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ORIGINAL ARTICLE

Fibroblasts from patients with major depressive disorder show distinct transcriptional response to metabolic stressors

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Major depressive disorder (MDD) is increasingly viewed as interplay of environmental stressors and genetic predisposition, and recent data suggest that the disease affects not only the brain, but the entire body. As a result, we aimed at determining whether patients with major depression have aberrant molecular responses to stress in peripheral tissues. We examined the effects of two metabolic stressors, galactose (GAL) or reduced lipids (RL), on the transcriptome and miRNome of human fibroblasts from 16 pairs of patients with MDD and matched healthy controls (CNTR). Our results demonstrate that both MDD and CNTR fibroblasts had a robust molecular response to GAL and RL challenges. Most importantly, a significant part (messenger RNAs (mRNAs): 26–33%; microRNAs (miRNAs): 81–90%) of the molecular response was only observed in MDD, but not in CNTR fibroblasts. The applied metabolic challenges uncovered mRNA and miRNA signatures, identifying responses to each stressor characteristic for the MDD fibroblasts. The distinct responses of MDD fibroblasts to GAL and RL revealed an aberrant engagement of molecular pathways, such as apoptosis, regulation of cell cycle, cell migration, metabolic control and energy production. In conclusion, the metabolic challenges evoked by GAL or RL in dermal fibroblasts exposed adaptive dysfunctions on mRNA and miRNA levels that are characteristic for MDD. This finding underscores the need to challenge biological systems to bring out disease-specific deficits, which otherwise might remain hidden under resting conditions.

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INTRODUCTION

Major depressive disorder (MDD) is a mental disorder characterized by persistent depressed mood, anhedonia, sleep and appetite disturbances, and feelings of worthlessness, guilt and hopelessness. It is increasingly viewed as an illness of the body as well as of the mind.[1] Both genetic and environmental factors have been associated with the etiology of the disease,[2] but their interplay remains unexplained to date. Stressful life events are associated with the onset and severity of major depression;[3] therefore, we speculated that aberrant adaptive responses to stressors would be also detectable at the cellular level.

Coordination of cellular responses to stress is largely accomplished by mitochondria.[4] Regardless of the type of stress a cell is experiencing (for example, exposure to harmful chemicals, changes of nutrient or oxygen supplies, dangerous levels of reactive oxygen species or even pathogen invasion), mitochondria utilize a wide array of mechanisms aimed at restoring cellular homeostasis.[5] Therefore, the health of these cellular organelles is critical not only for the fate of the individual cells, but for the well-being of the entire organism. Not surprisingly, abnormalities in mitochondrial functions have been implicated in neurological[6] and psychiatric disorders,[7–9] and specifically in MDD.[10] In addition, mitochondria are involved in multiple metabolic pathways and are the most prominent generators of energy in the cell. Energy is produced in the cytoplasm through glycolysis, or in the mitochondria through oxidative phosphorylation, also known as mitochondrial respiration. The reliance on energy produced by mitochondrial respiration, rather than glycolysis, can be experimentally shifted by changing the sugar source in the cell culture medium from glucose to galactose (GAL).[11] The shift to oxidative phosphorylation can reveal mitochondrial deficits intersecting control of carbohydrate metabolism and energy homeostasis. Energy production is also tightly interconnected with lipid metabolism through the key metabolite acetyl-CoA. Acetyl-CoA may remain in the mitochondria for energy production or may be exported for *de novo* fatty acid synthesis into the cytoplasm, depending mainly on the availability of fatty acids. This adaptive shift can be experimentally directed by limiting the fatty acid supply in the cell culture medium. Therefore, cellular adaptation to stress can be tested by simple manipulations of the carbohydrate and fatty acids source in the culture medium.

Molecular adaptation to stress in the context of mental disorders is difficult to address in the human brain. Alternatively, we used *in vitro* propagated peripheral cells to test the effects of metabolic stress. Dermal fibroblasts are an appealing cell-based model for studying peripheral events associated with brain disorders, based on the ease of establishing them from skin biopsies, and the ability to maintain in culture without additional reprograming.[12] In addition, the confounding variability in human samples on the basis of patients' hormones, life style or medication use, are virtually eliminated after several cell divisions. To date, skin fibroblast cultures have been used successfully to elucidate molecular mechanisms associated with schizophrenia[13,14] and developmental disorders,[15,16] and for discerning abnormalities in signal transduction pathways in MDD.[12,17–19] In addition, previous reports for dysregulations in

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lipid metabolism[20] and mitochondrial respiration,[21] suggest abnormalities in mitochondrial function in the periphery of MDD patients. Therefore, we aimed at elucidating whether MDD fibroblasts would in addition exhibit molecular deficits in response to metabolic stressors such as GAL and reduced lipids (RL) in the culture medium. In this manuscript, we present the experimental data gathered from metabolically challenged human dermal fibroblasts and discuss their relevance to MDD.

