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Analysis of Effects of the Herbal Preparation Circulat on Gene Expression Levels in Cultured Human Fibroblasts

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Circulat is a systemic standardized plant extract formulation that was developed for the prevention of severe manifestations of type 2 diabetes such as necrotic damage of the plantar foot. With the aim of revealing the molecular mechanisms underlying Circulat's biological activity, the effects of Circulat treatment on gene expression levels were examined in the cultured human fibroblast cell line MRC-5 using Affymetrix oligonucleotide microarrays. The analysis identified 187 genes, the expression levels of which underwent significant changes upon Circulat treatment. These include four genes (IL6, HMGA1, SLC19A2 and C4A) that have been implicated previously in the development of diabetes. A large proportion of the identified genes are involved in energy metabolism, protein synthesis, glucose metabolism and signaling pathways. Synergistic action of the Circulat components has also been revealed. Prospective applications of microarray analysis in phytopharmacology are discussed. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: gene expression; diabetes; adaptogens; systemic.

INTRODUCTION

Recent years have seen a steady growth of interest of modern medicine in the application of extracts from medicinal plants for the treatment of various functional disorders and diseases. To supervise these efforts, the National Center for Complementary and Alternative Medicine was established by the National Institutes of Health (Chesney and Straus, 2004). The first step in the objective science-based assessment of the usefulness of a particular extract requires one to establish whether the extract under study possesses any biological activity. Large-scale microarray-based gene expression analyses offer an excellent opportunity to answer this question as well as to obtain important data that may help to uncover molecular mechanisms of the extract's action. Studies of changes in gene expression levels in response to drug treatment aimed at an understanding of the molecular mechanisms of the drug action are one of the recent applications of genome-wide microarray analysis. It has been demonstrated, for example, that insulin treatment of type 2 diabetic patients results in significant changes in the abundance of multiple transcripts in skeletal muscles of the patients (Sreekumar *et al.*, 2002). Changes in gene expression levels have been registered for a number of genes in activated T lymphocytes treated with azathioprine (Thomas *et al.*, 2005). Recently it has been proposed to use gene expression analysis for the development of a screening system to determine the value of natural medicinal products (Katz *et al.*, 2006). Circulat is a systemic adaptogenic multi-component formulation developed at the Adaptogenic Medical Center (Caracas, Venezuela) as a remedy for the prevention of necrotic damage of the plantar foot in type 2 diabetic patients (Olalde Rangel *et al.*, 2005). While the effectiveness of Circulat (patent request number PCT/US06/60794) has been clinically proven, the molecular mechanisms underlying the therapeutic effects of the treatment are virtually unknown. This study examined the effects of treatment with Circulat and its components on gene expression levels in cultured human fibroblast cell line MRC-5 using Affymetrix oligonucleotide microarrays. The study identified a number of genes that are significantly affected by these treatments suggesting a possible molecular mechanism of Circulat action.

MATERIALS AND METHODS

Combined herbal extract Circulat. Circulat is composed of lyophilized ethanol/water extracts from 22 known medicinal plants in different ratios. The preparation is manufactured in the form of encapsulated powder by Nulab, Inc., Clearwater, FL 33765, USA. Four different samples were used in the experiments: total Circulat and three of its constituent parts (components 1, 2 and 3), which contained 6–8 extracts of different plants in the same proportions as found in Circulat as a whole. The composition of the three components added was

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identical to the composition of the total formula. Circulat and its constituents, which are produced in powder form, were extracted with 50% ethanol, lyophilized and added to the culture medium to a final concentration of 3 μg/mL. It corresponds to a concentration of 3–5 mg/kg body weight, which is approved by the Russian Pharmacopoeia (Mashkovsky, 1984). Such a concentration does not alter cell growth and, therefore, is physiological (Steinmann *et al.*, 2001).

Cell culture and RNA isolation. The human fibroblast cell line MRC-5 was grown under standard conditions in minimum essential medium (MEM, Gibco) supplemented with 10% fetal bovine serum (Gibco) at 37 °C. When the cells were close to confluence, the culture medium was replaced with medium containing Circulat or its components and the cells were grown for an additional 16 h. This period of time corresponds to one generation of cultivated cells. Control cells were grown in parallel in identical medium without the addition of any extracts.

Total RNA was extracted using Trizol (Invitrogen) reagent following the manufacturer's instructions. Control and experimental cell cultures were grown in triplicate and processed independently producing 15 RNA samples, each of which was analysed on an individual microarray chip. To ensure high data reproducibility and to enable data comparison across multiple experimental samples, the quality of total RNA prepared from each sample was analysed by electrophoresis (Fig. 1, panel a). The observed 28S:18S ratio of 2:1 demonstrated the high quality and the intactness of RNA. cRNA was prepared from 3 μg of total RNA and its yield and quality were assessed by electrophoretic and spectroscopic analyses (Fig. 1, panels b and c).

Microarray analysis and data processing. Microarray analysis was performed in accordance with standard procedures recommended by Affymetrix, Inc using Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays. Following hybridization and scanning, raw data in the form of image files were converted to gene expression values using Affymetrix GeneChip Operating Software (GCOS), which utilizes MAS 5.0 algorithm for data normalization, background subtraction, estimation of nonspecific binding, calculation of detection p-values and generation of 'presence' calls. Two-tailed Student's *t*-test assuming unequal sample variance was used to identify genes that displayed significant changes in the mean expression levels between control and each of the treated samples with the *t*-test *p*-value less than 0.05 and the mean fold change of at least 2. Only probes that were called 'present' in at least three of six independent measurements for each of the four treatment/control pairs were considered in this analysis. By comparing up- and down-regulated genes in each individual fraction and whole Circulat, several additional

