Gene Regulation and Systems Biology

ORIGINAL RESEARCH

Alpha-Tocopherol Modulates Transcriptional Activities that Affect Essential Biological Processes in Bovine Cells

Cong-jun Li, Robert W. Li and Theodore H. Elsasser

Bovine Functional Genomics Laboratory, Animal and Natural Resources Institute, ARS, USDA, 10300 Baltimore Ave, Beltsville, MD, 20705, USA. Corresponding author email: congjun.li@ars.usda.gov

Abstract: Using global expression profiling and pathway analysis on α-tocopherol-induced gene perturbation in bovine cells, this study has generated comprehensive information on the physiological functions of α-tocopherol. The data confirmed α-tocopherol is a potent regulator of gene expression and α-tocopherol possesses novel transcriptional activities that affect essential biological processes. The genes identified fall within a broad range of functional categories and provide the molecular basis for its distinctive effects. Enrichment analyses of gene regulatory networks indicate α-tocopherol alter the canonical pathway of lipid metabolism and transcription factors SREBP1 and SREBP2, (Sterol regulatory element binding proteins), which mediate the regulatory functions of lipid metabolism. Transcription factors HNF4-α (Hepatocyte nuclear factor 4), c-Myc, SP1 (Sp1 transcription factor), ESR1 (estrogen receptor 1, nuclear), and androgen receptor, along with several others, were centered as the hubs of transcription regulation networks. The data also provided direct evidence that α-tocopherol is involved in maintaining immuno-homeostasis through targeting the C3 (Complement Component 3) gene.

Keywords: α-tocopherol, bovine cells, ESR1, gene regulation, lipid metabolism

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Introduction

Vitamin E is present in plants in eight different forms with more or less equal antioxidant potential (α-, β-, γ, δ-tocopherol/tocotrienols). Nevertheless, in animals and humans, only α-tocopherol is preferentially retained, suggesting a specific mechanism for the uptake for this analogue,[1,2] despite the fact that this mechanism still remains elusive. It was discovered that α-tocopheryl succinate (α-TS) was a more effective form of vitamin E than the water soluble preparation of α-tocopherol or α-tocopheryl acetate for inducing differentiation, inhibition of cell proliferation, and inducing cell death in marine melanoma cells in culture.[3,4] However, after nearly 100 years of research and countless publications, the physiological functions of vitamin E remain mysterious to a certain degree. In recent years, it has been found that α-tocopherol is more than just a simple fat-soluble antioxidant. The ability of vitamin E to modulate signal transduction and gene expression has been observed in numerous studies.[5,6] Available evidence also suggests a role of vitamin E in cell homeostasis that occurs through the modulation of specific signaling pathways and genes involved in proliferation, metabolic, inflammatory, and antioxidant functions.

Improving nutrient efficiency of cattle is an important issue because feed costs are the greatest expense to dairy and beef production, at approximately 50% of production cost. The importance of nutrient supply on body composition and growth performance of calves has been evaluated extensively. However, as one of the most commonly used single nutrient supplements, potential mechanisms of effeteness of vitamin E on consequences of increased growth rate, associated with enhanced nutrition, have not been investigated. The objective of this study was to determine the effect of the α-tocopherol form of vitamin E in an established bovine cell line. In this study, potential biological roles of α-tocopherol were investigated using the established Madin-Darby bovine kidney epithelial cell line (MDBK). The study focused on determining whether normal bovine cells in a standard cell culture condition were sensitive to the biological effects of α-tocopherol. Here we report our findings regarding the functional category and pathway analysis of differential expressed genes in MDBK cells treated with α-tocopherol.

Materials and Methods

Cell culture and α-tocopherol treatments

The MDBK cells (American Type Culture Collection, Manassas, VA, and Catalog No. CCL-22) were cultured in Eagle's minimal essential medium and supplemented with 5% fetal bovine serum (Invitrogen, Carlsbad, CA) in 25 cm^2 flasks as described in our previous report.[7] At approximately 50% confluence (during the exponential phase), the cells were treated with α-tocopherol (designated concentration from 0 to 80 μM) for 24 h. (α-tocopherol acid succinate, Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). Stock solution of 10 mM α-tocopherol was prepared by dissolving α-tocopherol in 100% alcohol. For cells prepared for microarray study, treatment of 40 μM of α-tocopherol was selected for our experiments after titration for cell responses. This concentration induces biological effects of cell cycle arrest without inducing significant amount of cell death. It is also in a range of concentrations that were extensively used in many in vitro experiments.[8,4] Three replicate flasks of cells for both treatment and control groups (a total of 6 samples) were used for the microarray experiments. The control cells were treated with the same amount of solvent without α-tocopherol.

Flow cytometry analysis

To measure the DNA content, cells were stained with a fluorescent dye (propidium iodide) that is directly bound to the DNA in the nucleus of the cells. Measuring the fluorescence by flow cytometry provided a measurement of the amount of dye taken up by the cells and, indirectly, the amount of DNA content. To perform flow cytometry analysis, cells were collected by trypsinization, and then were washed and suspended with ice-cold PBS (phosphate-buffered saline, pH 7.4) buffer. Two volumes of ice-cold 100% ethanol were added in drops into tubes, and the cells were mixed in suspension through slow vortexing. After ethanol fixation, the cells were centrifuged (400 g, 5 min) and washed once in PBS buffer. The cells were then re-suspended at 10^6 cells/ml. Then RNase A was added to a final concentration of 50 μg/ml (Sigma Chemical Co., St. Louis, MO) to each sample, and the samples were incubated at 37 °C for 30 min. After incubation, 20 μg of propidium iodide (Sigma Chemical Co.,

St. Louise, MO) was added to each tube for at least 30 min to provide the nuclear signal for fluorescence-activated cell sorting (flow cytometry). The DNA content of the cells was analyzed using flow cytometry (FC500, Beckman Coulter Inc., Palatine, IL) and the collected data were analyzed with Cytomics RXP (Beckman Coulter Inc.). At least 10,000 cells per sample were analyzed.

Preparation of cell extracts and western blot analysis

Preparations of cells and cell extracts, SDS-PAGE, and Western blots were described in our previous publication.[7] Western blots were then scanned and analyzed with the Odyssey infrared imaging system (Li-COR Biosciences, Lincoln, NE) to quantify the density of the bands. Rabbit anti-ESR1 antibody (OriGene, Rockville, MD, Cat. No. TA300376), Mouse monoclonal IgG activated C3 antibody (Santa Cruz Biotech, Santa Cruz, CA, Cat. No. sc-55458) and rabbit anti-Erk1/Erk2 (extracellular signal-regulated kinases or mitogen-activated protein kinase being 3) antibody (Calbiochem, San Diego, Cat. No. 442704) were used for Western Blots. All three antibodies were validated against bovine cell extract before being used in the experiments.

Oligonucleotide microarray, hybridization, image acquisition, and data analysis

The bovine microarray platform used was described in our earlier paper.[9] A total of 86,191 unique 60-mer oligonucleotides were designed and synthesized in situ using photo deprotection chemistry.[10] Each unique oligonucleotide was repeated 4 times on the array (a total of ~340,000 features). These oligonucleotides represented 45,383 unique bovine sequences/genes, including 40,808 Tentative Consensus sequences (TCs) from the TIGR *Bos taurus* gene index (www.tigr.org) and 4,575 singletons. Hybridization, image acquisition, and data analysis was described in previous research.[11,12] The microarrays were scanned with an Axon GenePix 4000B scanner (Molecular Devices Corp., Union City, CA) at 5 μM resolution. The data was extracted from the raw images with NimbleScan software (NimbleGen, Madison, WI). The control and α-tocopherol treatment each had 3 replicates and a total of 6 microarrays were used in the experiment.

