Effect of serum from sub-healthy subjects with fatigue on the gene expression profile of skeletal muscle cells

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Abstract. To investigate the effect of serum from sub-healthy subjects with fatigue on the gene expression profile of skeletal muscle cells (HSkMCs), HSkMCs were cultured *in vitro* and treated with serum from healthy and sub-healthy subjects independently. Total RNA was extracted, purified and used for reverse transcription into cDNA. This was labeled with one of two fluorescence dyes, Cy3 or Cy5, and hybridized with a Human Genome Array carrying 8064 target genes. The differentially expressed genes were selected with the aid of software. There were 86 differentially expressed genes identified between the sub-healthy subjects and the healthy individuals, of which 60 were upregulated and 26 were down-regulated compared with those of the healthy individuals. These genes were mainly associated with the cell cycle, membrane channels, protein transport, energy metabolism and apoptosis. Our results suggest that serum from sub-healthy subjects with fatigue may change the gene expression profile of HSkMCs.

Introduction

In modern medicine, fatigue refers to the compromised function or response of cells, tissues and organs due to repeated loading and is usually characterized by functional decline and systemic discomfort. The production of fatigue is regarded as a combined response to multiple physiological and chemical changes. Christensen *et al* (1) proposed that physical fatigue may be defined as a state of disturbed homeostasis attributable to work and to the work environment. Studies have demonstrated that excessive energy consumption and generation of numerous metabolites are present in the state of fatigue. Certain metabolites may cause severe damage to the human body. These metabolites include lactic acid, ammonia, oxygen free radicals, peroxidated lipids and lipofuscin. These metabolites accumulate in the muscles resulting in disturbed homeostasis, damage to muscle cells, reduced synthesis of adenosine triphosphate, inhibition of membrane adenosine triphosphatase activity, compromised membrane function and reduction of sarcoplasmic reticulum calcium ion transport, which finally causes skeletal muscle fatigue (2-6). Fatigue is the most common symptom of sub-healthy subjects and is also a manifestation of compromised skeletal muscle cell functions. However, the pathogenesis of fatigue in sub-healthy subjects remains unclear. Our previous study revealed that the serum from sub-healthy subjects with fatigue was able to reduce the activity of mitochondrial cytochrome C oxidase (COX) and mitochondrial energy charge (EC) in human skeletal muscle cells (HSkMCs), which led to an energy metabolism disorder and changes in cellular ultrastructure (7). We speculated that these changes may play a significant role in the pathogenesis of fatigue in sub-healthy subjects. In addition, we also revealed that the serum from sub-healthy subjects with fatigue is able to affect the protein expression in HSkMCs (8). McCully *et al* identified that the blood flow and oxygen consumption in skeletal muscles remained unchanged in subjects with chronic fatigue but that the Mg^{2+} level increased significantly, which suggested that changes in certain substances in the blood may be attributed to the pathogenesis of fatigue (9-11). Numerous studies have revealed that sub-healthy subjects present abnormal serum levels of specific cytokines, including tumor necrosis factor α (TNF-α), interleukin (IL)-1, IL-4, IL-6, IL-10 and interferon, certain immunoglobulins, hormones, cortisol and adrenocorticotropic hormone (12-16). Our group has demonstrated the changes that occur in protein and gene expression in the serum of sub-healthy subjects (17-19). Cells, as the basic units of life, may be inevitably affected by numerous environmental, physical and chemical factors, which may lead to functional or organic changes. Thus, we speculate that certain substances in the serum of sub-healthy subjects are changed, resulting in the disorder of skeletal muscle cell functions and leading to the occurrence of fatigue.

Genes serve as the functional unit in genetics and carry genetic information in nucleic acids. They exhibit genomic functions through the encoding of functional proteins and

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consequently play a determinant role in life, with a direct or indirect correlation with almost all diseases and health states. Increasing numbers of studies have revealed that changes in gene expression determine the occurrence and development of diseases in humans. In recent years, microarray technology has provided an effective tool for the large-scale analysis of gene expression. In the present study, a microarray was employed to investigate the effect of serum from sub-healthy subjects with fatigue on the gene expression of HSkMCs *in vitro*. Through this experiment differentially expressed genes were identified with an aim to provide evidence for the pathogenesis of fatigue in sub-healthy subjects.