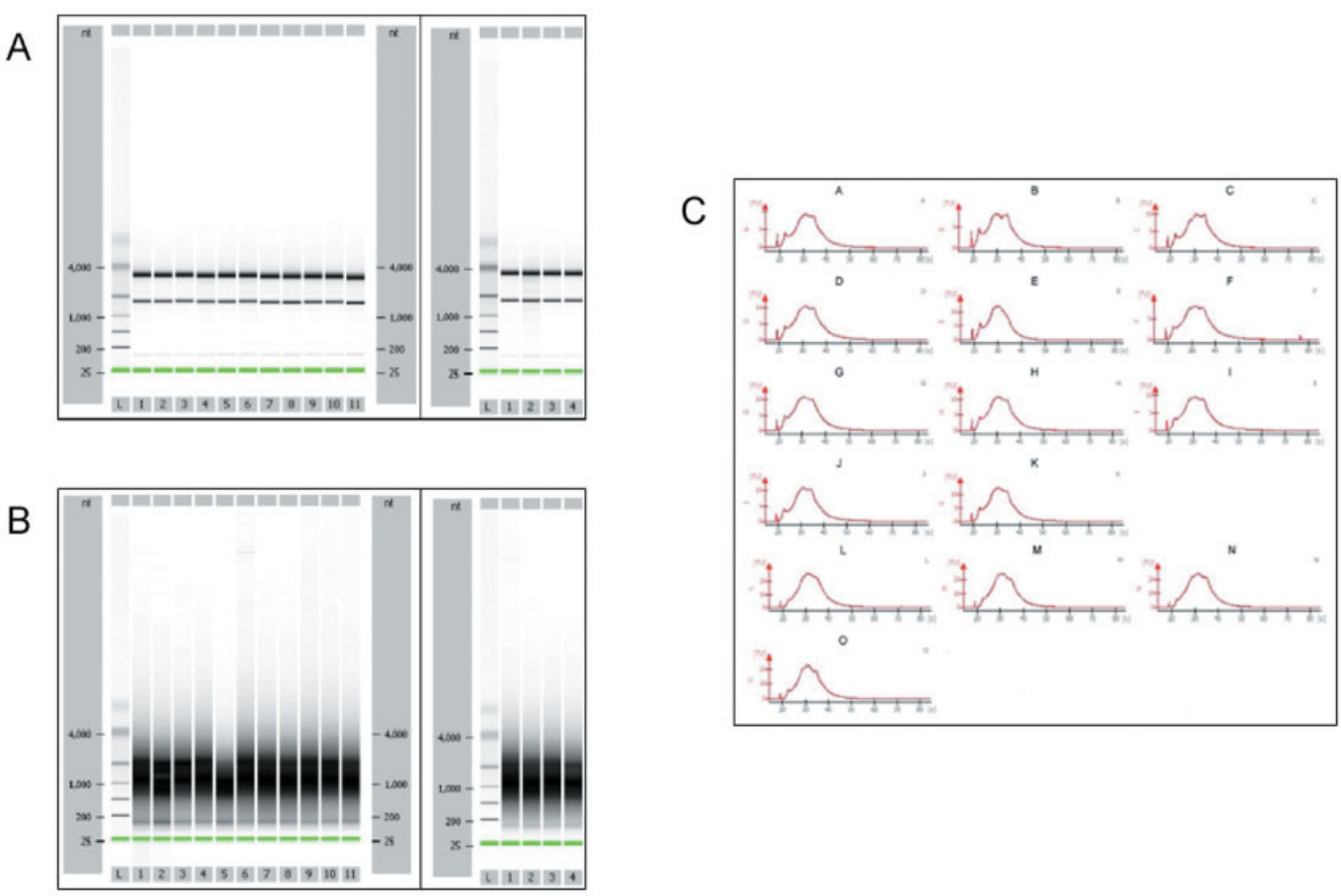

Figure 1. Comparison of RNA preparations used for microarray analyses. (A) Quality of total RNA isolated in triplicates from control samples as well as from samples treated with component 1–3 and whole Circulat, respectively, was assessed by electrophoresis. The prominent bands correspond to 28S and 18S ribosomal RNA. The observed relative 28S:18S ratio of 2:1 for all samples indicates intact RNA. (B) cRNA was prepared from 3 μg of total RNA and its yield and quality were assessed by electrophoretic (B) and spectroscopic (C) analyses.

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genes were identified that were regulated between 1.5 and 2 fold in the whole preparation and followed the same trend as in individual fractions, where they were up- or down-regulated by at least 2 fold. Together, these analyses resulted in a list of 87, 96, 24 and 187 genes (probes) that were significantly up- or down-regulated upon treatment with components 1, 2, 3 and whole Circulat, respectively.

RESULTS AND DISCUSSION

To gain insight into the molecular mechanisms of Circulat's action, the study carried out whole-genome microarray-based comparison of the gene expression levels in Circulat or one of its components-treated and control human fibroblast cell lines MRC-5. Both Circulat-treated and control experiments were carried out in triplicate yielding 15 samples that were processed independently as described in Materials and Methods. Microarray analysis was performed using Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays that provide full genomic coverage and contain probes for more than 47 000 unique transcripts corresponding to more than 38 500 human genes. This allowed us to monitor simultaneously the expression levels of practically all annotated genes of the human genome in an unbiased manner. Unlike earlier microarray platforms, Affymetrix GeneChip represents the state of the art in microarray design and features both perfectly matched and off-by-one probes that together with sophisticated processing algorithms allow one to distinguish precisely between specific and non-specific hybridization signals. It has been proven to produce highly reliable data, which in combination with the high quality of starting RNA and sufficient number of replicates virtually eliminates false-positives.

Two-tailed Student's *t*-test assuming unequal sample variance was used to identify genes that displayed significant changes in the mean expression levels between control and treated samples resulting in a list of 87, 96, 24 and187 genes (probes) that were significantly up- or down-regulated upon treatment with components 1, 2, 3 and whole Circulat, respectively. A complete list of Circulat-responsive genes with their descriptions, ratios of RNA abundances in experimental vs control samples and the calculated *t*-test values is presented in Table 1.

Comparison of genes regulated by individual components and whole Circulat

As expected, the genes regulated by individual components and by Circulat as a whole show significant

Table 1. Gene names and descriptions

Id	Ratio	*t*-test	Gene symbol	Gene title
239546_at	4.35	0.01	PQLC2	PQ loop repeat containing 2
215730_at	3.22	0.02	VGLL1	Vestigial like 1 (Drosophila)
232186_at	3.17	0.01	C20orf142	Chromosome 20 open reading frame 142
1556462_a_at	2.93	0.01	KLF12	Kruppel-like factor 12
216387_x_at	2.8	0	---	---
1552266_at	2.77	0.05	ADAM32	ADAM metallopeptidase domain 32
234137_s_at	2.72	0.01	RANBP17	RAN binding protein 17
232963_at	2.67	0.02	RFWD2	Ring finger and WD repeat domain 2
1569385_s_at	2.65	0.01	FLJ20032	Hypothetical protein FLJ20032
240625_at	2.65	0.03	UBE2E3	Ubiquitin-conjugating enzyme E2E 3 (UBC4/5 homolog, yeast)
231640_at	2.65	0.03	LOC144363	Hypothetical protein LOC144363
220166_at	2.61	0.02	CNNM1	Cyclin M1
232786_at	2.49	0.01	COG6	Component of oligomeric golgi complex 6
1558428_at	2.45	0.02	ALS2CR11	Amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 11
235955_at	2.43	0.03	MARVELD2	MARVEL domain containing 2
242766_at	2.41	0.03	LOC391269	Similar to ankyrin repeat domain 20 family, member A2
206497_at	2.4	0.01	FLJ10803	Hypothetical protein FLJ10803
216555_at	2.35	0.04	RP4-694E4.2	Hypothetical protein MGC50372
221703_at	2.26	0.02	BRIP1	BRCA1 interacting protein *C*-terminal helicase 1
202549_at	2.21	0.05	VAPB	VAMP (vesicle-associated membrane protein)-associated protein B and C
239734_at	2.21	0.05	LOC401320	Hypothetical LOC401320
1558934_a_at	2.2	0.01	---	Clone 27609 defective mariner transposon Hsmar2 mRNA sequence
1562836_at	2.18	0	---	CDNA FLJ11653 fis, clone HEMBA1004538
215591_at	2.16	0.01	SATB2	SATB family member 2
241777_x_at	2.11	0.05	DIP13B	DIP13 beta
223501_at	2.09	0	---	---
205699_at	2.08	0.02	---	---
1554113_a_at	2.08	0.01	SLC4A8	Solute carrier family 4, sodium bicarbonate cotransporter, member 8
242398_x_at	2.08	0.03	ATP5F1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1
240118_at	2.07	0.04	TXNDC11	Thioredoxin domain containing 11
210457_x_at	2.07	0.05	HMGA1	High mobility group AT-hook 1
1557218_s_at	2.07	0.03	FANCB	Fanconi anemia, complementation group B
1562648_at	2.07	0.02	KIAA1212	KIAA1212
220397_at	2.07	0	MDM1	Mdm4, transformed 3T3 cell double minute 1, p53 binding protein (mouse)
1562056_at	2.06	0.01	---	CDNA FLJ35091 fis, clone PLACE6005786

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Table 1. (*Continued*)

