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Xenohormetic and anti-aging activity of secoiridoid polyphenols present in extra virgin olive oil

A new family of gerosuppressant agents

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Aging can be viewed as a quasi-programmed phenomenon driven by the overactivation of the nutrient-sensing mTOR gerogene. mTOR-driven aging can be triggered or accelerated by a decline or loss of responsiveness to activation of the energy-sensing protein AMPK, a critical gerosuppressor of mTOR. The occurrence of age-related diseases, therefore, reflects the synergistic interaction between our evolutionary path to sedentarism, which chronically increases a number of mTOR activating gero-promoters (e.g., food, growth factors, cytokines and insulin) and the "defective design" of central metabolic integrators such as mTOR and AMPK. Our laboratories at the Bioactive Food Component Platform in Spain have initiated a systematic approach to molecularly elucidate and clinically explore whether the "xenohormesis hypothesis," which states that stress-induced synthesis of plant polyphenols and many other phytochemicals provides an environmental chemical signature that upregulates stress-resistance pathways in plant consumers, can be explained in terms of the reactivity of the AMPK/mTOR-axis to so-called xenohormetins. Here, we explore the AMPK/mTOR-xenohormetic nature of complex polyphenols naturally present in extra virgin olive oil (EVOO), a pivotal component of the Mediterranean style diet that has been repeatedly associated with a reduction in age-related morbid conditions and longer life expectancy. Using crude EVOO phenolic extracts highly enriched in the secoiridoids oleuropein aglycon and decarboxymethyl oleuropein aglycon, we show for the first time that: (1) The anticancer activity of EVOO secoiridoids is related to the activation of anti-aging/cellular stress-like gene signatures, including endoplasmic reticulum (ER) stress and the unfolded protein response, spermidine and polyamine metabolism, sirtuin-1 (SIRT1) and NRF2 signaling; (2) EVOO secoiridoids activate AMPK and suppress crucial genes involved in the Warburg effect and the self-renewal capacity of "immortal" cancer stem cells; (3) EVOO secoiridoids prevent age-related changes in the cell size, morphological heterogeneity, arrayed cell arrangement and senescence-associated β-galactosidase staining of normal diploid human

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fibroblasts at the end of their proliferative lifespans. EVOO secoiridoids, which provide an effective defense against plant attack by herbivores and pathogens, are bona fide xenohormetins that are able to activate the gerosuppressor AMPK and trigger numerous resveratrol-like anti-aging transcriptomic signatures. As such, EVOO secoiridoids constitute a new family of plant-produced gerosuppressant agents that molecularly "repair" the aimless (and harmful) AMPK/mTOR-driven quasi-program that leads to aging and aging-related diseases, including cancer.

Plants have been used for medicinal purposes for thousands of years. A third of the 20 most widely sold drugs on the market are plant-derived, and new molecules that may be beneficial for health are rapidly being discovered.[1-4] The global economy and human health both depend in part on the discovery of new and effective medicines. Surprisingly, little effort has been focused on plants that are known to synthesize molecules beneficial to the health of other organisms. One of the reasons for this lack of attention is the ease of patenting new synthetic drugs (known as "new chemical entities"); another is the "impurity" (non-specificity) of plant-derived biocompounds. A compound is considered "non-specific" if it interacts with a number of endogenous proteins. A priori, a plant-derived compound that interacts with several molecular targets may have an imperceptible effect (or even an adverse effect) compared with a pure molecule that interacts specifically with a particular protein.[1-4] However, a number of plant molecules interact with enzymes and receptors in ways that are not harmful. By the 5th century B.C.E., Hippocrates had described salicylic acid as "a bitter powder extracted from the willow that relieves pain and reduces fevers."[5,6] In 1763, Reverend Edward Stone experimented with the bark of the white willow (*Salix alba*) to treat fever and concluded that it was "a very effective remedy."[6,7] Since then, a variety of salicylates have been isolated from plants and used in the treatment of goiter, rheumatic fever, pain and arthritis. Today, 45,000 t of aspirin, an acetylated salicylic acid derivative, is produced each year. Aspirin is just one example of the dozens of plant-derived compounds that are now known to be beneficial to human health and that, furthermore, interact with more than one molecular target. Curiously, salicylate has been shown to activate adenosine monophosphate-activated protein kinase (AMPK),[8,9] which is a key therapeutic target for the treatment of obesity, type 2 diabetes and cancer due to its role as a central regulator of lipid and glucose metabolism[10-12] and as a critical modulator of aging through its interactions with mTOR, SIRT1 and the sestrins.[13-24]

Hormesis

Living organisms continually face adverse situations or harmful stimuli. Adaptation to these external aggressors, whether chemical, physical, biological or social, is paramount to survival. In addition, mild exposure to a stimulus that could be harmful at high concentrations might confer subsequent resistance or tolerance to some aggression, even one brought about by the stimulus itself. This adaptive response to stress has been identified as an evolutionarily conserved process. In toxicology, the term hormesis is used to define a two-phase nonlinear biological response in which exposure to a low dose of or weak stimulus by an environmental toxin or harmful substance produces a potentially beneficial effect, while a high dose leads to adverse effects.[25-30] In the biomedical field, hormesis refers to an adaptive response of cells and organisms to a moderate or intermittent stressor.[31-34] Thus, hormesis could be defined as a process in which exposure to a low dose of an environmental factor or chemical compound that is harmful at high concentrations has a beneficial and adaptive effect on the cell or organism. Furthermore, hormesis represents an essential concept in evolution, because it offers a possible explanation for how life on this planet has adapted to an environment that is at times particularly aggressive. To overcome environmental stresses, organisms might have developed a variety of cell signaling pathways that mediate hormetic responses.[35-38] These include transcription factors and the kinases that regulate them, which modulate the expression of genes that encode cytoprotective and stress-response proteins such as chaperones and antioxidant and detoxifying enzymes, among others.

Many animal studies involving dietary restriction (DR) regimens such as caloric restriction (CR), total-nutrient restriction, alternate-day fasting and short-term fasting have shown that DR can increase the resistance of the cells of these animals to various types of stress.[39-50] For example, mortality due to natural causes or induced by temperature or specific toxins is significantly reduced in animals subjected to CR compared with animals consuming a normal diet. Reduced caloric intake may also protect animals from various types of cancer, including pancreatic, mammary and prostate cancer.[51-57] In humans, alternate-day fasting improves symptoms and reduces markers of inflammation and oxidative stress in asthma patients, retards the growth of tumors and sensitizes a range of cancer cell types to chemotherapy.[58-60] By considering CR without malnutrition to be a "mild dietary stress," the ability of CR to prevent or lessen the severity of cancer, stroke, coronary heart disease, autoimmune disease, allergy, Parkinson disease and Alzheimer disease has been largely considered to represent an "overcompensation" resulting from hormetic mechanisms.[61]

Xenohormesis

Another well-known example of a hormetic process is exposure to low concentrations of certain phytochemicals. Organisms appear to have evolved the ability to detect stress markers produced by other species in their habitats. In this way, organisms might prepare themselves in anticipation of potential adverse environmental conditions. This inter-species hormesis is known as xenohormesis, the phenomenon in which an organism detects the chemical signals of another species regarding the state of the immediate environment or the availability of food.[62-66] This hormetic process generates beneficial effects for the organism. The existence of xenohormesis might explain how chemical compounds produced by plants and other autotrophs to defend against adverse environmental conditions can produce beneficial effects in the heterotrophs (animals and fungi) that consume them. Animals take

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advantage of the information contained in specific compounds produced by plants in response to stress. In fact, the majority of the known beneficial health effects of edible plants are attributed to molecules produced in response to stress.

Plant stress responses have evolved over millions of years. Because most plants cannot move physically, they must tolerate environmental stresses that may appear at any moment. This type of "sedentary lifestyle" may explain the complexity of the stress response in plants. Plants produce toxins to protect themselves against fungi, insects and predators. Plants cultivated for consumption contain fewer natural toxins than their wild counterparts. When plants grow under aggressive conditions, one observes an increase in the production of natural pesticides (biopesticides) that can produce acute intoxication in humans. Some studies have estimated that more than 90% of pesticides present in the human diet are chemical compounds that are produced by plants to protect themselves. Therefore, xenohormesis could explain how the sophisticated stress response that has evolved as a result of the stationary lifestyle of plants can confer stress resistance and survival benefits to animals that consume bioactive compounds produced by environmentally stressed plants.[63] While xenohormetic compounds are harmful to insects and microorganisms, the subtoxic levels at which humans ingest them appear to result in moderate cellular stress responses. This, in turn, might activate stress-response adaptation pathways, leading to increased expression of genes that encode cytoprotective proteins such as antioxidant enzymes, chaperones, growth factors, phase 2 detoxification enzymes and mitochondrial proteins. In this scenario, the ability of a combination of antioxidant/anti-inflammatory polyphenols found in many fruits and vegetables to slow aging[67] can be explained by molecular mechanisms that are largely unrelated to any potential antioxidant properties. For example, dietary flavonoids such as quercetin and blueberry polyphenols, among others, have been shown to modulate the lifespan of simple model organisms by activating molecular mechanisms independent of their antioxidant capacity.[68-74] Polyphenols found in tea and curcumin interact with dozens of molecular targets, providing many health benefits unrelated to their antioxidant properties.[75-85] In this regard, the natural polyphenolic compound resveratrol (3,5,4'-trihydroxystilbene) has emerged as a still-debatable mediator of longevity that certainly delays or attenuates many age-related chronic diseases in animal models.[86-106] Currently, activation of AMPK[15,107-110] rather than activation of the deacetylase SIRT1 seems to be a/the major effect of resveratrol, providing a plausible explanation for many of the health benefits of this compound that have been reported to date.

Extra Virgin Olive Oil (EVOO) Polyphenols And Xenohormesis: A Forgotten Scenario

We are beginning to accumulate epidemiological, clinical and experimental evidence suggesting that consumption of phenolic-enriched fruits, vegetables and herbs might reduce the risk of chronic diseases, including human malignancies.[111-113] In this regard, it has been repeatedly suggested that the ability of the so-called "Mediterranean diet" (i.e., the dietary patterns found in olive-growing areas of the Mediterranean basin) to significantly reduce the incidence of atherosclerosis and cardiovascular disease and decrease the risk of several types of human carcinomas, including breast cancer,[114-118] can be largely attributed to the unique characteristics of extra virgin olive oil (EVOO), which is an integral ingredient of the traditional Mediterranean diet and is the juice of the olive obtained solely by mechanical means and consumed without any further refining process other than washing, filtration, decantation or centrifugation. Apart from the health benefits that can be expected from EVOO as the richest source of the monounsaturated fatty acid (MUFA) oleic acid (OA; 18:1n-9),[119] cold-pressed EVOO includes minor components such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds and several antioxidants.[120-126] Although tocopherols and carotenes are also present, hydrophilic phenolics represent the most abundant family of bioactive EVOO compounds.

