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Gene Expression Changes in the Human Fibroblast Induced by *Centella asiatica* Triterpenoids

Abstract

The molecular pathways underlying the diverse biological activity of the triterpeniod compounds isolated from the tropical medicinal plant *Centella asiatica* were studied with gene microarrays and real-time reverse transcription polymerase chain reaction (real-time RT-PCR) to quantify the expression of 1053 human genes in human fibroblasts. Fibroblast cells grown in culture were used as a model system to evaluate the stimulation of wound healing by titrated extract from *Centella asiatica* (TECA) as well as by the four principal triterpenoid components of *Centella*. TECA treatment effects the expression of genes involved in angiogenesis and the remodeling of extracellular matrix, as well as diverse growth factor genes. The extent of expression change of TNFAIP6, an extracellular hyaluronan binding protein, was found to be largely dose-dependent, to respond most strongly to the free acids asiatic acid and madecassic acid, and to increase in expression over 48 hours of treatment. These results show that *Centella* triterpenes evoke a gene-expression response consistent with their prevailing medical uses in the treatment of connective tissue disorders such as wound healing and microangiopathy. The identification of genes modulated by these compounds provides the basis for a molecular understanding of *Centella*'s bioactivity, and opportunities for the quantitative correlation of this activity with clinical effectiveness at a molecular level.

Key words
Centella asiatica · Apiaceae · asiatic acid · madecassic acid · wound healing · gene expression

Abbreviations

TNFAIP6:	tumor necrosis factor alpha, induced protein 6
ECM:	extracellular matrix
AA:	asiatic acid
MA:	madecassic acid
AS:	asiaticoside
MS:	madecassoside
TECA:	titrated extract of *Centella asiatica*
MTT:	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium
GAPDH:	glyceraldehyde-3-phosphate dehydrogenase

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Introduction

Centella asiatica (L.) Urb. (Apiaceae) is a widely-used medicinal plant of Southeast Asian origin and is commonly known as Pegaga or Pennywort (reviewed in [1]). While *Centella asiatica* extracts have been investigated for their purported sedative, antiviral and anti-microbial qualities, most studies have focused on biological activity associated with connective tissue function. Wound healing is an exceedingly complex biological phenomenon involving numerous cell types and consisting of processes with timescales from seconds to minutes (coagulation), minutes to hours (inflammation) hours to days (migration and proliferation) and days to months (scar formation and remodeling). Triterpenoids of *Centella asiatica* (Fig. **1**) have been shown to affect two of these processes: the synthesis of collagen associated with extracellular matrix remodeling [2], [3], and the modulation of microcirculatory function [4], [5]. We have applied transcriptional profiling with gene microarrays and real-time RT-PCR for an understanding of *Centella asiatica* bioactivity at the molecular level using a fibroblast cell culture model system.

Centella asiatica products are comprised of a mixture of four related triterpenoids: asiatic acid (AA), madecassic acid (MA), asiaticoside (AS) and madecassoside (MS), these structures are shown in Fig. **1**. Each of these compounds is present in the field-grown plant, and preliminary experiments have shown that the relative concentration of each of these compounds in the aqueous or alcoholic plant extract varies greatly with the ecotype and tissue employed (data not shown). For this study we have employed the commercially available mixture of AA, MA, and MS marketed as "Titrated Extract of *Centella asiatica*" (TECA) and widely employed in topical preparations and cosmetics. The application of these compounds to human fibroblast cells has previously been shown to increase collagen synthesis in a dose-dependant fashion [6], but the molecular details of this augmentation and its context with respect to the clinical uses of *Centella* have remained unclear.

Microarray-based methods for the quantitative study of gene expression provide an exciting opportunity for drug discovery, the study of drug functions [7] and have been extended to include the study of natural product drugs [8]. Transcriptional profiling allows the researcher to test a wide range of potential bioactivities efficiently, and can produce the rigorously quantitative data necessary for the determination of active purified chemical principals as well as the qualification of superior plant accessions and preparations.

Materials and Methods

Cell lines

Normal human fibroblasts (ATCC CRL-2450) were obtained from the American Type Culture Collection (Manassas, VA) and passaged fewer than 6 times in Iscove's modified Dulbecco's medium with 10% FBS, 2 mM glutamine, and penicillin-streptomycin (at 100 IU/mL and 100 μg/mL, respectively) in humidified 5% CO_2. For each treatment with triterpenoids, cells were grown to fresh confluence in 10 cm circular plates. Media were renewed 2 days prior to the start of treatment, and dissolved triterpenoids (or vehicle control) was added as a 0.1% v/v solution with fresh media at the start of treatment.

