliferation, apoptosis, mitochondrial degradation, and survivin and caspase expressions were examined. In addition, global protein expression profiles were analyzed using proteomic analysis. Results demonstrated significant decrease in proliferation and increase in apoptosis in cells exposed to farnesol and *C. albicans* culture media. Concurrently, protein expression analysis demonstrated a significant decrease in survivin and an increase in cleaved-caspase expression, whereas fluorescent microscopy revealed the presence of active caspases with mitochondrial degradation in exposed cells. A total of 36 differentially expressed proteins were identified by proteomic analysis. Among the 26 up-regulated proteins were those involved in the inhibition of carcinogenesis, proliferation suppression, and aging. Most notable among the 10 down-regulated proteins were those involved in the inhibition of apoptosis and proteins overexpressed in epithelial carcinomas. This study demonstrates that farnesol significantly inhibits the proliferation of OSCCs and promotes apoptosis *in vitro* through both the intrinsic and extrinsic apoptotic signaling pathways. In addition, we report for the first time the ability of *Candida*-produced farnesol to induce a similar apoptotic response through the same pathways. The capability of farnesol to trigger apoptosis in cancer cells makes it a potential tool for studying tumor progression and an attractive candidate as a therapeutic agent.

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## Introduction

Apoptosis, or programmed cell death, is a gene-controlled, tightly regulated process, which occurs naturally during embryogenesis and in development and is used to control the proliferation of undesired or genetically altered mammalian cells without the induction of an inflammatory response [1,2]. The inhibition or deregulation of this process, combined with altered cell proliferation, provides the basis for many cancerous diseases such as oral squamous cell carcinoma (OSCC), accounting for more than 90% of head and neck cancers, with an estimated 35,310 new cases and 7590 deaths expected in 2008 in the United States alone [3].

In mammalian cells, apoptotic cell death can be triggered by many different extracellular and intracellular stimuli resulting in the activation of extrinsic and intrinsic apoptotic signaling pathways involving both the death domain pathways and the mitochondria, as well as various downstream caspases [4,5]. Mitochondrial degradation and fragmentation of the nuclear genome are major cellular responses

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characteristic of apoptosis conducted by multiple nucleases activated by apoptotic signaling pathways [4,5].

Caspases are a class of cysteine–aspartic acid proteases regulated at the posttranslational level, which, when cleaved, convey an apoptotic signal in a proteolytic cascade that induces apoptosis and leads to cell death [4,5]. Caspase 3, in particular, is the major effector caspase in apoptotic cells, which, on activation, is cleaved into a large subunit and a smaller subunit resulting in a pivotal event in the execution of apoptosis [4,5].

Survivin, a member of the inhibitor of apoptosis family of proteins, is shown to be associated with carcinogenesis including oral, tonsillar, esophageal, and laryngeal OSCC, and its expression is correlated with an adverse prognosis and poor survival rates [2,6,7]. In addition, survivin has been implicated in the regulation of both the extrinsic and intrinsic apoptotic signaling pathways, interacting directly and indirectly with caspase 3 and 9 [4].

Among the recently identified extracellular stimuli of apoptosis in human tumor cells is farnesol, a naturally available sesquiterpene alcohol and a key intermediate in *de novo* synthesis of cholesterol in all mammalian cells [8–11]. Interestingly, although farnesol was shown to induce apoptosis in different types of human cancer cells, it seems to preferentially induce apoptosis in neoplastic cells *versus* nonmalignant cell [8–12].

Studies on the apoptotic effects of farnesol on certain human tumor cells demonstrated that the addition of farnesol resulted in the rapid inhibition of phosphatidylcholine (PC) synthesis due to the inhibition at the cholinephosphotransferase step [1]. Phosphatidylcholine is the most abundant lipid present in eukaryotic cell membranes comprising ~50% of cellular phospholipid mass. Diacylglycerol (DAG), a protein kinase C activator, is a lipid second messenger that acts as a signaling molecule and regulates cell growth and apoptosis [1]. In those studies, the exogenous administration of DAG significantly reduced farnesol-induced apoptosis but did not rescue PC synthesis [1]. It was concluded from these observations that farnesol-induced apoptosis is likely due to farnesol's activation of a DAG-mediated process that results in the induction of apoptosis or inhibition of a DAG-dependent process that is required for cell proliferation [11].

In addition to its identification as an inducer of apoptosis in mammalian tumor cells, farnesol was recently identified as a quorum-sensing molecule produced and extracellularly secreted by the most important human fungal pathogen *Candida albicans* [13,14]. In *C. albicans*, farnesol is endogenously generated in the cell by enzymatic dephosphorylation of farnesyl diphosphate, a precursor for the synthesis of sterols in the sterol biosynthesis pathway [12,15].

*Candida albicans* is a dimorphic species capable of changing its morphology from the yeast form to the hyphal form, a transition critical to its pathogenesis, which is triggered by quorum-sensing molecules [13,14]. In addition to its involvement in morphogenesis, quorum sensing is a strategy of cell-cell communication benefiting a microbial community by controlling unnecessary overpopulation with important implications for the infectious process.

The involvement of quorum sensing in *C. albicans* morphogenesis was demonstrated by us and others through the recent identification of farnesol, where both exogenous and *Candida*-produced farnesol was shown to suppress hyphal formation [13,14]. Our studies, however, also demonstrated that farnesol is increasingly produced by *Candida* in culture in a manner proportional to cell density and age of culture; and at certain concentrations, is capable of killing the fungal cell [15]. Therefore, although the phenomenon of apoptosis in *C. albicans* has not been previously investigated, it is conceivable that *C. albicans* may use farnesol as a strategy for programmed cell death to control cell proliferation. The hypothesis of this current study was to determine the existence of a *Candida*-induced apoptotic mechanism on human OSCCs by way of farnesol. Specifically, using primary OSCC lines as a model, this study was designed to characterize a farnesol-induced apoptotic axis and to determine whether the *C. albicans*–secreted quorum-sensing molecule, identified as farnesol, triggers apoptosis. Further, we sought to identify potential pathways and proteins involved in this process.