MATERIALS AND METHODS

Human fibroblasts

This study was approved by the Vanderbilt University Institutional Review Board. Participants were diagnosed with a current major depressive episode according to the Structured Clinical Interview for DSM-IV-TR[22] with an exclusion criteria of other primary axis I DSM-IV diagnosis. Procedures for recruitment and diagnosis have been described previously.[12,18] A written informed consent was obtained from all the participants before any procedures were conducted. Sixteen healthy controls (CNTR) were matched by age, race and gender to MDD patients (Supplementary Material 1). The MDD and CNTR subject groups had similar average age (MDD = 34.9, CNTR = 35.2), representation of gender (12 female and four male) and race (12 white and four African American). Fibroblast cultures were established from skin biopsies according to a protocol previously described.[12,18]

Briefly, fibroblast cultures underwent 5–10 passages with DMEM (Dulbecco's Modified Eagle's medium; Mediatech, Manassas, VA, USA) containing 250 mM glucose and 1 mM sodium pyruvate, supplemented with 2 mM L-glutamine (Mediatech), 10% fetal bovine serum (Thermo Scientific HyClone, Logan, UT, USA) and antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA, USA). Cultures were maintained at 37 °C and 5% CO_2. A cell line from each patient was divided into three plates (plating density 1.2×10^6 cells per plate) and after overnight adherence, each plate received one of the following formulations: (1) standard (STD) medium: DMEM containing 25 mM glucose; (2) galactose-containing (GAL) medium: DMEM with no glucose, supplemented with 10 mM GAL (Sigma-Aldrich, St. Louis, MO, USA); (3) RL medium: DMEM containing 25 mM glucose, supplemented with lipid-reduced fetal bovine serum (Thermo Scientific HyClone). All the cultures were grown for 7 days, refreshing the media every 2 days. The cells were collected after washing with ice-cold phosphate-buffered saline twice, and then trypsinized, pelleted (700 g for 8 min at 18 °C) and stored at −80 °C until RNA isolation. In all the tested cell lines, neither GAL nor RL exhibited a detectable effect on cell growth and mortality.

RNA isolation and analyses

Total RNA and small RNA fractions were isolated from frozen fibroblasts using mirVana microRNA (miRNA) isolation Kit (Ambion, Austin, TX, USA) according to manufacturer's instruction. Both total RNA and small RNA were eluted with 100 µl Elution Solution. Agilent 2100 Bioanalyzer was used to determine the quality and size of the RNA preps. Total RNA integrity number for all messenger RNA (mRNA) samples ranged from 9.4 to 10, and was similar between the MDD and CNTR groups and various conditions. The samples were stored at −80 °C until further use.

The mRNA levels in each fibroblast culture were examined with the GeneChip HT HG-U133+ PM Array Plate (Affymetrix, Santa Clara, CA, USA). The complementary DNA synthesis from total RNA, labeling and hybridization were performed according to manufacturer's protocol. The segmented images from each GeneChip were normalized and log2 transformed using GC-robust multi-array analysis.[23] The average logarithmic ratio ($ALR = Mean_{MDD} - Mean_{CNTR}$) was calculated for each gene probe, as an indicator for magnitude of change and Student's paired and group two-tailed *P*-values as indicators for significance of change. Significance was established according to the dual criteria of $|ALR| > 0.3785$ (30% change) and both pairwise and groupwise $P \leqslant 0.01$. Quantitaive PCR validation of the microarray data was performed with RT^2 custom Profiler PCR Arrays (Qiagen, Valencia, CA, USA), designed to test the mRNA expression level of 10 genes involved in the regulation of lipid metabolism: stearoyl-CoA desaturase (delta-9-desaturase), fatty acid binding protein 3, 7-dehydrocholesterol reductase, insulin induced gene 1, quinolinate phosphoribosyltransferase, 3-hydroxy-3-methylglutaryl-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA synthase 1, low-density lipoprotein receptor, fatty acid desaturase 1, fatty acid desaturase 2. The differentially expressed genes were subjected to a two-way hierarchical clustering analysis using GenePattern software.[24] In addition, for each gene probe, we applied a linear statistical model with gene expression values as the outcome variable and group, stressor and group × stressor as fixed effects (groups: CNTR, MDD; stressors: GAL, RL). Data were analyzed with procedure MIXED in SAS software (version 9.3, SAS Institute, Cary, NC, USA). Gene set enrichment analysis (GSEA) based on pre-defined gene classes were carried out with the GenePattern software.[25] BioCarta defined set of genes were considered significantly overrepresented at $P \leqslant 0.05$.