Isolation of total RNA

Total RNA was extracted with TRIzol by following the manufacturer's recommendations (Invitrogen). Trace genomic DNA in the crude total RNA samples was removed by incubation with 4–10 units DNase I per 100 μg total RNA (Ambion, Austin, TX) at 37 °C for 30 min. Total RNA was further purified with an RNeasy Mini kit (Qiagen, Valencia, CA). The concentration of the total RNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE) and RNA integrity was verified with a Bioanalyzer 1000 (Agilent, Palo Alto, CA).

Generation of biotin-labeled cRNA

Biotin-labeled cRNA was generated with a modified procedure of the Superscript Choice System (Invitrogen) for double-strand (ds) cDNA synthesis followed by in vitro transcription. Briefly, the 1st strand cDNA was synthesized from 4.0 μg total RNA with 1.0 unit SuperScript II reverse transcriptase (Invitrogen) in the presence of 100 pmoles T7 promoter Oligo dT primer. After 2nd strand synthesis, the DNA was purified with a DNA Clean and Concentrator-5 kit (Zymo Research, Orange, CA) and eluted with 8 to 16 μl of deionized (dd) H_2O. The recovered ds cDNA was further concentrated down to 3 μl by a speed vacuum device. The cRNA was synthesized with a MEGAscript in vitro Transcription kit (Ambion). The in vitro transcription reaction was carried out in a total volume of 23.0 μl, which consisted of 3.0 μl ds cDNA, 2.3 μl 10X Ambion reaction buffer, 2.3 μl 10X Ambion T7 enzyme mix, and 15.4 μl NTP labeling mix (7.5 mM ATP, 7.5 mM GTP, 5.625 mM UTP, 5.625 mM CTP, and 1.875 mM biotin-16-UTP and 1.875 mM biotin-11 CTP). The in vitro transcription reaction was incubated at 37 °C for 16 hours in a thermocycler. The cRNA was purified with an RNeasy mini-kit (Qiagen). Generally, 40 to 60 μg of cRNA can be obtained from 4.0 μg of total input RNA. The size range of the cRNA, expected to be between 300 to 3000 bp with the maximum intensity centered at least at 1000 bp, was verified using a Bioanalyzer 1000. The biotinylated cRNA was fragmented into 50 to 200 bp pieces by heating the cRNA in a buffer

consisting of 40 mM Tris-acetate, pH 8.0, 100 mM potassium acetate, and 30 mM magnesium acetate at 95 °C for 35 min.

Functional and pathways analyses

To understand the molecular processes initiated following stimulation by α-tocopherol, the expression data were input into MetaCore (the MetaCore analytical suite, Version 4.7, GeneGo, St. Joseph, MI, www.GeneGO.com) and analyzed for transcription regulatory networks enriched with the expression data. MetaCore is a web-based suite for functional analysis of experimental data in the context of a manually curated database containing the probability of having the protein interactions, protein-DNA interactions, canonical pathways, signaling pathways, and knowledge base ontologies of cellular processes, diseases, and toxicology. The 1183 genes altered with regard to expression were used as a list of input nodes and subjected to the Analyzed Networks (AN) Transcription regulation algorithm, AN Transcription factors algorithm, and AN Receptors algorithm.

Enrichment analysis consists of matching gene IDs for the common, similar, and unique sets of the uploaded files with gene IDs in functional ontologies in MetaCore. Enrichment analysis and gene regulatory networks were generated with the MetaCore analytical suite. The experimental data in MetaCore can be subjected to enrichment analysis[13] in six functional ontologies: gene ontology processes (GO), GeneGo process networks, diseases, GeneGo diseases, canonical pathway maps, and metabolic processes. Enrichment analysis in GO processes was used in this study. Enrichment analysis consists of matching gene IDs for common, similar, and unique sets of uploaded files with gene IDs in functional ontologies in MetaCore. The ontologies include canonical pathway maps, GeneGo cellular processes, GO cellular processes, and disease categories. The degree of relevance to different categories in the uploaded datasets is defined by *P*-values so that the lower *P*-value gets higher priority. Both enrichment analysis and the calculation of the statistical significance of networks are based on *P*-values, which are defined as the probability of a given number of genes from the input list matching a certain number of genes in the ontology folder. The *P*-values can also be defined, as described in the supplementary files of Shipitsin et al[14] as the probability of the network's assembly from a random set of nodes (genes) that are the same size as the input list. The whole data set was used to build networks using the Analyze Networks (AN) (transcription regulation) algorithm and the AN (receptor, transcription regulation, and transcription factors) networks algorithm, which generate sub-networks centered on transcription factors and receptors respectively. The sub-networks were scored and prioritized based on relative enrichment using the data from the input list and saturation with 'canonical pathways' while using *P*-values and z-scores as statistical metrics.[14] Networks of interest were also expanded by merging different networks.

Results

Flow cytometry analysis

Prior to microarray analysis, the DNA contents of the α-tocopherol treated cells were measured by flow cytometry. As shown in Figure 1, after α-tocopherol treatment for 24 h, cell population profiles changed significantly. There was a significant increase in the number of cells in G1 (cells with 2 copies (2C) of DNA contents) whereas those in the S phase (cells falling between the 2C and 4 copies (4C) DNA contents) were decreased. This response was also seen in a dose-dependent manner. Nevertheless, there was a notable shift in the G1/GO peak in α-tocopheryl-treated cells indicating the accumulation of cells that contain more DNA than that of G1/G0 cells.

α-tocopherol induces profound changes in gene expression on MDBK cells

After applying two differential layers of filtering to the normalized data, we identified 910 induced sequences that were enhanced greater than 2.0 fold and 273 sequences repressed greater than 1.5 fold in three biological replicates by α-tocopherol. Some redundant sequences on the microarray are apparent. Out of the genes surveyed (45,383), 1183 (910 + 273) were affected by α-tocopherol. This is approximately 2.60% (1183/45383) of the total genes surveyed. The genes are listed in the Supplement Table S1.

To verify the microarray findings, we used Western blotting to detect three selected gene products: ESR1, C3 (activated) and Erk1/2. The level of ESR1 (Fig. 2A) detected by Western blot confirmed