Id	Ratio	*t*-test	Gene symbol	Gene title
225877_at	2.05	0.04	TYSND1	Trypsin domain containing 1
216361_s_at	2.05	0.01	MYST3	MYST histone acetyltransferase (monocytic leukemia) 3
229399_at	2.05	0.01	C10orf118	Chromosome 10 open reading frame 118
231393_x_at	2.05	0.03	---	Transcribed locus, strongly similar to XP_528433.1
204317_at	2.04	0.02	GTSE1	G-2 and S-phase expressed 1
1553157_at	2.04	0.02	LHX4	LIM homeobox 4
219468_s_at	2.03	0.01	CUEDC1	CUE domain containing 1
1561442_at	2.03	0.02	LOC283585	Hypothetical protein LOC283585
1557217_a_at	2	0.01	FANCB	Fanconi anemia, complementation group B
1558469_at	1.92	0	LPP	LIM domain containing preferred translocation partner in lipoma
1554785_at	1.88	0.01	CCDC82	Coiled-coil domain containing 82
212525_s_at	1.86	0.01	H2AFX	H2A histone family, member X
213120_at	1.71	0	KIAA0701	KIAA0701 protein
1569201_a_at	1.69	0.01	SEC15L2	SEC15-like 2 (S. cerevisiae)
232048_at	1.61	0	FAM76B	Family with sequence similarity 76, member B
204159_at	1.55	0.01	CDKN2C	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
238281_at	0.66	5.96E-005	RBMS1	RNA binding motif, single stranded interacting protein 1
209721_s_at	0.66	0.01	HOM-TES-103	Hypothetical protein LOC25900, isoform 3
232429_at	0.66	0.01	---	MRNA; cDNA DKFZp434G1972 (from clone DKFZp434G1972)
206360_s_at	0.66	0.01	SOCS3	Suppressor of cytokine signaling 3
214696_at	0.65	0.01	MGC14376	Hypothetical protein MGC14376
212980_at	0.64	0.01	AHSA2	AHA1, activator of heat shock 90kDa protein ATPase homolog 2 (yeast)
233539_at	0.64	0	NAPE-PLD	*N*-acyl-phosphatidylethanolamine-hydrolysing phospholipase D
220153_at	0.63	0	ENTPD7	Ectonucleoside triphosphate diphosphohydrolase 7
208957_at	0.63	0	TXNDC4	Thioredoxin domain containing 4 (endoplasmic reticulum)
230454_at	0.63	0.01	ICA1L	Islet cell autoantigen 1,69kDa-like
227015_at	0.63	0	ASPHD2	Aspartate beta-hydroxylase domain containing 2
223802_s_at	0.62	0	RBBP6	Retinoblastoma binding protein 6
227602_at	0.61	0.01	RAB7	RAB7, member RAS oncogene family
228218_at	0.6	0	---	CDNA clone IMAGE:5284125
223764_x_at	0.6	0.01	NIPSNAP3B	Nipsnap homolog 3B (*C. elegans*)
1569690_at	0.6	0.01	CCDC36	Coiled-coil domain containing 36
235666_at	0.6	0.01	---	---
241413_at	0.59	0.01	RNF111	Ring finger protein 111
231212_x_at	0.58	0.01	TPST1	Tyrosylprotein sulfotransferase 1
226125_at	0.56	0	SLC9A3	Solute carrier family 9 (sodium/hydrogen exchanger), member 3
235165_at	0.55	0.01	PARD6B	par-6 partitioning defective 6 homolog beta (*C. elegans*)
1554786_at	0.55	0.01	C20orf32	Chromosome 20 open reading frame 32
210247_at	0.51	0.01	SYN2	Synapsin II
234020_x_at	0.5	0.02	COMMD1	Copper metabolism (Murr1) domain containing 1
236490_at	0.5	0.04	---	---
237515_at	0.5	0.04	TMEM56	Transmembrane protein 56
242681_at	0.5	0.02	NQO1	NAD(P)H dehydrogenase, quinone 1
1560818_at	0.5	0.03	LOC387895	Hypothetical gene supported by BC040060
240773_at	0.5	0.02	TRIO	Triple functional domain (PTPRF interacting)
239247_at	0.49	0.01	LOC401577	Hypothetical gene supported by AK125149
232918_at	0.49	0.05	LOC541471	Hypothetical LOC541471
235967_at	0.49	0.03	LOC641819	Hypothetical protein LOC641819
222380_s_at	0.49	0.04	PDCD6	Programmed cell death 6
230520_at	0.49	0.03	AIG1	Androgen-induced 1
238456_at	0.49	0.01	---	CDNA FLJ36202 fis, clone TESTI2028296
1555938_x_at	0.49	0.01	VIM	Vimentin
243947_s_at	0.49	0.01	---	Transcribed locus
238972_at	0.49	0.02	---	Transcribed locus
1564658_at	0.49	0	FLJ39237	FLJ39237 protein
201839_s_at	0.49	0.01	TACSTD1	Tumor-associated calcium signal transducer 1
202940_at	0.49	0.01	WNK1	WNK lysine deficient protein kinase 1
236499_at	0.48	0.03	C1orf86	Chromosome 1 open reading frame 86
235856_at	0.48	0.02	C4A	Complement component 4A (Rodgers blood group)
215816_at	0.48	0.04	LOC91316	Similar to bK246H3.1 (immunoglobulin lambda-like polypeptide 1, pre-B-cell specific)
230609_at	0.48	0.01	ENTH	Enthoprotin
201041_s_at	0.48	0	DUSP1	Dual specificity phosphatase 1
236610_at	0.47	0.01	PDE4D	Phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog)
234360_at	0.47	0.05	---	---
232555_at	0.47	0.03	CREB5	CAMP responsive element binding protein 5
244700_at	0.47	0.02	SEC61B	Sec61 beta subunit
242421_at	0.47	0.04	---	Transcribed locus

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Table 1. (*Continued*)