The miRNA levels were interrogated with miRNome miScript miRNA PCR Arrays (Qiagen). The complementary DNA was prepared from small RNA preps with miScript HiSpec Buffer according to miScript II RT Kit (Qiagen) instructions. The complementary DNA from each sample was quantified and proportionately combined into four pools of samples according to age and gender (Supplementary Material 1). Creating multiple sample pools from closely matched individuals allowed us a reduced work load and cost, while still retaining the ability to establish statistical significance in the downstream analyses. However, to avoid any pooling biases and outlier effects, the outcome of these data had to be validated in the individual samples. As a result, significantly changed miRNA levels in each individual fibroblast sample were examined with custom miScript miRNA PCR Arrays. (Qiagen), containing assays for the following miRNAs: miR-146b-5p, miR-550a, miR-214, miR-132*, miR-376c, miR-19a, miR-181a*, miR-486-5p, miR-424*, miR-542-3p, miR-22, miR-376b, miR-29b, miR-564. The selection of the 14 miRNA assays was based on a statistically significant difference in response to GAL or RL in the MDD or the CNTR group. The Ct values for each miRNA from both miRNome and custom qPCR arrays were normalized to the average Ct from a set of housekeeping genes and thus ΔCt values were generated. At $Ct > 35$, a miRNA level was considered non-detectable. In cases where the levels of a miRNA were non-detectable in two or more samples within a group, the miRNA was not used for further comparisons. The miRNA difference of expression between groups was measured by $\Delta\Delta Ct_{RL} = Mean\Delta Ct_{RL} - Mean\Delta Ct_{STD}$ and $\Delta\Delta Ct_{GAL} = Mean\Delta Ct_{GAL} - Mean\Delta Ct_{STD}$. A miRNA was considered differentially expressed when $|\Delta\Delta Ct| > 0.3785$ and pairwise $P \leqslant 0.05$. Group × stressor interaction was determined by the dual criteria of $|\Delta\Delta\Delta Ct| > 0.3785$ (30% difference) and groupwise $P \leqslant 0.05$. $\Delta\Delta\Delta Ct_{GAL} = \Delta\Delta Ct_{MDDGAL} - \Delta\Delta Ct_{CNTRGAL}$; $\Delta\Delta\Delta Ct_{RL} = \Delta\Delta Ct_{MDDRL} - \Delta\Delta Ct_{CNTRRL}$.

RESULTS

MDD fibroblast response to metabolic stress: mRNA changes

Metabolic stress response in MDD and CNTR fibroblasts from 32 subjects (16 MDD and 16 CNTR) was achieved by exposure to culture media containing GAL or RL (Supplementary Material 2). Analysis of mRNA species by oligonucleotide microarrays revealed that GAL substitution of glucose in the growth medium resulted in robust changes in the transcriptome of both MDD and CNTR fibroblasts. Using dual criteria for establishing significant expression differences ($|ALR_{GAL-STD}| > 0.3785$ (30% change) and both group- and pairwise $P \leqslant 0.01$), we identified 1196 transcripts changed in GAL-treated MDD fibroblasts (Supplementary Material 3A). Of those, 975 were upregulated as a result of GAL treatment. According to the same criteria, 1111 probes were changed in CNTR fibroblasts, 733 of which were upregulated. In comparison with the CNTR fibroblasts, MDD fibroblasts revealed a distinct GAL-induced mRNA pattern; of the total number of mRNAs, 310 (26%) were only exhibited in MDD (Supplementary Material 4A).

RL treatment also resulted in pronounced mRNA changes in both MDD and CNTR cells. Differential expression in RL conditions (compared with STD media) was noted for 312 transcripts in the MDD: 262 upregulated and 50 downregulated mRNAs. Similarly, in CNTR fibroblast, 360 mRNA species (261 upregulated, 99 downregulated) were differentially expressed in RL compared with STD conditions (Supplementary Material 3B). Importantly, the MDD fibroblasts responded to RL condition differently than CNTR; 103 (33.4%) of all mRNA changes induced by RL were only exhibited in MDD (Supplementary Material 4B).

It was remarkable that both GAL and RL treatments resulted in distinct mRNA changes in MDD fibroblasts that were not present