Chemicals

TECA {a defined mixture of AS (40% w/w), AA (30% w/w), and MA (30% w/w), [6]} and asiatic acid were obtained from MMP (South Plainfield, NJ), the American distributor for the Serdex division of Roche Nicolas S.A. Madecassic acid, madecassoside and asiaticoside were obtained from ChromaDex (Irvine, CA). The stated purity of each triterpenoid was greater than 95% pure; this was verified by reverse phase HPLC, as was the composition of the TECA sample. Solutions for addition to cell culture were prepared by dissolving triterpenoids in DMSO and ethanol (1 : 1) followed by sterile filtration using a Millex-LG 0.20 μm filter membrane (Millipore, Bedford, MA). These stock solutions were diluted such that the desired triterpeniod concentration was obtained from the addition of 0.1% v/v triterpenoid solution to the culture medium.

RNA isolation

When the treatment periods had elapsed, fibroblasts were lysed and RNA isolated using the RNEasy (Qiagen, Valencia CA) in-plate lysis strategy. Low levels of co-purified DNA were removed by treatment with DNase I during purification. Yield and RNA integrity were verified by formaldehyde-agarose gel electrophoresis.

Cell viability assay

The antiproliferative effect of *Centella asiatica* triterpenoids on the human fibroblast cell line CRL-2450 was determined using the modified MTT dye reduction test [9]. Approximately 10,000 cells were plated per well using 96-well tissue culture plates with added test compounds, each sample in quadruplicate. The compounds were dissolved in DMSO and ethanol (1 : 1); the final concentration of solvent in the culture medium was 0.2%. Following 72 h of exposure to the test compounds at 37 °C and 5% CO_2 the culture supernatants were removed and 100 μL MTT in

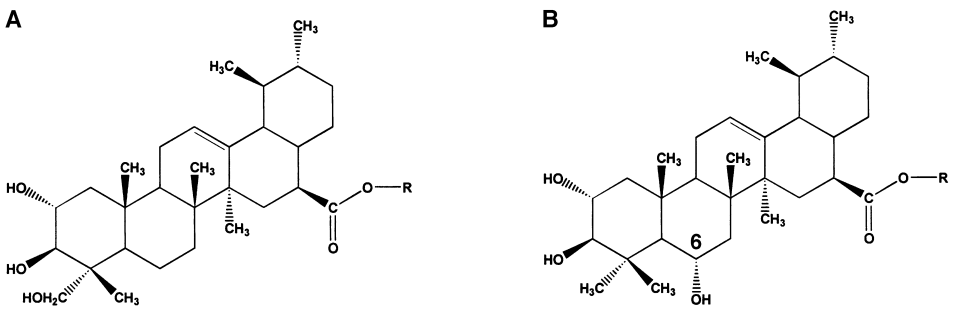

Fig. **1** Triterpenoids of *Centella asiatica*. **A**: asiatic acid (AA) (R = H) and asiaticoside (AS) (R = Glc-Glc-Rha). **B**: madecassic acid (MA) (R = H) and madecassoside (MS) (R = Glc-Glc-Rha). Both 6α- and 6β-configurations of MA and MS are present.

PBS (0.5 mg/mL) were added to each sample. After further 4 h incubation, the medium was removed, and the dye precipitate was dissolved with 200 μL of DMSO containing 10% (v/v) of 0.1 M-glycine buffer (pH 10.6). Optical density at 570 nm was measured with a Bio-Tek GL311 plate reader, and the results expressed in relative absorbance units. The IC_{90} values were derived from the dose-response curves.

Microarrays

The spotted cDNA microarrays used in this study were produced from 1156 I.M.A.G.E. sequence verified clones [10] using standard methods for bacterial culture, PCR amplification of cDNA inserts, amplicon purification, and printing [11], [12]. A complete list of the clones on these arrays is available at http://minihelix.mit.edu/malaysia/library/Clone_list.xls. Purified DNA was dissolved at a concentration ranging from 0.10 μg/μL to 0.15 μg/μL in 50% v/v DMSO and spotted in duplicate onto GAPS II slides using a Genetic Micro Systems 417 robot (Affymetrix, CA) under controlled conditions of 45 – 55% relative humidity and 20 – 22 °C. DNA was cross-linked to the slides with ultraviolet light (160 mJ) and blocked with succinic anhydride solution. RNA was labeled via incorporation of aminoallyl-dUTP (AA-dUTP) into cDNA using the FairPlay labeling kit (Stratagene, La Jolla CA) and coupled with Cy3 or Cy5 monofuctional NHS-ester dye (Amersham, Piscataway, NJ). Total RNA (20 μg) from either treated or control fibroblasts was reverse-transcribed and precipitated. Treated and control cDNA pools were resuspended, split and coupled with either Cy3 or Cy5 according to the FairPlay kit instructions. Coupling was stopped by the addition of 4.5 μL of 4 M hydroxylamine, opposing dye samples were mixed, and unincorporated dye removed using a DyeEx column (Qiagen). Cy-labeled cDNA samples were then dried and resuspended in 22 μL hybridization buffer [25% formamide, 5 × SSC, 0.1% SDS, 1 μg/μL Cot-1 DNA, 1 μg/μL poly(dA) DNA]), denatured at 95 °C for 3 min, cooled to 45 °C, loaded onto the array and hybridized for 20 hours at 45 °C. Slides were washed according to standard protocols [12] and imaged on an ArrayWoRx confocal microscope scanner (Applied Precision, Issaquah, WA). Fluorescence intensity was quantified using the ArrayWoRx software and further processed using the SNOMAD [13] tool set, employing a global background and global mean normalization, and a local mean normalization across element intensity. Subsequent to this normalization the resulting data were reduced: replicate spots and arrays were combined, and the resulting 4 replicates per data point are expressed as the mean ± standard deviation.