Advances in proteomic technology offer great promise in the understanding and treatment of the molecular basis of disease. Specifically, the study of dynamic protein expression has culminated in the identification of many disease-related biomarkers and potential new drug targets. Therefore, in addition to the assessment of classic apoptotic markers in human tumor cells, a global proteomic approach was used to elucidate the mechanisms underlying the antitumor activity of farnesol and to unravel altered protein expression after farnesol treatment.

## Materials and Methods

### *Cell Lines and Cell Cultures*

All experiments were performed using two established primary human tongue squamous cell carcinoma cell lines (OSCC 9 and OSCC 25) from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in 1:1 mix of Ham's F12 and Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 U of penicillin, 100 μg/ml streptomycin, and 0.4 g/ml hydrocortisone (Sigma Chemical Co., St. Louis, MO). The cells were cultured at 37°C in a 5% $CO_2$ air atmosphere until confluent and subcultured using a disaggregration assay with Trypsin (0.1%) and EDTA (0.01%) in phosphate-buffered saline (PBS) pH 7.5. For all experiments, cells were grown in 6- or 24-well plates at $5 \times 10^4$ cells per well and grown to 80% confluence. Control cells for all experiments were treated with the vehicle alone (0.1% methanol) in normal medium (NM). All experiments were performed on three separate occasions.

*Candida albicans* SC5314 and a non–farnesol-producing strain *C. albicans* (ATCC 10231) [16] were maintained on Sabouraud dextrose agar (BBL, Cockeysville, MD) and cultured in YNB [0.67% yeast nitrogen base (pH 7.0), 50 mM glucose]. Cultures were grown overnight in an orbital shaker at 30°C under aerobic conditions. Cells were harvested and washed twice in sterile PBS. To obtain *C. albicans* spent culture medium (CA), *C. albicans* biofilms were formed on the surfaces of 75-$cm^2$ tissue culture flasks in RPMI-1640 supplemented with L-glutamine and buffered with HEPES (Invitrogen, Grand Island, NY) to a cell density of $1 \times 10^6$ cells/ml and grown for 48 and 72 hours. Culture supernatants were then collected, filter-sterilized, and diluted 1:1 with fresh RPMI-1640 [13]. In all experiments, fresh media were included as negative controls.

### *Farnesol Treatment of OSCCs*

Farnesol (Sigma) was obtained as a 3-M stock solution and diluted to a 30-mM solution in 0.1% methanol. Previous experiments had shown that methanol did not affect cell viability at the concentration used in these experiments [13]. Cells grown were then either left

untreated or treated with synthetic farnesol at 30- and 60-μM final concentrations. Similarly, cells were also grown in the sterilized *C. albicans* spent CA from both the farnesol-producing and non–farnesol-producing strains of *C. albicans*. Simultaneously, experiments were performed as described above with a DAG analog (1,2-dioctanyl-*sn*-glycerol; Sigma) added, and plates were incubated for 48 hours.

### *Cell Proliferation*

For cell proliferation assays, cells were treated with increasing concentrations (10, 30, 50, and 60 μM) of synthetic farnesol to determine $IC_{50}$. For all other experiments, cells were treated with 30 or 60 μM farnesol or with conditioned media from farnesol- or non–farnesol-producing *C. albicans* as described above for 48 hours. The cells were removed enzymatically and counted using a Coulter Counter (Model ZI; Coulter, Miami, FL). The percent of cell growth was determined by setting as 100% the growth of cells treated with the vehicle alone (NM; 0.1% methanol) and calculating all others to this level. All analyses were performed in triplicate.

### *Flow Cytometry (Apoptosis Analysis)*

Following the same treatments as described above, apoptosis was evaluated using Annexin V–FITC methods. Cells were washed with PBS, followed by lysis using Trypsin (0.1%) and EDTA (0.01%) in PBS at pH 7.5. The cells were washed with normal medium and cold PBS and resuspended in 1× binding buffer (BD-Pharmingen Biosciences, San Diego, CA). Five microliters of Annexin and 5 μl propidium iodide were added to cells, vortexed, and incubated for 15 minutes in the dark. Finally, 400 μl of 1× binding buffer was added, and samples were evaluated by flow cytometry. The percent of apoptosis was determined by setting as 0% the apoptosis of cells treated with the vehicle alone (NM; 0.1% methanol) and calculating all others to this level. All analyses were performed in triplicate.

### *Western Blot*

Following the same treatments as described above, cells were incubated and washed twice with ice-cold PBS, followed by lysis using radioimmunoprecipitation assay buffer [50 μM Tris (pH 7.4), 150 μM NaCl, 1% Triton X-100, 1% deoxycholic acid, 150 μM sodium salt, 0.1% SDS, 100 μg/ml phenylmethysulfonyl flouride, 1 μg/ml aprotinin, 1 mM dithiothreitol (DTT), 1 mM sodium orthovanadate] for 10 minutes at 4°C. The wells were then scraped, and cells were recovered and centrifuged at 40,000*g* for 15 minutes at 4°C. The recovered proteins were measured and equalized using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA) per manufacturer's instructions and were electrophoresed on a 15% acrylamide gel. Western blot analysis was performed using a 1:1000 dilution of monoclonal primary antibodies for survivin (Abcam, Cambridge, UK), cleaved-caspase 3 (Cell Signaling, Beverly, MA), and cleaved-caspase 9 (Cell Signaling), with β-actin (Sigma) used as a loading control. Blots were processed with lumiGLO (KPL, Upstate, Waltham, MA) exposed for radiographic imaging, then stripped, and reprobed with β-actin antibody. All analyses were performed in triplicate.

### *Confocal Laser Microscopy*

Active caspases were detected microscopically using FLICA Apoptosis Detection Kits (Immunochemistry Technologies, LLC, Bloomington, MN) according to manufacturer's recommendation. Apoptotic cells fluoresce green, whereas nonapoptotic cells appear unstained. Similarly, mitochondrial degradation was evaluated using Mitochondrial Permeability Transition Detection Kit MitoPT 924 (Immunochemistry Technologies) according to the manufacturer's recommendation. Apoptotic cells treated with MitoPT reagent appear green; nonapoptotic cells appear red. Processed samples were observed with a confocal microscope (with video capture system, automatic camera, and image analysis hardware software; Axiovert 100; Zeiss, Berlin, Germany) using 20×, 40×, and 100× oil immersion objectives. Confocal images of green and red fluorescence were collected simultaneously using the Z-stack mode. Imaging of stained cells were accomplished by using a protocol with an excitation wavelength of 488 nm and emission peaks at 528 and 617 nm for green and red, respectively. Images were processed for display by using Axiovision 3.x software (Zeiss).