As for many plant-derived polyphenols, it has been largely assumed that EVOO-derived complex phenols such as lignans, flavonoids and secoiridoids[127-130] provide health benefits, primarily due to their antioxidant activity.[131-133] However, the antioxidant capacity of EVOO polyphenols does not directly correlate with their efficacy in terms of bioactivity (e.g., toxicity against cultured cancer cells). Moreover, when EVOO is provided in the diet, plasma concentrations of polyphenols are often lower than the levels required for protection against oxidation. Although the metabolites of EVOO polyphenols may reach concentrations in the bloodstream that are several-fold higher than that found in EVOO, EVOO polyphenol-derived compounds tend to have significantly decreased antioxidant activity compared with the parental compounds.[63,134] As an alternative to general mechanisms related to the antioxidant and/or trapping activity of oxygen radicals commonly observed with many plant-derived phenolics, recent studies have demonstrated that complex polyphenols can exert anti-carcinogenic effects by directly modulating the activities of various types of oncoproteins.[135-140] The results from our laboratory support the idea that EVOO-derived complex polyphenols constitute a previously unrecognized family of anticancer phytochemicals that have a significant impact on the proliferation and survival of cancer cells, at least in part through the specific suppression of protein activities, gene expression and/or signal transduction events closely related to the malignant phenotype.[129,141-149]

Although a Mediterranean diet and, more specifically, EVOO consumption have both been associated with increased longevity in the human population,[150-156] few studies have attempted to explore in depth the ultimate molecular mechanisms by which EVOO may influence longevity; for the most part, it has been assumed that these effects are the result of the antioxidant potential of its phenolic compounds and other free-radical scavengers, such as vitamin E.[157] However, if it is accepted that aging is not caused by reactive oxygen species (ROS), which are instead associated with longevity,[40,158-178] why have scientists not yet rejected the commonly accepted

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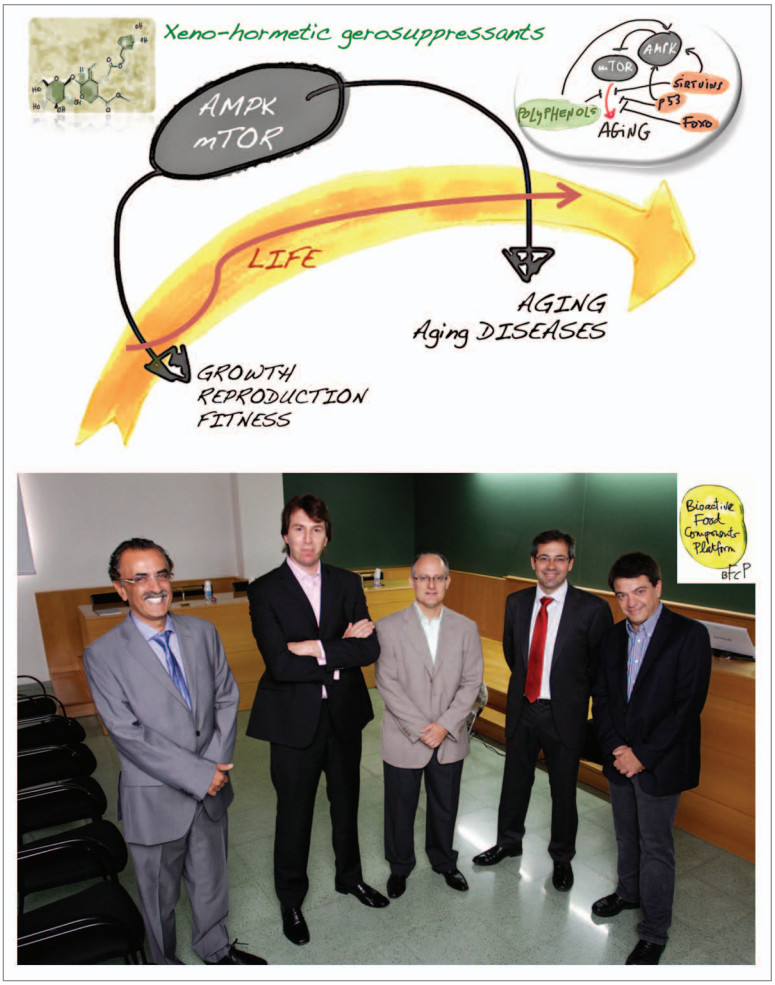

Figure 1. The Bioactive Food Component Platform (BFCP), Spain. The Spanish BFCP has two main goals: first, to molecularly elucidate the cellular and physiological abilities of humans to take advantage of the health benefits chemically encrypted within plant-derived biocompounds; second, to translate the sophisticated stress response of plants, which has evolved as a result of their stationary lifestyle, to the clinical arena to combat human aging and age-related diseases. Both goals of the BFCP revolve around the assumption that age-related diseases (e.g., atherosclerosis, diabetes, cancer, and others) reflect the synergistic interaction between our evolutionary path to sedentarism, which chronically increases a number of mTOR activating gero-promoting factors (e.g., nutrients, growth factors, cytokines, insulin), and the "defective design" of central metabolic integrators such as mTOR and AMPK. Design defects in the metabolic nature of the antagonistic pleiotropy model of aging involve both the ability of the mTOR gerogene to continue, in an aimless (and harmful) manner, a developmental program that was beneficial early in life but was not switched off upon its completion, and the necessary weakness of gerosuppressor genes such as AMPK that antagonize the gerogenic mTOR pathway (i.e., the responsiveness of AMPK signaling should clearly decline with aging, because robust, continuous activation of AMPK in response to cellular stresses will result in accelerated aging).[354,355] The BFCP therefore aims to revisit the xenohormesis hypothesis in terms of clinically valuable plant-produced gerosuppressant agents that molecularly "repair" the aimless (and harmful) AMPK/mTOR-driven quasi-program of aging and aging-related diseases (top panel). The BFCP integrates five multidisciplinary teams of biologists, biochemists, chemists, pharmacists, physicians, and engineers to research, design, and develop anti-aging biomedical strategies based on plant-derived gerosuppressants. From left to right in the bottom photograph are Dr Jorge Joven (Universitat Rovira I Virgili, Reus, Spain), Dr Javier A. Menendez (Catalan Institute of Oncology, Girona, Spain), Dr Vicente Micol (Miguel Hernández University, Elche, Spain), Dr Antonio Segura-Carretero (University of Granada, Granada, Spain), and Dr Carlos Alonso-Villaverde (Universitat Rovira i Virgili, Reus, Spain). (The original painting in the top panel is from Dr Jorge Joven based on Fig. 3, ref. 311 by Dr Mikhail V. Blagosklonny; the BCFP team photograph in the bottom panel is by photographer Pere Ferré, Tarragona, Spain).

view that the anti-aging benefits of EVOO phenolics are simply due to their antioxidant potential? Even more intriguing is the fact that, despite the structural resemblance of EVOO complex polyphenols to some of the hormetic polyphenols mentioned above (e.g., resveratrol), none of the phenolic components naturally present in EVOO have been characterized in terms of their potential to extend lifespan. An exception is a recently published study by Cañuelo and colleagues[179] suggesting that tyrosol, a phenol present in EVOO, may increase lifespan and stress resistance in *Caenorhabditis elegans*, likely through the activation of hormetic mechanisms.

Our laboratories at the Bioactive Food Component Platform (BFCP) in Spain (**Fig. 1**) have recently begun a systematic approach to evaluate for the first time whether secoiridoids, a family of complex phenols found in Oleacea plants that structurally resemble well-known anti-aging molecules such as resveratrol, are bona fide xenohormetic compounds that significantly impact pivotal signal-transduction pathway(s) (i.e., gerogenes and/or gerosuppressors) that drive(s) most, if not all, aging-related diseases. Dr Blagosklonny has recently proposed that "hormesis does not make sense except in the light of TOR-driven aging."[180] Instead of purposely reconciling hormesis with the conventional view on aging (i.e., aging is a decline and/or a deterioration due to the accumulation of random molecular and cellular damage), Dr Blagosklonny proposes that, because aging is an aimless quasi-programmed phenomenon that is driven by overactivated gerogenes belonging to the nutrient-sensing mTOR (mammalian target of rapamycin) pathway (e.g., mTOR, S6K), the mTOR pathway limits lifespan by accelerating age-related diseases (**Fig. 1**). Therefore, in humans (and other mammals), age-related diseases represent hyperfunctional phenotypes of mTOR-driven aging that actually limit lifespan. Understandably, if mTOR gerogene activity limits lifespan by accelerating the progression of age-related diseases, such as atherosclerosis or cancer, direct or indirect pharmacological suppression of mTOR-driven aging via the activation of mTOR gerosuppressors such as AMPK would be expected to increase healthy lifespan. In this scenario,

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Dr Blagosklonny differentiates two types of hormesis, namely, "increasing aging tolerance" or "hormesis B," which does not affect the aging process itself, and "slowing-down aging" or "hormesis A," which does affect the aging process by directly inhibiting mTOR activity (e.g., CR, rapamycin, resveratrol, metformin) or by imitating mTOR inhibition (e.g., heat shock). We obviously rejected the idea that EVOO secoiridoids could increase aging tolerance, which may allow an organism to survive catastrophes caused by aging-related diseases. In a "hormesis A" scenario, we hypothesized the following: (1) The anticancer activity of EVOO secoiridoid polyphenols results from the activation of anti-aging-like gene signatures in cancer cells (i.e., the enhancement of cellular stress mechanisms suppresses the hyperfunctional phenotype of immortal cells). (2) EVOO-derived secoiridoids activate the energy-sensing AMPK gerosuppressor (i.e., EVOO secoiridoids operates as AMPK-activating low-energy mimickers). (3) Chronic exposure to EVOO secoiridoid polyphenols efficiently delays the senescence phenotype in normal diploid human fibroblasts (i.e., the agonistic activity of EVOO secoiridoids toward the AMPK gerosuppressor improves the structural and functional integrity of normal cells without promoting their entrance into a potentially deleterious hyperproliferative mode).

In this paper, we present the first body of experimental evidence suggesting that EVOO secoiridoid polyphenols, by acting as biocompounds that belong to the recently defined group of "hormesis A" compounds, can efficiently promote cytotoxicity in human cancer cells through the paradoxical activation of anti-aging/cellular stress-like gene signatures, which, in turn, significantly weaken age-related effects (e.g., cellular senescence) in normal human diploid fibroblasts.