Real-time RT-PCR

Confirmation of array results and extension to other experimental samples was done with real-time RT-PCR [14] using SYBR Green chemistry on an Opticon (MJ research, Waltham, MA) thermocycler. Primers for TNFAIP6 (5′-TCCATATGGCTTGAACGAGCAGC-3′, sense and 5′-CTTAGCTTCTGCGTAGGTGAGC-3′, antisense) and GAPDH (5′-GAAGGTGAAGGTCGGAGTC-3′, sense and 5′-GAAGATGGTGATGGGATTTC-3′, antisense) were purchased from MWG (High Point, NC). Reverse transcription and PCR reagents were from Qiagen and SYBR Green dye was from Molecular Probes (Eugene, OR).

Results

Effective concentrations for the treatment of fibroblast cells with *Centella asiatica* derived triterpenoids were determined from the IC_{90} values summarized in Table **1**. From these values a range of concentrations for the treatment of cells in culture was specified incorporating 12% or 50% of IC_{90}, with treatment times of 4, 24, and 48 hours. These concentrations and times were designed to elicit strong, robust gene expression responses. The values chosen agree with literature results [2], [6] that suggest that these concentrations and times are appropriate for the detection of any transcriptionally controlled element of the augmentation of collagen and fibronectin accumulation. Vitamin C is a well studied stimulator of collagen synthesis [15]. As a positive control for the stimulation of collagen synthesis, cells were treated with 60 nM Vitamin C for 48 hours. Extracellular collagen discharged into the culture medium was determined using a competitive inhibition ELISA as described by Grimaud [16] and shown to increase by 60%.

From the matrix of concentrations and times chosen for this study, the RNA sample corresponding to treatment with 160 μg/mL TECA for 24 hours was selected for the quantification of gene expression changes using the cDNA microarray. Fig. **2** shows the raw data for one such hybridization; a qualitatively similar result was obtained for a "dye swapped" microarray in which the same set of experimental sample and control were labeled with the opposite pairing of Cy dye (i.e., vehicle control sample labeled with Cy5 and TECA-treated sample labeled with Cy3). The resulting two sets of hybridization intensities were quantified and normalized separately to correct for systematic bias [12], and then reduced together, yielding a single pair of values for each gene

Table **1** IC_{90} values of *Centella asiatica* derived triterpenoids in the fibroblast cell culture system, and triterpenoid concentrations chosen for analysis

	IC_{90}	Low dose (~12% of IC_{90})	High dose (~50% of IC_{90})
TECA	250 ± 20 μg/mL	40 μg/mL (56 μM)	160 μg/mL (225 μM)
Asiatic Acid	60 ± 5 μg/mL	8 μg/mL (16 μM)	30 μg/mL (61 μM)
Asiaticoside	> 400 μg/mL	50 μg/mL (48 μM)	200 μg/mL (192 μM)
Madecassic Acid	175 ± 20 μg/mL	30 μg/mL (61 μM)	110 μg/mL (225 μM)
Madecassoside	> 400 μg/mL	50 μg/mL (48 μM)	200 μg/mL (192 μM)
Vitamin C	N.D.	10 ng/mL (60 nM)	N.D.

TECA molar concentration is calculated as moles of triterpenoids, based on the determined composition (40% asiaticoside, 30% asiatic acid, 30% madecassic acid).

based on the measured intensity in each of four independent two channel hybridization features (spots). This collection of mean values is plotted in Fig. **3** and is available for download at http://minihelix.mit.edu/malaysia/library/Ratio_data.xls .