### *Reversal of Apoptosis by Exogenous DAG*

Cell proliferation and caspase protein expression assays were also performed in the presence of DAG. Cells were grown with synthetic farnesol (0, 30, or 60 μM) and in *C. albicans* spent culture media as previously described in the presence and absence of 30 μg/ml DAG analog for 24 hours.

### *Proteomic Analysis*

Two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI–ToF MS) were used to compare the proteomes of treated *versus* untreated OSCCs as described in previous studies [17–20].

### *Reagents*

Urea, thiourea, trichloroacetic acid, iodoacetamide, and phenylmethysulfonyl flouride were obtained from Sigma-Aldrich Chemical Inc. (St. Louis, MO); the Immobiline Dry Strips (pH 3-10 nonlinear), Pharmalytes (3-10), DTT, 3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid, the Multiphor II isoelectric focuser, and Höefer DALT Vertical System were obtained from Amersham Biosciences (Piscataway, NJ).

### *Two-dimensional Gel Electrophoresis*

Two-dimensional gel electrophoresis was conducted as outlined by Gorg et al. [21]. Cytosolic proteins were extracted from cell lines as previously described. To accomplish rehydration of the protein, 500 μg of crude protein was extracted by adding a 1/10 volume of an ice-cold 1:10 mixture of trichloroacetic acid and acetone (Sigma). The resulting pellet was then directly solubilized in rehydration buffer (0.1 mM urea, 25 μM thiourea, 0.35 μM DTT, 0.5% w/v CHAPS, and 1.6% Pharmalyte 3-10). These samples were applied to 18-cm, pH 3-10, NL Immobiline Dry-Strips (GE Healthcare, Piscataway, NJ). Isoelectric focusing, which separated the proteins based on their p*I*, was performed using a Multiphor II from Amersham (Amersham Biosciences) as per manufacturer's directions. Before the second dimension, the immobilized pH gradient strips were equilibrated (as per manufacturer's directions) and subsequently applied to SDS-PAGE gels. For the resolution of crude protein extracts in the second dimension, a 26 × 20-$cm^2$ two-dimensional gel system from Höefer DALT Vertical System (Amersham Biosciences) was used. Crude protein extracts were separated at 10°C on an 11% resolving gel, which was then nondestructively silver-stained. As previously mentioned, each treatment was performed in three independent

replicates. In addition, duplicate runs were performed on each replicate to ensure the accuracy of analyses. Stained gels were scanned (300-dpi resolution), and gel images were analyzed with PDQuest version 7.0 (Bio-Rad Laboratories). Differentially expressed proteins were selected for identification. Spots of interest were excised and subjected to trypsinization.

### *MALDI–ToF MS Analysis*

Only those spots which changed consistently and significantly (>1.5-fold) were selected for analysis by mass spectrometry (MS). Spots of interest were excised and subjected to trypsinization. Peptides were extracted and analyzed using MALDI–ToF MS. An Applied Biosystems Voyager-DE STR MALDI–ToF MS was used, functioning in a positive-ion mode with α-cyano-4-hydroxycinnamic acid matrix for ionization. At least 100 laser shots per spectrum were averaged. Mass spectral peaks with a signal-to-noise ratio greater than 5:1 were deisotoped, and the resulting monoisotopic masses were used for protein identification using mass fingerprint analysis. The software used for protein identification was the Profound search engine using the Genomic Solution's Knexus software (ver 2004.03.15), and the database used was the latest NCBI nonredundant database obtained from the National Institutes of Health.

### *Statistical Analysis*

For all measurements as needed, a Student's *t* test was used to assess the statistical significance of treated groups *versus* control groups along with SE. A statistically significant difference was considered to be present at $P < .05$. Protein spots were considered to represent differentially expressed proteins if they were up- or down-regulated ≥1.5-fold in three independent experiments for two-dimensional gel electrophoresis experiments. The protein spots that were identified as differentially expressed were positively identified by MALDI–ToF MS and database comparison if they received an expectation score of $1 \times 10^{-3}$ or less.

## Results

Results from all experiments performed were consistent for both cell lines, and therefore, only data from one of the cell lines used (OSCC 9) are presented.

### *Farnesol-Induced Cell Growth Inhibition and Apoptosis*

*Determination of cell proliferation.* Cell proliferation data indicated that exposure to 30 to 60 μM synthetic farnesol (F30 or F60) for 48 hours resulted in a significant 56.25% and 53.7% decrease in proliferation of OSCCs compared to vehicle only–treated cells (NM), respectively ($P < .05$; Figure 1*A*). Similarly, exposure to *C. albicans* spent culture media grown for 48 or 72 hours (CA48h or CA72h) and treated for 48 hours on OSCCs decreased cell proliferation significantly by 30% to 35% ($P < .05$; Figure 1*B*). Interestingly, no significant differences in proliferation were observed between the different *C. albicans* growth culture incubation periods (CA48h or CA72h) or between farnesol 30- and 60-μM treatments ($P < .05$). Further, exposure to spent culture media of the *C. albicans* non–farnesol-producing strain cultured at 48 or 72 hours (NF48h or NF72h) and treated for 48 hours on OSCCs did not affect cell proliferation (Figure 1*B*).