Secoiridoid-Rich EVOO Phenolic Fractions Activate Resveratrol-Like Anti-Aging Transcriptomic Signatures in Cancer Cells

We previously reported that the cytotoxic potencies of individual phenolic extracts (PE) from a variety of EVOO monovarietals were positively related to the relative content of secoiridoids, a group of complex polyphenols.[145,146] Highly active EVOO PEs were notably enriched in secoiridoids (**Fig. 2**), whereas substitution of secoiridoids by other complex polyphenols, such as lignans, in PE mixtures was related to a loss of tumoricidal activity. To identify the key pathways and functions associated with the anti-tumoral activity of crude PE isolated from individual EVOO monovarietals, we performed genome-wide analyses in which we compared the global transcriptomic profiles of JIMT1 breast cancer cells using whole human genome microarrays. RNA was extracted and prepared from metastatic JIMT1 breast cancer cells that had been cultured for 6 h at 70% confluence in the absence or presence of four different EVOO PEs exhibiting the following cytotoxic potencies: EVOO-PE7 > EVOO-PE3 > EVOO-PE10 > > EVOO-PE12, as determined by MTT-based cell viability assays after 5 d exposure to EVOO PEs.[145,146] After RNA hybridization to an Agilent 44K (double-density) Whole Human Genome Oligo Microarray containing 45,220 features (probes) representing 41,000 unique human genes and transcripts, the normalized and filtered data from all experimental groups were analyzed simultaneously using the SAM algorithm. We set the significance cut-off at a median FDR of < 5.0%. When we used a 2.0-fold change cut-off relative to the transcriptome of untreated control cells to identify specific effects of EVOO PEs on gene expression, we observed that JIMT1 cancer cells treated with the EVOO PE with the lowest secoiridoid content (PE12) had the lowest number of altered genes (**Fig. 3**). Of note, while the total number of altered genes was similar (~400 to 600) after exposure to EVOO PEs with higher secoiridoid content (EVOO-PE7, EVOO-PE3 and EVOO-PE10), there was a trend toward enhanced levels of transcripts of more genes in response to EVOO PEs with higher secoiridoid contents. Intriguingly, the majority of the altered genes (~84%) were upregulated following treatment with EVOO-PE7, the phenolic extract with the highest relative secoiridoid content (**Fig. 2**; **Table S1**) and the highest anti-tumoral activity.[146]

When we previously screened the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database by performing Gene Set Enrichment Analysis (GSEA) to identify key pathways and functions potentially associated with the anti-tumoral activity of EVOO PEs, we observed that the highly active EVOO-PE7 had a dramatic differential impact on the expression of the *GADD45* stress-response gene family; by contrast, the expression of this family of genes remained largely unchanged upon treatment with EVOO-PE3 or EVOO-PE10.[146] We thus speculated that naturally occurring phenolic mixtures highly enriched in the complex polyphenols oleuropein aglycon (OA) and decarboxymethyl oleuropein aglycon (DOA) (**Fig. 2**) could lead to enhanced transcript levels of genes that are upregulated by stress. To test this hypothesis, we utilized the "core analysis" function included in the analysis software package ingenuity pathway analysis (IPA, Ingenuity Systems Inc.) to interpret EVOO-PE7-induced global transcriptomic profiles in the context of biological processes, networks and pathways. The IPA software algorithmically generates networks of up- and downregulated functionally related annotated genes based on their connectivity and assigns a score (i.e., a numerical value that takes into consideration both the number of focus genes in a network and the size of the network to approximate how relevant each network is to the original list of focus genes). **Figure 4** illustrates graphically the two gene network functions that were most significantly (score ≥ 3) upregulated (red) and downregulated (green) within the EVOO secoiridoid-induced stress transcriptomic signature in human breast cancer cells.

EVOO secoiridoids activate endoplasmic reticulum (ER) stress chaperones and unfolded protein response (UPR) genes. The primary function of the gene networks that were upregulated by EVOO secoiridoids was related to "cellular function and maintenance and cellular compromise" (score = 49). These gene networks include numerous genes encoding isoforms of constitutevely expressed and stress-induced 70-kDa heat shock proteins (Hsp70s), which are chaperones

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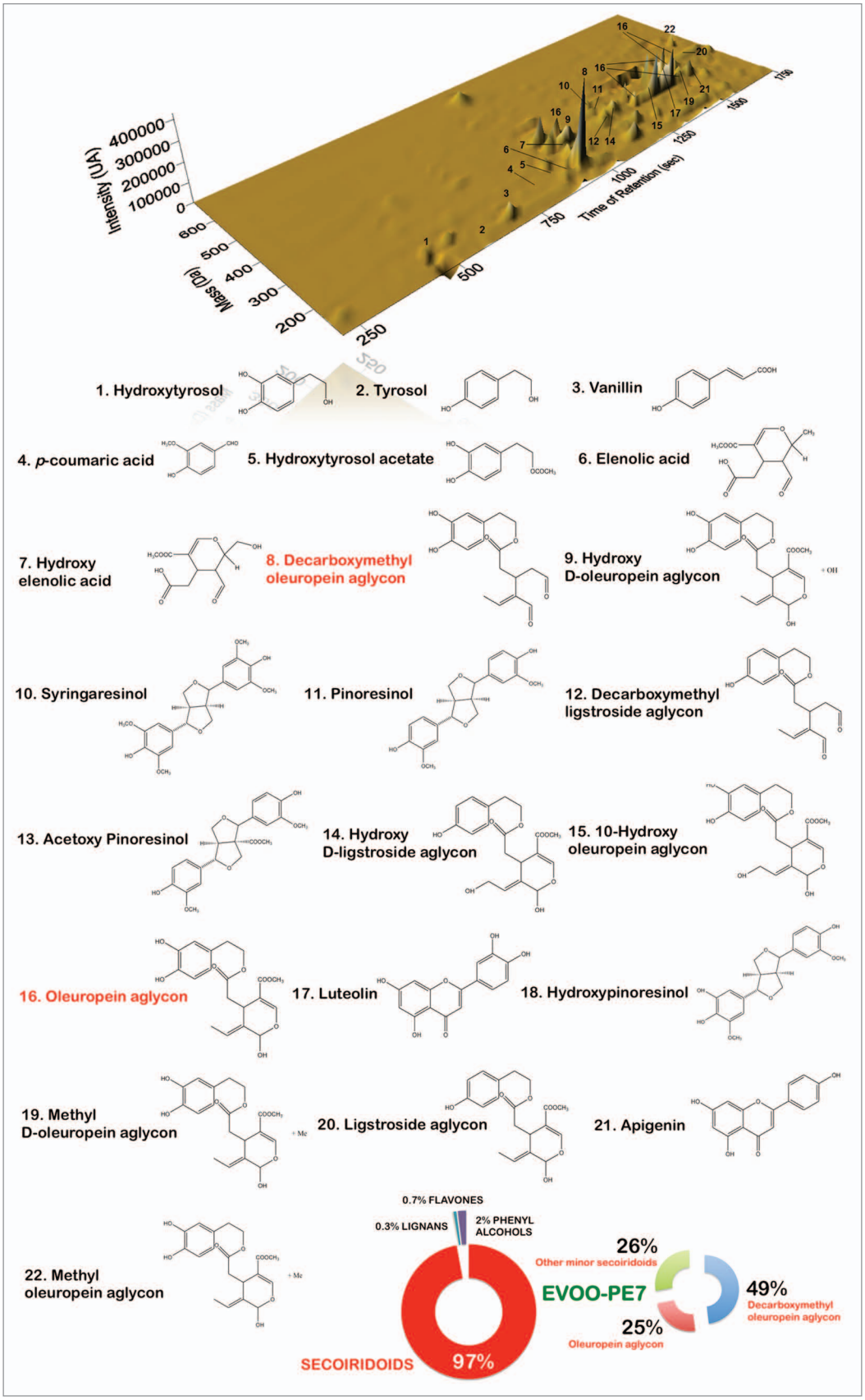

Figure 2 . Main phenolic compounds identified in EVOO phenolic extracts by HPLC-DAD-ESI-TOF. The figure shows the chemical structures of the main phenolic compounds compounds identified in secoiridoids-rich Picual EVOO variety following protocols described in reference 147. The figure shows also the percent distribution of the main phenolic families identified in the Picual EVOO-PE7.

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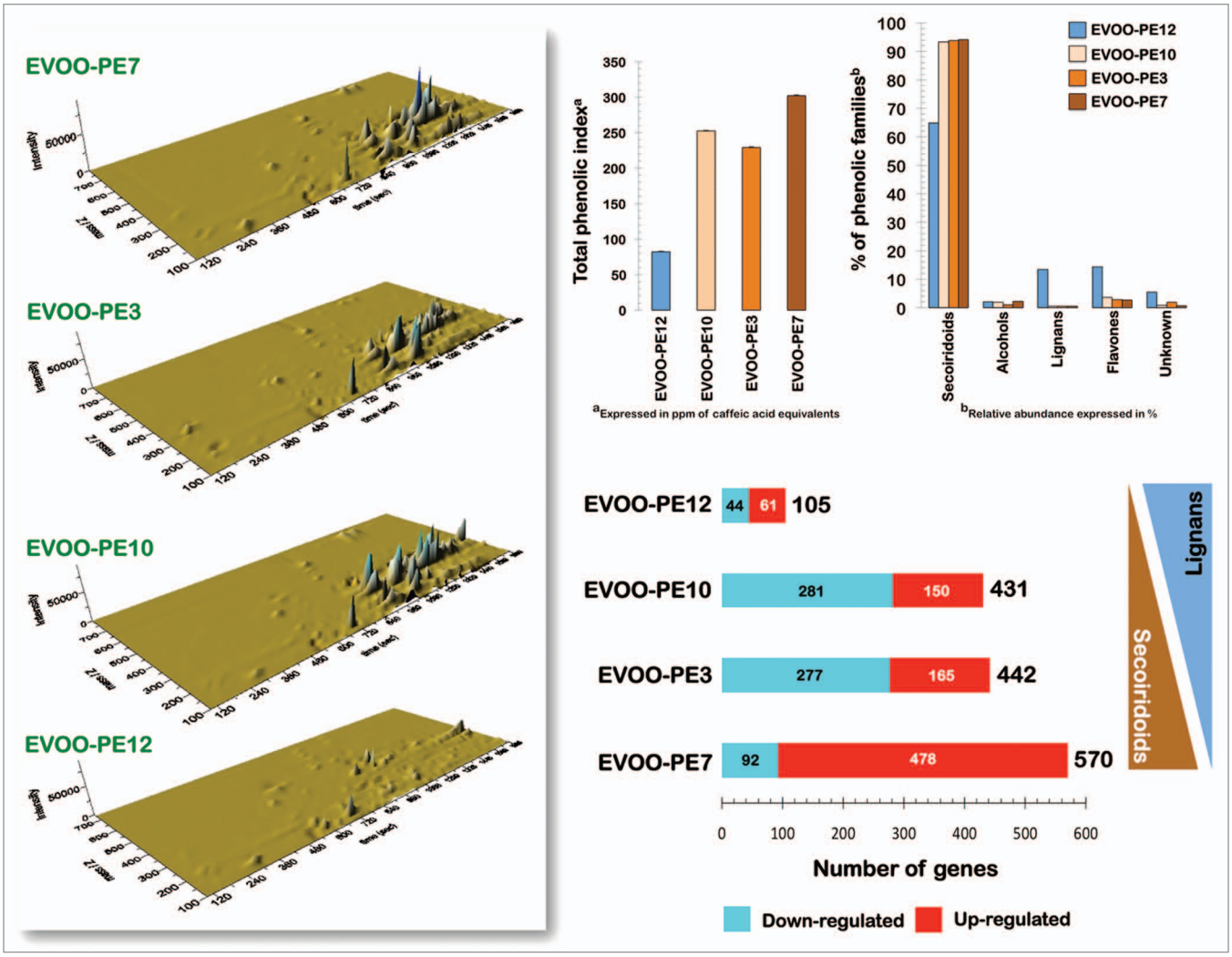