From the initial set of 1156 clones on the microarray, 1053 passed a clone verification test prior to printing and are therefore included in the analysis. Clones were considered to be verified if the expected size of the insert in each of the cDNAs amplified for printing was comparable to the size observed upon electrophoresis of the amplified insert (data not shown). In addition, 12 (14%) of the clones listed in Tables **2** and **3** were verified by sequencing plasmid DNA isolated from the original clone stock. The overall success rate for this clone set is 1053 out of 1156 clones, or 91% of the spots printed on this microarray. This is within the 95% confidence interval (between 83% and 91%) reported for this portion of the I.M.A.G.E. library [17]. The expression ratio (r) values for these genes ranged from r = 0.35 ± 0.04 (mean ± standard deviation) for thrombospondin 1, to r = 11.77 ± 1.60 for TNFAIP6. The average standard deviation for this set of 1053 ratios is σ_{ave} = 0.16; we set a nominal threshold for significance of 1 ± 2.5σ_{ave} (r > 1.40 indicates a significantly up-regulated transcript, r < 0.59 indicates significant down-regulation). By these criteria, the up-regulation of 58 transcripts and the down-regulation of 24 transcripts were found to result from TECA treatment (Tables **2** and **3**). Expression levels for fibronectin (FN1), collagen (COL1A2, COL2A1, COL3A1, COL6A2) and collagen processing enzymes (P4HA1, P4HB) were measured; none were significantly modulated by TECA (see also Fig. **3**).

The validation of microarray results can be divided into two separate tasks: the verification of the identity of the gene printed on the array and the independent demonstration of expression change by another method. The microarray-detected expression change in TNFAIP6 resulting from TECA treatment at 160 μg/mL for 24 hours was further verified by real-time RT-PCR, and the change in TNFAIP6 expression elicited by each of the purified triterpenoids over a range of concentration and treatment duration was also measured using this assay (Fig. **4**). Real-time RT-PCR results are the mean of four measurements, and are expressed as the mean ± standard deviation of the ratio of TNFAIP6 to GAPDH, normalized to the same ratio from a vehicle-treated control.

Discussion

TECA elicits a strong and consistent change in the levels of gene expression in fibroblast cell culture. Of 1053 genes analyzed, 82 (7.8%) were found to have statistically recognizable changes in expression; considering the direction of this change along with the role these genes play in cellular function provides insight into the biological activity of TECA. Furthermore, the phenomenon of expression modulation itself is a useful assay for the identification of active principals.

The classification of microarray-detected gene expression changes as "significant" requires the consideration of both the precision of the microarray data as well as the nature of the biological change it is presumed to represent. It is conceivable that numerically significant changes in the expression of a particular gene

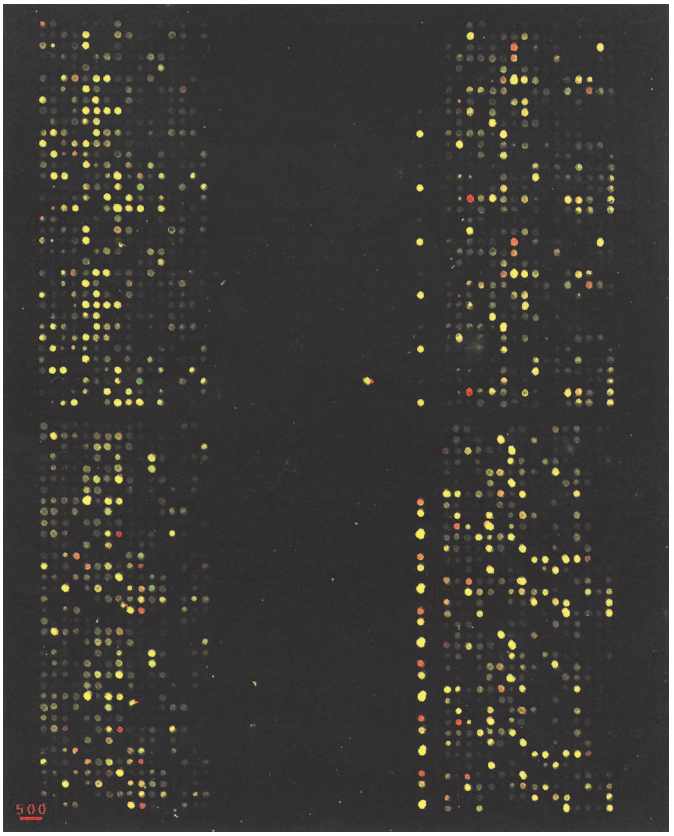

Fig. **2** False color image of a single expression microarray hybridized with cDNA derived from vehicle control (Cy3, green) and treated (Cy5, red) fibroblast cells, treatment details: TECA 160 μg/mL, 24 hour duration. Spots representing genes with increased expression levels appear red, while those with decreased expression appear green.