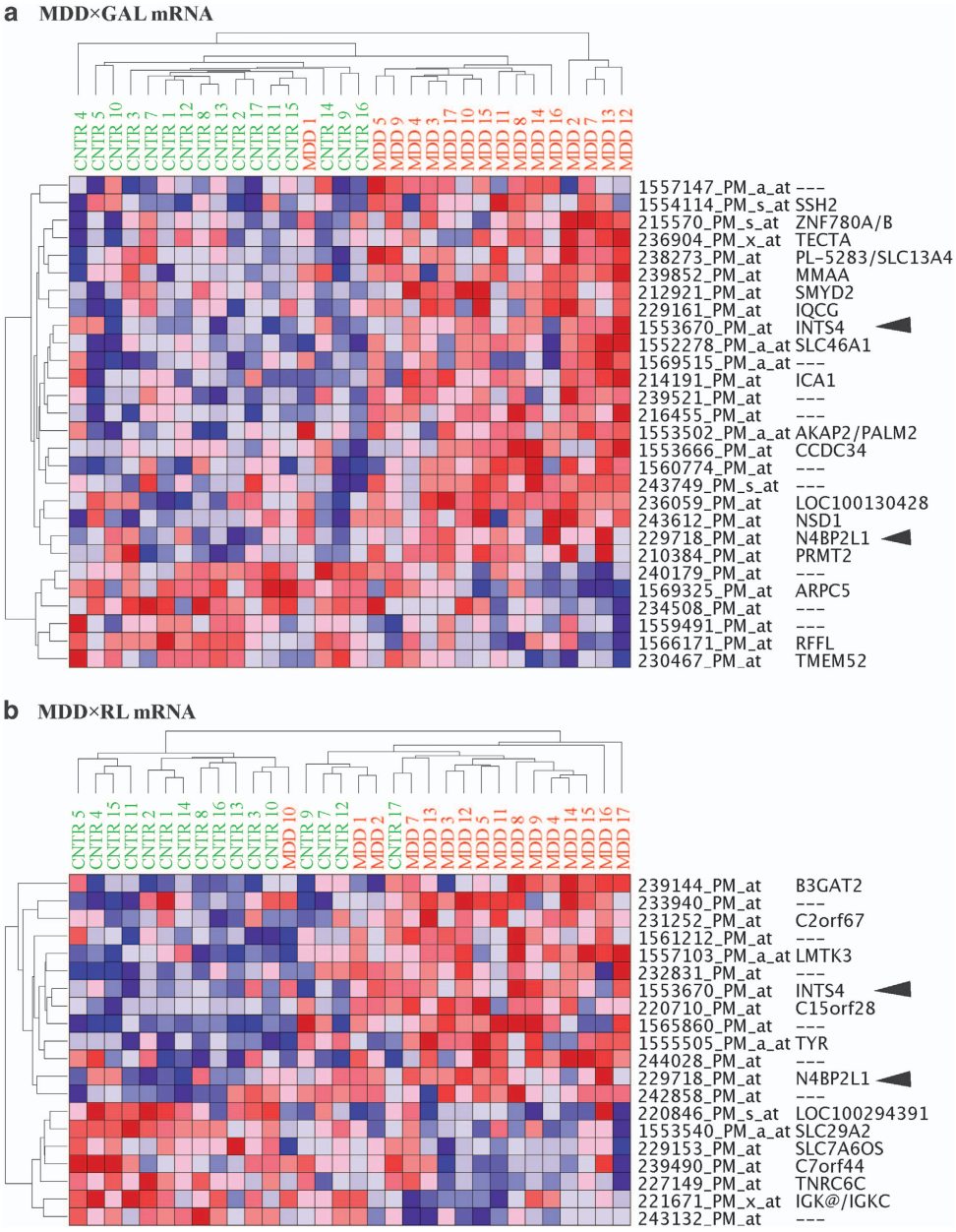

Figure 1. Disease × stressor interaction mRNA signatures. (**a**) MDD × GAL and (**b**) MDD × RL. The ALR ($Mean_{RL} - Mean_{STD}$) of the probes with significant disease × challenge interaction were subjected to unsupervised hierarchical clustering. The colored squares represent the increase (red) or decrease (blue) of each ALR from the mean. Color intensity is proportional to magnitude of change. Clear separation of MDD and CNTR groups was observed. Furthermore, two genes (INTS4 and N4BP2L1—denoted by arrows) are commonly present in both signatures. CNTR, control; GAL, galactose; MDD, major depressive disorder; mRNA, messenger RNA; RL, reduced lipid; STD, standard.

in CNTR, therefore, we further sought to identify the interaction effect between disease state and metabolic challenge (group × stressor) by applying a linear statistical model. We used a dual significance cutoff criterion of $|\Delta ALR| > 0.3785$ (30% change) and $P \leqslant 0.01$. We found that the characteristic MDD signature in response to GAL (MDD × GAL) was defined by 28 (Figure 1a), and in response to RL (MDD × RL) was defined by 20 mRNA species (Figure 1b). Two of the genes (integrator complex subunit 4; INTS4 and NEDD4 binding protein 2-like 1; N4BP2L1) overlapped in the MDD × GAL and MDD × RL response-defining pattern.

Custom qPCR arrays were used to validate the microarray-detected mRNA changes in response to GAL and RL. The arrays were designed to test the mRNA expression level of 10 genes involved in lipid metabolism with significantly changed expression in MDD and in CNTR samples in GAL vs STD and in RL vs STD conditions. The statistical significance of the microarray-detected expression changes for all 10 genes was confirmed by qPCR (Figure 2). In addition, the microarray's ALRs had a high correlation with the qPCR's $-\Delta\Delta Ct$ in both GAL vs STD and RL vs STD comparisons.

To better understand what molecular pathways are differently engaged in MDD compared with CNTR in response to metabolic stress, we performed GSEA with predetermined gene sets by BioCarta. Significantly enriched gene sets, representing molecular pathways, were identified for both MDD and CNTR in GAL and RL environment (Table 1, GSEA). GAL treatment induced enrichment of 21 pathways in MDD and 17 in CNTR. Nine of the 21 gene sets (43%) were only enriched in MDD and are involved in cell cycle

	CONTROL							
	GALACTOSE				REDUCED LIPID			
	microarray		qPCR		microarray		qPCR	
Gene	ALR(GAL-STD)	p-value	-ΔΔCt (GAL-STD)	p-value	ALR(RL-STD)	p-value	-ΔΔCt (GAL-STD)	p-value
SCD	2.02	0.00000	2.66	0.00040	1.82	0.00000	2.23	0.00007
FABP3	1.86	0.00001	2.80	0.00397	1.89	0.00000	3.10	0.00014
DHCR7	1.18	0.00000	1.42	0.00021	1.82	0.00000	2.49	0.00000
INSIG1	1.43	0.00000	1.79	0.00571	1.61	0.00000	2.16	0.00004
QPRT	1.42	0.00000	2.03	0.00125	1.96	0.00000	2.71	0.00036
HMGCR	0.70	0.00017	0.43	0.64146	1.50	0.00000	1.18	0.19592
HMGCS1	1.17	0.00010	1.20	0.00937	1.64	0.00000	2.40	0.00000
LDLR	1.08	0.00000	1.46	0.04054	1.55	0.00000	2.35	0.00020
FADS1	1.08	0.00000	1.36	0.00531	1.22	0.00000	1.57	0.00001
FADS2	1.00	0.00000	1.84	0.00143	1.51	0.00000	2.50	0.00000