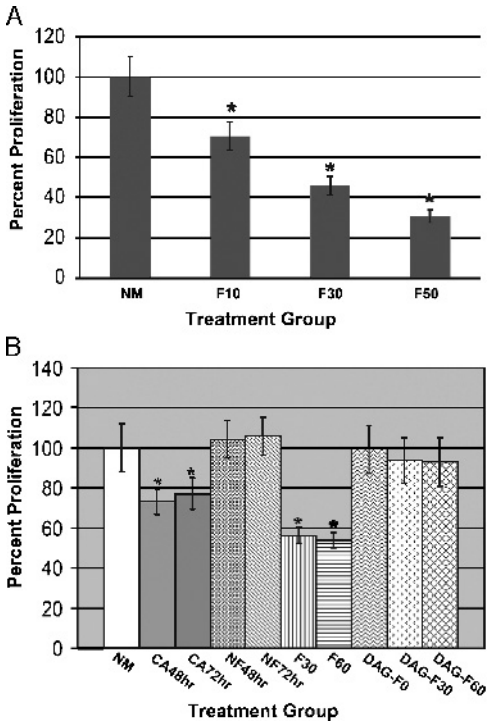



**Figure 1.** Cell proliferation assay: Assessment of OSCC 9 cell proliferation, all after 48 hours of treatment compared to control-vehicle–treated cells (NM). Decrease in tumor cell proliferation after exposure to (A) synthetic 30- to 60-μM farnesol (F30, F60; >57%) or (B) *C. albicans* spent culture media grown for 48 to 72 hours (CA48h, CA72h; >35%) without a change in proliferation with the non–farnesol-producing *C. albicans* spent culture media grown for 48 to 72 hours (NF48h, NF72h). Additionally, a reversal of the proliferation inhibition is seen by the addition of DAG combined with F30 and F60 (DAG-F30, DAG-F60), with DAG by itself (DAG) inducing no effect. The growth of control-vehicle–treated cells (NM) has been set to 100%, to which all other measures were calculated. Error bars, SEM. *Statistically significant ($P <$ .05) differences compared to control cells, non–farnesol-producing *C. albicans* and to the addition of DAG, respectively.

*Reversal of apoptosis by exogenous DAG.* Proliferation assays were also performed in the presence of DAG to determine whether DAG was able to salvage OSCCs from apoptosis induced by farnesol. These studies indicated that the addition of DAG (DAG-F30 or DAG-F60) was able to maintain cell proliferation at 100% in the presence of 30- to 60-μM farnesol treated for 48 hours (Figure 1*B*).

*Flow cytometry (apoptosis analysis).* The effect of farnesol on cell proliferation was corroborated by the data obtained from Annexin V apoptosis studies after treatment of OSCCs for 48 hours with 30- to 60-μM farnesol or 48- to 72-hour-old *C. albicans* spent culture media. In these studies, cells exposed to synthetic and *C. albicans* farnesol exhibited 7.3% to 10.06% apoptosis compared to unexposed cells ($P < .05$; Figure 2).

*Western blot analysis.* Proteins were extracted from OSCCs after 48 hours of exposure to 30- to 60-μM synthetic farnesol (F30 or F60) or *C. albicans* spent culture media grown from 24 to 72 hours from farnesol-producing (CA48h or CA-72h) and non–farnesol-producing (NF24h, NF48h, or NF72h) *C. albicans* strains and

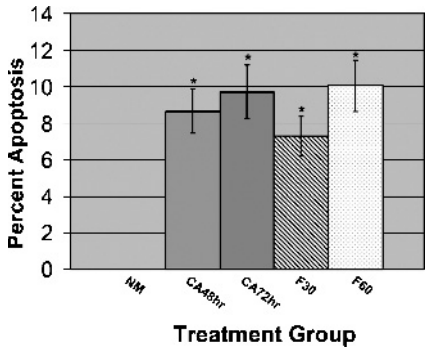



**Figure 2.** Annexin V apoptosis assay: Assessment of OSCC 9 cell apoptosis, all after 48 hours of treatment compared to control-vehicle–treated cells (NM). Results demonstrated an increase in apoptosis with synthetic 30- to 60-$\mu$M farnesol-treated cells (F30, F60) or *C. albicans* spent culture media grown for 48 to 72 hours (CA48h, CA72h). The apoptosis of control-vehicle–treated cells (NM) has been set to 0%, to which all other measures were calculated. Error bars, SEM. *Statistically significant ($P < .05$) differences compared to control (NM) cells.

treated for 48 hours on OSCCs. After SDS-PAGE and Western blot analysis, membranes were probed with antibodies to survivin, cleaved-caspase 3, and cleaved-caspase 9. These studies demonstrated a significant decrease in survivin expression in the synthetic and *Candida*-produced farnesol-exposed OSCCs but no change in survivin expression with the non–farnesol-producing *C. albicans* strain (Figure 3*A*). Analysis of cleaved-caspases 3 and 9, however, demonstrated an increase in caspase expression in the farnesol and *C. albicans* spent culture media–exposed cells. Similar to the proliferation studies, however, caspase expression was restored to baseline levels in the presence of DAG treatment for 48 hours (Figure 3*B*).

*Fluorescent microscopy.* To confirm the active caspase protein expression findings and the presence of apoptosis, fluorescent microscopic assays were performed using apoptosis and mitochondrial degradation FLICA Apoptosis Detection Kits that detect the presence of intracellular active caspases and mitochondrial damage. Microscopic images revealed the presence of green fluorescence in the synthetic farnesol–exposed cells indicating the presence of active caspases (Figure 4*B*). Similarly, intracellular red fluorescence indicative of mitochondrial degradation was seen in these farnesol-exposed cells (Figure 4; similar images were obtained with 30 $\mu$M farnesol).

*Proteomic analysis.* Three independent proteomic analyses showed high reproducibility in demonstrating a significant number of proteins to be differentially regulated on 48 hours of treatment with either synthetic (F30 or 60) or *C. albicans* spent culture medium farnesol (CA48h or CA72h; Figure 5). Of these 36 proteins, 26 were consistently up-regulated and 10 were down-regulated in the three independent cell culture experiments. The 36 differentially expressed spots were subjected to MALDI–ToF MS analysis and database searching, which resulted in the identification of most these proteins (25/36). The proteins identified and the detailed peptide data are provided in Table 1.

Most notable among the down-regulated proteins involved in OSCC are as follows: glutathione *S*-transferase, a protein involved in hypermethylation; heat shock protein 27 kDa (HSP27), an inhibitor of apoptosis; RAN, a contributor to genetic instability and cell cycle progression; and various keratins overexpressed in epithelial carcinomas and dysplastic lesions (Table 1*A*).