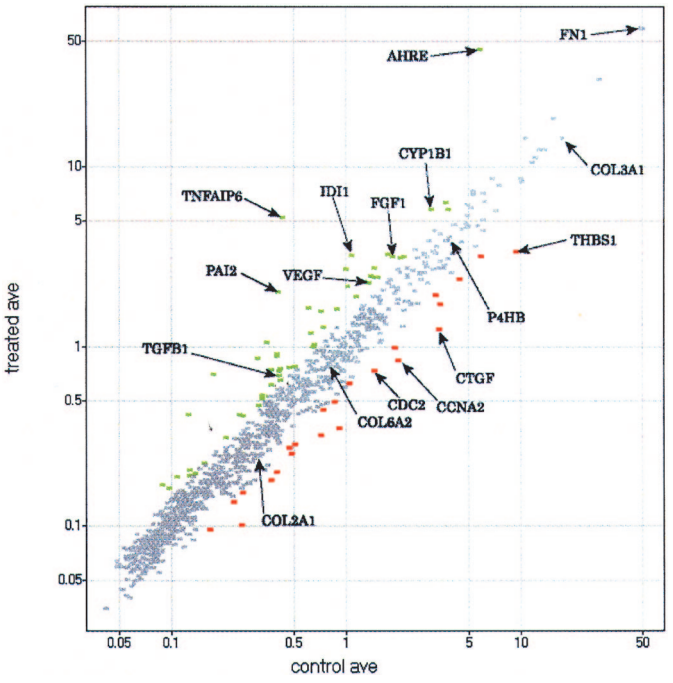

Fig. **3** Scatter plot of mean fluorescence intensity on a log-log scale. Intensities from treated fibroblast samples (TECA, 160 μg/mL, 24 hours) are on the vertical axis, those from vehicle control samples (1:1 DMSO:ethanol, 0.1% v/v) are on the horizontal axis. Green symbols denote genes determined to be significantly up-regulated; red symbols denote down-regulated genes.

Table **2** Transcripts up-regulated in fibroblast cells upon treatment with TECA at 160 μg/mL, 24 hours. Mean ratio r > (1 + 2.5σ)