	MDD							
	GALACTOSE				REDUCED LIPID			
	microarray		qPCR		microarray		qPCR	
Gene	ALR(GAL-STD)	p-value	-ΔΔCt (GAL-STD)	p-value	ALR(RL-STD)	p-value	-ΔΔCt (GAL-STD)	p-value
SCD	2.13	0.00001	2.37	0.00005	1.71	0.00000	2.21	0.00004
FABP3	1.88	0.00000	3.33	0.00028	1.55	0.00003	3.36	0.00003
DHCR7	1.24	0.00000	1.76	0.00172	1.99	0.00000	2.57	0.00000
INSIG1	1.44	0.00000	1.76	0.01071	1.59	0.00000	2.41	0.00008
QPRT	1.67	0.00000	2.07	0.00313	2.02	0.00000	2.65	0.00043
HMGCR	0.75	0.00004	0.98	0.01540	1.50	0.00000	1.96	0.00000
HMGCS1	1.21	0.00070	1.46	0.02899	1.73	0.00000	2.30	0.00011
LDLR	1.24	0.00000	1.75	0.00665	1.70	0.00000	2.44	0.00002
FADS1	1.08	0.00003	1.36	0.00239	1.23	0.00000	1.58	0.00000
FADS2	0.76	0.00100	1.78	0.00342	1.27	0.00000	2.34	0.00010

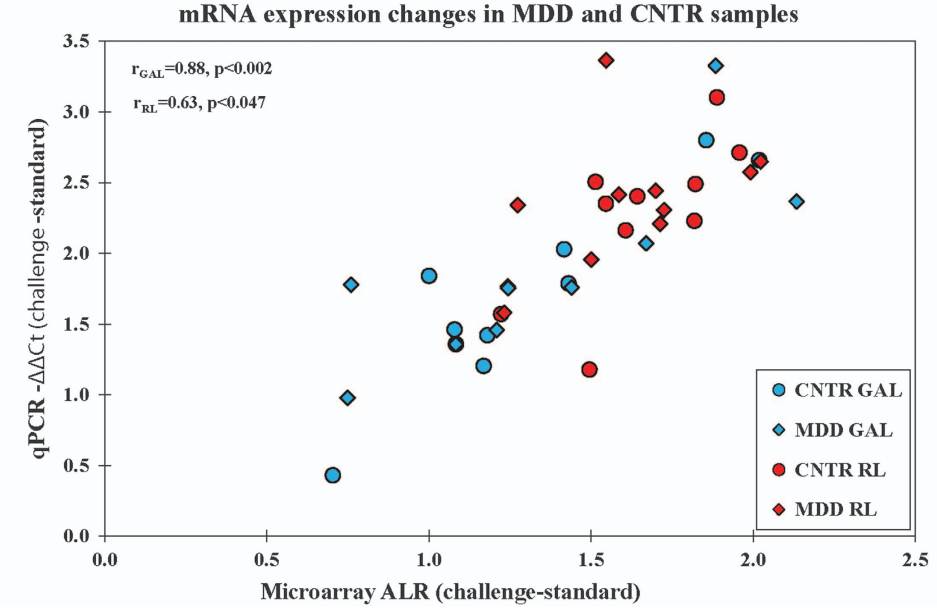

Figure 2. qPCR validation of the differential mRNA expression detected with microarrays. The differential expression of 10 mRNAs, detected with microarrays, was validated with custom qPCR arrays (groups: MDD, CNTR; culture conditions: STD, GAL, RL). ALR ($ALR_{GAL} = Mean_{GAL} - Mean_{STD}$, blue, $ALR_{RL} = Mean_{RL} - Mean_{STD}$, pink) was used as an estimate for the microarray expression changes and was plotted on the x axis. ΔΔCt ($\Delta\Delta Ct_{GAL} = \Delta Ct_{GAL} - \Delta Ct_{STD}$, blue; $\Delta\Delta Ct_{RL} = \Delta Ct_{RL} - \Delta Ct_{STD,}$ pink) was used for a qPCR expression changes estimate and was plotted as − ΔΔCt on the y axis. Note that the estimates from both analyses in each comparison were highly correlated. CNTR, control; GAL, galactose; MDD, major depressive disorder; mRNA, messenger RNA; qPCR, quantitative PCR; RL, reduced lipid; STD, standard.

regulation (PTC1, MPR, ACAP95, ARF), apoptosis (HSP27, SET, BAD), anti-inflammation (IL10) and cell survival (IL7). RL resulted in enrichment of 19 pathways in MDD and 15 in CNTR. Ten of the 19 gene sets (53%) were only enriched in MDD and are involved in cell repair (TFF), survival (TRKA), migration (ECM) and proliferation (CDMAC, SPRY), and also in regulation of metabolism (GH) and energy production (FEEDER, GLYCOLYSIS). Overall, GSEA revealed that the specific changes in MDD evoked by both metabolic stressors are represented by approximately half of all detected molecular pathways, indicating that MDD fibroblasts utilize different strategies for adaptation to the metabolic stressors. Moreover, challenging the MDD fibroblasts with GAL and RL exposed characteristic molecular signatures undetectable at STD conditions.