Id	Ratio	*t*-test	Gene symbol	Gene title
206115_at	0.47	0.01	EGR3	Early growth response 3
235564_at	0.46	0.03	ZNF117	Zinc finger protein 117 (HPF9)
243378_at	0.46	0.01	---	Transcribed locus, moderately similar to NP_060312.1 hypothetical protein FLJ20489
207575_at	0.46	0.02	GOLGA	Golgin-like protein
243395_at	0.46	0.03	CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)
205266_at	0.46	0.01	LIF	Leukemia inhibitory factor (cholinergic differentiation factor)
1554812_at	0.46	0.01	CLDN20	Claudin 20
222074_at	0.46	0.02	UROD	Uroporphyrinogen decarboxylase
231996_at	0.45	0.03	N4BP2	Nedd4 binding protein 2
201169_s_at	0.45	0	BHLHB2	Basic helix-loop-helix domain containing, class B, 2
215995_x_at	0.45	0.01	PLEKHQ1	Pleckstrin homology domain containing, family Q member 1
230333_at	0.45	0	SAT	Spermidine/spermine N1-acetyltransferase
202241_at	0.45	0	TRIB1	Tribbles homolog 1 (*Drosophila*)
223681_s_at	0.45	0.03	INADL	InaD-like (*Drosophila*)
242904_x_at	0.45	0	---	---
242861_at	0.45	0.02	---	---
233156_at	0.44	0.01	DLEU8	Deleted in lymphocytic leukemia 8
238501_at	0.44	0	---	Transcribed locus
207850_at	0.44	0	CXCL3	Chemokine (C-X-C motif) ligand 3
216263_s_at	0.44	0.01	C14orf120	Chromosome 14 open reading frame 120
1560817_at	0.44	0.03	---	CDNA FLJ20814 fis, clone ADSE01064
241280_at	0.44	0.02	ALDOB	Aldolase B, fructose-bisphosphate
210222_s_at	0.44	0.01	RTN1	Reticulon 1
221841_s_at	0.44	0	KLF4	Kruppel-like factor 4 (gut)
1562550_at	0.44	0.04	PITPNM2	Phosphatidylinositol transfer protein, membrane-associated 2
241928_at	0.44	0.03	CDKL1	Cyclin-dependent kinase-like 1 (CDC2-related kinase)
237494_at	0.44	0.01	---	---
1554309_at	0.44	0.01	EIF4G3	Eukaryotic translation initiation factor 4 gamma, 3
1556602_at	0.43	0.03	SLC19A2	Solute carrier family 19 (thiamine transporter), member 2
232392_at	0.43	0.03	SFRS3	Splicing factor, arginine/serine-rich 3
229455_at	0.43	0	---	Full length insert cDNA clone YZ04E02
202388_at	0.42	0	RGS2	Regulator of G-protein signalling 2, 24kDa
237996_at	0.42	0.03	---	---
207826_s_at	0.42	0	ID3	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein
228285_at	0.42	0.01	TDRD9	Tudor domain containing 9
205207_at	0.42	0.02	IL6	Interleukin 6 (interferon, beta 2)
207130_at	0.42	0.03	PRKCBP1	Protein kinase C binding protein 1
238320_at	0.41	0.05	TncRNA	Trophoblast-derived noncoding RNA
235750_at	0.41	0.04	C17orf65	Chromosome 17 open reading frame 65
239527_at	0.41	0.03	RAB3GAP1	RAB3 GTPase activating protein subunit 1 (catalytic)
234611_at	0.41	0	LOC645721	Hypothetical protein LOC645721
241795_at	0.41	0.01	ABP1	Amiloride binding protein 1 (amine oxidase (copper-containing))
242116_x_at	0.4	0.05	ANKRD17	Ankyrin repeat domain 17
204748_at	0.4	0.01	PTGS2	Prostaglandin-endoperoxide synthase 2
242741_x_at	0.4	0.01	NSUN4	NOL1/NOP2/Sun domain family, member 4
201739_at	0.39	0.01	SGK	Serum/glucocorticoid regulated kinase
211916_s_at	0.39	0.03	MYO1A	Myosin IA
220266_s_at	0.39	0.01	KLF4	Kruppel-like factor 4 (gut)
230581_at	0.39	0	---	CDNA FLJ32217 fis, clone PLACE6003771
235462_at	0.38	0	CPEB2	Cytoplasmic polyadenylation element binding protein 2
209774_x_at	0.38	0.02	CXCL2	Chemokine (C-X-C motif) ligand 2
235555_at	0.38	0.01	---	Transcribed locus, moderately similar to NP_659411.1 hypothetical protein FLJ31846
244677_at	0.38	0.04	PER1	Period homolog 1 (*Drosophila*)
242836_at	0.37	0.04	ATP1B3	ATPase, Na+/K+ transporting, beta 3 polypeptide
1559127_x_at	0.37	0.03	KIAA0690	KIAA0690
222303_at	0.37	0.01	---	---
242714_at	0.36	0.01	---	---
243993_at	0.36	0.04	PCTK2	PCTAIRE protein kinase 2
226444_at	0.35	0.02	SLC39A10	Solute carrier family 39 (zinc transporter), member 10
201531_at	0.35	0.01	ZFP36	Zinc finger protein 36, C3H type, homolog (mouse)
1558365_at	0.35	0	PGK1	Phosphoglycerate kinase 1
1559747_at	0.35	0.05	KIAA1840	KIAA1840
242784_at	0.34	0.02	ETS2	V-ets erythroblastosis virus E26 oncogene homolog 2 (avian)
208078_s_at	0.34	0.05	SNF1LK	SNF1-like kinase
209291_at	0.33	0	ID4	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein

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Table 1. (*Continued*)

Id	Ratio	*t*-test	Gene symbol	Gene title
233613_x_at	0.33	0.03	REXO2	REX2, RNA exonuclease 2 homolog (*S. cerevisiae*)
232461_at	0.32	0.03	AHI1	Abelson helper integration site 1
244025_at	0.31	0.02	---	---
218541_s_at	0.31	0	C8orf4	Chromosome 8 open reading frame 4
1554997_a_at	0.3	0	PTGS2	Prostaglandin-endoperoxide synthase 2
201044_x_at	0.27	0	DUSP1	Dual specificity phosphatase 1
209292_at	0.26	0	ID4	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein
1555847_a_at	0.25	0	LOC284454	Hypothetical protein LOC284454
242482_at	0.25	0.02	PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha
1556520_at	0.25	0.01	---	CDNA clone IMAGE:5296510
202768_at	0.24	0	FOSB	FBJ murine osteosarcoma viral oncogene homolog B
213931_at	0.24	0	ID2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein
203394_s_at	0.24	0.03	HES1	Hairy and enhancer of split 1, (*Drosophila*)
243296_at	0.23	0.03	PBEF1	Pre-B-cell colony enhancing factor 1
1554420_at	0.21	0	ATF3	Activating transcription factor 3
1554980_a_at	0.21	0.01	ATF3	Activating transcription factor 3
1566696_at	0.21	0.01	---	CDNA clone IMAGE:5289071
203395_s_at	0.21	0.01	HES1	Hairy and enhancer of split 1, (*Drosophila*)
216979_at	0.19	0.03	NR4A3	Nuclear receptor subfamily 4, group A, member 3
227099_s_at	0.16	0.01	LOC387763	Hypothetical LOC387763

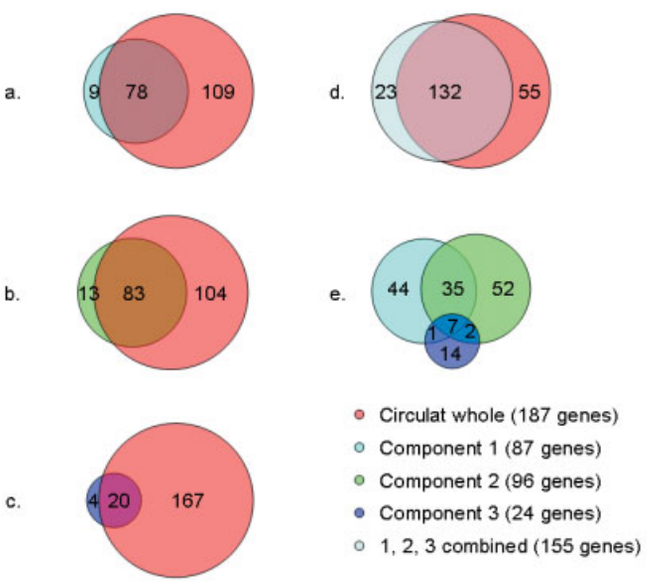

Figure 2. Comparison of genes affected by individual components and Circulat as a whole. (a–c) Components 1–3 vs Circulat, respectively. (d) Combination of three components *vs.* Circulat. (e) Relationship between the components. Numbers of genes belonging to a particular class (specific to a particular set or shared by two or more sets) are indicated.

overlap: more than 80% of genes affected by individual parts were also affected by the whole preparation (Fig. 2, panels a–c). Analyses of data for each of the three components also identified a sizable number (23) of genes that did not show up in the whole Circulat analysis. On the other hand, 55 genes the expression levels of which changed significantly after Circulat treatment were not observed with any of the three fractions (Fig. 2, panel d, Table 2). The regulation of these genes by Circulat is most likely due to interaction between active ingredients of the three components that produce a synergistic effect on gene regulation. Taken together, the data demonstrate that (1) treatment of human fibroblast cells with either Circulat or its components results in marked changes in gene expression patterns; (2) significant interactions between the active ingredients of Circulat exist resulting in a more complex pattern of gene expression in the complete preparation compared with those of isolated components which can be understood to be synergetic.