Mean Ratio	*Ratios*	*Gene Symbol*	*Unigene #*	*GenBank ID*	*Description*
11.77	1.60	*TNFAIP6*	Hs.29352	W93163	tumor necrosis factor, alpha-induced protein 6
7.51	0.25	*ARHE*	Hs.6838	AA443302	ras homolog gene family, member E
4.83	0.65	*PAI2*	Hs.75716	T49159	plasminogen activator inhibitor, type II (arginine-serpin)
3.89	0.48	*THBD*	Hs.2030	H59861	Thrombomodulin
3.24	0.30	*H2BFQ*	Hs.2178	AA456695	H2B histone family, member Q
2.93	0.34	*GEM*	Hs.79022	AA418077	GTP-binding protein overexpressed in skeletal muscle
2.90	0.07	*IDI1*	Hs.76038	H08899	isopentenyl-diphosphate delta isomerase
2.65	0.19	*CYP51*	Hs.226213	AA477893	cytochrome P450, 51 (lanosterol 14-alpha-demethylase)
2.62	0.17	*CKMT2*	Hs.80691	AA460480	creatine kinase, mitochondrial 2 (sarcomeric)
2.20	0.12	no data	Hs.182638	H98218	ESTs, Highly similar to HIGH MOBILITY GROUP PROTEIN HMGI-C [*H.sapiens*]
2.16	0.13	*SAT*	Hs.28491	AA011215	spermidine/spermine N1-acetyltransferase
2.14	0.17	*HU-K4*	Hs.74573	H15746	similar to vaccinia virus HindIII K4L ORF
2.07	0.08	*ARHB*	Hs.204354	AA495846	ras homolog gene family, member B
1.94	0.27	no data	Hs.24385	R31168	Human hbc647 mRNA sequence
1.86	0.61	no data	Hs.81795	AA490688	ESTs
1.86	0.19	*NKG7*	Hs.10306	T57859	natural killer cell group 7 sequence
1.84	0.19	*FDPS*	Hs.77393	T65907	farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase)
1.84	0.06	*SLC2A3*	Hs.7594	AA406551	solute carrier family 2 (facilitated glucose transporter), member 3
1.82	0.07	*GBE1*	Hs.1691	R09069	glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme)
1.77	0.30	*H2BFL*	Hs.239884	H70775	H2B histone family, member L
1.76	0.15	no data	Hs.14553	R07295	ESTs
1.75	0.19	*MAPK6*	Hs.75465	H17504	mitogen-activated protein kinase 6
1.75	0.31	*EIF5*	Hs.184242	AA291494	eukaryotic translation initiation factor 5
1.72	0.16	*ENO2*	Hs.146580	AA450189	enolase 2, (gamma, neuronal)
1.69	0.48	no data	Hs.116078	AA625791	EST
1.68	0.30	*FGF1*	Hs.75297	AA015793	fibroblast growth factor 1 (acidic)
1.67	0.22	*SRD5A1*	Hs.552	R36874	steroid-5-alpha-reductase, alpha polypeptide 1
1.65	0.27	*BRF1*	Hs.85155	AA424743	butyrate response factor 1 (EGF-response factor 1)
1.64	0.13	no data	Hs.220108	AA708784	ESTs, Moderately similar to 67A9.b [*D. melanogaster*]
1.64	0.03	no data	Hs.123641	H18633	ESTs
1.63	0.10	*VEGF*	Hs.73793	R19956	vascular endothelial growth factor
1.63	0.11	*FGF2*	Hs.56066	R38539	fibroblast growth factor 2 (basic)
1.62	0.09	*ZFP103*	Hs.155968	AA429297	zinc finger protein homologous to Zfp103 in mouse
1.62	0.16	*TGFB1*	Hs.1103	R36467	transforming growth factor, beta 1
1.61	0.05	*KIAA0470*	Hs.25132	AA421472	KIAA0470 gene product
1.60	0.12	*BTEB1*	Hs.150557	N80235	basic transcription element binding protein 1
1.59	0.10	*RPS6KA3*	Hs.173965	H55921	ribosomal protein S6 kinase, 90kD, polypeptide 3
1.58	0.12	*TP53BP2*	Hs.44585	H69153	tumor protein p53-binding protein, 2
1.57	0.07	*GLCLC*	Hs.151393	H56069	glutamate-cysteine ligase (gamma-glutamylcysteine synthetase), catalytic (72.8kD)
1.56	0.06	*PTPN12*	Hs.62	AA429969	protein tyrosine phosphatase, non-receptor type 12
1.55	0.23	*UTX*	Hs.13980	H90287	ubiquitously transcribed tetratricopeptide repeat gene, X chromosome
1.52	0.15	no data	Hs.72200	AA157911	ESTs
1.51	0.18	*FXR2*	Hs.52788	AA489729	fragile X mental retardation, autosomal homolog 2
1.50	0.12	*POLA2*	Hs.81942	AA486289	polymerase (DNA-directed), alpha (70kD)
1.50	0.22	*CD58*	Hs.75626	AA136359	CD58 antigen, (lymphocyte function-associated antigen 3)
1.48	0.15	*MAN1A1*	Hs.2750	T85698	mannosidase, alpha, class 1A, member 1
1.48	0.13	*GTF2B*	Hs.255464	H23978	general transcription factor IIB
1.48	0.26	*HE1*	Hs.119529	AA630449	epididymal secretory protein (19.5kD)
1.47	0.03	no data	Hs.146388	R77251	*Homo sapiens* microtubule-associated protein 7 (MAP7), mRNA
1.47	0.22	*CSF2RA*	Hs.182378	N92646	colony stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage)
1.47	0.13	*CYP1B1*	Hs.154654	AA448157	cytochrome P450, subfamily I (dioxin-inducible), polypeptide 1
1.46	0.19	*ZNF259*	Hs.7165	AI343293	zinc finger protein 259
1.46	0.08	*CCND1*	Hs.82932	AA487700	cyclin D1 (PRAD1: parathyroid adenomatosis 1)
1.46	0.20	*MAFG*	Hs.255546	AA102591	v-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein G
1.45	0.08	*ISGF3G*	Hs.1706	AA291577	interferon-stimulated transcription factor 3, gamma (48kD)
1.42	0.15	*DDR1*	Hs.75562	AA487526	discoidin domain receptor family, member 1
1.42	0.09	*LIM*	Hs.154103	H12466	LIM protein (similar to rat protein kinase C-binding enigma)
1.41	0.20	*FOLR1*	Hs.73769	R24635	folate receptor 1 (adult)

Table **3** Transcripts down-regulated in fibroblast cells upon treatment with TECA at 160 μg/mL, 24 hours. Mean ratio $r < (1 - 2.5\sigma)$