MDD fibroblast response to metabolic stress: miRNA changes

The miRNAs are important regulators of mRNA function and they are heavily involved in control of metabolism and energy homeostasis.[26] Therefore, we sought to identify the miRNA response to metabolic challenges in the same MDD fibroblasts in which differential mRNA expression patterns had been established.

The miRNA samples, isolated individually from the 16 MDD and 16 CNTR fibroblast cultures, were pooled into four sets according to the patients' age and gender (Supplementary Material 1). Human miRNome miScript miRNA PCR Arrays (Qiagen) were used to profile the expression of the 1008 most abundantly expressed miRNA species in the human miRNA genome (miRNome).

Table 1. BioCarta GSEA enrichment of mRNA expression in response to GAL and RL challenges

Pathway	*Size*	*NES*	P-*value*
Enriched in CNTR GAL			
RACCYCD	26	1.89	0.0076
CELLCYCLE	23	1.81	0.0000
TEL	18	1.74	0.0040
SRCRPTP	11	1.72	0.0000
P53	16	1.66	0.0078
CERAMIDE	22	1.65	0.0115
CFTR	12	1.62	0.0000
CHREBP2	42	1.61	0.0275
G2	24	1.61	0.0199
ATRBRCA	21	1.58	0.0159
CARM_ER	34	1.58	0.0373
STATHMIN	19	1.56	0.0233
ATM	20	1.55	0.0492
CHEMICAL	22	1.53	0.0497
RB	13	1.53	0.0346
AKAPCENTROSOME	15	1.52	0.0471
PLCE	12	1.51	0.0299
Enriched in CNTR RL			
PPARA	56	1.83	0.0000
RACCYCD	26	1.71	0.0229
CHREBP2	42	1.65	0.0136
CD40	15	1.59	0.0301
ARF	17	1.57	0.0359
VIP	26	1.57	0.0169
P38MAPK	39	1.53	0.0076
HSP27	15	1.50	0.0389
BAD	26	1.50	0.0217
GCR	19	1.47	0.0360
LYM	11	1.43	0.0368
PLCE	12	1.43	0.0443
IL22BP	16	−1.53	0.0300
DNAFRAGMENT	10	−1.54	0.0237
AHSP	11	−1.56	0.0412
Enriched in MDD GAL			
TEL	18	1.98	0.0000
SRCRPTP	11	1.73	0.0020
PTC1	11	1.73	0.0000
G2	24	1.66	0.0021
CARM_ER	34	1.66	0.0056
HSP27	15	1.65	0.0116
RB	13	1.62	0.0062
CERAMIDE	22	1.60	0.0212
P53	16	1.58	0.0220
SET	11	1.58	0.0328
CHEMICAL	22	1.58	0.0243
ATRBRCA	21	1.57	0.0080
MPR	34	1.56	0.0413
CELLCYCLE	23	1.56	0.0305
AKAP95	12	1.56	0.0469
CHREBP2	42	1.56	0.0222
IL10	17	1.53	0.0320
ARF	17	1.50	0.0382
PLCE	12	1.47	0.0301
IL7	17	1.46	0.0258
BAD	26	1.43	0.0138
Enriched in MDD RL			
RACCYCD	26	1.90	0.0040
TFF	21	1.88	0.0064
CD40	15	1.78	0.0102
TRKA	12	1.74	0.0084
ECM	24	1.67	0.0234
PPARA	56	1.63	0.0066
HSP27	15	1.62	0.0176
GH	28	1.60	0.0204
GLEEVEC	23	1.58	0.0361
ARF	17	1.54	0.0463
EXTRINSIC	13	1.53	0.0140
CHREBP2	42	1.53	0.0246
CDMAC	16	1.52	0.0355
BAD	26	1.48	0.0118
SPRY	18	1.45	0.0417
FEEDER	9	−1.53	0.0236
DNAFRAGMENT	10	−1.54	0.0488
AHSP	11	−1.56	0.0354
GLYCOLYSIS	10	−1.63	0.0040

Abbreviations: CNTR, control; GAL, galactose; GSEA, gene set enrichment analysis; MDD, major depressive disorder; mRNA, messenger RNA; RL, reduced lipid. Molecular pathway enrichment of mRNA in MDD and CNTR. Pathways enriched only in one group (MDD or CNTR) are in bold.

Approximately 50% of the assayed miRNAs were detected in human cultured dermal fibroblasts, regardless of disease status or culture condition. Significant expression differences in metabolic stress vs STD conditions were defined when change was larger than 30% ($|\Delta\Delta Ct| > 0.3785$) and paired *t*-test $P \leqslant 0.05$. In response to GAL challenge, MDD fibroblasts revealed expression changes in 63 miRNAs (Supplementary Material 5A). The number of miRNAs changed by GAL challenge in CNTR samples was substantially lower, 38 miRNAs. In addition, 81% of the miRNAs changed in GAL were specific for MDD fibroblasts.