Materials and methods

Main instrument and materials. A Leica inverted microscope (Wetzlar, Germany), Sigma 3K30 centrifuge (Sigma, St. Louis, MO, USA), incubator (Sun Co., Ltd., Japan), clean bench (Sunshine Experimental Instrument Co., Ltd., Shanghai, China), Gen III Microarray Spotter (Amersham Pharmacia Biotech Ltd., Foster City, CA, USA), Gen III Microarray Scanner (Amersham Pharmacia Biotech, Ltd.), Shel Lab general purpose incubator (Shel Lab, Ltd.), Bio-Rad Mini-Sub GT System (Bio-Rad Ltd., Hercules, CA, USA), DU 520 UV/Vis spectrophotometer (Beckman Coulter Ltd., Miami, FL, USA), Vilber Gel documentation system (Vilber, Ltd.), UV Crosslinker (Vilber Lourmat, France) and MIDAS (Chipscreen Biosciences, Ltd.) were used in the present study. A Qiagen RNeasy mini kit (Qiagen, Inc., Hilden, Germany), Qiagen PCR Purification kit (Qiagen, Inc.), CyScribe cDNA Labeling kit (Amersham Pharmacia Biotech, Ltd.), TRIzol reagent (Invitrogen, Carlsbad, CA, USA), DEPC (Amersham Pharmacia Biotech, Ltd.), DMEM/F12, fetal bovine serum (FBS; HyClone, Logan, UT, USA), 0.25% trypsin, serum from sub-healthy subjects with fatigue and normal serum (our lab) were also used in the present study. Human skeletal muscle cells (HSkMCs) were purchased from ScienCell (Carlsbad, CA, USA). The study was approved by the ethics committee of Nanfang Hospital, Southern Medical University.

Preparation of serum from fatigued sub-healthy and healthy subjects. The diagnosis of fatigue in sub-healthy subjects was based on fatigue as the main manifestation lasting for >3 months, but not meeting the criteria for the clinical and sub-clinical diagnosis of diseases. A total of 10 sub-healthy subjects with fatigue aged 25-40 years old were recruited and informed consent was obtained prior to the study. Serum was prepared according to the following procedures: i) Serum from sub-healthy subjects with fatigue; non-anti-coagulated fasting venous blood (20 ml) was collected from the cubital vein at 10:00 a.m. and kept at room temperature for 1 h followed by centrifugation at 3,000 rpm for 10 min. The supernatant was collected under aseptic conditions and then heated in a water bath at 65˚C for 30 min during which gentle agitation was performed, aiming to inactivate the serum. Aliquots of serum were prepared after filtration with a disposable aseptic filter (Millipore, Billerica, MA, USA) and stored at -20˚C; ii) serum from healthy subjects; ten sub-healthy subjects with fatigue which resolved after treatment were recruited at least 1 week after treatment discontinuation. Fasting venous blood (20 ml) was collected from the cubital vein at 10:00 a.m. and the blood was processed as described above.

Cell culture and grouping. The HSkMCs were thawed and maintained in a 75-cm^2 flask. When the cell confluence reached >80%, cells were divided into four 75-cm^2 flasks and were randomly assigned into the healthy (n=2) and the sub-healthy groups (n=2). Cells in the healthy and sub-healthy groups were maintained in DMEM/F12 containing 30% healthy serum and 30% sub-healthy serum, respectively, for 72 h. The cells were then collected and washed in PBS and the total RNA was extracted.

Design and preparation of the human genome microarray. The human genome microarray was purchased from Shenzhen Weixin Biotech Co., Ltd (Shenzhen, China). The microarray was generated by directly adding the cDNA template and included 8,064 genes, the majority of which were from IMAGE human cDNA library (Invitrogen). There were 7,488 cloned fragments including 7,458 expressed gene fragments (>500 bases in length) and 30 blank vector fragments. The reference standard gene samples were purchased from Amersham Pharmacia Biotech Ltd., and included 120 external and 132 internal reference standard samples and 132 negative and positive control genes. Other gene fragments were cloned and purified with RT-PCR and added to the samples in the microarray by Shenzhen Weixin Biotech Ltd. The cDNA samples were dissolved in spotting buffer (Amersham Pharmacia Biotech Ltd.) followed by automatic spotting with an Array Spotter (Amersham Pharmacia Biotech Ltd.). Subsequently, the cDNA was adhered to the plate by ultraviolet cross-linking and then the microarray was stored in a dry environment.