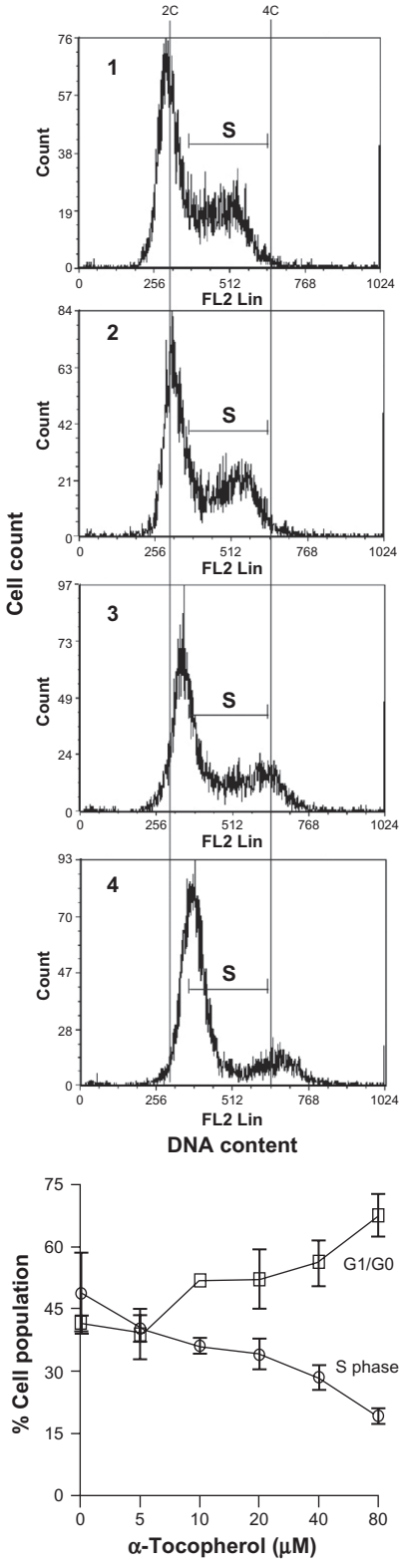

Figure 1. Alpha-tocopherol induces inhabitation of cell proliferation in Madin-Darby bovine kidney (MDBK) epithelial cells. **A1**–**A4**) Histogram plot of flow cytometry analysis of MDBK cells sorted by their DNA content in cells treated with 0, 10, 20 and 40 μM alpha-tocopherol respectively. (S: S-phase-DNA or the synthesis phase of the cell cycle. Synthesis occurs in the S phase and chromosomes are duplicated) **B**) Quantized cell population. G1/G0: cells in G1/G0 cell cycle phases; S: cells in S cell cycle phase.

that the ESR1 gene expression was up-regulated by α-tocopherol not only at the mRNA level but also significantly increased at the protein level. We also confirmed that levels of activated C3 (40 kDa fragment) (Fig. 2B) and Erk1/2 proteins (Fig. 2C)

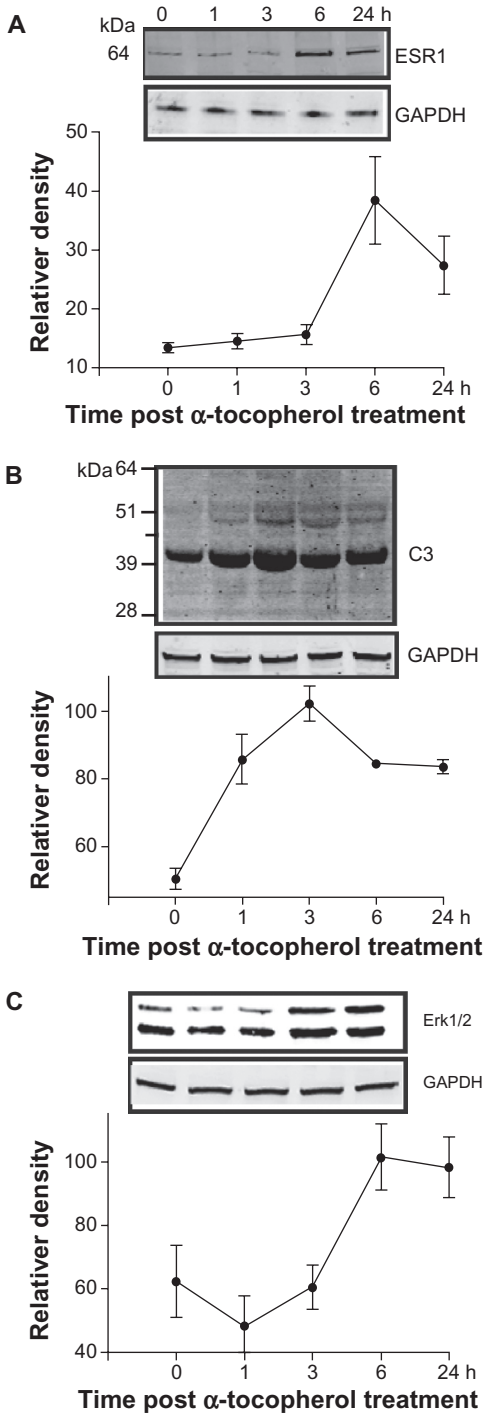

Figure 2. Western blotting of proteins. This figure is representative of two experiments. Equal amount of protein from different samples were separated by SDS PAGE 4 to 20% polyacrylamide gradient gels and was transferred to a membrane for Western blotting. The graphic represent quantitation from two Western blots.

increased upon α-tocopherol treatment. All three proteins (ESR1, C3, and Erk1/2) apparently reached the peak after 3 to 6 hr of α-tocopherol treatment and leveled off thereafter. There are significant changes ($P < 0.05$) for all three proteins from time 0 to the highest level during α-tocopherol treatment.

Enrichment analysis of gene regulatory networks

Canonical pathway maps represent a set of about 650 signaling and metabolic maps covering human biology in a comprehensive way. The top ten most significant pathways, based on the overrepresentation analysis ($P < 0.05$), are shown in Figure 3. The five most significant pathways were 1) regulation of lipid metabolism—regulation of lipid metabolism via LXR, NF-Y, and SREBP; 2) development—role of IL-8 in angiogenesis; 3) cytoskeleton remodeling; 4) cell adhesion—endothelia, cell contacts by junctional mechanisms; and 5) transcription—Sin3 and NuRD in transcription regulation. Many of these pathways are involved in cell metabolisms, transcriptional regulation, and cell proliferation regulation. The top scored map (map with the lowest *P*-value) based on the enrichment distribution sorted by 'statistically significant maps' is shown in Figure 4. It is the regulation pathway of lipid metabolism. In this pathway, transcription factors SREBP1 and SREBP2 mediate many established responses to sterols.

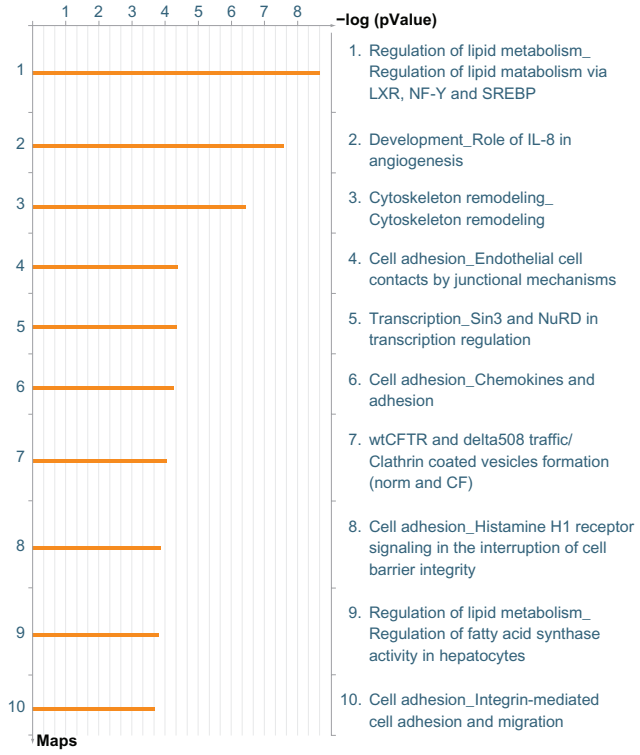

Figure 3. The top 10 most significant pathways, based on the overrepresentation analysis ($P < 0.05$) induced by α-topopherol treatment.