Among the significantly up-regulated are various proteins involved in the inhibition of carcinogenesis (Table 1, *B* and *C*). Most notable are proteins involved in tumor immune function (Met enkephalin), proliferation suppression (Mdm-2, laminin-binding, Annexin A2, transketolase), and the aging-associated protein YWHAZ.

## Discussion

Apoptosis is a naturally occurring developmental process triggered by various extracellular and intracellular stimuli and is divided classically into extrinsic and intrinsic pathways [7]. The extrinsic pathway typically involves the activation of death domain receptors and

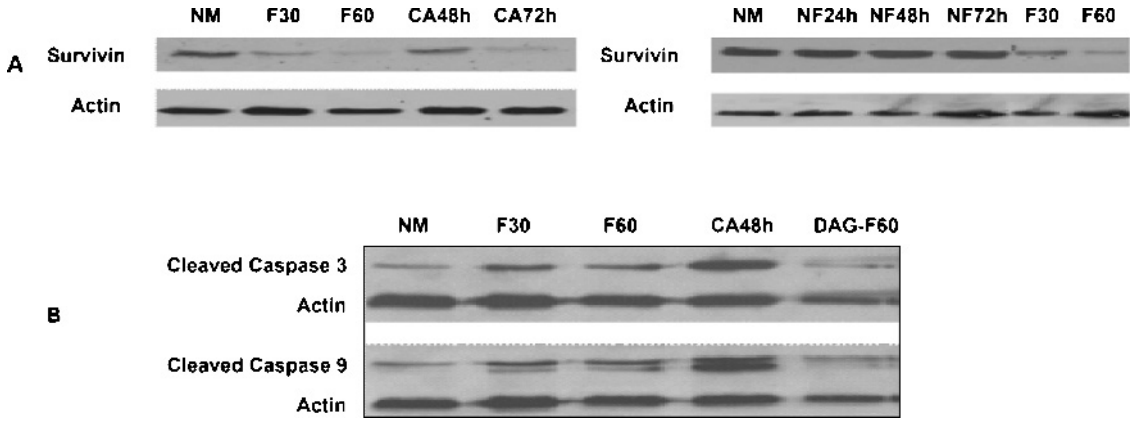



**Figure 3.** Western blot analysis: Assessment of OSCC 9 cell protein expression, all after 48 hours of treatment, demonstrating (A) a decrease in survivin expression and (B) an increase in cleaved-caspase 3 and cleaved-caspase 9 expression in cells exposed to synthetic 30- to 60-$\mu$M farnesol (F30, F60) or *C. albicans* spent culture media grown for 48 to 72 hours (CA48h, CA72h), without a change in expression with the non–farnesol-producing *C. albicans* spent culture media grown for 24 to 72 hours (NF24, NF48h, NF72h). The restoration of caspase expression to baseline levels is seen in the presence of 48 hours of combined DAG and farnesol (DAG-F60) treatment. Actin was used as the loading control.

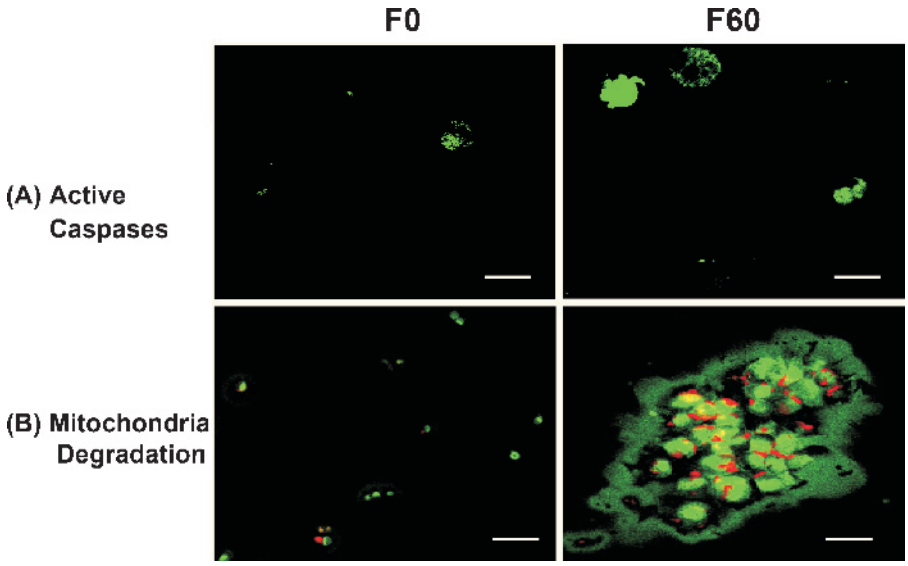



**Figure 4.** Fluorescent microscopic images revealing the presence of (A) active caspases and (B) mitochondrial degradation in the 60-$\mu$M farnesol-treated cells (F60) compared to control (F0). Active caspases are seen as green fluorescence, whereas mitochondrial degradation appears as red fluorescence. Minimal fluorescence was observed in the untreated-control cells (0F). Bar, 20 $\mu$m.

the downstream regulation and activation of caspase 3 [4,5]. The intrinsic pathway elicits the degradation of the mitochondria and activation of the intermediary caspase 9, which, when cleaved, induces caspase 3 activation, a terminal caspase for both pathways [4,5].

Among the recently identified extracellular stimuli of apoptosis in mammalian tumor cells is farnesol, an extracellularly secreted quorum-sensing molecule by the fungal species *C. albicans* [1]. In this investigation, studies were designed to examine and compare the apoptotic effects of both synthetic farnesol and *C. albicans*–produced farnesol secreted into culture medium on OSCCs. To that end, two primary OSCC lines were used as a model, and analyses of apoptotic markers and two-dimensional–based proteomics were undertaken to annotate the altered proteins in the cells before and after exposure to farnesol.