Extraction, identification and purification of total RNA. Total RNA was extracted from cells in each group with TRIzol followed by purification of the mRNA. The purity and content of the mRNA were determined following electrophoresis of a fraction of RNA.

Hybridization of microarray. Labeling and purification of the probes and hybridization and washing of the microarray were performed by Shenzhen Weixin Biotech Co., Ltd.

Detection and data processing. The microarray was scanned and the acquired images were transformed into digital signals representing fluorescence intensity. This was followed by data analysis and processing.

Results

Quality and purity of RNA from HSkMCs. The total RNA from the 2 groups presented as three bands at 28S, 18S and 5S. The ratio of 28S to 18S was approximately 2:1 demonstrating favorable integrity. As shown in Table I, the quality, concentration and content of total RNA met the requirements for further experiments.

Gene expression profile analysis. The gene expression profile is exhibited in Fig. 1 and met the requirements for further

Table I. A260, A280, A260A:280 ratio, RNA concentration and RNA content in two groups.

No.	Group	A260	A280	A260:A280 ratio	RNA concentration (μg/μl)	RNA content (μg)
1	Healthy	0.675	0.341	1.98	2.70	54.00
2	Sub-healthy	0.822	0.415	1.98	3.29	65.80

Figure 1. Gene expression profile in the healthy and sub-healthy group (microarray no. 2000067473). Green shows the expression signal of the healthy individuals was higher than that of sub-healthy individuals; yellow shows equal expression signal; red shows expression signal of the healthy individuals was lower than that of sub-healthy individuals.

analysis. The signal density was high, the background was even and no clear defects were observed.

Analysis of microarray quality. The correlation coefficient (R^2) of the hybridization signals between the two groups was ≥0.85, which suggested that there was no significant difference in the biological properties between the samples. The coefficient of variation was low for the internal and external reference standard genes, and the positive and negative control genes had favorable reproducibility indicating the excellent quality of the microarray. This and subsequent processing of the samples did not result in artificial bias and the results were reliable.

Analysis of data and biological information

Screening of data. When the signal intensity of a spot was higher than the sum of the mean signal intensity of the negative controls and 3 times its standard deviation, this spot was regarded as an applicable spot. The determination of a significant difference in gene expression was based on step screening. When the fluorescence signals met the above criteria in two channels, the above criteria were directly applied (ratio >2 or <0.5); when the fluorescence signals met the above criteria in only one channel (CY3 OR CY5), the criteria for statistical significance were defined as ratio >3 or <0.3.

Changes in gene expression. In the two channels of microarray 2000067473, there were 2,979 applicable genes. Significant differences in gene expression were identified in 71 genes, including 53 that revealed upregulation and 18 with downregulation in the sub-healthy group. A total of 567 applicable genes were identified in only one channel with a significant difference in the gene expression identified in 15 genes, including 7 that revealed upregulation and 8 with downregulation in the sub-healthy group (Table II).

Classification of genes with differential expression. Primary data analysis revealed that the genes differentially expressed between healthy subjects and sub-healthy subjects were mainly associated with skeletal muscle development, energy metabolism (transport, phosphorylation and hydrolysis), cell proliferation and differentiation, oxidative stress, cell apoptosis and signal transduction. Thus, the damage of skeletal muscle cells by serum from sub-healthy subjects with fatigue may be associated with the genes involved in skeletal muscle development, energy metabolism (transport, phosphorylation and hydrolysis) and cell proliferation and differentiation.

Discussion

Gene expression refers to how the genetic information in genes is transformed into RNA via transcription and into proteins via translation. In organisms, specific genes are switched on or switched off using their own regulatory mechanisms. The final result of gene expression is the harmonious and integral activity of life. In humans, each cell possesses 2-4x10^4 functional genes, the expression of which determines the functional state. Thus, results from the analysis of information in RNA involve not only the gene potentials in organisms, but the dynamic changes in their functional state. In modern medicine, it has been accepted that gene expression is regulated by genetic and environmental factors. These genetic factors are also known as gene regulatory sequences and include promoter, enhancer and splice junction sites. Even the slightest changes in these factors may significantly affect gene expression. In addition, gene

Table II. Differentially expressed genes between healthy subjects and sub-healthy subjects (microarray no. 2000067473).