Mean Ratio	*Ratio sd*	*Gene Symbol*	*Unigene #*	*GenBank ID*	*Description*
0.35	0.04	*THBS1*	Hs.87409	AA464630	thrombospondin 1
0.35	0.05	*CTGF*	Hs.75511	AA598794	connective tissue growth factor
0.37	0.04	*WNT2*	Hs.89791	T99653	wingless-type MMTV integration site family member 2
0.39	0.09	no data	Hs.253620	H23378	ESTs
0.41	0.07	*CCNA2*	Hs.85137	AA608568	cyclin A2
0.43	0.03	*GBP1*	Hs.62661	AA486849	guanylate binding protein 1, interferon-inducible, 67kD
0.47	0.07	no data	Hs.251179	H15552	ESTs
0.48	0.06	*KRTHA4*	Hs.89359	AA284260	keratin, hair, acidic, 4
0.48	0.04	*ENG*	Hs.76753	AA446108	endoglin (Osler-Rendu-Weber syndrome 1)
0.49	0.09	*CDC2*	Hs.184572	AA598974	cell division cycle 2, G1 to S and G2 to M
0.50	0.04	*CHN1*	Hs.169965	AA598668	chimerin (chimaerin) 1
0.51	0.05	no data	Hs.24176	R27671	ESTs
0.52	0.04	*KIAA0069*	Hs.75249	H20652	KIAA0069 protein
0.52	0.02	*FBN2*	Hs.79432	T98152	fibrillin 2 (congenital contractural arachnodactyly)
0.52	0.05	no data	Hs.76822	W58368	ESTs, Weakly similar to translation initiation factor EIF-2B gamma subunit
0.54	0.03	*KIAA0074*	Hs.1192	N54344	KIAA0074 protein
0.55	0.12	no data	Hs.2094	AA490477	Smooth muscle myosin heavy chain isoform SMem
0.56	0.11	no data	Hs.115617	AA287695	ESTs
0.56	0.06	*DSCR1L1*	Hs.156007	H19439	Down syndrome candidate region 1-like 1
0.57	0.05	*DUT*	Hs.82113	AA489219	dUTP pyrophosphatase
0.58	0.10	*TTK*	Hs.169840	AI337292	TTK protein kinase
0.58	0.06	*SMARCA3*	Hs.3068	AA459632	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3
0.58	0.11	*KIAA0042*	Hs.3104	AA477501	KIAA0042 gene product
0.58	0.07	*TJP1*	Hs.74614	H50377	tight junction protein 1 (zona occludens 1)

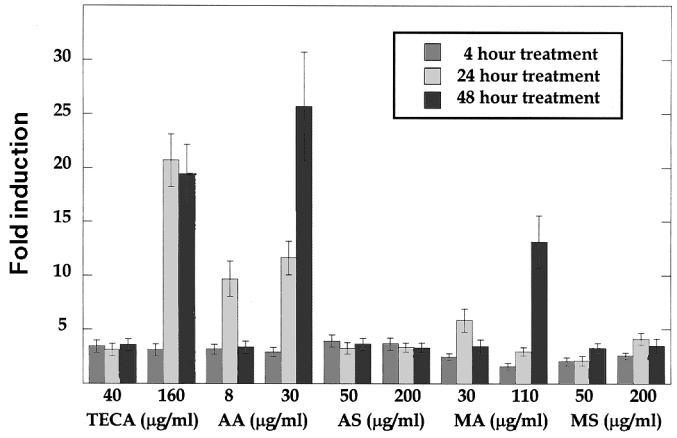

Fig. **4** Effect of triterpenoids from *Centella asiatica* on the expression of TNFAIP6 in human fibroblasts as determined by real-time RT-PCR. Each bar represents the average of four replicate measurements, and fold induction is reported relative to GAPDH expression and normalized to vehicle control

may have no biological consequence; it is also plausible that small fluctuations in the expression of key genes may have biological significance but be numerically undetected. One key aim in our use of microarray technology is to identify differentially expressed genes for use as markers in subsequent higher throughput assays where sample size is more limited, including *in vivo* and clinical samples. For this reason we have adopted a significance criteria of $1 \pm 2.5\sigma_{ave}$ which is expected to lead to an acceptably low (approximately 1 %) false discovery rate.

Our transcript profiling results support previous studies that suggest *Centella asiatica* triterpenoids affect the properties of the ECM in dermal tissues, and provide additional insight into the molecular nature of this effect. Of the 1053 genes analyzed, TNFAIP6 shows the largest expression change upon treatment with TECA: an 11.77 ± 1.60-fold increase. TNFAIP6 is a secreted hyaladherin with a central role in ECM remodeling and the modulation of inflammation [18]; it has anti-inflammatory properties, and its expression is elevated in osteoarthritic joints [19], the ripening cervix [20], and other environments of extensive ECM remodeling. While some aspects of TNFAIP6 biochemistry remain unknown, it has been shown to inhibit matrix metalloprotease activity via a stable complex with inter-α-inhibitor [18].

Each of the *Centella asiatica* triterpenoids activates TNFAIP6 to a different extent (Fig. **4**). Among the components of TECA, AA is the most potent in the induction of TNFAIP6; 24 hour treatment with AA increases expression by 9.96 ± 1.05-fold at only 8 μg/mL and 25.73 ± 3.64-fold after 48 hours at 30 μg/mL. MA shows less stimulatory activity, and requires 48 hours at 110 μg/mL to produce a 13.11 ± 2.44-fold increase in TNFAIP6. Neither glycoside (AS and MS) caused greater than a 5-fold increase in expression of this gene even at concentrations up to 200 μg/mL. The relative activities of the purified triterpenoids and TECA are notably non-additive. The high dose of TECA (160 μg/mL) contains 48 μg/mL

AA and 48 μg/mL MA, yet the induction of TNFAIP6 expression by this sample at 48 hours is lower than the induction by 30 μg/mL of purified AA. The real-time RT-PCR data for treatment with TECA at 160 μg/mL for 24 hours is consistent with microarray data for the same sample. The microarray-generated value, (11.77 ± 1.60-fold induction), is lower than the real-time RT-PCR value (20.69 ± 2.40-fold induction); however the approximate 1.7-fold difference may be due to control gene normalization issues.