To date, the exact mechanism of farnesol cytotoxicity is yet to be elucidated. However, it has been suggested from limited studies that farnesol's effect may be due to interference with a phosphatidylinositol-type signaling secondary to decreased levels of intracellular DAG. The result inducing the triggering of caspases and inhibition of survivin, leading to cell disassembly and growth inhibition [2]. Although the exogenous administration of DAG in those studies prevented farnesol-induced inhibition of cell proliferation, it did not relieve the farnesol-induced block in PC synthesis [2]. Thus, the execution of apoptosis by farnesol seems to be a separate and distinct event from farnesol-induced inhibition of PC biosynthesis and instead likely occurs through a DAG-mediated process that is downstream of PC synthesis [2]. These observations were corroborated by our study, where the addition of DAG reversed the inhibitory effect of farnesol on cell proliferation and on caspase expression (Figures 1 and 3).

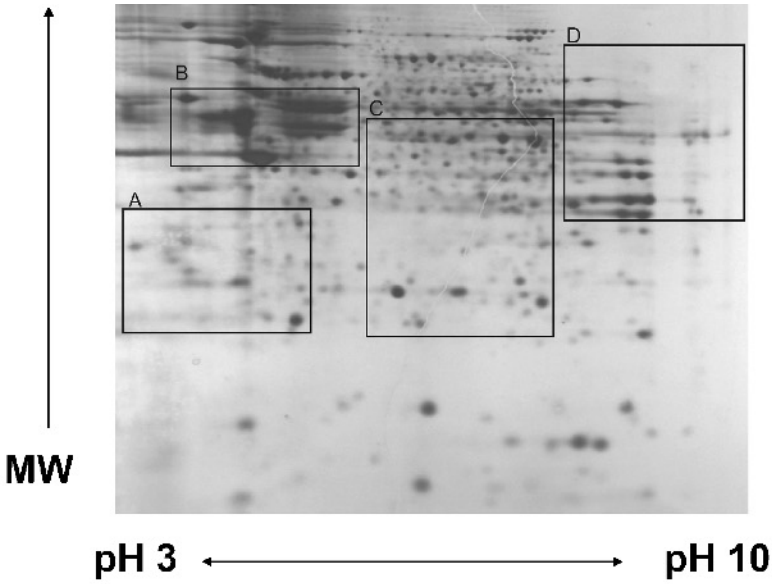
Farnesol has been reported to induce cell growth inhibition and/or apoptosis in a variety of tumor cells; however, the reported $IC_{50}$ values varied widely for different tumor types and different cell lines [22]. In our study, we observed an $IC_{50}$ value of 30 to 60 $\mu$M for farnesol on the OSCC lines in our study.

Our cell proliferation studies indicated that the exposure to synthetic or *Candida*-produced farnesol resulted in a significant decrease in the proliferation of OSCCs compared to unexposed cells. To validate our findings, a non–farnesol-producing *Candida* strain was included. In these experiments, exposure of OSCCs to *C. albicans* spent culture medium from this strain had no effect on cell proliferation or apoptosis (Figures 1 and 3).

To elucidate the pathways involved in farnesol-mediated apoptosis, Western blot and fluorescent microscopic analyses were performed. Fluorescent images demonstrated the intracellular presence of mitochondrial degradation and activated caspases in the farnesol-exposed cells. In addition, the overexpression of terminal cleaved-caspases and the presence of cleaved activated subunits were also demonstrated in the farnesol-exposed cells, along with a decrease in the expression of the intermediary apoptosis–inhibiting protein survivin (Figure 3). These findings indicate that both the intrinsic and extrinsic apoptotic signaling pathways are activated using farnesol.

The involvement of both apoptotic pathways was further demonstrated through proteomic analysis. The induction of death domain receptors at the cell surface induces the activation of caspase 8, which cleaves and activates caspase 3 inducing apoptosis [23]. However, HSP27, a protein associated with a wide range of human cancers and associated with a poor prognosis and resistance to therapy, has been shown to prevent apoptosis by suppressing the activation of procaspase 9 [24]. Interestingly, HSP27 was found to be underexpressed after farnesol treatment, as shown by our proteomic analysis supporting the hypothesis that farnesol *in vitro* triggers apoptosis in OSCCs through both intrinsic and extrinsic apoptotic signaling pathways.

Through global proteomic analyses of farnesol-exposed and non–farnesol-exposed cells, a total of 58 altered proteins were visualized by two-dimensional gel electrophoresis and Coomassie brilliant blue staining. Of those, 36 proteins were excised and analyzed by MALDI–ToF MS, where 25 proteins were positively identified, including those which function in diverse biologic processes, such as