Gene	Gene tag	Cy3/Cy5 ratio
CD36 antigen (collagen type I receptor, thrombospondin receptor)	CD36	0.02
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, β	NFKBIB	3.95
Ring finger protein 24	RNF24	0.03
Heat shock transcription factor 2 binding protein	HSF2BP	3.88
Proteasome (prosome, macropain) 26S subunit, ATPase, 3	PSMC3	2.95
Homeo box B3	HOXB3	6.26
SCAN domain-containing 1	SCAND1	6.97
Neurogenic differentiation 2	NEUROD2	0.33
Zinc finger protein 7 (KOX 4, clone HF.16)	ZNF7	4.78
Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	ID1	2.57
Mitogen-activated protein kinase 6	MAPK6	3.71
Claudin 9	CLDN9	27.57
Human T-cell receptor active α-chain mRNA from JM cell line, complete cds	TRA	4.97
HLA-G histocompatibility antigen, class I, G	HLA-G	3.12
Insulin-like growth factor binding protein 3	IGFBP3	3.23
Cytokine receptor-like factor 1	CRLF1	4.70
Pim-1 oncogene	PIM1	6.06
Transforming, acidic coiled-coil containing protein 1	TACC1	0.31
Paraoxonase 1	PON1	9.02
Heat shock 27 kDa protein 1	HSPB1	2.01
Calcium channel, voltage-dependent, β 3 subunit	CACNB3	8.36
Chloride intracellular channel 1	CLIC1	3.28
Solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	SLC7A7	3.56
Hyperpolarization activated cyclic nucleotide-gated potassium channel 2	HCN2	3.80
Solute carrier family 29 (nucleoside transporters), member 2	SLC29A2	7.64
Solute carrier family 23 (nucleobase transporters), member 2	SLC23A2	4.97
Elastin microfibril interface located protein	EMILIN	4.89
Endoplasmic reticulum glycoprotein	GP36B	2.31
A kinase (PRKA) anchor protein 4	AKAP4	21.49
Lipoprotein lipase	LPL	13.89
Uridine phosphorylase	UP	0.34
Aldolase C, fructose-bisphosphate	ALDOC	2.85
Phosphoglycerate kinase 1	PGK1	2.44
Aspartoacylase (aminoacylase 2, Canavan disease)	ASPA	0.37
Succinate-CoA ligase, GDP-forming, β subunit	SUCLG2	0.28
Hydroxysteroid (17-β) dehydrogenase 2	HSD17B2	5.67
Peripheral myelin protein 2	PMP2	0.32
Cytochrome P450, subfamily VIIB (oxysterol 7 α-hydroxylase), polypeptide 1	CYP7B1	6.17
Alcohol dehydrogenase 3 (class I), γ polypeptide	ADH3	0.11
Lysozyme homolog	LOC57151	3.88
Enolase 3 (β, muscle)	ENO3	3.33
Phosphatidylserine decarboxylase	PISD	3.30
Phosphoglycerate dehydrogenase	PHGDH	2.10
Cholinephosphotransferase 1	LOC56994	10.78
Glycogenin	GYG	0.45
Ubiquitin-conjugating enzyme E2E 1 (homologous to yeast UBC4/5)	UBE2E1	0.06
Diptheria toxin resistance protein required for diphthamide biosynthesis (*Saccharomyces*)-like 2	DPH2L2	4.44
Tubulin-specific chaperone c	TBCC	0.05
Protein geranylgeranyltransferase type I, β subunit	PGGT1B	3.49
Eukaryotic translation elongation factor 1 δ (guanine nucleotide exchange protein)	EEF1D	2.52
Caspase 5, apoptosis-related cysteine protease	CASP5	0.28

Table II. Continued.