Figure 3. Relationship between the distribution of phenolic families in EVOO-PEs and their impact on the whole-genome transcription profile of human breast cancer cells. Total RNA isolated from JIMT1 cells grown in the absence or presence of EVOO-PE12, EVOO-PE10, EVOO-PE3 or EVOO-PE7 (2 μg/mL) for 6 h was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA quantity and quality were determined using the RNA 6000 Nano Assay kit on an Agilent 2100 BioAnalyzer (Agilent Technologies) as recommended. Whole Human Genome Oligo Microarrays (G4112F) were then hybridized. Briefly, 500 ng of total RNA from each sample was amplified by Oligo-dT-T7 reverse transcription and labeled by in vitro transcription with T7 RNA polymerase in the presence of Cy5-CTP or Cy3-CTP using the Quick Amp Labeling Kit (Agilent) and purified using RNAeasy columns (Qiagen). After fragmentation, 825 ng of labeled cRNA from each of the two samples were co-hybridized in in situ hybridization buffer (Agilent) for 17 h at 65°C and washed at room temperature for 1 min in Gene Expression Wash Buffer 1 (Agilent) and 1 min at 37°C in Gene Expression Wash Buffer 2 (Agilent). The images were generated on a confocal microarray scanner (G2565BA, Agilent) at 5 μm resolution and quantified using GenePix 6.0 (Molecular Dynamics). Spots with signal intensities of at least twice the local background that were not saturated and not flagged by GenePix were considered reliable. Extracted intensities were background-corrected, and the log2 ratios were normalized in an intensity-dependent fashion by the global LOWESS method (intra-chip normalization). Normalized log2 ratios were scaled between arrays to make all data comparable. Raw data were processed using MMARGE, a web implementation of LIMMA, a microarray analysis library developed within the Bioconductor project in the R statistical environment. To identify genes that are differentially expressed, the multiclass SAM procedure (significance analysis of microarrays) was applied. Genes with a q-value (FDR) below 5% and a fold change exceeding 2.0 in absolute value were selected as relevant (see also **Table S2**).

involved in crucial cellular functions in all kingdoms of life.[181-184] While constitutively expressed Hsp70 chaperones have housekeeping functions (e.g., folding of nascent polypeptides, protein translocation between cellular compartments and degradation of unstable and misfolded proteins), stress-induced Hsp70s prevent the accumulation of proteins that have become denatured in response to various cellular stresses (e.g., heat stress, radiation, ischemia, heavy metals or other stimuli that activate stress transcription factors). Treatment with the secoiridoid-rich EVOO-PE7 extract markedly (~11-fold) upregulated the expression of the *HSPA6* (Hsp70B') gene, which is strictly inducible with no detectable basal expression.[185,186] Indeed, HSPA6 protein induction is a sensitive biomarker of cellular stress that appears transiently in response to heat stress, whereas levels of *HSPA1A* (Hsp72), which was also induced by EVOO-PE7, persist for days.[187] EVOO-PE7

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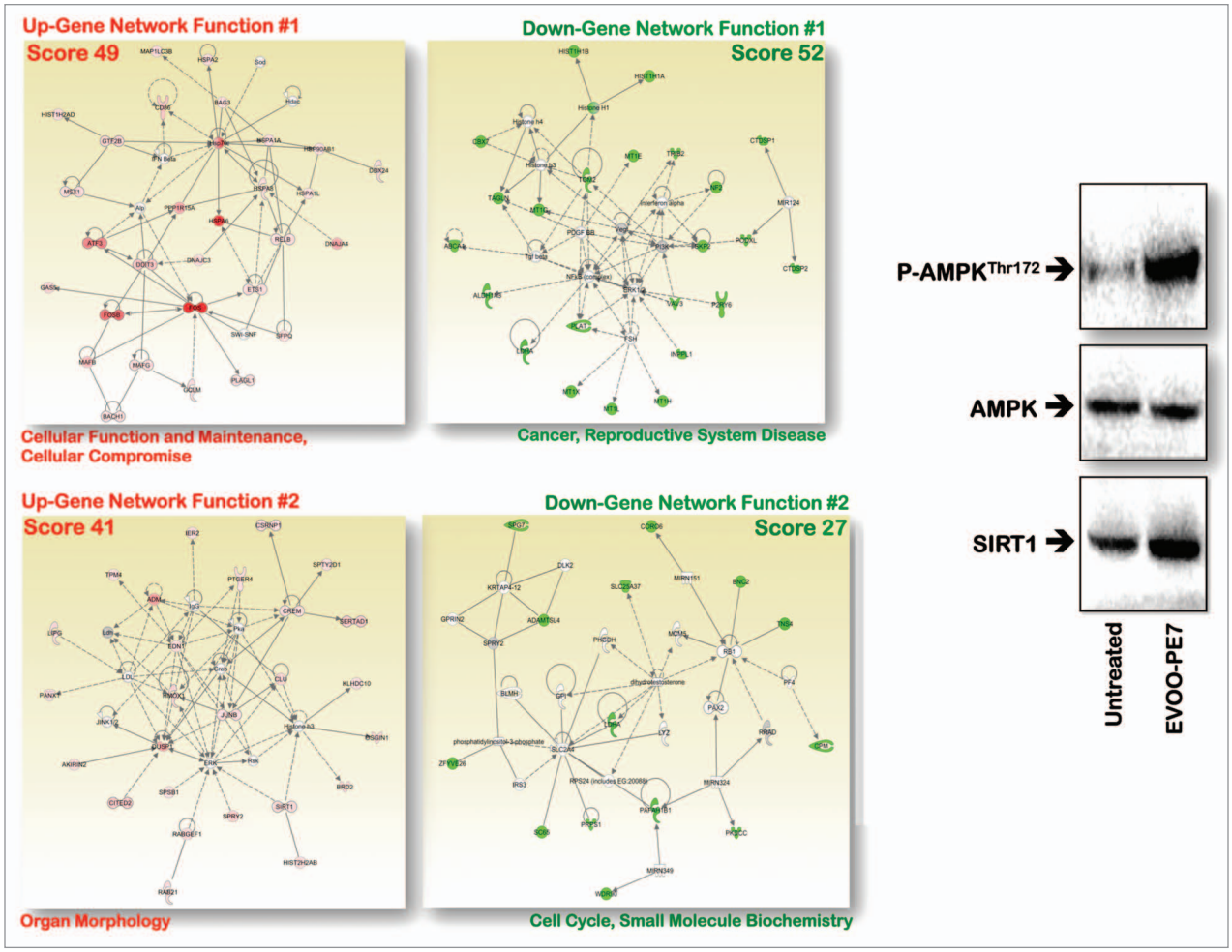

Figure 4. Network analysis of EVOO secoiridoids-regulated genes in human breast cancer cells. Left: Gene networks were constructed using ingenuity pathway analysis (Ingenuity® Systems). Data sets containing identifiers of genes with > 2.0-fold up- or downregulatory changes were uploaded into the application. These "focus genes" were overlaid onto a global molecular network developed from information contained in the ingenuity pathway knowledge base. Networks of these "focus genes" (nodes) are algorithmically generated based on the principle that highly connected gene networks are most biologically meaningful. All edges are supported by at least one reference from the literature stored in the ingenuity pathway knowledge base (the IPA interaction database is manually curated by scientists and updated quarterly). Briefly, the user-input or "'focus genes" gene list is compared with the "global molecular network" (GMN) database, which consists of thousands of genes and interactions. The focus genes are sorted based on highest to lowest connectivity within the GMN; networks of approximately 35 genes are then constructed beginning with the most highly connected focus gene. IPA assigns a p valuefor a network of size *n* and an input focus gene list of size *f* by calculating the probability of identifying *f* or more focus genes in a randomly selected set of *n* genes from the GMN. The intensity of the node color indicates the degree of expression (green scale for downregulated nodes; red scale for upregulated nodes). The score indicates the likelihood that the genes in a network are found together by random chance. Using a 99% confidence interval, scores of ≥ 3 are significant. Nodes are displayed using various shapes that represent the functional class of the gene product (diamonds, enzymes; ovals, transcription factors; triangles, kinases; circles, others). A solid line indicates a direct interaction; a dashed line indicates an indirect interaction. A line without an arrowhead indicates binding, and a plus sign indicates that other networks contain this gene product. Figure shows up- and downregulated networks with the two highest IPA score (a composite measure that indicates statistical significance that molecules depicted in the network are interconnected). Right: Representative western blot analyses of SIRT1, total AMPK, and phosphorylated AMPK (fosfo-AMPKα^{Thr172}) in EVOO untreated (control) and secoiridoids-treated JIMT1 breast cancer cells.

secoiridoids enhanced the expression of the *HSPA1L* gene (Hsp70-hom or Hsp70t), which encodes a constitutively expressed, non-inducible cytosolic protein that is highly abundant in testis,[189,190] and of *HSPA2* (Hsp70-2), a constitutively expressed gene that is expressed at high levels in testis, is essential for the maturation of male gametocytes and is linked to male infertility.[191-194] Importantly, EVOO-PE7 secoiridoids also upregulated the transcriptional expression of the *DNAJA4* and *DNAJC3* genes, which encodes the endoplasmic reticulum (ER)-localized DnaJ family of proteins (ERdj proteins).[195] The induced transcription of DNAJ genes is part of a specific pathway that is collectively termed the "unfolded protein response" (UPR) and is evoked when unfolded proteins accumulate in the ER.[196-199] The UPR ultimately leads to reduced import of proteins into the ER and upregulation of genes encoding ER chaperones and other components of the ER-associated degradation pathway.[200]