Analysis of processes and pathway affected by Circulat

Analysis of the biological process subset of Gene Ontology (GO) terms (Ashburner *et al.*, 2000) associated with each gene and their distribution revealed that the genes affected by Circulat are involved in a variety of cellular processes including protein, nucleic acid, lipid and carbohydrate metabolism, regulation of transcription, response to endogenous and external stimuli and stress, signal transduction and cell communication, cell growth and proliferation, development, protein modification and biosynthesis, generation of precursor metabolites and energy, etc. (Fig. 3). The broad spectrum of processes potentially regulated by Circulat is consistent

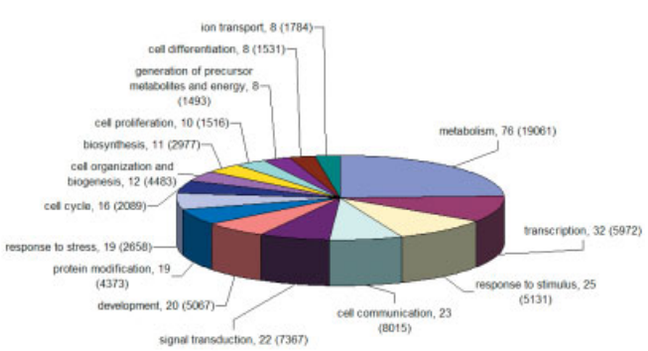

Figure 3. Major biological processes affected by Circulat treatment. Number of genes involved in a particular process that were affected by Circulat treatment is indicated. Numbers in parentheses correspond to the number of probes on Affymetrix GeneChip Human Genome U133 Plus 2.0 array that are annotated with a particular process. Total number of probes on array is 54613.

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Table 2. Genes affected by Circulat as a whole but by not individual components

Id	Ratio	*t*-test	Gene symbol	Gene title
239546_at	4.35	0.01	PQLC2	PQ loop repeat containing 2
1552266_at	2.77	0.05	ADAM32	ADAM metallopeptidase domain 32
232963_at	2.67	0.02	RFWD2	Ring finger and WD repeat domain 2
240625_at	2.65	0.03	UBE2E3	Ubiquitin-conjugating enzyme E2E 3 (UBC4/5 homolog, yeast)
220166_at	2.61	0.02	CNNM1	Cyclin M1
221703_at	2.26	0.02	BRIP1	BRCA1 interacting protein *C*-terminal helicase 1
239734_at	2.21	0.05	LOC401320	Hypothetical LOC401320
215591_at	2.16	0.01	SATB2	SATB family member 2
223501_at	2.09	0	---	---
242398_x_at	2.08	0.03	ATP5F1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1
210457_x_at	2.07	0.05	HMGA1	High mobility group AT-hook 1
204317_at	2.04	0.02	GTSE1	G-2 and S-phase expressed 1
1557217_a_at	2	0.01	FANCB	Fanconi anemia, complementation group B
1558469_at	1.92	0	LPP	LIM domain containing preferred translocation partner in lipoma
1554785_at	1.88	0.01	CCDC82	Coiled-coil domain containing 82
212525_s_at	1.86	0.01	H2AFX	H2A histone family, member X
213120_at	1.71	0	KIAA0701	KIAA0701 protein
1569201_a_at	1.69	0.01	SEC15L2	SEC15-like 2 (S. cerevisiae)
232048_at	1.61	0	FAM76B	Family with sequence similarity 76, member B
204159_at	1.55	0.01	CDKN2C	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
232429_at	0.66	0.01	---	MRNA; cDNA DKFZp434G1972 (from clone DKFZp434G1972)
209721_s_at	0.66	0.01	HOM-TES-103	Hypothetical protein LOC25900, isoform 3
238281_at	0.66	5.96E-005	RBMS1	RNA binding motif, single stranded interacting protein 1
206360_s_at	0.66	0.01	SOCS3	Suppressor of cytokine signaling 3
214696_at	0.65	0.01	MGC14376	Hypothetical protein MGC14376
233539_at	0.64	0	NAPE-PLD	*N*-acyl-phosphatidylethanolamine-hydrolysing phospholipase D
212980_at	0.64	0.01	AHSA2	AHA1, activator of heat shock 90kDa protein ATPase homolog 2 (yeast)
230454_at	0.63	0.01	ICA1L	Islet cell autoantigen 1,69kDa-like
208957_at	0.63	0	TXNDC4	Thioredoxin domain containing 4 (endoplasmic reticulum)
220153_at	0.63	0	ENTPD7	Ectonucleoside triphosphate diphosphohydrolase 7
227015_at	0.63	0	ASPHD2	Aspartate beta-hydroxylase domain containing 2
223802_s_at	0.62	0	RBBP6	Retinoblastoma binding protein 6
227602_at	0.61	0.01	RAB7	RAB7, member RAS oncogene family
223764_x_at	0.6	0.01	NIPSNAP3B	Nipsnap homolog 3B (*C. elegans*)
235666_at	0.6	0.01	---	---
1569690_at	0.6	0.01	CCDC36	Coiled-coil domain containing 36
228218_at	0.6	0	---	CDNA clone IMAGE:5284125
241413_at	0.59	0.01	RNF111	Ring finger protein 111
231212_x_at	0.58	0.01	TPST1	Tyrosylprotein sulfotransferase 1
226125_at	0.56	0	SLC9A3	Solute carrier family 9 (sodium/hydrogen exchanger), member 3
1554786_at	0.55	0.01	C20orf32	Chromosome 20 open reading frame 32
235165_at	0.55	0.01	PARD6B	par-6 partitioning defective 6 homolog beta (*C. elegans*)
210247_at	0.51	0.01	SYN2	Synapsin II
242681_at	0.5	0.02	NQO1	NAD(P)H dehydrogenase, quinone 1
238972_at	0.49	0.02	---	Transcribed locus
230520_at	0.49	0.03	AIG1	Androgen-induced 1
244700_at	0.47	0.02	SEC61B	Sec61 beta subunit
235564_at	0.46	0.03	ZNF117	Zinc finger protein 117 (HPF9)
243378_at	0.46	0.01	---	Transcribed locus, moderately similar to NP_060312.1
210222_s_at	0.44	0.01	RTN1	Reticulon 1
229455_at	0.43	0	---	Full length insert cDNA clone YZ04E02
211916_s_at	0.39	0.03	MYO1A	Myosin IA
235462_at	0.38	0	CPEB2	Cytoplasmic polyadenylation element binding protein 2
1558365_at	0.35	0	PGK1	Phosphoglycerate kinase 1
1559747_at	0.35	0.05	KIAA1840	KIAA1840

with the established ability of Circulat to prevent the development of severe manifestations of type 2 diabetes, which is a complex syndrome involving many intracellular signaling cascades and pathways as well as cell–cell interactions. Many of those pathways are involved in energy generation and are regulated on both transcriptional and post-transcriptional levels in response to endogenous or exogenous stimuli. A high proportion of genes identified in our experiments are implicated in the regulation of transcription (e.g. ATF3, Kruppel-like factor 4, Zinc finger protein 117, SATB2), energy production and glucose metabolism (thioredoxin domain containing 11, ATP5F1, PGK1) and signal transduction corroborating the hypothesis that Circulat is capable of normalizing molecular processes perturbed in the course of type 2 diabetes. Specifically, Circulat has been proven to be effective in the treatment of diabetic gangrene, which is often resistant to common treatments. Previous studies have suggested that impaired fibroblast function such as proliferation, cell migration,