Gene	Gene tag	Cy3/Cy5 ratio
BCL2/adenovirus E1B 19 kDa-interacting protein 1	BNIP1	3.48
Etoposide-induced mRNA	PIG8	124.99
Programmed cell death 8 (apoptosis-inducing factor)	PDCD8	2.91
2'-5' oligoadenylate synthetase 2	OAS2	3.96
Tumor necrosis factor (ligand) superfamily, member 11	TNFSF11	19.54
Neuromedin B receptor	NMBR	3.00
Integrin, β-like 1 (with EGF-like repeat domains)	ITGBL1	3.01
Inhibin, β A (activin A, activin AB α polypeptide)	INHBA	4.13
Insulin-like growth factor 2 (somatomedin A)	IGF2	0.39
FYN oncogene related to SRC, FGR, YES	FYN	0.32
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	YWHAH	2.63
TRAF family member-associated NFKB activator	TANK	0.10
Regulator of G-protein signaling 10	RGS10	0.06
CDC14 (cell division cycle 14, *S. cerevisiae*) homolog B	CDC14B	0.06
RAB26, member RAS oncogene family	RAB26	0.04
ADP-ribosylation factor 1	ARF1	2.40
Triple functional domain (PTPRF interacting)	TRIO	3.45
COP9 homolog	COP9	0.19
Dipeptidylpeptidase VI	DPP6	3.66
Protein tyrosine phosphatase, receptor type, N	PTPRN	0.37
Troponin C, slow	TNNC1	6.81
Profilin 2	PFN2	0.02
Actin, γ 2, smooth muscle, enteric	ACTG2	2.29
Tropomyosin 2 (β)	TPM2	2.74
FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1 (chondrocyte-derived)	FARP1	0.05
Periplakin	PPL	0.30
Myosin-binding protein C, slow-type	MYBPC1	3.06
Hypothetical protein FLJ11184	FLJ11184	0.05
Putative gene product	13CDNA73	0.39
Type I transmembrane protein Fn14	FN14	2.35
Tumor necrosis factor α-inducible cellular protein containing leucine zipper domains; Huntingtin-interacting protein L; transcription factor IIIA-interacting protein	FIP2	3.16
Soc-2 (suppressor of clear, *C. elegans*) homolog	SHOC2	0.27
JM27 protein	JM27	9.20
Replication factor C (activator 1) 5 (36.5 kDa)	RFC5	14.97
Origin recognition complex, subunit 5 (yeast homolog)-like	ORC5L	0.25

Data with a ratio <1 represent downregulation.

expression is also affected by certain environmental factors, including temperature, light, mood, other factors causing changes in endogenous hormones or other substances with involvement in signal transduction. Studies have demonstrated the special changes that occur in mRNA following heat shock, pharmacotherapy, metabolism and disease (20-22). Chen *et al* revealed that the expression of more than 40 genes was markedly changed in hepatocytes undergoing hypoxia, and that these genes were involved in metabolism, signal transduction and defense (23). Shi *et al* revealed that the expression of more than 180 genes was markedly changed in the skeletal muscle cells of mice undergoing exhaustive exercise and that these genes were involved in ion transport, energy metabolism, transcription and translation, the cell cycle, cell regulatory proteins, skeletal muscle development, signal transduction and phosphorylation of proteins and amino acids (24).

Whistler *et al* identified that the abnormal expression of 839 genes in peripheral blood mononuclear cells (PBMCs) was correlated with fatigue in subjects with chronic fatigue of unknown cause, and that these genes were involved in oxidative phosphorylation, gluconeogenesis, lipid metabolism and signal transduction (25). Vernon *et al* also identified 8 differentially

expressed genes in PBMCs in subjects with chronic fatigue syndrome which were mainly involved in immune function and included CMRF35 antigen, IL-8 and HD protein (26). Kaushik *et al* identified 35 differentially expressed genes in PBMCs in subjects with chronic fatigue syndrome, of which 15 were revealed to be upregulated (ABCD4, PRKCL1, MRPL23, CD2BP2, GSN, NTE, POLR2G, PEX16, EIF2B4, EIF4G1, ANAPC11, PDCD2, KHSRP, BRMS1 and GABARAPL1) and one downregulated (IL-10RA) demonstrated by RT-PCR (27). Smith *et al* applied genomics in the investigation of gene expression profiles and single nucleotide polymorphisms (SNPs) in the PBMCs of subjects with chronic fatigue syndrome. Their results revealed 65 SNPs and 165 differentially expressed genes which were associated with chronic fatigue syndrome (28). These findings confirm that the abnormal expression of specific genes is closely associated with the occurrence and development of fatigue.