The previously unrecognized ability of EVOO secoiridoids to upregulate a set of

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genes involved in the ER stress response to unfolded proteins may appear to conflict with the demonstrated ability of these compounds to strongly inhibit the growth of highly aggressive breast cancer cells.[145,146] The UPR is the major protective and compensatory mechanism that enables cells to survive during ER stress. While UPR induction initially results in a general decrease in protein synthesis, which reduces the influx of nascent proteins into the ER, activation of the UPR also results in the enhanced transcription of ER resident chaperones, folding enzymes and other components of the protein degradation machinery, thus preventing aggregation of the accumulating misfolded proteins. This cell protective mechanism, which is also elicited upon induction of Hsp70s,[201,202] results in a transient induction of cell cycle arrest and in the accumulation of molecular chaperones that bind and recover unfolded proteins. However, prolonged exposure of cells to ER stress can induce a switch from cell survival to cell death, because the protective function of these mechanisms appears to be temporally restricted.[203,204] In this scenario, it is reasonable to suggest that exposure to EVOO secoiridoids promotes cell death-UPR branch signaling by impeding the alleviation of ER stress. Moreover, the coupling of EVOO secoiridoid-activated ER stress and UPR with EVOO secoiridoid-induced cytotoxicity in cancer cells appears to recapitulate the molecular mechanism by which the well-known defense molecule resveratrol simultaneously exerts anti-proliferative and chemopreventive effects.[205,206] First, induction of *GADD153/CHOP (DDIT3)*, one of the pivotal components of the ER stress pathway that is significantly upregulated by EVOO secoiridoids, has been shown to be involved in resveratrol-induced cell death in cancer cells.[207] Second, resveratrol has been shown to upregulate genes involved in the ER stress response to unfolded proteins.[208] Third, because resveratrol can trigger ER stress-induced cell death, UPR could be a potential mechanism of resveratrol cytotoxicity.[206,209] EVOO secoiridoids and resveratrol could also share mechanism(s) through which they activate ER stress-like responses. Like resveratrol, EVOO secoiridoid polyphenols paradoxically have a propensity to stimulate the formation of ROS,[210-213] which can cause oxidation of nascent proteins, leading to misfolding of proteins and ER stress.[214] In addition, EVOO secoiridoid polyphenols can operate in a resveratrol-like manner to molecularly mimic a CR-like situation involving ATP deficiency.[15,107-110,215-217]

EVOO secoiridoids induce c-Fos and modify the expression of genes related to polyamine metabolism. The most prominent EVOO secoiridoid-activated "cellular function and maintenance and cellular compromise" gene network involves not only the chaperone genes *HSPA6* and *Hsp70,* but also *c-Fos*, a key resveratrol-targeted proto-oncogene.[218,219] Indeed, the gene whose expression was most enhanced after treatment with EVOO secoiridoids was *c-Fos* (FOS; ~20-fold). *FOSB*, another member of the Fos family of transcription factors, which includes *c-Fos*, *FosB*, *Fra1* and *Fra2*, was also one of the 10 most upregulated genes (~11-fold). The Fos family proteins heterodimerize with Jun family proteins (c-Jun, JunB and JunD) to form active AP-1 (activator protein-1) transcription factors, which bind to AP-1 sites present in the promoters of certain genes and regulate their transcription. Of note, treatment of human breast cancer cells with EVOO secoiridoids significantly (~3-fold) upregulated the expression of *JUNB*. There is increasing evidence that the AP-1 complex plays an important role not only in the proliferation but also in the differentiation of several cell types; several chemopreventive agents (e.g., 1,25-dihydroxyvitamin D3 and butyrate) stimulate cell differentiation in an AP-1-dependent manner.[220,221] Resveratrol also stimulates the AP-1 constituents c-Fos and c-Jun to inhibit cancer cell growth.[218] The data imply that EVOO secoiridoid-induced upregulation of AP-1 is not associated with tumorigenesis, but rather with growth inhibition and/or differentiation of breast cancer cells.

Because resveratrol-induced *c-Fos* is functionally related to resveratrol's ability to modify polyamine metabolism,[218] we speculated that the previously unrecognized ability of EVOO secoiridoids to induce *c-Fos* might involve changes in the expression of genes associated with polyamine synthesis and/or polyamine catabolism. Similar to resveratrol, EVOO secoiridoids significantly upregulated (~4-fold) the expression of spermidine/spermine N^1-acetyltransferase (*SSAT*), the rate-limiting enzyme in polyamine catabolism (**Table S1**). This enzyme converts spermine to spermidine and the latter to putrescine in cooperation with polyamine oxidase (*PAOX*). Increased polyamine catabolism in response to EVOO secoiridoids was also suggested by the significant upregulation of the spermine oxidase (*SMO*) gene. Unlike resveratrol, EVOO secoiridoids downregulated *PAOX* gene expression, whereas they upregulated some genes involved in polyamine biosynthesis, such as arginase (*ARG2*) and ornithine decarboxylase (*ODC*). Although we lacked experimental approaches for measuring the intracellular levels of spermine, spermidine, putrescine and acetyl-spermidine following exposure to EVOO secoiridoids, our data indicate that the mechanism of the growth inhibitory action of EVOO-derived complex polyphenols likely involves an increase in polyamine catabolism with simultaneous induction of *c-Fos* and its AP-1-related DNA binding activity.

The previously unrecognized ability of EVOO secoiridoids to upregulate key genes directly involved in the conversion of arginine to ornithine (i.e., arginase) and in the conversion of ornithine to putrescine (i.e., ornithine decarboxylase) suggests that the augmentation of polyamine catabolism observed after exposure of cells to EVOO secoiridoids could be related to growth inhibition processes, whereas the augmentation of polyamine synthesis could be related to bona fide anti-aging effects. This appears likely, because polyamine levels decline continuously with age and polyamine (spermidine or high-polyamine diet) supplementation increases life span in model organisms.[101,222-231] Because autophagy is required for the cytoprotective and/or anti-aging effects of resveratrol and spermidine, experiments are currently underway in our laboratories at the Bioactive Food Component Platform in Spain to determine if regulation of polyamine metabolism by EVOO-derived secoiridoids differentially impacts the fitness of cancer vs. normal cells undergoing

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metabolic stress. Because pro-autophagic polyphenols have been shown to reduce the acetylation of cytoplasmic proteins,[232] we are also investigating whether EVOO secoiridoids might impact the activation status of autophagy while differentially affecting the acetylproteome of cancer vs. normal cells.

EVOO secoiridoids upregulate SIRT1 and inhibit cancer-promoting genes. In the above-mentioned transcriptome scenario and considering that resveratrol and spermidine increase lifespan by activating the histone deacetylase Sirtuin 1 (SIRT1) and inhibiting histone acetylases, respectively,[101] we determined if the resveratrol-like actions of EVOO secoiridoids involve changes in the expression of the *SIRT1* gene. Of note, not only was SIRT1 significantly upregulated by EVOO secoiridoids, *SIRT1* was also part of the second most significant gene network activated by EVOO secoiridoids, the "organ morphology" gene network (score = 41) (**Fig. 4**). Although *SIRT1* has long been thought to play a role in cancer, the debate regarding its role as an oncogene or tumor suppressor continues.[19,232,233] As an inducer of cell survival, it might appear reasonable to suggest that *SIRT1* fits the definition of an oncogene; conversely, because *SIRT1* is considered important in organism survival, a tumor suppressor function might also be anticipated. Genetic and drug-induced activation of SIRT1 has been shown to inhibit growth and/or induce apoptosis in certain cancer models,[234,235] while super-*SIRT1* mice exhibiting moderate SIRT1 overexpression (a ~3-fold increase) are generally healthier than control mice and are partially protected from certain solid tumors.[236-239]

Because neoplastic cells are thought to recapitulate many stem cell characteristics, including metabolic ones,[17,20,240-245] the oncogenic vs. tumor-suppressive activities of SIRT1 can be viewed in terms of the specific contribution of SIRT1 to maintaining or impeding "stemness-like" status in cell populations involved in tissue regeneration or cancer tissue heterogeneity, respectively. To preliminarily assess whether EVOO secoiridoid-induced upregulation of SIRT1 is related to the activation of onco-suppressive transcriptional events in highly aggressive cancer cells, we evaluated whether well-known oncogenes were among the 92 genes that were significantly downregulated by EVOO secoiridoids (**Table S1**; **Fig. 4**). Interestingly, the EVOO secoiridoids most frequently downregulated gene networks related to "cancer and reproductive system disease" (score = 52), including numerous metallothionein (MT) gene isoforms (*MT1E*, *MT1G*, *MT1X*, *MT1L*, *MT1H*). MT belongs to a family of metal-binding proteins whose roles range from heavy metal detoxification to the promotion of tumorigenesis. MT has been reported to be highly expressed in many tumors, including breast cancer, and is known to regulate key processes such as cell proliferation, apoptosis and even chemoresistance.[246-250] Because the role of MT in metal ion homeostasis is fundamental for controlling the activation of stem/progenitor cells, we speculated that MT downregulation by EVOO secoiridoids might be part of a broader genetic network involving key cancer stem cell (CSC)-related genes. Consistent with this hypothesis, the "cancer and reproductive system disease" gene network downregulated by EVOO secoiridoids includes the *ALDH1A3* gene, a biomarker of primitive normal human mammary luminal cells that shows high activity specifically in breast carcinomas. In such tumors, expression of the *ALDH1A3* gene identifies the tumorigenic cell fraction that is capable of self-renewal and of generating tumors by recapitulating the heterogeneity of the parental tumor (i.e., breast CSCs).[251-254] We are currently investigating whether treatment with EVOO secoiridoids impedes the propensity of breast CSCs to form multicellular "microtumors" under non-adherent and non-differentiating conditions (i.e., mammospheres).

In addition to the CSC marker *ALDH1A3*, treatment with EVOO secoiridoids notably downregulated the expression of *SKP2*, the gene that encodes the F-box protein S-phase kinase-associated protein 2 (Skp2). Skp2 belongs to the ubiquitin-proteasome system (UPS), which plays a vital role in regulating many biological processes by controlling the timely turnover of proteins. Because Skp2 is responsible for the degradation of several tumor suppressor proteins (e.g., p27, p57, p21, p130, FOXO1), it is thought to function as an oncoprotein that interacts with other major signaling pathways (e.g., PI3K/Akt, mTOR, PPARγ, ERK, FoxP3 and IGF) in breast cancer.[255-259] As such, there is renewed interest in developing Skp2 inhibitors as a general approach for cancer prevention and therapy.[259] Although specific drugs that inactivate Skp2 in cancer cells have not been identified, it is noteworthy that several naturally occurring compounds (1,2,3,4,6-penta-O-galloyl-β-D-glucose [PGG], gallic acid, epigallocatechin-gallate [EGCG], quercetin, curcumin, and lycopene) downregulate Skp2 expression in human cancers, including breast cancer.[260-262] We now add the complex polyphenols secoiridoids to the growing list of natural polyphenols that can function as potent inhibitors of Skp2.