Analysis of transcription regulation networks

The gene content of the perturbed genes detected by the microarray was used as the input list for the generation of biological networks using the Transcription Regulation algorithm with default settings. These networks were built on the fly and were unique to our experimental data. Analysis of the gene expression data in MetaCore with the transcription regulation algorithm resulted in 20 sub networks with a center of various transcription factors. These networks described the functional relationships between gene products based on the known interaction reported in the literature. The 20 networks consisted of transcription factors that directly regulated the expression of downstream genes with a total network size of 134 to 58 genes and *P*-values for network significance ranging from $7.17e^{-265}$ to $2.23e^{-111}$. Transcription factors, such as HNF4-alpha, c-Myc, SP1, ESR1 (nuclear), and androgen receptor, were centered as the hubs of the networks. All networks generated from AN transcription regulation algorithm are listed in Table 1. To visualize the networks, Figure 5 shows a representative network, in which transcription factors NF-κB functioned as the key object of the network (both edge in and edge out). Genes that were down-regulated due to α-tocopherol treatment are marked with a blue dot while the up-regulated genes are marked with a red dot. This network appears to function in some very important GO processes, such as the regulation of cellular biosynthesis, and many other metabolic processes, such as positive regulation of DNA-dependent transcription, (30.6%; $P = 6.831e^{-13}$), positive regulation of RNA metabolic processes (30.6%; $P = 7.635e^{-13}$), positive regulation of transcription (32.3%; $P = 8.750e^{-13}$), positive regulation of gene expression (32.3%; $P = 1.209e^{-12}$), and the positive regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolic processes (32.3%; $P = 5.569e^{-12}$). This network caught our attention also because the key object of the network, ESR1, is a novel transcriptional modification target

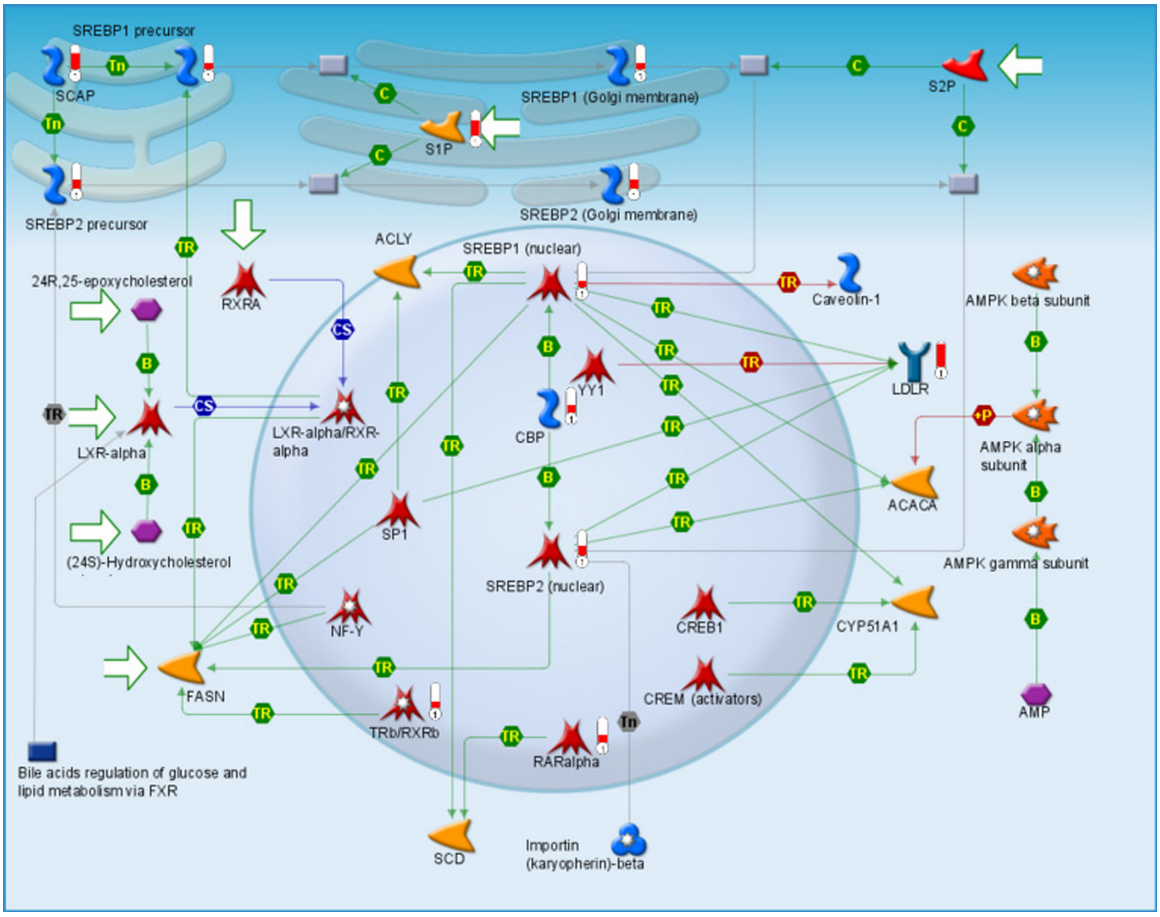

Figure 4. GeneGO Pathway Maps. The top scored map (map with the lowest *P*-value) based on the enrichment distribution sorted by 'Statistically significant Maps' set with the lowest *P*-value and ordered by –log10 of the *P*-value of the hypergeometric distribution: Regulation of lipid metabolism-regulation of lipid metabolism through LXR, NF-Y and SREBP pathway. Experimental data is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

of α-tocopherol. Two gene products (ESR1 and C3) from this network were confirmed by western blot (see Fig. 2).

Analysis of GeneGo process classification

There are about 117 cellular and molecular processes whose content is defined and annotated by GeneGo. Each process represents a pre-set network of protein interactions characteristic to the process. In order to explore the biological significance of α-tocopherol induced gene expression in bovine cells, the GO classification was analyzed using MetaCore. The most highly represented process networks, sorted by statistical significance, include 1) development—regulation of angiogenesis; 2) development-neurogenesis—Axonal guidance; 3) cell adhesion—cell junctions; 4) Inflammation—MIF signaling; and 5) transcription—nuclear receptors transcriptional regulation. There are also the original GO cellular processes represented at GeneGo. Since most of GO processes have no gene/protein content, the 'empty terms' are excluded from the *P*-value calculations. GO processes sorted by the 'statistically significant processes' are shown in Figure 6. The GO processes affected by α-tocopherol, such as the cellular metabolic process and the primary metabolic process, indicate that α-tocopherol possesses important transcriptional activities that regulate functional biological processes of cells, especially the cell metabolic processes.

The gene content of the experimental data was used as the input list for the generation of biological networks with the AN algorithm on default settings. This is a variant of the shortest paths algorithm with the main parameters: 1) relative enrichment of the

Table 1. α-tocopheryl modulates biological networks generated using transcription regulation algorithm.