In modern medicine, disorders of the nerve-secretion-immune network may be the major cause of occurrence and development of sub-health. Studies have demonstrated the presence of abnormal metabolites in the blood of sub-healthy subjects, which may cause damage to homeostasis thus affecting cellular physiological functions (7,8). Our previous study revealed that the serum from sub-healthy subjects with fatigue are able to affect the structure and function of skeletal cells *in vitro*. In the present study, our findings revealed 86 differentially expressed genes in HSkMCs from sub-healthy subjects with fatigue undergoing treatment with 30% serum for 72 h. Of these genes, 60 were revealed to be upregulated and 26 downregulated. These genes were predominantly involved in cell growth and development (MFGE8, HLA-F, IGFBP6, IGFBP3, C3, MAPK6, PIG11, CNN2, MAP4, CDC20, TFCP2, DAG1, ID1), membrane-related ion channels (CLIC1, KCNJ15, CAANB3, HCN2, SLC23A, SLC7A7), energy metabolism (TPI1, LPL, PTGS1, LDHA, PGK1, HSD17B2, ADH3, COX8, ALDOC), stress (HSPB1, HSF2BP), cell signal transduction (GAS6, IGF2) and apoptosis (SIVA, PDCD8) and the majority were upregulated. Abnormal expression of these genes may cause aberrant cell development and changes in ion channels in the cell membranes. This may affect ion exchange and lead to an increase of intracellular Ca^{2+} and an abnormal membrane potential, causing an energy metabolism disorder, damage to cellular structures and functions and impairment of cell viability.

Heat shock proteins (HSPs) are a group of highly conserved proteins possessing significant physiological functions. Physiological, pathological and environmental factors may induce the production of HSPs which thus are also known as stress proteins. These physiological functions protect cells against damage induced by various stimuli and have also been revealed to be involved in the regulation of cell proliferation, differentiation and apoptosis (29). In addition, HSPs have been demonstrated to play significant roles in the maintenance of homeostasis, defense against oxidative stress, aiding protein synthesis and in the repair of misfolded proteins. Stress is the basic cause of increased synthesis of HSPs in cells (30-35). Sun *et al* identified that HSP-27 expression was increased in the serum of sub-healthy subjects (19). In the present study, our findings suggest that the serum from sub-healthy subjects with fatigue was able to upregulate the HSP genes. This may be attributed to the theory that certain metabolites (including peroxidized lipids) in the blood of sub-healthy subjects with fatigue may cause damage to human skeletal muscle cells, which may lead to disorder of the cytoskeleton in these cells resulting in activation of genes, including HSPB1 and HSF2BP, and the subsequent increase of HSP expression. These changes are essential for the enhancement of tolerance to stress and the maintenance of cell homeostasis.

Energy depletion in skeletal muscle cells is one cause of fatigue. Our previous study revealed that mitochondrial membrane cytochrome C oxidase activity and the mitochondrial energy load were reduced in *in vitro* human skeletal muscle cells undergoing treatment with serum from sub-healthy subjects with fatigue; this may cause a disorder of the energy metabolism in the cells (7). Glucose and fat are the main sources of energy. PISD, LOC56994, ALDOC, AKAP4, LPL, PHGDH and DPP6 are the major genes regulating the metabolism of glucose and fat. In the present study, the expression of these genes was markedly increased in sub-healthy subjects with fatigue compared with the healthy subjects. This may be attributed to the theory that the serum from sub-healthy subjects with fatigue causes an energy metabolism disorder in skeletal muscle cells. This may induce the expression of genes associated with energy metabolism in order to meet the requirement for energy which is essential for cell homeostasis and cellular physiological functions.

Taking all these results together, we speculate that the serum from sub-healthy subjects with fatigue may alter the expression of certain genes in human skeletal muscle cells, which provides evidence for the pathogenesis of fatigue at a cellular level.

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