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growth factor production and collagen synthesis may be part of the mechanism of diabetic necrotic damage. Fibroblasts are central to the processes of extracellular matrix deposition and remodeling that take place during tissue repair process. It functions both as a synthetic cell, depositing a collagen-rich matrix, and as a signaling cell, secreting the growth factors important for cell–cell communication during the tissue repair process. As shown in Table 3, many Circulat-regulated genes play roles in signal transduction and cell communication (ADAM32, TRIO, RAB7), response to endogenous and exogenous stimuli, an essential component in wound healing (FANCB, SLC19A2, C4A), growth factor-mediated signaling and cell motility (IL6, CXCL2, VIM), cell proliferation and biosynthesis (PBEF1, ABP1, PTGS2) corroborating Circulat's effectiveness in tissue repair. Furthermore, tissue remodeling involves both the generation of new cell types as well as regulated cell death, and several genes important for cell differentiation and apoptosis were identified in our experiments (KLF4, RTN1, PDCD6). It was also observed that significantly down-regulated genes are

Table 3. Major functional classes of genes affected by Circulat

Transcription regulation Gene symbol	Gene title
LHX4	LIM homeobox 4
MYST3	MYST histone acetyltransferase (monocytic leukemia) 3
HMGA1	High mobility group AT-hook 1
SATB2	SATB family member 2
BRIP1	BRCA1 interacting protein *C*-terminal helicase 1
KLF12	Kruppel-like factor 12
VGLL1	Vestigial like 1 (*Drosophila*)
NR4A3	Nuclear receptor subfamily 4, group A, member 3
ATF3	Activating transcription factor 3
HES1	Hairy and enhancer of split 1, (*Drosophila*)
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
ID2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein
HES1	Hairy and enhancer of split 1, (*Drosophila*)
PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)
ID4	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein
ETS2	V-ets erythroblastosis virus E26 oncogene homolog 2 (avian)
SNF1LK	SNF1-like kinase
PER1	Period homolog 1 (*Drosophila*)
KLF4	Kruppel-like factor 4 (gut)
ABP1	Amiloride binding protein 1 (amine oxidase (copper-containing))
ID3	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein
PRKCBP1	Protein kinase C binding protein 1
KLF4	Kruppel-like factor 4 (gut)
BHLHB2	Basic helix-loop-helix domain containing, class B, 2
ZNF117	Zinc finger protein 117 (HPF9)
CREB5	CAMP responsive element binding protein 5
EGR3	Early growth response 3
PDCD6	Programmed cell death 6
NQO1	NAD(P)H dehydrogenase, quinone 1
CCNL2	Cyclin L2
Response to stimulus	
H2AFX	H2A histone family, member X
FANCB	Fanconi anemia, complementation group B
GTSE1	G-2 and S-phase expressed 1
FANCB	Fanconi anemia, complementation group B
BRIP1	BRCA1 interacting protein *C*-terminal helicase 1
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
DUSP1	Dual specificity phosphatase 1
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
SNF1LK	SNF1-like kinase
CXCL2	Chemokine (C-X-C motif) ligand 2
MYO1A	Myosin IA
SGK	Serum/glucocorticoid regulated kinase
ANKRD17	Ankyrin repeat domain 17
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
ABP1	Amiloride binding protein 1 (amine oxidase (copper-containing))
IL6	Interleukin 6 (interferon, beta 2)
SLC19A2	Solute carrier family 19 (thiamine transporter), member 2
CXCL3	Chemokine (C-X-C motif) ligand 3
CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)
LIF	Leukemia inhibitory factor (cholinergic differentiation factor)
C4A	Complement component 4A (Rodgers blood group)

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Table 3. (*Continued*)

Transcription regulation Gene symbol	Gene title
DUSP1	Dual specificity phosphatase 1
NQO1	NAD(P)H dehydrogenase, quinone 1
TPST1	Tyrosylprotein sulfotransferase 1
TXNDC4	Thioredoxin domain containing 4 (endoplasmic reticulum)
Cell communication	
HMGA1	High mobility group AT-hook 1
DIP13B	DIP13 beta
ADAM32	ADAM metallopeptidase domain 32
PBEF1	Pre-B-cell colony enhancing factor 1
PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)
SNF1LK	SNF1-like kinase
CXCL2	Chemokine (C-X-C motif) ligand 2
PER1	Period homolog 1 (*Drosophila*)
ABP1	Amiloride binding protein 1 (amine oxidase (copper-containing))
RGS2	Regulator of G-protein signalling 2, 24kDa
IL6	Interleukin 6 (interferon, beta 2)
CXCL3	Chemokine (C-X-C motif) ligand 3
RTN1	Reticulon 1
LIF	Leukemia inhibitory factor (cholinergic differentiation factor)
PDE4D	Phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, *Drosophila*)
LOC91316	Similar to bK246H3.1 (immunoglobulin lambda-like polypeptide 1, pre-B-cell specific)
WNK1	WNK lysine deficient protein kinase 1
PDCD6	Programmed cell death 6
TRIO	Triple functional domain (PTPRF interacting)
NQO1	NAD(P)H dehydrogenase, quinone 1
SYN2	Synapsin II
RAB7	RAB7, member RAS oncogene family
SOCS3	Suppressor of cytokine signaling 3
Signal transduction	
HMGA1	High mobility group AT-hook 1
DIP13B	DIP13 beta
ADAM32	ADAM metallopeptidase domain 32
PBEF1	Pre-B-cell colony enhancing factor 1
PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)
SNF1LK	SNF1-like kinase
CXCL2	Chemokine (C-X-C motif) ligand 2
PER1	Period homolog 1 (*Drosophila*)
ABP1	Amiloride binding protein 1 (amine oxidase (copper-containing))
RGS2	Regulator of G-protein signalling 2, 24kDa
IL6	Interleukin 6 (interferon, beta 2)
CXCL3	Chemokine (C-X-C motif) ligand 3
RTN1	Reticulon 1
LIF	Leukemia inhibitory factor (cholinergic differentiation factor)
PDE4D	Phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, *Drosophila*)
LOC91316	Similar to bK246H3.1 (immunoglobulin lambda-like polypeptide 1, pre-B-cell specific)
WNK1	WNK lysine deficient protein kinase 1
PDCD6	Programmed cell death 6
TRIO	Triple functional domain (PTPRF interacting)
NQO1	NAD(P)H dehydrogenase, quinone 1
RAB7	RAB7, member RAS oncogene family
SOCS3	Suppressor of cytokine signaling 3
Cell surface receptor-linked signal transduction	
HMGA1	High mobility group AT-hook 1
ADAM32	ADAM metallopeptidase domain 32
CXCL2	Chemokine (C-X-C motif) ligand 2
RGS2	Regulator of G-protein signalling 2, 24kDa
IL6	Interleukin 6 (interferon, beta 2)
CXCL3	Chemokine (C-X-C motif) ligand 3
LIF	Leukemia inhibitory factor (cholinergic differentiation factor)
TRIO	Triple functional domain (PTPRF interacting)
NQO1	NAD(P)H dehydrogenase, quinone 1
Cell motility	
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
ABP1	Amiloride binding protein 1 (amine oxidase (copper-containing))
VIM	Vimentin
PARD6B	par-6 partitioning defective 6 homolog beta (*C. elegans*)

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Table 3. (*Continued*)