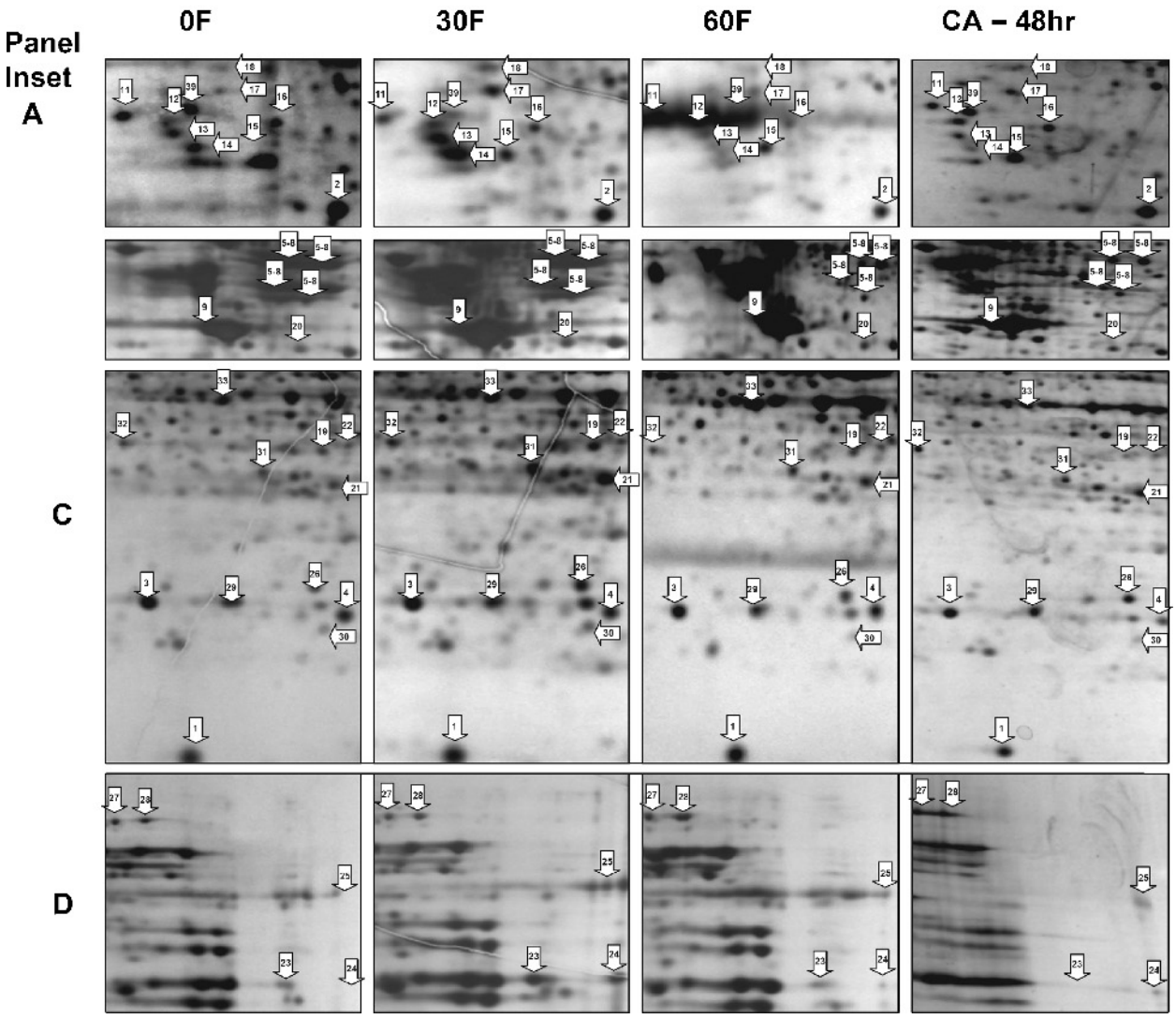




**Figure 5.** Representative two-dimensional gels of extracted proteins from OSCC 9 cells, all after 48 hours of treatment and grown in the presence of 30- to 60-$\mu$M farnesol (F30, F60) or *C. albicans* spent culture media grown for 48 hours (CA48h) demonstrating differential protein expression. Thirty-six protein spots (marked with *arrows* and *numbers*) displayed consistent alterations on farnesol treatment. Information on the identities and functions of these spots is listed in Table 1.

**Table 1.** Proteins Differentially Regulated with Farnesol.

| Reference | Spot | MW (kDa) | p*I* | Identity | Peptide Matches* | Protein Score,† Confidence Interval (%) | Accession # | Function‡ |
|---|---|---|---|---|---|---|---|---|
| **(A) Proteins down-regulated with farnesol** | | | | | | | | |
| [34] | 1 | 17,028.9 | 8.54 | Cofilin-1 p18 isoform | 4 | 96.3 | 73983054 | Expressed in leukemias, role in metastasis |
| [30] | 2 | 23,595.1 | 5.43 | Glutathione *S*-transferase | 4 | 99.5 | 2204207 | Associated with carcinogenesis through hypermethylation |
| [27] | 3 | 22,825.5 | 5.98 | Heat shock protein 27 kDa | 9 | 100 | 4504517 | Expressed in squamous cell carcinomas and leukemias, associated with poor prognosis, up-regulated with chemotherapy to inhibit apoptosis |
| [25] | 4 | 25,378 | 7.16 | RAN protein | 5 | 99.9 | 32425497 | Contributes to genetic instability characterizing many cancer cells, transformation and cell cycle progression |
| [28] | 5 | 60,323.4 | 7.59 | Keratin 5A | 15 | 100 | 46812692 | Overexpressed in epithelial carcinomas, progression, and metastasis |
| [28] | 5 | 60,323.4 | 7.59 | Keratin 6A | 16 | 100 | 46812692 | Overexpressed in epithelial carcinomas |
| [28] | 7 | 51,874.5 | 5.09 | Keratin 14 | 14 | 100 | 15431310 | Overexpressed in epithelial carcinomas, metastasis |
| [28] | 8 | 48,361.1 | 4.97 | Keratin 17 | 7 | 100 | 4557701 | Early expression in all dysplastic lesions, metastasis |
| [33] | 9 | 47,797.1 | 5.61 | ARP3 actin-related protein 3 homolog | 6 | 100 | 27882036 | Involved in actin polymerization and cell motility |
| **(B) Proteins up-regulated with farnesol** | | | | | | | | |
| [31] | 11 | 18,554 | 4.93 | Met Enkephalin | 3 | 95.2 | 223431 | Suppresses immune function |
| [29] | 13 | 25,249.8 | 4.87 | Mdm2-B | 4 | 100 | 992679 | Growth-inhibitory functions, overexpressed with mutant *p53* |
| [26] | 14 | 30,099.9 | 4.72 | YWHAZ | 4 | 99.9 | 49119653 | Role in human aging, metastasis, poor prognosis in cancer |
| [48] | 15 | 23,235.7 | 5.02 | Rho GDP dissociation inhibitor | 9 | 100 | 36038 | Overexpressed in cancer, resistance to treatment |
| [39] | 16 | 26,509.4 | 4.95 | Chloride intracellular channel-1 | 9 | 100 | 55961619 | Overexpressed in cancer |
| [35] | 17 | 35,840.4 | 4.94 | Annexin V (Chain-C) | 6 | 100 | 809190 | Overexpressed in cancer, marker of apoptosis |
| [38] | 18 | 31,888 | 4.84 | Laminin-binding protein | 5 | 95.1 | 34234 | Overexpressed in cancer, role in cell adhesion, mitogenesis, differentiation, and metastasis |
| [36] | 19 | 29,431.7 | 4.86 | Similar to 40S ribosomal protein SA | 5 | 96.5 | 109041487 | Lamin receptor, multidrug resistance |
| [42] | 21 | 38,807.9 | 7.57 | Annexin A2 | 6 | 100 | 50845386 | Overexpressed in cancer, proliferation, differentiation, and metastasis |
| [51] | 23 | 36,197.5 | 8.57 | Aging-associated gene 9 protein | 6 | 100 | 54303910 | Involved in programmed cell death |
| [43] | 26 | 29,002.9 | 6.67 | Phosphoglycerate mutase 1 | 5 | 100 | 89035672 | Inhibitor of enzyme in glycolytic pathway, cell immortalization |
| [37] | 28 | 68,519 | 7.58 | Transketolase | 11 | 100 | 14602962 | Nucleic acid ribose synthesis → proliferation and survival |
| **(C) Proteins up-regulated with 60 μM farnesol** | | | | | | | | |
| [50] | 34 | 24,536.5 | 4.85 | Alpha-S1-casein | 6 | 100 | 162794 | Activation of protein kinase $p40^{TAK}$ a casein kinase 2 in epithelial cancer |
| [53] | 35 | 27,386.9 | 4.71 | Tropomyosin 3 | 18 | 100 | 47938162 | Cytoskeletal microfilaments, implicated in cancer as a possible oncogene |
| [28] | 36 | 66,198.1 | 8.16 | Keratin 1 | 13 | 100 | 17318569 | Squamous cell differentiation, overexpressed in epithelial cancer |