Network	GO processes	Total nodes	Root nodes	*P*-value
HNF4-alpha	macromolecule metabolic process (66.7%; 5.187e-10), biopolymer metabolic process (65.0%; 1.209e-09), cellular macromolecule metabolic process (65.0%; 2.383e-09), cellular biopolymer metabolic process (64.2%; 2.510e-09), regulation of macromolecule metabolic process (45.5%; 2.126e-08)	134	133	7.17E-265
c-Myc	regulation of gene expression (52.2%; 6.050e-15), regulation of macromolecule metabolic process (55.7%; 2.406e-14), regulation of macromolecule biosynthetic process (50.4%; 1.164e-13), regulation of metabolic process (56.5%; 1.356e-13), regulation of transcription (47.0%; 6.305e-13)	121	120	2.99E-238
SP1	positive regulation of transcription, DNA-dependent (22.6%; 1.014e-12), positive regulation of RNA metabolic process (22.6%; 1.160e-12), positive regulation of transcription (23.6%; 3.335e-12), positive regulation of gene expression (23.6%; 4.892e-12), positive regulation of transcription from RNA polymerase II promoter (19.8%; 6.495e-12)	109	108	8.17E-214
ESR1 (nuclear)	negative regulation of cellular process (42.1%; 7.653e-11), positive regulation of transcription (25.0%; 4.580e-10), positive regulation of gene expression (25.0%; 6.145e-10), negative regulation of biological process (42.1%; 6.848e-10), positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (25.0%; 2.455e-09)	80	80	3.39E-159
p53	negative regulation of cellular process (48.5%; 6.970e-13), negative regulation of biological process (48.5%; 7.026e-12), regulation of developmental process (42.4%; 5.969e-11), positive regulation of cellular process (45.5%; 1.403e-10), positive regulation of transcription, DNA-dependent (25.8%; 2.406e-10)	71	70	2.84E-137
Androgen receptor	positive regulation of cellular process (48.5%; 1.745e-12), negative regulation of metabolic process (32.4%; 9.730e-12), negative regulation of cellular metabolic process (30.9%; 2.354e-11), positive regulation of biological process (48.5%; 2.739e-11), positive regulation of transcription, DNA-dependent (26.5%; 4.319e-11)	71	70	2.84E-137
E2F1	regulation of metabolic process (69.4%; 8.766e-14), regulation of gene expression (62.9%; 1.042e-13), regulation of RNA metabolic process (58.1%; 2.208e-13), regulation of cellular process (91.9%; 4.013e-13), regulation of macromolecule metabolic process (66.1%; 4.112e-13)	65	65	4.12E-129
AP-1	negative regulation of cellular process (50.8%; 3.143e-13), negative regulation of biological process (50.8%; 3.053e-12), negative regulation of transcription (27.9%; 8.447e-12), negative regulation of macromolecule biosynthetic process (29.5%; 1.464e-11), negative regulation of gene expression (27.9%; 1.829e-11)	65	65	4.12E-129
NF-kB	positive regulation of transcription, DNA-dependent (30.6%; 6.831e-13), positive regulation of RNA metabolic process (30.6%; 7.635e-13), positive regulation of transcription (32.3%; 8.750e-13), positive regulation of gene expression (32.3%; 1.209e-12), positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (32.3%; 5.569e-12)	65	64	2.65E-125
C/EBPbeta	positive regulation of transcription (33.3%; 4.329e-13), positive regulation of gene expression (33.3%; 5.990e-13), positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (33.3%; 2.780e-12), positive regulation of macromolecule metabolic process (36.7%; 3.061e-12), positive regulation of transcription, DNA-dependent (30.0%; 4.164e-12)	64	63	2.60E-123

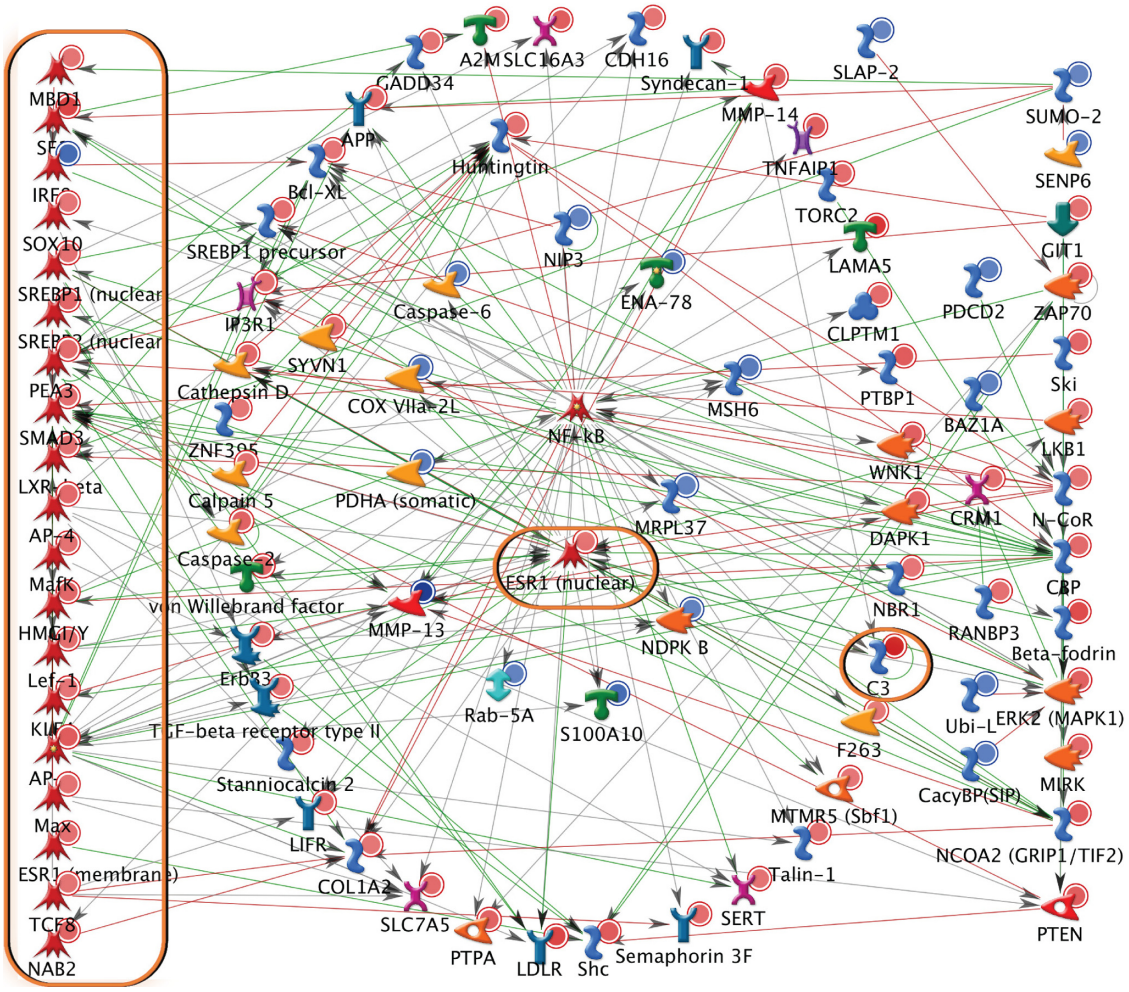

Figure 5. Representative biological network generated using the Analyze Networks Transcription regulation Algorithm. Up-regulated genes are marked with red circles and down-regulated genes are marked with blue circles. The network is centered with NF-κB and ESR1. All other transcription factors are shown in the left panel. All the symbols in the network are listed in Supplement Figure S1.

uploaded data and 2) relative saturation of networks with canonical pathways. These networks are built on the fly and are unique to the uploaded data. Most relevant biological networks are listed in Table 2.

Comparison of up- and down-regulated genes and gene networks

In order to look into exactly which pathways or networks are up- or down-regulated by α-tocopherol, we compared the genes that are up regulated by α-tocopherol to genes that are down-regulated. Using MetaCore Compare Experiment Workflow, the most relevant biological networks that are unique to either up- or down-regulated networks were generated (Table 3). In this workflow, the networks are prioritized based on the number of fragments of canonical pathways on the networks. To visualize the networks, the third network unique to up-regulated genes is presented in Figure 7 as the representative network. This network involves many important biological processes, such as response to stress (45.8%), cellular response to stimulus (31.2%), and immune system development (20.8%).