Transcription regulation Gene symbol	Gene title
Cell proliferation	
CDKN2C	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
DIP13B	DIP13 beta
PBEF1	Pre-B-cell colony enhancing factor 1
SNF1LK	SNF1-like kinase
KLF4	Kruppel-like factor 4 (gut)
ABP1	Amiloride binding protein 1 (amine oxidase (copper-containing))
IL6	Interleukin 6 (interferon, beta 2)
KLF4	Kruppel-like factor 4 (gut)
LIF	Leukemia inhibitory factor (cholinergic differentiation factor)
NQO1	NAD(P)H dehydrogenase, quinone 1
Cell differentiation	
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
SNF1LK	SNF1-like kinase
KLF4	Kruppel-like factor 4 (gut)
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
ABP1	Amiloride binding protein 1 (amine oxidase (copper-containing))
RTN1	Reticulon 1
KLF4	Kruppel-like factor 4 (gut)
PARD6B	par-6 partitioning defective 6 homolog beta (*C. elegans*)
Biosynthesis	
MRPL34	Mitochondrial ribosomal protein L34
ATP5F1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1
PBEF1	Pre-B-cell colony enhancing factor 1
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
ABP1	Amiloride binding protein 1 (amine oxidase (copper-containing))
IL6	Interleukin 6 (interferon, beta 2)
EIF4G3	Eukaryotic translation initiation factor 4 gamma, 3
UROD	Uroporphyrinogen decarboxylase
NQO1	NAD(P)H dehydrogenase, quinone 1
RBMS1	RNA binding motif, single stranded interacting protein 1
Cell death	
SGK	Serum/glucocorticoid regulated kinase
ABP1	Amiloride binding protein 1 (amine oxidase (copper-containing))
IL6	Interleukin 6 (interferon, beta 2)
PDCD6	Programmed cell death 6
SOCS3	Suppressor of cytokine signaling 3
Generation of precursor metabolites and energy	
TXNDC11	Thioredoxin domain containing 11
ATP5F1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1
PGK1	Phosphoglycerate kinase 1
ABP1	Amiloride binding protein 1 (amine oxidase (copper-containing))
ALDOB	Aldolase B, fructose-bisphosphate
C4A	Complement component 4A (Rodgers blood group)
NQO1	NAD(P)H dehydrogenase, quinone 1
TXNDC4	Thioredoxin domain containing 4 (endoplasmic reticulum)

enriched for members of signaling cascades known to regulate transcription and progression through the mitotic cycle such as FOSB, SNF1-like kinase and PRKAR1A (cAMP-dependent protein kinase regulatory subunit), cyclin L2. Up-regulated genes also contain a high number of genes involved in progression through the cell cycle as well as in DNA damage response, including cyclin M, GTSE1, FANCB and BRIP1 (BRCA1 interacting protein *C*-terminal helicase 1), cyclin-dependent kinase inhibitor C2 (CDKN2C). A number of transcription factors and members of protein degradation machinery (ubiquitin-conjugating enzyme E2E 3, Ring finger and WD repeat domain 2) are also present among those genes. These data suggest that one of the reasons for Circulat therapeutic effects is derived from its ability to stimulate cellular activities that respond to internal and external stress by slowing or arresting the cell cycle to allow repair of cellular components that could be damaged in the course of the disease, such as DNA and proteins, to be carried out by repair enzymes. Another prominent feature of the dataset is the presence of genes that have been shown to participate in muscle hypertrophy and contraction (SNF1LK, ATF3, NR4A3, ABP1, IL6, RGS2). It was shown that four genes, IL6, HMGA1, SLC19A2 and C4A, that are known to be involved in the development and progression diabetes are strongly regulated by Circulat. This not only validates our experimental approach, but also allows us for the first time to suggest an explanation for the well-established clinical effectiveness of Circulat at the molecular level. The role of interleukin-6 (IL6) in diabetes type 1 and 2

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is thoroughly documented (for a recent review see Kristiansen and Mandrup-Poulsen, 2005). Low-grade inflammation has been proposed to be involved in the pathogenic processes causing type 2 diabetes and inflammatory mechanisms are known to play a key role in the pathogenesis of type 1 diabetes. As a mediator of inflammation, IL6 is thought to be involved in events causing both types of the disease when present at elevated levels. In addition, IL6 can also regulate glucose homeostasis and metabolism directly and indirectly by both increasing the destruction of insulin producing β-cells by promoting apoptosis and playing a role in mounting insulin resistance in skeletal muscle, adipocytes and other tissues. Since elevated levels of IL6 are the known predisposition factors for development of diabetes, reduction of IL6 concentration should have the opposite therapeutic effect. The results demonstrate that Circulat treatment reduces IL6 expression more than two fold (0.42, $p = 0.02$) providing a solid link between the molecular events and the clinical manifestations taking place upon Circulat treatment. HMGA1 expression levels were elevated by more than two fold (2.07, $p = 0.05$) in samples treated with Circulat. Mutations in this small nuclear protein that acts as an architectural transcription factor have recently been detected in individuals suffering from type 2 diabetes (Foti *et al.*, 2005). This correlated with the reduction of expression of the insulin receptor and consequent development of insulin resistance. Deletion of HMGA1 gene in mice resulted in almost a complete loss of insulin receptor expression, development of insulin resistance and type 2 diabetes-like symptoms. HMGA1 thus plays a crucial role in glucose homeostasis and its increased expression promoted by Circulat may counteract deleterious effects caused by the loss of insulin receptor observed in type 2 diabetic patients. Two other genes, SLC19A2 and C4A, that are affected by Circulat (0.43, $p = 0.03$ and 0.48, $p = 0.02$, respectively) have also been unequivocally linked to diabetes. SLC19A2 encodes a thiamine transporter protein and causes thiamine-responsive megaloblastic anaemia (TRMA), also known as Rogers syndrome, when mutated (Labay *et al.*, 1999). Diabetes (both type 1 and 2) is the primary disease that defines the syndrome. C4A encodes the acidic form of complement factor 4, part of the classical activation pathway. Deficiency of this protein is associated with systemic lupus erythematosus and type 1 diabetes mellitus (Palsdottir *et al.*, 1983). While no direct link between C4A and type 2 diabetes has been found thus far, it has been suggested that the two types of the disease share many of the underlying processes thus making C4A involvement in type 2 diabetes a real possibility. Precise molecular mechanisms of SLC19A2 and C4A involvement in the disease development are not established as well as for IL6 and HMGA1. Nevertheless, their link to diabetes is indisputable and the ability of Circulat to influence their expression suggests additional possible mechanisms of Circulat action. Analysis of genes affected by Circulat also reveals that 26 of them have been implicated in many human diseases other than diabetes (Table 4). It is possible that some of those diseases and syndromes

Table 4. Disease-associated genes affected by Circulat treatment

Gene symbol	OMIM disease description
	Hemolytic anemia due to PGK deficiency
PGK1	Myoglobinuria/hemolysis due to PGK deficiency
MYO1A	Deafness, autosomal dominant nonsyndromic sensorineural
PRKAR1A	Adrenocortical tumor, somatic
	Carney complex, type 1
	Myxoma, intracardiac
	Pigmented adrenocortical disease, primary isolated
	Thyroid carcinoma, papillary
AHI1	Joubert syndrome-3
NQO1	Benzene toxicity, susceptibility to
	Leukemia, post-chemotherapy, susceptibility to
SYN2	Schizophrenia, susceptibility to
C4A	C4 deficiency
	Blood group, Rodgers
	Diabetes type 1
UROD	Porphyria cutanea tarda
	Porphyria, hepatoerythropoietic
WNK1	Pseudohypoaldosteronism, type II
NR4A3	Chondrosarcoma, extraskeletal myxoid
RAB7	Charcot-Marie-Tooth disease, type 2B
IL6	Diabetes type 1 and 2
	Osteopenia/osteoporosis
	Kaposi sarcoma, susceptibility to
SAT	Keratosis follicularis spinulosa decalvans
SOCS3	Dermatitis, atopic, 4
PDE4D	Stroke, susceptibility to, 1
SLC19A2	Diabetes type 2
	Thiamine-responsive megaloblastic anemia syndrome
RAB3GAP1	Warburg micro syndrome 1
ENTH	Schizophrenia, susceptibility to
CD55	Blood group Cromer

DOI: 10.1002/ptr

Table 4. (*Continued*)