RL elicited much stronger miRNA response in MDD (147 miRNAs), compared with CNTR (38 miRNA) (Supplementary Material 5B), leading us to believe that MDD fibroblasts are experiencing the RL environment as much more stressful than CNTRs. Furthermore, 90% of the miRNA RL-response pattern was observed only in MDD, but not in CNTR.

Validation of miRNome analyses on pooled samples was performed with custom qPCR array of 14 miRNAs (SABiosciences, Qiagen) on individual samples. The ΔΔCt ($\Delta\Delta Ct_{GAL} = \Delta Ct_{GAL} - \Delta Ct_{STD}$, $\Delta\Delta Ct_{RL} = \Delta Ct_{RL} - \Delta Ct_{STD}$) from the pooled samples for each miRNA was correlated with the corresponding ΔΔCt from the individual samples (Figure 3). Both GAL- and RL-induced miRNA response demonstrated high correlation between pooled and individual samples with coefficients of $r_{GAL} = 0.920$ and $r_{RL} = 0.785$. In addition, 85% of the miRNA changes in the individual samples were significantly different in the predicted direction ($P \leqslant 0.05$), providing a strong validation for the miRNome data.

In addition, in an attempt to identify the miRNA changes that characterize the specific response of MDD to each metabolic challenge, we used a group × challenge linear statistical model and the dual significance criterion of $|\Delta\Delta\Delta Ct| > 0.3785$ and $P \leqslant 0.05$. Thus, we derived two miRNA signatures: (1) representing MDD × GAL interaction (16 miRNAs) and (2) representing MDD × RL interaction (36 miRNAs, Figure 4). It is noteworthy that miR-7, miR-382, miR-296-5p and miR-3176 were common for both signatures.

Literature search in PubMed for the roles of the MDD × GAL and MDD × RL signature miRNAs revealed an interesting trend toward extensive miRNA involvement in the regulation of metabolism, cell proliferation, survival and migration (Table 2). In relation to control of metabolism, we found miRNAs regulating insulin levels and secretion, obesity and starvation in both MDD × GAL (38%) and MDD × RL (37%) panels. In addition, eight miRNAs from the MDD × RL panel: miR-3613-3p, miR-33a, miR-192, miR-26a, miR--34a, miR-370, miR-15b and miR-296-5p, have been described in the literature as regulators of lipid metabolism. With regard to cell proliferation and apoptosis, 56% of the MDD × GAL and 61% of the MDD × RL miRNAs are known controllers of various signaling

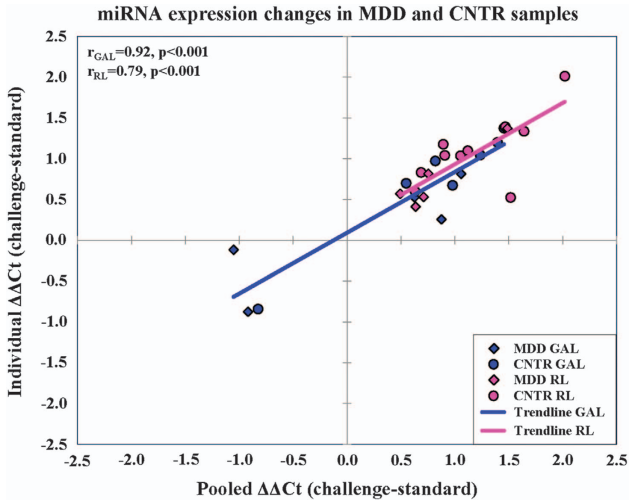

Figure 3. Differential miRNA expression in pooled samples is validated in individual samples. The expression level of 14 miRNAs, detected in miRNome analyses of pooled samples, was validated with independent qPCR arrays of individual samples (groups: MDD, CNTR; culture conditions: STD, GAL, RL). The expression changes, estimated with ΔΔCt ($\Delta\Delta Ct_{GAL} = \Delta Ct_{GAL} - \Delta Ct_{STD}$, blue; $\Delta\Delta Ct_{RL} = \Delta Ct_{RL} - \Delta Ct_{STD}$, pink), from the pooled samples are plotted on the x axis, and from the individual samples on the y axis. Note that the values from each comparison were highly correlated. CNTR, control; GAL, galactose; MDD, major depressive disorder; miRNA, microRNA; qPCR, quantitative PCR; RL, reduced lipid; STD, standard.

cascades, out of which 13% (GAL) and 33% (RL) affect or are affected by the tumor suppressor protein p53. As p53 is not only a modulator of apoptosis, but is also involved in regulation of metabolism,[27] some overlap between these categories is expected. Furthermore, 25% (MDD × GAL) and 39% (MDD × RL) of the signature miRNAs are known to regulate cell motility. Interestingly, three of them: miR-34a, miR-34b and miR-199a-3p, are recognized as regulators of the hepatocyte growth factor receptor, c-Met,[28,29] a key controller of cell migration.