The gene content of the perturbed genes detected by the microarray was also divided into up- and down-regulated groups and used as the input list for the generation of biological networks with the transcription regulation algorithm on default settings. The representative up-regulated network of ESR1 is shown in Figure 8A. In this network, ESR1 is the divergence and convergence hub of the network. This network involves the regulation of cellular biosynthetic processes (47.5%; $P = 1.410e^{-09}$), transcription (38.6%; $P = 1.455e^{-09}$), regulation of metabolic processes

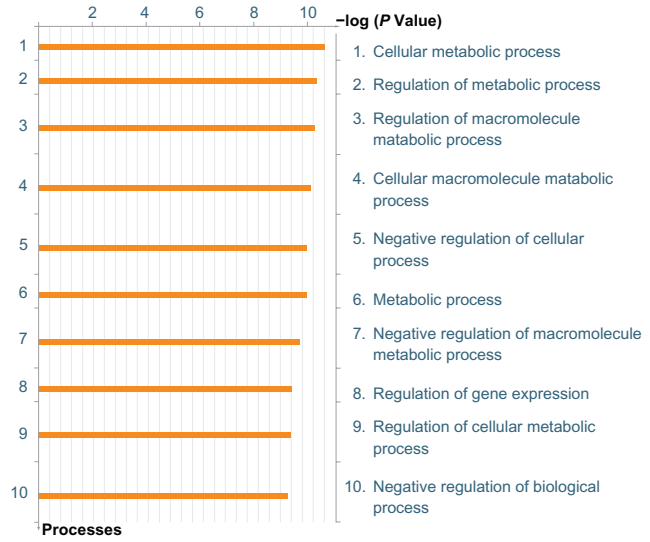

Figure 6. GeneGo process networks: representation of the most significant biological process networks induced by α-tocopherol, sorted by statistically significant Networks, ordered by $-\log^{10}$ of the *P*-value of the hypergeometric distribution.

(53.5%; $P = 1.621e^{-09}$), regulation of biosynthetic processes (47.5%; $P = 1.836e^{-09}$), and regulation of cellular metabolic processes (51.5%; $P = 2.592e^{-09}$). The important components of this network, ESR1, C3 and Erk1/2 are detected by western blots. Three down-regulated transcription regulation networks were merged and are presented in Figure 8B. The c-Myc network involves the GO-processes of cellular metabolic processes (84.8%), translation (21.7%), gene expression (52.2%), metabolic processes (84.8%), and cellular macromolecule metabolic processes (67.4%). The network of SP1 involves the GO-processes of nucleoside diphosphate phosphorylation (5.9%), nucleotide phosphorylation (5.9%), positive regulation of helicase activity (5.9%), purine ribonucleoside triphosphate biosynthetic processes (11.8%), and purine nucleoside triphosphate biosynthetic processes (11.8%). and The third network, ESP1 network, involves acute inflammatory response to non-antigenic stimulus (6.7%), selenium metabolic processes (6.7%), positive regulation of retinal cell programmed cell death (6.7%), regulation of apoptosis (33.3%), and the regulation of programmed cell death.

Discussion

Vitamin E was discovered almost 100 years ago by Evans and Bishop[15] as a new nutritional factor and was identified as α-tocopherol. After years of research, the physiological functions of vitamin E still remain mysterious in many ways. Nevertheless, vitamin E is one of the most commonly used single nutrient supplements. Recent data has suggested that α-tocopherol is not only an antioxidant but also a regulator of gene expression through its binding to nuclear receptors.[16,17] The precise mechanism in which it regulates gene expression is still unknown. In this study, we found that α-tocopherol induces cell cycle arrest at the G1/S boundary by flow cytometry analysis of α-tocopherol treatment of cells. Flow cytometry analysis is based on the idea that proliferating cells go through phases to increase their DNA content from the original amount (2C: two copies) in the G1 phase to twice the amount (4C: four copies) in the post-replication M/G2 phases. DNA content in S-phase

Table 2. Most relevant networks generated using Analyze Networks (AN) algorithm.

No	Processes	Size	Target	Pathways	*P*-value
1	Organ development (80.0%), system development (84.0%), anatomical structure development (84.0%)	50	7	196	9.21e-06
2	Cellular component movement (44.0%), anatomical structure morphogenesis (58.0%), regulation of cellular component organization (40.0%)	50	14	128	1.40e-14
3	Response to organic substance (56.2%), positive regulation of cellular process (70.8%), regulation of multicellular organismal process (58.3%)	50	13	85	2.67e-13
4	Cellular response to chemical stimulus (29.7%), positive regulation of biological process (56.8%), cAMP biosynthetic process (10.8%)	50	19	57	2.15e-21
5	Organ morphogenesis (56.5%), anatomical structure morphogenesis (63.0%), regulation of developmental process (54.3%)	50	17	38	4.80e-19

Table 3. The functional networks unique for up- or down-regulated by α-tocopherol.

No	Processes	Size	Target	*P*-value
Unique for down-regulated by α-tocopherol				
1	Actin cytoskeleton organization (36.7%), actin filament-based process (36.7%), cytoskeleton organization (40.8%)	50	15	3.27E-23
2	Translation (23.3%), RNA processing (25.6%), Ribonucleoprotein complex biogenesis (16.3%)	50	29	1.66E-56
3	Response to stress (45.8%), cellular response to stimulus (31.2%), immune system development (20.8%)	51	13	2.32E-19
Unique for up-regulated by α-tocopherol				
1	Cellular component organization (87.5%), cellular component movement (54.2%), cell projection organization (50.0%)	52	13	7.85E-13
2	Organ development (80.0%), system development (82.0%), Organ morphogenesis (56.0%)	50	8	1.18E-06
3	Response to organic substance (54.2%), response to endogenous stimulus (41.7%), positive regulation of biological process (62.5%)	50	16	3.00E-17

cells will fall in between the two measurements. The cell responses were concentration-dependent. This result indicates that α-tocopherol has a biological effect of inhibition of cell proliferation. There was a shift in the G1/GO peak in α-tocopherol- treated cells (see Fig. 1A). This shift indicates that α-tocopherol does not simply stop DNA synthesis altogether, rather it just slows down the process of DNA synthesis. The result of such slowing down is the accumulation of cells that contain more DNA content than that of