The function of the second most downregulated gene network in response to EVOO secoiridoids is related to "cell cycle, amino acid metabolism and small molecule biochemistry" (score = 27) and was identified based on *LDHA*, a gene that was also overrepresented in the most downregulated gene network described above (**Fig. 4**). Lactate dehydrogenase (LDH) acts at a critical branch point in the metabolism of major nutrients; it is also active in the tricarboxylic acid (TCA) cycle and in determining tumor pH.[263] Glucose and glutamine are the major carbon sources for rapidly proliferating tumors and provide precursors for nucleic acids, proteins and lipids as well as reducing capability (NADPH). Pyruvate is largely derived from glucose and glutamine metabolism; it can be converted to lactate by the LDH complex and/or enter the TCA cycle for conversion to CO_2 and ATP. The conversion of pyruvate to lactate is also catalyzed by LDH in a reversible reaction that results in the formation of NAD^+, which is necessary for further glycolysis. LDH is a tetrameric enzyme containing two major subunits (A and B) that are coded by the *LDHA* and *LDHB* genes; together, these subunits form five different isoenzymes.[264] Although all five isoenzymes can catalyze the forward and backward conversion of pyruvate and lactate, LDHA kinetically favors the conversion of pyruvate to lactate, whereas LDHB

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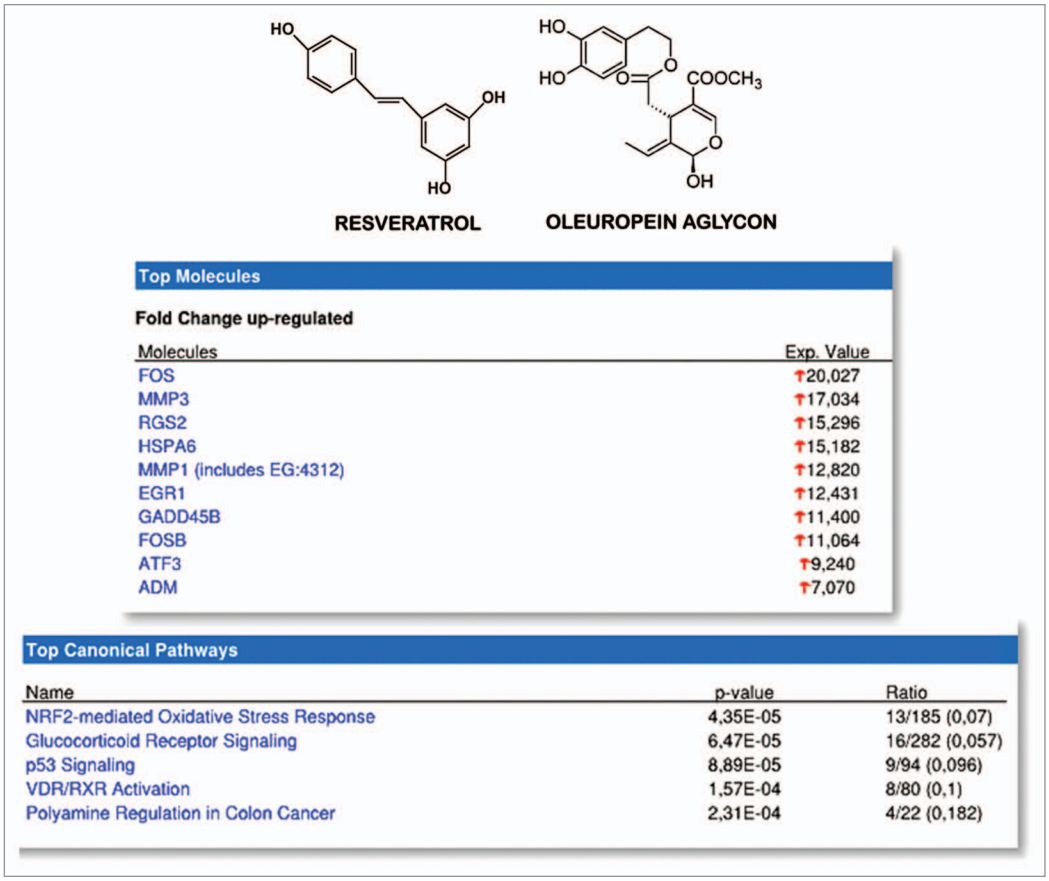

Top Molecules

Fold Change up-regulated

Molecules	Exp. Value
FOS	↑20,027
MMP3	↑17,034
RGS2	↑15,296
HSPA6	↑15,182
MMP1 (includes EG:4312)	↑12,820
EGR1	↑12,431
GADD45B	↑11,400
FOSB	↑11,064
ATF3	↑9,240
ADM	↑7,070

Top Canonical Pathways

Name	p-value	Ratio
NRF2-mediated Oxidative Stress Response	4,35E-05	13/185 (0,07)
Glucocorticoid Receptor Signaling	6,47E-05	16/282 (0,057)
p53 Signaling	8,89E-05	9/94 (0,096)
VDR/RXR Activation	1,57E-04	8/80 (0,1)
Polyamine Regulation in Colon Cancer	2,31E-04	4/22 (0,182)

Figure 5. Top: Structural similarities between resveratrol and the EVOO secoiridoid oleuropein aglycon. Bottom: IPA-identified top individual genes and top canonical pathways affected by EVOO secoiridoids.

predominantly converts lactate to pyruvate, which is further oxidized through the TCA cycle. Serum LDH levels are often increased in cancer patients, and *LDHA* protein expression is often upregulated in tumors.[265-273] Like high lactate levels, which are a key feature of the aerobic glycolysis (Warburg effect) in tumor cells and are associated with the subsequent development of metastases,[274,275] the presence of high LDH levels in tumors has been linked to poor prognosis and greater metastatic potential. Because the LDHA protein is required for the maintenance and progression of many tumors, it also represents a potential target for cancer therapy.[276] Our findings suggest a potential inhibitory role of EVOO secoiridoids against the Warburg effect in tumor cells.

EVOO secoiridoids are resveratrol transcriptional mimickers that activate the energy sensor AMPK. Previous studies have shown that resveratrol efficiently induces changes in the transcriptional profiles of key metabolic tissues that closely resemble the changes induced by CR.[91,277] The data presented here demonstrate that EVOO secoiridoids appear to mimic key features of resveratrol-induced gene expression patterns to inhibit the growth of cancer cells, whose aberrant bioenergetic and biosynthetic metabolism is unambiguously required for proliferation and/or survival. To further confirm that administration of EVOO secoiridoids functionally mimics resveratrol via activation of AMPK-related stress signaling pathways, we employed two additional complementary approaches. First, systematic and integrative analyses of EVOO secoiridoid-regulated gene lists were conducted using the DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatics resource (National Institute of Allergy and Infectious Diseases, NIH), a web-based public database capable of uncovering biological features and meaning associated with large gene lists, regardless of which genomic platform or software package was used to generate the list (http://david.abcc.ncifcrf.gov/). DAVID uses a set of fuzzy classification algorithms to group genes based on their co-occurrence in annotation terms and ranks the gene groups using an internal (EASE) score.[278,279] DAVID was used to evaluate the enrichment distribution across the "biological processes" in the gene ontology (GO) tree. The threshold value of the enrichment score was set at 1.0 instead of 1.3, thereby avoiding the loss of important information. The gene list was organized and condensed into biologically meaningful modules using the DAVID gene functional classification tool at the medium level of statistical stringency. When ranking the importance of annotation groups with enrichment scores ≥ 1.0, DAVID term-centric modular enrichment analysis revealed that the "biological modules" significantly enhanced by EVOO

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secoiridoids paradoxically included positive regulation of "developmental and biological processes," "response to stress," "organ morphogenesis," "response to chemical stimulus (unfolded protein)," "response to wounding" and "chromatin assembly," among others (**Table S2**). The activation of anti-aging biological modules was concomitant with the significant downregulation of "hexose catabolic processes," "cell cycle" and "cellular carbohydrate metabolic processes," among others (**Table S2**). Therefore, highly aggressive cancer cells appear to react to EVOO secoiridoid-triggered cellular stress signals by evoking cell survival programs that ultimately result in cancer cell death.

Second, to unambiguously determine whether the crucial signaling pathways that are significantly altered in the presence of EVOO secoiridoids are similar to those previously recognized for resveratrol, we used the "canonical pathway analysis" function included in the IPA analysis software. This analysis associates probe sets with the canonical pathways included in Ingenuity's Knowledge Base and returns two measures of association: (1) the ratio of the number of genes from the list that map to the pathway to the total number of genes that map to the same pathway, and (2) a p valuebased on Fisher's exact test to ascertain enrichment. Notably, when the canonical pathways induced by EVOO secoiridoids were ordered by p value($p < 0.05$; the ratio value is also shown), all of the molecular mechanisms underlying resveratrol's recognized anti-aging effects were over-represented in the five canonical pathways that were most significantly upregulated by EVOO secoiridoids in cancer cells (**Fig. 5**). First, the above-mentioned resveratrol-induced FOS-dependent inhibition of polyamine synthesis and increased polyamine catabolism[218,219] was mirrored in the EVOO secoiridoid-induced "polyamine regulation in colon cancer" pathway (p value= 2.31E-04). Second, the resveratrol-related vitamin-D/retinoic acid-like differentiation-induced effects[280-284] were mirrored in the EVOO secoiridoid-induced "VDR/RXR activation" pathway (p value= 1.57E-04). Third, the resveratrol-induced p53-related engagement of cell cycle arrest and/or apoptotic signals[285-290] was mirrored in the EVOO secoiridoid-induced "p53 signaling" pathway (p value= 8.89E-05). Fourth, resveratrol's anti-inflammatory effects related to epigenetic and chaperone-dependent activation of the glucocorticoid receptor[134,291-294] were mirrored in the EVOO secoiridoid-induced "glucocorticoid receptor signaling" pathway (p value= 6.47E-05). Fifth, resveratrol's ability to protect against oxidative stress damage by modulating nuclear redox factor 2 (NRF2) signaling[295-302] was mirrored in the EVOO secoiridoid-induced "NRF2-mediated oxidative stress response" pathway (p value= 4.35E-05). Considering that most of the beneficial effects of CR on the carcinogenic process are likely mediated by NRF2[303,304] and that recent studies have shown that a diet rich in EVOO phenolics (e.g., hydroxytyrosol, which is mainly formed from the hydrolysis of the secoiridoid oleuropein aglycone) induces SIRT1 and NRF2-dependent gene expression of anti-stress targets [e.g., glutathione-S-transferase (GST), γ-glutamyl cysteine synthetase (γ-GCS), nicotinamide adenine dinucleotide phosphate [NAD(P)H]:quinone oxidoreductase (NQO1) and paraoxonase-2 (PON2) mRNAs as well as paraoxonase-1 (PON1) activity] in senescence-accelerated (SAMP8) mice,[305] our findings strongly support the idea that the ability of secoiridoids to activate NRF2 signaling in somatic cells constitutes a mechanism through which EVOO complex polyphenols could lead to a delay in or the prevention of the onset of some forms of human cancers (e.g., breast cancer) and subsequently contribute to improved human health and lifespan.