Gene symbol	OMIM disease description
ALDOB	Fructose intolerance
LHX4	Short stature, pituitary and cerebellar defects, and small sella turcica\
VAPB	Amyotrophic lateral sclerosis 8
	Spinal muscular atrophy, late-onset, Finkel type
LPP	Leukemia, acute myeloid
	Lipoma
HMGA1	Diabetes type 2
	Lipoma
FANCB	Fanconi anemia, complementation group B
BRIP1	Breast cancer, early-onset
	Fanconi anemia, complementation group J

could be caused by mis-regulation of the same (or similar) genetic pathways that are perturbed in type 2 diabetes, which could be one of the explanations for this observation. On the other hand, it raises an exciting possibility that Circulat could be effective for treatment of conditions other than type 2 diabetes. For instance, five genes (PRKAR1A, UROD, SAT, FANCB, BRIP1) have been linked to various disorders affecting skin (and may have additional manifestations) such as Carney complex, Porphyria cutanea tarda, Keratosis follicularis spinulosa decalvans and Fanconi anemia, suggesting a possible common target that may be affected by Circulat. LPP and HMGA1 have been implicated in fat deposition disorder (lipoma), while VAPB has been demonstrated to be instrumental in the development of Amyotrophic lateral sclerosis and Spinal muscular atrophy, both of which are muscle-affecting syndromes. This is particularly intriguing given the high number of genes required for proper muscle function and development that have been identified in our experiments, as mentioned above. The ability of Circulat to regulate those genes suggests a possibility that Circulat may be useful for treatment of some muscle-affecting diseases. Both extensive clinical studies and laboratory experiments will be required to test these hypotheses in the future. Results of our experiments provide a molecular foundation for the clinically observed effectiveness of Circulat components (Table 5) in treating severe manifestations of type 2 diabetes, such as the necrotic damage of the plantar foot, and demonstrate the potential of this preparation for normalization of gene expression altered in type 2 diabetes. They are validated by the fact that four genes that had been previously shown to be involved in the development of diabetes were among the genes regulated by Circulat. The large number of genes previously implicated in a variety of human diseases revealed by this study suggests some possible new uses for the preparation as well. Finally, analysis of genes regulated by the total Circulat and its individual components demonstrated the existence of significant interactions between the active ingredients of Circulat suggesting that the full therapeutic effects can only be achieved by administration of the complete preparation. The application of full-genome expression analyses to phytopharmacology opens new horizons for carrying out scientific studies of herbal remedies and integration of herbal-based treatments into mainstream medicine. Using this approach, the physiologically active fractions of effective herbal extracts can be isolated and their specific activities can be determined. Such separation of different activities of a particular extract may enable researchers to selectively regulate the expression of specific genes (or gene groups) by varying the composition of the herbal preparation. It is likely therefore, that expression-profiling-based approaches to studies of herbal medicines will become standard in phytopharmacology in the near future.

Table 5. Components of Circulat

Phytomedicine
Energy enhancers
Eleutherococcus senticosus
Rhaponticum carthamoides
Panax ginseng root
Panax quinquefolius
Pfaffia paniculata (Suma)
Rhodiola rosea
Bio-intelligence modulators
Echinacea angustifolia
Echinacea purpurea
Ganoderma lucidum
Grifola frondosa
Hydrastis canadensis
Petiveria alliacea
Sutherlandia frutescens
Tabebuia avellanedae
Uncaria tomentosa
Organization improvers
Angelica sinensis
Crataegus oxyacantha
Croton lechleri
Ginkgo biloba
Hydrocotile asiatica
Ruscus aculeatus
Vaccinium myrtillus

Note: Ratios – concentration of active ingredient over the natural state – type of extract (plant, root, bark leaf . . .) and amounts – mg of extract – are also part of Circulat's patent application (PCT/US06/60794).

Results support Systemic Theory (ST)

A novel aspect of Circulat is its design according to ST, that any formula for maximum effectiveness should carry three categories (fractions) of tonic adaptogens

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(Olalde Rangel *et al.*, 2005) that correspond to (1) energy (ATP enhancers) (2) bio-intelligence (neural, endocrine, immune enhancers) and (3) organizational (structure and function enhancers), respectively. From the viewpoint of pure data analysis, these results support Circulat´s formulation according to ST, and are also consistent with TCM, in that the three categories of herbals mentioned, when acting together couple in an ensemble, to produce the desired result. In the case of Circulat, four significant genes related to diabetes would not have been regulated by the whole preparation, had this rule been left aside when formulating the product. As follows:

- HMGA1 up-regulation resulted from the synergetic interaction of the three fractions; i.e. it does not appear up-regulated in any of the individual fractions.
- IL6 was down-regulated by each, fraction 1 and 2. This is significant because their addition makes a difference in results, coinciding not only with ST, but also with millenary TCM, which includes chi herbals (ATP enhancing) in most formulations for chronic degenerative disease.
- C4A was down-regulated by the extracts that correspond to fraction 1 only, also in support of ST and TCM.
- SLC19A2 was down-regulated by the extracts that correspond to fraction 2, which supports ST.

As a relevant issue for further research, it should be noted that fractions 1 (energy enhancers) and 2 (bio-intelligence enhancers) significantly influenced more genes than fraction 3 (organizational herbals). It should also be highlighted that fraction 2 modulated more genes than any other. This suggests that the treatment of pathologies with substances that act only on function and structure may be an incomplete approach. Whether these findings may be generalized in confirmation of the systemic (E, I, O) approach is something that should be researched by medical science since it may pave the way for a new integral vision of therapeutics.

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REFERENCES

Ashburner M, Ball CA, Blake JA *et al.* 2000. The Gene Ontology Consortium. Gene ontology: tool for the unification of biology. *Nature Genet* **25**: 25–29.

Chesney MA, Straus SE. 2004. Complementary and alternative medicine: the convergence of public interest and science in the United States. *Med J Aust* **181**: 335–336.

Foti D, Chiefari E, Fedele M *et al.* 2005. Lack of the architectural factor HMGA1 causes insulin resistance and diabetes in humans and mice. *Nat Med* **11**: 765–773.

Katz S, Harris R, Lau JT, Chau A. 2006. The use of gene expression analysis and proteomic databases in the development of a screening system to determine the value of natural medicinal products. *Evid Based Complement Alternat Med* **3**: 65–70.

Kristiansen OP, Mandrup-Poulsen T. 2005. Interleukin-6 and diabetes: the good, the bad, or the indifferent? *Diabetes* (Suppl) **2**: S114–S124.

Labay V, Raz T, Baron D *et al.* 1999. Mutations in SLC19A2 cause thiamine-responsive megaloblastic anaemia associated with diabetes mellitus and deafness. *Nat Genet* **22**: 300–304.

Mashkovsky MD. 1984. *Medicinal Remedies*. Medicina: Moscow, Volume 1, 139–141.

Olalde Rangel JA, Magarici M, Amendola F, del Castillo O. 2005. The systemic theory of living systems. Part IV: Systemic medicine – the praxis. *Evid Based Complement Alternat Med* **2**: 429–439.

Palsdottir A, Cross SJ, Edwards JH, Carroll MC. 1983. Correlation between a DNA restriction fragment length polymorphism and C4A6 protein. *Nature* **306(5943)**: 615–616.

Sreekumar R, Halvatsiotis P, Schimke JC, Nair KS. 2002. Gene expression profile in skeletal muscle of type 2 diabetes and the effect of insulin treatment. *Diabetes* **51**: 1913–1920.

Steinmann GG, Esprester A, Joller P. 2001. Immunopharmacological *in vitro* effects of *Eleutherococcus senticosus* extract. *Arzneimittelforschung* **51**: 76–83.

Thomas CW, Myhre GM, Tshumper R *et al.* 2005. Selective inhibition of inflammatory gene expression in activated T lymphocytes: a mechanism of immune suppression by thiopurines. *J Pharmacol Exp Ther* **312**: 537–545.