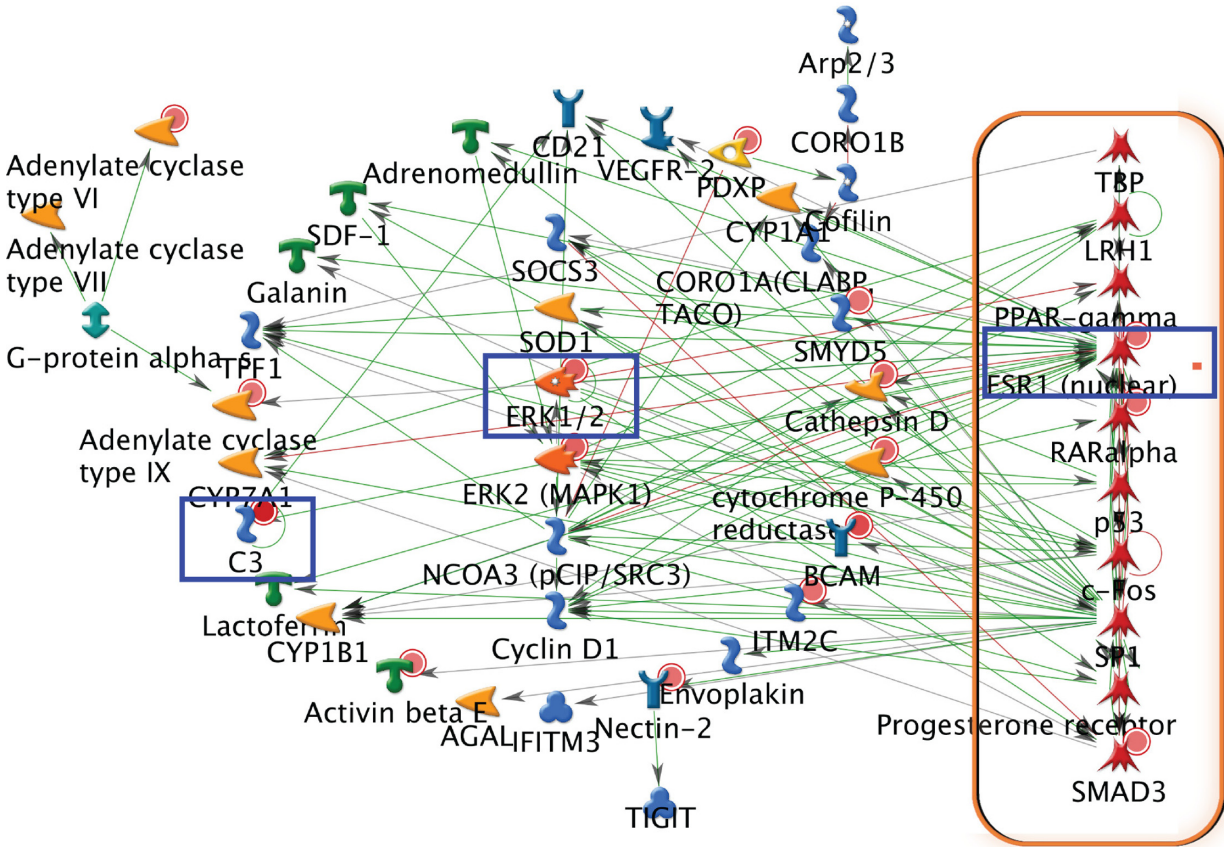

Figure 7. The representative network of the most relevant biological networks that is unique to up-regulated networks using the Analyze Networks Transcription regulation Algorithm. This is a variant of the shortest paths algorithm with relative enrichment and relative saturation of networks with canonical pathways. Key network objects include ESR1, C3, Erk1/2 (shown in blue rectangles). Large orange rectangle highlights the transcription factors.

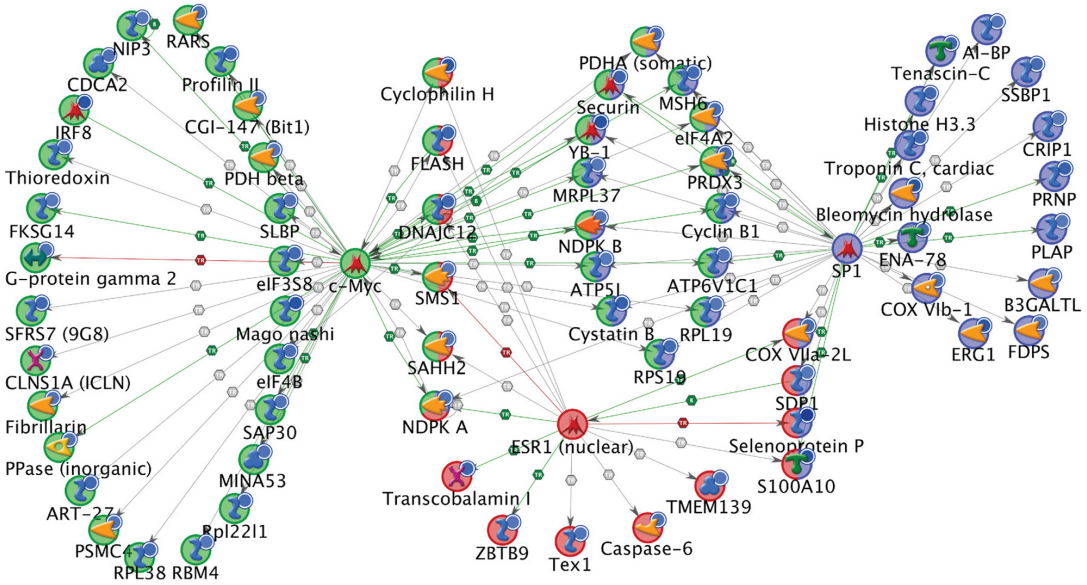

Figure 8. The representative networks of the most relevant biological networks that are unique to down-regulated networks using the Analyze Networks Transcription regulation Algorithm. This is a variant of the shortest paths algorithm with relative enrichment and relative saturation of networks with canonical pathways. The figure shows three (green, purple and red) merged down-regulated networks.

G1/G0 cells. However, those cells did not move forward to fully replicate the DNA. We also noticed that a higher concentration (up to 80 μM) of α-tocopherol induces apoptosis in cell culture (data not show), indicating that α-tocopherol may have biological function of modulation of apoptosis.

To look into the possible mechanisms for the cell cycle arrest induced by α-tocopherol, global gene expression profiles of the bovine kidney epithelial cells regulated by α-tocopherol were investigated with high-density oligonucleotide microarrays. The gene expression profiles generated by the microarray technique were further analyzed by pathway analysis technologies to map gene expression data into relevant pathways based on their functional annotation and known molecular interactions. To examine the molecular functions and genetic networks, the data that we generated from microarray was explored using MetaCore. MetaCore is a web-based suite for the functional analysis of experimental data in the context of a manually curated database containing the probability of having the protein interactions, protein-DNA interactions, canonical pathways, and signaling pathways as well as knowledge base ontologies of cellular processes, diseases, and toxicology.

This study, being the first global expression profiling and pathway analysis of α-tocopherol-induced gene perturbation and the functional genomic study of α-tocopherol in bovine cells, has generated comprehensive information on the physiological functions of α-tocopherol. Using global expression profiling and pathway analysis, we identified specific genomic activities induced by α-tocopherol and found insights into the molecular mechanisms that mediate these activities. Overall, our data confirmed that α-tocopherol is a potent regulator of gene expression and α-tocopherol possesses novel transcriptional activities that affect essential biological processes. The genes that we identified (Supplementary Table S1), which fall within a broad range of functional categories, appear to provide the molecular basis for its distinctive biological effects. These data may reflect one of the characteristics of α-tocopherol and those genes perturbed by α-tocopherol treatment may play very important roles in biological functions.

Pathway analysis of the α-tocopherol-induced genes in MDBK cells with the MetaCore Analysis tool successfully identified remarkable changes in genetic networks related to fundamental cell functions. This study suggests underlying mechanisms for biological effects such as inhibition of cell proliferation in MDBK cells induced by α-tocopherol, as well as the protective effects of α-tocopherol in living animals against infections (unpublished data from our lab). Alpha-tocopherol exerts a very broad range of effects on many biological pathways. The genes we

identified (Supplementary Table 1) fall within a broad range of functional categories. This study successfully identified remarkable changes in expression of genes related to cell cycle, nucleic acid metabolism, and DNA replication. Among down-regulated genes, CDC42, NUF2, CCNB1, AGK, PRNP, PTTG1, and MTBP are directly related to regulation of cell cycle and DNA replication. These changes may be directly responsible for the inhabitation of proliferation activity of α-tocopherol. Moreover, there are also 11 genes (ATP5B, ATP5 J, ENPP1, GUCY2C, KIF20B, NME1, NME2, PDE7B, POLD4, PRIM2, and PSMC4) that are related to purine metabolism and down regulated by α-tocopherol. These changes may underlie indirect mechanisms for the cell cycle arrest.