*Peptide count for peptide in the spectra of tryptic digests that matched the proteins identified.
†Proteins identified by mass spectrometry are assigned a MOWSE scoring algorithm expressed as a protein score for a peptide mass fingerprint. Protein scores higher than 65 indicate with 95% confidence [protein score confidence interval (CI)] that the proteins were not identified by random matches of peptide mass data. The closer the protein score of % CI value is to 100%, the more likely the protein is correctly identified.
‡Large reviewed computational analysis by The FANTOM Consortium and RIKEN Genome Exploration Research Group and Genome Science Group (Genome Network Project Core Group).

protein binding and folding, metabolism, proteolysis, signal transduction, carcinogenesis, aging, and programmed cell death (see Table 1) [25–55]. Among the 36 proteins found with differential regulation, 10 were down-regulated (Table 1*A*) and 26 were up-regulated by farnesol treatment (Table 1, *B* and *C*). Most notable among the down-regulated proteins, cofilin 1 is assumed to play a role in leukemias and metastasis [40]. The down-regulation of these proteins may play a role in farnesol-mediated growth inhibition of OSCCs and in the induction of apoptosis.

Among the 26 proteins displaying significant overexpression, 15 proteins were identified (Table 1, *B* and *C*). Among those, three were found to be up-regulated with only 60-μM farnesol (Table 1*C*). Although not quite clear, this could possibly be related to a greater threshold needed to overcome counteracting inhibitory factors, native to the cancer cells. In addition to proteins involved in programmed cell death (an aging-associated gene 9 protein), both Annexin A2 (ANXA2), a calcium-binding protein, and Annexin V, a calcium-dependent phospholipid-binding protein, members of the ANX family, were among the up-regulated proteins [35,42,45,47]. Both of these proteins have been shown to be up-regulated in human tumors and play a role in cell growth and cell signaling [35,42]. ANXA2 is a multifunctional $Ca^{2+}$, lipid, and actin-binding protein, which is implicated in a number of intracellular functions such as cell signaling, membrane trafficking, mRNA transport, and regulation of membrane/cytoskeleton contacts and extracellular functions [42]. The precise biologic function of Annexin V, although a widely distributed protein, is yet to be established. However, it is reported to be an intracellular inhibitor of protein kinase C (PKC) [35]. Interestingly, the activation of conventional PKC groups are dependent on the presence of $Ca^{+}$, phosphatidylserine (PS), and DAG that are inhibited by Annexin V and, in turn, counteract the phosphorylation by PKC by Annexin I, Annexin II, and histone IIIS [56].

A comparison between the protein expression in cells grown in synthetic farnesol and those grown in 48- and 72-hour-old *C. albicans*

spent culture media demonstrated a similar pattern of differentially expressed proteins indicating the presence of a substance in the media exerting similar effect on OSCCs to those observed with synthetic farnesol. Although the pattern of expression was similar between growth in 48- and 72-hour-old *C. albicans* spent culture media, the level of expression was more pronounced in cells grown in the 48-hour-old *C. albicans* spent culture media, which more closely resembled the intensity of protein expression pattern of the cells grown in 30-μM synthetic farnesol. Farnesol has been shown to be increasingly produced with the age of the culture at a reported estimated concentration of 35 μM in 24- to 48-hour-old cultures, consistent with the comparable effects of the *C. albicans* spent culture media to that of 30-μM farnesol on the OSCCs observed in our study [15].

Although the clinical implications of the *Candida*-induced apoptotic effect on OSCCs are not yet clear, the incidence of OSCC with chronic mucocutaneous candidiasis and polyendocrinopathy-candidiasis-ectodermal dystrophy has been previously described [57,58]. These interesting observations warrant further explorations to determine a correlation between farnesol production by *C. albicans* and induction of apoptosis, because it may shed light on the mechanisms that *C. albicans* uses to colonize and potentially modify oral dysplastic and cancerous lesions.

The capability of farnesol to trigger apoptosis in mammalian cancer cells makes it a potential tool for studying tumor progression and an attractive candidate as a therapeutic agent with antitumorigenic properties. However, the mechanism by which farnesol preferentially exerts its proapoptotic effect on cancer cells is not yet fully known. What is known is that cancer cells are defective in some of their signaling pathways secondary to genetic/epigenetic alterations of certain genes, which could explain why tumor cells tend to be more sensitive to farnesol. Hence, the precise pathway by which farnesol causes apoptosis warrants further investigation. Future studies using our proteomic analysis could potentially delineate how farnesol preferentially targets transformed cells.

In conclusion, the combined findings from our study demonstrate that farnesol induces an inhibition of cell growth and promotes apoptosis in OSCCs through the induction of caspases, inhibition of survivin, and down-regulation of various cellular metabolic modalities. In addition, we report for the first time that the ability of a *Candida*-secreted substance to induce apoptosis is OSCCs through the same pathways as synthetic farnesol. This study will enable a better understanding of the molecular mechanisms underlying farnesol-mediated antitumor effects at the protein level.

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