Finally, we sought to investigate the unexplored possibility that EVOO secoiridoids might attenuate the adaptable aging-accelerating mTOR signaling pathway in cancer cells. Incubation of JIMT1 breast cancer cells (**Fig. 4**) and PC9 lung carcinoma cells (data not shown) with increasing concentrations of an EVOO PE rich in secoiridoids resulted in increasing activation of the mTOR gerosuppressor AMPK.[306] Activation of AMPK was associated with phosphorylation of the α-catalytic subunit of the enzyme at Thr-172, as assessed using a phosphospecific antibody. Minimal changes in total AMPK protein levels were detected with anti-α1/-α2 AMPK antibodies. When EVOO phenolic extracts highly enriched with secoiridoids were replaced by EVOO phenolic extracts with a similar content of total polyphenols but enriched with lignans, we observed a drastic decrease in the ability of the EVOO PEs to activate AMPK (data not shown). These results demonstrate for the first time that AMPK becomes significantly activated by EVOO-derived phenolic extracts when the amount of complex polyphenol secoiridoids but not lignans exceeds a critical threshold.

EVOO Secoiridoid Polyphenols Induce Senescence Delay in Human Diploid Fibroblasts

Well-accepted hormetic strategies such as repeated mild heat stress (RMHS) significantly affect several age-related phenomena in human skin fibroblasts, e.g., cell size and cell morphology, but do not modify the proliferative capacity of these cells.[32,33,78] We decided to investigate whether repeated exposure to non-cytotoxic concentrations of EVOO secoiridoids might improve the structural and functional integrity of skin fibroblasts in vitro without promoting entrance of these cells into a potentially deleterious hyperproliferative mode. Thus, using the well-established in vitro senescence model of human diploid fibroblasts (HDFs), we determined for the first time whether senescence-associated changes occur in response to chronic exposure to crude EVOO PEs. Cell viability (MTT assays) confirmed that, at the concentration employed in our studies, EVOO secoiridoids did not exhibit highly toxic effects. Compared with untreated control fibroblasts, we observed almost no cell death of young HDFs after 72 h of incubation with 200 ng/mL EVOO-PE7. After 10 d of treatment, however, 20–25% of the cells cultured in the presence of the secoiridoid-rich EVOO PE were metabolically nonviable (data not shown).

Low-passage $p16^{INK4a}$-positive WI-38 fetal lung HDFs and $p16^{INK4a}$-negative BJ-1 neonatal foreskin HDFs were exposed to low concentrations of EVOO secoiridoids or to the same volume of vehicle twice a week during serial

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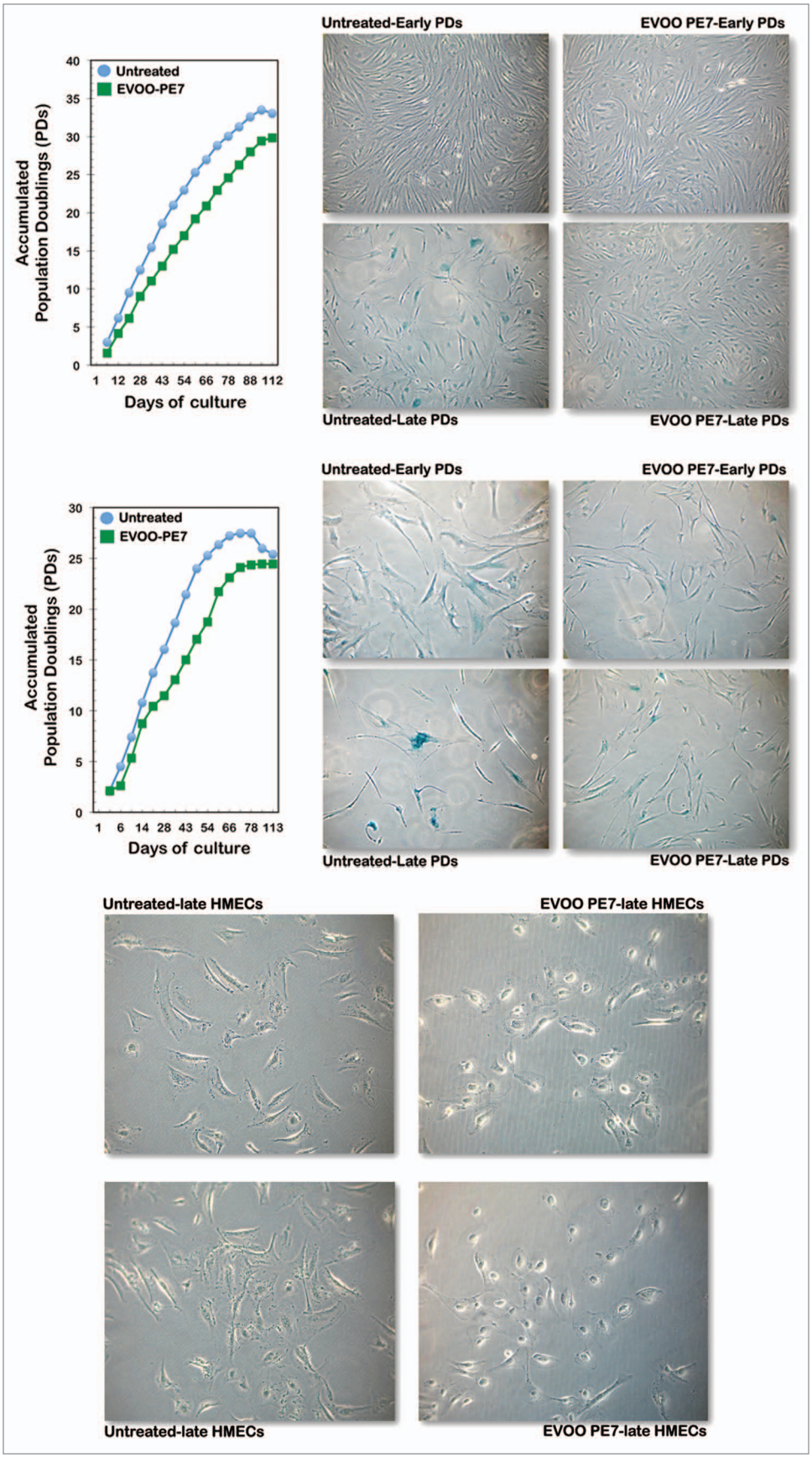

Figure 6. For figure legend, see page 568.

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Figure 6 (See previous page). Impact of chronic exposure to EVOO secoiridoids in age-related changes of cultured human diploid fibroblasts (HDFs) and human mammary epithelial cells (HMECs). Left: Graphs showing cumulative population doubling for BJ-1 (top) and WI-38 (bottom) HDFs continuously cultured in the absence or presence of 200 ng/mL EVOO-PE7. Representative microphotographs illustrate the differential acquisition of age-related biomarkers including changes in cell morphology and SA-β-gal activity (blue staining) in response to EVOO secoiridoids. Right: Representative microphotographs illustrate the impact of short-term treatment (3 d) with EVOO secoiridoids in the vacuolization and abundant accumulation of cell debris in pre-senescent HMECs.

passaging throughout their entire replicative life spans. Chronic exposure to EVOO secoiridoids failed to lengthen the proliferative lifespans of WI-38 and BJ-1 HDFs (**Fig. 6**). However, whereas the growth rates, population doubling rates and cumulative population doubling (PD) levels of the cells were mostly unaffected by repeated exposure to EVOO secoiridoids, age-related changes in cell size, cellular morphology and senescence-associated β-galactosidase (SA-β-gal) staining were significantly altered. Age-related alterations in the morphology of fibroblasts, which is one of the most obvious changes that occurs during cellular aging, was significantly reduced in EVOO secoiridoid-treated HDFs. At the end of their proliferative lifespans, untreated control cultures underwent a significant increase in cell size, taking on a flattened appearance; their morphological heterogeneity also increased, and they suffered a complete loss of arrayed arrangement and accumulated significant amounts of intracellular and extracellular debris, with concomitant increases in the sizes of their nuclei and nucleoli (**Fig. 6**, left). Indeed, a short-term treatment (3 d) with EVOO secoiridoids notably ameliorated the intense vacuolization and abundante accumulation of cell debris in nearly senescent human mammary epithelial cells (HMECs) (**Fig. 6**, right). Moreover, we noted significantly higher proportions of β-gal-positive WI-38 and BJ-1 cells in old HDF cultures than in young HDF cultures. At the end of their proliferative lifespans, HDF cultures grown continuously in the presence of EVOO secoiridoids demonstrated significantly reduced age-related morphological alterations and displayed relatively young-like morphologies. Old HDF cultures chronically exposed to EVOO secoiridoids did not undergo significant cell enlargement and largely maintained the thin, long, spindle shapes observed in younger HDF cell populations. In contrast to control cultures, EVOO secoiridoid-treated HDFs maintained an arrayed arrangement of morphologically homogeneous cells (**Fig. 6**), with reduced accumulation of lysosomal residual bodies and an almost complete absence of multinucleated cells. When the proliferation rate of the untreated control cultures began to decrease as cellular senescence approached, SA-β-gal activity was measured; we notably observed significantly fewer β-gal-positive cells in EVOO secoiridoid-treated HDFs than in vehicle-treated HDFs (**Fig. 6**).