We also showed that treating cultured bovine cells with α-tocopherol alters the expression of multiple genes and these genes, such as TRb/RXRb, RAPalpha, SREBP1, and SREBP2, are the imperative components of the canonical pathway of lipid metabolism. SREBP1 and SREBP2 are sterol-responsive transcription factors that bind to the sterol regulatory element DNA sequence.[18,19] The data indicate that α-tocopherol modulates the expression of SREBP-1 and SREBP-2 and may mediate specific genomic responses to vitamin E. These novel transcriptional activities of α-tocopherol reveal that α-tocopherol may regulate lipid metabolism in cells through SREBP1 and SREBP2, which may have selective roles for either cholesterol or fatty acid metabolism.[19–22] In addition, SREBPs possess highly unique functions as key regulators of nutritional homeostasis.[21] This is a very interesting result and currently we are following up with a more in-depth study to confirm and understand the interaction between α-tocopherol and its role in regulating lipid metabolism.

Another group of genes revealed by the pathway analysis, ESR1, C3, and Erk1/2, are also novel targets of the transcriptional activities of α-tocopherol. The protein levels of these gene products were confirmed by western blots. There is a possibility that translation regulation may also be involved in the changes of the protein level. However, the consistency of the levels of mRNA and proteins is more important as we can drew a conclusion that α-tocopherol positively regulates the expression of these three genes. ESR1 belongs to the nuclear hormone receptor family of transcription factors.[23] The steroid hormones and their receptors are involved in the regulation of eukaryotic gene expression and affect cellular proliferation and differentiation in target tissues. This gene encodes an estrogen receptor, a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding, and activation of transcription. The cellular signaling of estrogens is mediated through ESR1 and ESR2. ESRs contain conserved structure and five distinctive functional domains.[24] Estrogen receptors are also involved in pathological processes, including breast cancer, endometrial cancer, and osteoporosis. The ability of vitamin E to modulate signal transduction and gene expression has been observed in numerous studies[25] and references therein. However, this is the first time, to our best knowledge, that the expression of the ESR1 gene has been linked to α-tocopherol. This data suggest that α-tocopherol is not only an antioxidant but also a regulator of gene expression through its biological effects on nuclear factors and transcription factors, which may include not only binding to nuclear factors,[16] but also up-regulating expressions of those nuclear factors/receptors. The elucidation of this novel molecular event affected by α-tocopherol could reveal the detailed molecular mechanisms of the essentiality of vitamin E in biological functions.

C3 is a 190 kDa glycoprotein essential for eliciting the complement response. This protein is activated through proteolysis by specific enzyme complexes.[26] We used a monoclonal anti-C3 antibody in our western blot and detected a major protein band of about 40 kDa. We suspect that this is the C3 target-bound fragment of C3dg.[27] The mammalian complement system has a major role in innate and adaptive immunity. Activation of C3 is central to the three complement pathways and results in inflammation and the elimination of self and non-self targets.[27] There has been some evidence that suggests a role of α-tocopherol in cell homeostasis, which is induced through the modulation of specific signaling pathways and genes involved in proliferation, metabolism, and inflammation pathways.[5] Our data reported here provides direct evidence that α-tocopherol is involved in maintaining immuno-homeostasis through targeting the C3 gene.

This observation also indicated that the key roles of vitamin E in maintaining and supporting animal and human health may be mediated by its ability to regulate the transcription activities of target genes.

The mechanisms that underlie the gene regulatory activity of vitamin E (α-tocopherol), however, are still unclear. It is important for us to understand the mechanism(s) of how α-tocopherol targets these genes and causes these changes in gene expressions. However, such an understanding will require great effort and is certainly beyond the scope of this report.

In conclusion, global gene expression profiling and computational pathway analyses provide a detailed knowledge of changes in gene expression induced by α-tocopherol. This detailed knowledge will provide a basis for understanding the molecular mechanisms of the biological effects of α-tocopherol in normal bovine cells. Understanding the signaling and gene expression effects of α-tocopherol could facilitate our knowledge of the physiological and functional roles of this vitamin.

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Disclosures

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Supplementary figure

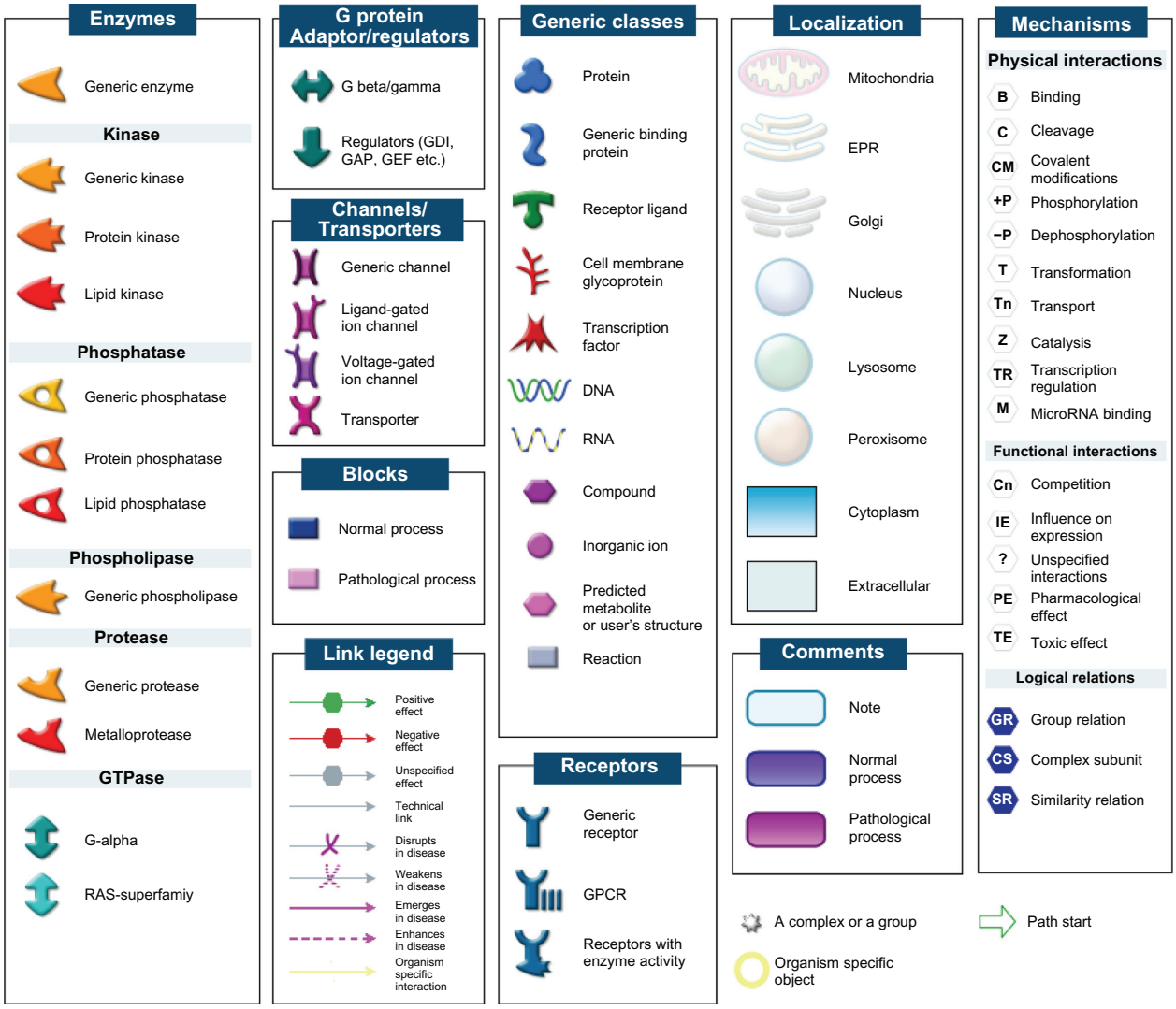

Figure S1. Legends for gene network presentation (www.GeneGO.com).