No change in the expression levels of the genes encoding fibronectin (FN1), collagen (COL1A2, COL2A1, COL6A2, COL3A1) and the collagen modifying enzyme proline 4-hydroxylase (P4HA1, P4HB) was detected in TECA treated cells relative to control cells, however a large increase in the level of transcript for the plasminogen activator inhibitor (PAI2, 4.83 ± 0.65-fold increase) was seen. PAIs are secreted inhibitors of the thrombolytic cascade: increase in PAI levels are expected to reduce plasminogen activator activity, and in turn reduce plasmin activity with a coincident decrease in collagenase activity [21], [22]. The end result of this cascade is expected to be a decreased rate of collagen hydrolysis, and therefore an increased accumulation of extracellular collagen. Additional support for increased collagen accumulation comes from transcript levels of transforming growth factor beta, which were seen to increase (1.62 ± 0.16-fold increase); this is expected to have the direct affect of increasing collagen levels [23], an activity which may be mitigated by the decreased level of thrombospondin (THBS1, down 2.86 ± 0.25-fold). Thus the gene expression changes that we observe following treatment with TECA may relate directly to previous observations regarding the modulation of collagen levels, and can be seen as supporting the contention that striking changes in the ECM are affected by these triterpenoids.

Changes in the expression levels of PAI2 as well as a number of cytokine growth factors present a likely scenario for increased angiogenic potential following TECA application. PAI2 increase, as well as an increases in vascular epithelial growth factor (VEGF) expression (1.63 ± 0.10-fold increase), and a decrease in thrombospondin (THBS1, down 2.86 ± 0.25-fold) could be expected to shift the cytokine balance toward angiogenesis [24]. Additionally, expression levels of both heparin-binding fibroblast growth factors FGF1 and FGF2 increase (1.68 ± 0.30-fold and 1.63 ± 0.11-fold, respectively), both have potent mitogenic and angiogenic activities. This is consistent with reports of the successful use of *Centella asiatica* triterpenoids in treatments for venous insufficiency [25], as well as microangiopathy and edema [5], [26]. Our studies indicate a preliminary correlation of these expression changes to the clinical effectiveness of triterpenoid-based treatments, and present an appealing opportunity for the connection of natural product-based medical treatments to quantitative functional genomics.

TECA has been shown to exhibit significant cytotoxicity *in vitro* at the concentration chosen for microarray analysis; changes in the expression of several cell cycle-related genes and genes encoding detoxifying enzymes may reflect this effect. The cytochromes P450 isotypes 51 and 1B1 were both found to increase in expression (CYP51, 2.65 ± 0.19-fold, CYP1B1 1.47 ± 0.13-fold), both belong to one of the principal classes of enzymes responsible for xenobiotic detoxification [27]. Genes involved in cell cycle progression are also modulated in a manner that is consistent with cytotoxicity: lowered expression of cell division cycle 2 (CDC2, down 2.04 ± 0.34-fold) and cyclin A2 (CCNA2, down 2.43 ± 0.38-fold) and elevated levels of RhoB and RhoE (up 2.07 ± 0.08-fold and 7.51 ± 0.25-fold), as well as increased capacity for prenylation (isopentyl-diphosphate delta isomerase, IDI1 up 2.90 ± 0.07-fold and farnesol diphosphate synthase FDPS, up 1.84 ± 0.19-fold) may be indicative of the cellular response to triterpenoid cytotoxicity.

In conclusion, the triterpenes present in *Centella asiatica* are shown to modulate gene expression when applied to human fibroblast cells in culture, and several changes in gene expression have been identified and quantified in this study, which provides a more fundamental understanding of the biological activity of these compounds. These changes are consistent with previous clinical and biochemical data supporting their use in connective tissue-related applications. TECA has been shown to drive changes in hyaladherin and cytokine expression which may be expected to lower the rate of proteolysis in the ECM, and thereby support the accumulation of collagen and fibronectin. Proangiogenic changes in the expression of a number of growth factors were also detected. TECA does not directly affect the expression levels of collagen or fibronectin genes. This information has provided a number of new tools for expression-based bioactivity assays, and the changes in the expression of the hyaladherin TNFAIP6 may be used to suggest that the free acids in TECA (AA and MA) have greater biological activity than the glycosides (AS and MS). It is expected that this or other expression-based assays will be useful for clinical studies of *Centella asiatica* products as well as the development of superior plant accessions and preparations.

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