Taken together, these findings demonstrate for the first time that complex mixtures of crude EVOO PEs antagonize cellular senescence without modifying the proliferative capacity of HDFs. Katsiki and colleagues[307] previously reported that oleuropein-treated cultures of normal human fibroblasts exhibited a significant delay in the appearance of senescence morphology. In their hands, however, oleuropein treatment of human embryonic fibroblasts conferred a life span extension of approximately 15%. It is plausible that the presence of numerous phenolic molecules within a crude EVOO PE would not preclude the ability of "diluted" secoiridoids to suppress senescence as efficiently as a single purified secoiridoid (e.g., oleuropein) and that, at concentrations such as those used in our experiment, the slightly cytotoxic effects of the crude EVOO phenolic mixture would prevent a plausible anti-aging (preservation of proliferative capacity) effect. Of note, when older HDFs chronically cultured in the presence of 200 ng/mL EVOO-PE7 were challenged with higher concentrations of the same PE, they were notably refractory to the cytotoxic effects observed when EVOO secoiridoid-naive young HDFs were treated with the same high dose of polyphenols (data not shown). This finding supports the idea that continuous exposure to hormetic stresses (e.g., low-dose secoiridoids) can protect cells from stronger stresses (e.g., high-dose secoiridoids) but that these stronger stresses do not cause aging, as aging is not caused by any stress.[180]

EVOO Secoiridoid Polyphenols: A New Family of "Xenohormetic" Compounds

The previously unrecognized ability of EVOO secoiridoids to activate endogenous cellular defense pathways (e.g., the evolutionarily conserved NAD-dependent deacetylase sirtuin-1 and NRF2 pathways) that integrate the adaptive stress response and positively control the expression of a battery of stress response proteins in human cells support the original "xenohormesis hypothesis" of Howitz and Sinclair[63,89,134] invoking the interspecies communication of stress signals. The literature on sirtuin focuses on pharmacological activators of SIRT1 (e.g., resveratrol, SRT1720), which have been proposed as therapeutics for diabetes, neurodegeneration, inflammation and other diseases. However, many compounds may have been identified as SIRT1 activators due to artifacts in the assay methodology (i.e., the use of fluorescently tagged substrates). By performing the first comprehensive analysis of gene expression and transcriptome dynamics of human breast cancer cells grown in the presence of crude phenolic EVOO extracts, we present compelling data that suggest that the stress response of Oleacea plants, which has evolved as a result of their stationary lifestyle, might confer stress resistance and "anti-aging benefits" to animals such as humans that consume bioactive secoiridoids produced by Oleacea.

In highly proliferative cancer cells that possess aberrant bioenergetic and biosynthetic metabolism, EVOO secoiridoid-imposed metabolic reprogramming would be expected to promote growth inhibition and cell death; however, the ability of

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relatively non-toxic secoiridoids to upregulate a variety of transcriptomic programs involved in regulating stress responses should result in increased longevity of normal cells. This apparent metabolic paradox can easily be resolved in the context of an evolutionary view of the "AMPK/mTOR-xenohormetic" model. AMPK, whose ancestral role may have been related to the response to starvation for the preferred carbon source, glucose, appears to have arisen very early during eukaryotic evolution. Rapid cell growth requires the active synthesis of proteins, rRNA and lipids, all of which are switched off by the activation of AMPK (and, likely, by downstream inactivation of mTOR). Indeed, one reason for the high glycolytic rate of rapidly proliferating cells, including tumor cells, is that the TCA cycle ceases to be a purely catabolic pathway and becomes at least partially anabolic, actively providing precursors for biosynthesis, particularly citrate for lipid synthesis.[275] Accordingly, both tumor cells and viruses (and likely other pathogens) appear to have developed mechanisms to downregulate the energy sensor AMPK and escape from its restraining influence on growth and biosynthesis.[9,10,107] In an "AMPK/mTOR-xenohormetic" model, there is no need to assume that animals and fungi have retained an ability to be activated by certain plant stress molecules, because they provide useful advance warning of a deteriorating environment or food supply. Soil bacteria do not produce the macrocyclic lactone rapamycin as an anticancer drug or a pro-longevity medicine but as an antibiotic that inhibits the growth of fungal competitors. The French lilac or goat's rue (*Galega officinalis*) produces galegine, the bioactive starting material from which metformin was developed, as a defense compound to deter grazing by herbivores, not as a gerosuppressant that delays aging and suppresses tumorigenesis. Grapes produce resveratrol in response to fungal infection but not as a longevity nutrient with anticancer properties. Similarly, upon activation and conversion to oleuropein aglycon by deglycosylation, phenolic secoiridoid glycosides such as oleuropein can induce a loss of nutritive value via the loss of lysine and inactivation of enzymes by functioning as a unique multivalent alkylator that acts as a protein crosslinker, providing plants with an effective defense against attack by herbivores and possibly by pathogens.[307-310] The fact that numerous and apparently unrelated "nutraceuticals," "xenobiotics" and other biocompounds derived from traditional herbal medicines act as xenohormetic compounds merely reflects their common ability to inhibit the aging-driven activity of mTOR gerogenes and/or to activate key gerosuppressors of the mTOR pathway (i.e., AMPK).[180,311-322] Considering that mitochondria became the main cellular power source during the evolutionary development of eukaryotes,[323] AMPK plausibly arose very early during eukaryotic evolution due to the requirement for sensing energy status in the cytoplasm and providing a signal to modulate mitochondrial function. Indeed, the ancestral function of AMPK in plants and animals was likely to orchestrate resistance responses to the effects of carbohydrate starvation (e.g., to trigger a switch back to oxidative metabolism in response to deprivation of the preferred carbon source, glucose). Of note, most of the potent activators of AMPK are plant defense compounds that inhibit mitochondrial ATP synthesis. Forthcoming studies should definitively elucidate whether EVOO secoiridoids and other xenohormetic compounds impact both mitochondrial functionality and AMPK-like metabolic sensors across different species (e.g., olive and human) during times of stress; in this scenario, xenohormesis should be viewed as providing a shared ability to molecularly connect mitochondria and AMPK during evolution. Curiously, SIRT1 has been shown to play an essential role in the ability of moderate doses of resveratrol to stimulate AMPK and improve mitochondrial function both in vitro and in vivo.[324]

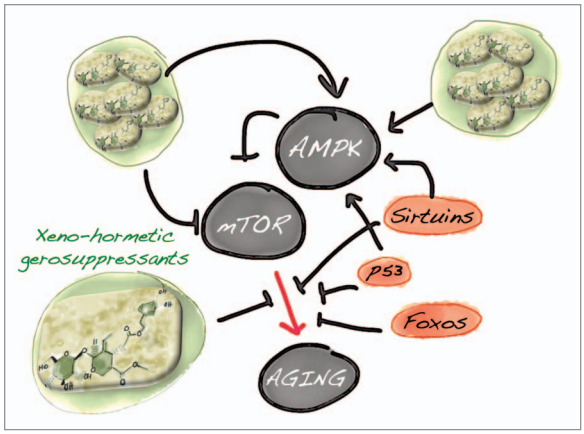

Figure 7. EVOO secoiridoids: A new family of plant-produced gerosuppressant agents that molecularly "repair" the aimless (and harmful) AMPK/mTOR-driven quasi-program that leads to aging and aging-related diseases, including cancer.

EVOO Secoiridoid Polyphenols: A New Family of "Gerosuppressant" Compounds

Despite the high degree of structural resemblance between EVOO-derived complex polyphenols and well-recognized CR-like polyphenols that are known to experimentally extend lifespan (i.e., resveratrol), no studies have explored the actual molecular function of EVOO secoiridoids in retarding human aging. As for many other polyphenols, it has been erroneously assumed that EVOO-derived complex phenols provide health benefits, including higher longevity, largely because of their antioxidant activity. Our laboratories at the Spanish BFCP have been studying for the first time whether secoiridoids, a family of complex phenolics characteristic of Oleacea plants, by functioning as biocompounds belonging to the recently defined group of "hormesis A" compounds (i.e., inhibitors of the pro-aging activity of mTOR gerogenes and/or activators of mTOR gerosuppressors such as AMPK) can, like resveratrol, affect anti-aging signaling pathways in ways that significantly promote cytotoxicity in immortal tumor cells and that weaken

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age-related pro-senescence effects in normal cells. Because changes in the expression of significant numbers of genes have been linked to the anticancer and lifespan effects of all known lifespan interventions (CR- and the so-called CR-mimetics), we hypothesized that global changes in the human transcriptome detectable by the use of high-density microarrays could be used for the preliminary identification of candidate EVOO secoiridoids-induced anti-aging/anticancer gene signatures. The strength of evidence supporting the xenohormetic activity of EVOO secoiridoids was tested by assuming that their tumoricidal activity results from the paradoxical activation of cellular stress-like, anti-aging transcriptomic signatures in cancer cells. By following this genome-wide analysis approach in highly aggressive human breast cancer cells that were briefly exposed to crude EVOO phenolic extracts highly enriched in the secoiridoids oleuropein aglycone and decarboxymethyl oleuropein aglycone, we demonstrated that Oleacea plant defense molecules, which are able to exert strong protein-denaturing/protein-crosslinking/lysine-alkylating activities against herbivores, can efficiently induce in human cells intracellular signaling pathways that may respond to biological stress at the molecular/cellular level. We confirmed that the stress pathways activated by EVOO secoiridoids might defend cells and tissues in a hormetic-like manner, because they regulate energy metabolism in a way that would be expected to enhance cellular survival during times of stress. Thus, the anticancer activity of EVOO secoiridoids was found to be related to the activation of anti-aging/cellular stress-like gene signatures, including endoplasmic reticulum (ER) stress and the unfolded protein response, spermidine and polyamine metabolism, sirtuin-1 (SIRT1),[325-351] and NRF2 signaling. EVOO secoiridoids activated the gerosuppressor AMPK and inhibited crucial metabolic genes involved in the Warburg effect and the self-renewal capacity of "immortal" cancer stem cells and EVOO secoiridoids significantly prevented age-related changes in cell size, morphological heterogeneity, arrayed arrangement and senescence-associated β-galactosidase staining of normal diploid human fibroblasts at the end of their proliferative lifespan.

Aging can be viewed as a quasi-programmed phenomenon driven by the overactivation of the nutrient-sensing mTOR gerogene. Complementing this idea, emerging studies indicate that the responsiveness of AMPK signaling clearly declines with aging.[306] The loss of sensitivity of AMPK activation to cellular stress impairs metabolic regulation, increases oxidative stress and reduces autophagic clearance. Not surprisingly, very recent studies have illuminated the central role that loss of the AMPK gerosuppressor plays in dictating the unique metabotype that drives tumorigenesis.[352,353] Given that AMPK is a crucial gerosuppressor and tumor-suppressor that suppresses mTOR-driven geroconversion (as well as mTOR oncogenic transformation), age-related diseases reflect a synergistic interaction between our evolutionary path to sedentarism, which chronically increases a number of gero-promoting factors, e.g., mTOR activators such as nutrients (glucose, amino acids, fatty acids), growth factors, cytokines and insulin and the "defective design" of central metabolic integrators such as AMPK and mTOR. In this scenario, xenohormesis should be viewed in terms of plant-produced gerosuppressants that molecularly "repair" the aimless (and harmful) AMPK/mTOR-driven quasi-program of aging and aging-related diseases (**Fig. 7**).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
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