DISCUSSION

Our results demonstrate that the metabolic challenges evoked by substitution of glucose with GAL or reducing the abundance of lipids in the growth media of fibroblast cultures result in robust changes of mRNA and miRNA expression. MDD fibroblasts responded to the metabolic stress with alternate expression of 1196 (GAL) and 312 (RL) transcripts. A substantial fraction of these mRNA changes, 26% (GAL) and 33% (RL) were only observed in MDD, but not in CNTR. The specific response of MDD fibroblasts to each challenge was represented by 20 (MDD × GAL) and 28 (MDD × RL) mRNAs with significant group by challenge interaction. The characteristic response of MDD fibroblasts to the metabolic stressors was even more pronounced on the miRNA level; 81% (GAL) and 90% (RL) of the total miRNA changes were only observed in MDD, but not in CNTR. The miRNA signature response of MDD fibroblasts was defined by 16 (MDD × GAL) and 36 (MDD × RL) miRNAs. The stress-induced changes, observed on both mRNA and miRNA level, revealed MDD-associated impairments in molecular pathways involved in the control of metabolism and energy production, cell survival, proliferation and migration. These impairments are likely to be present in all the somatic cells, and therefore are informative of the disease process. In fact, the results are consistent with our previous findings in human postmortem brain tissue, as both studies implicated disturbances in apoptotic molecular pathways.[30] Impairments in any of the detected molecular pathways could be critical for the development and plasticity of the brain and can result in the disease state of MDD.[31] In a previous study, MDD fibroblasts cultured in STD condition revealed deficits in cell proliferation and cell motility.[32] In this study, we demonstrated that molecular pathways involved in the same processes were also impaired in response to metabolic challenges with GAL and RL, and revealed previously unknown disruption of pathways regulating metabolism and energy production. Presenting metabolic challenges was instrumental for defining distinct MDD mRNA and miRNA response signatures that might also contribute to patients' aberrant responses to life stressors.

GAL and RL treatments elicited common responses in MDD fibroblasts. The commonality was demonstrated on both mRNA and miRNA level. On mRNA level, the overlap between MDD × GAL and MDD × RL signatures was defined by the integrator complex subunit 4 (INTS4), and the NEDD4 binding protein 2-like 1 (N4BP2L1). The INTS4 associates with RNA polymerase II, and therefore potentially has a general effect on regulation of gene transcription and RNA processing.[33] The N4BP2L1 is a paralog of N4BP2 and is likely involved in cell survival.[34] Evidence for MDD impairment in the regulation of cell survival and proliferation streamed from the molecular pathway analyses as well. The HSP27, BAD and ARF molecular pathways were the commonly enriched pathways in MDD during GAL and RL challenges; all of which affect cell survival and proliferation. Furthermore, 38% (GAL) and 17% (RL) of the MDD-enriched gene sets (PTC1, MPR, ACAP95, ARF, HSP27, SET, BAD, IL7, TRKA, CDMAC, SPRY), contribute to the regulation of cell survival and proliferation. On the miRNA level, three of the commonly changed miRNAs are regulators of cell proliferation and apoptosis.[35–37] In addition, a striking 56–61% of the miRNAs from the MDD × GAL and MDD × RL signatures have roles in cell cycle and survival regulation. Overall, these data provide an overwhelming support for the aberrant engagement of mechanisms regulating cell proliferation and survival by MDD fibroblasts when exposed to two distinct metabolic challenges. Commonality between GAL- and RL-induced responses in MDD was also detected in their respective miRNA signatures, where at least 38% (GAL) and 37% (RL) of the miRNAs were regulators of metabolism or energy homeostasis, indicating deficiencies in those signaling cascades in MDD as well. In summary, the challenges presented by GAL and RL in MDD fibroblasts affected cellular processes, such as control of cell proliferation and survival, regulation of metabolism and energy production. The molecular machinery for regulation of these processes is located mainly in the mitochondria. Therefore, our data strongly support the disruption of mitochondrial functions in patients with MDD, as previously suggested.[10]

The GAL and RL treatment also evoked different responses in MDD fibroblasts. The number of mRNAs with changed expression in GAL was four times the number of mRNAs changed in RL. In contrast, the mRNA signatures characterizing the specific response of MDD to each stress, have similar number of mRNAs (MDD × GAL = 28, MDD × RL = 20). Therefore, the proportion of the specific mRNAs from the total mRNA changes (GAL = 1196, RL = 360) is in favor of RL (5.6 vs 2.3%). Consequently, we concluded that RL vs GAL environment more strongly illuminates the molecular deficits of MDD. Furthermore, additional data, obtained from RL treatment, reveal abnormalities in MDD, which were not apparent in GAL. These are the eight miRNAs in the MDD × RL miRNA signature known to control lipid metabolism, pointing toward the possibility of fatty acid metabolism dysregulation in patients with MDD, a phenomenon studied in many different ways during the last couple of decades.[38–42] Another interesting fact revealed only in RL environment is the presence of six hypoxamirs in the MDD × RL miRNA signature. Hypoxamirs have been identified as miRNAs contributing to mitochondrial respiration arrest in a low oxygen environment.[43] In our study, CNTR fibroblasts upregulated six hypoxamirs (let-7b*, miR-192, miR-26a, miR-98, miR-23a and

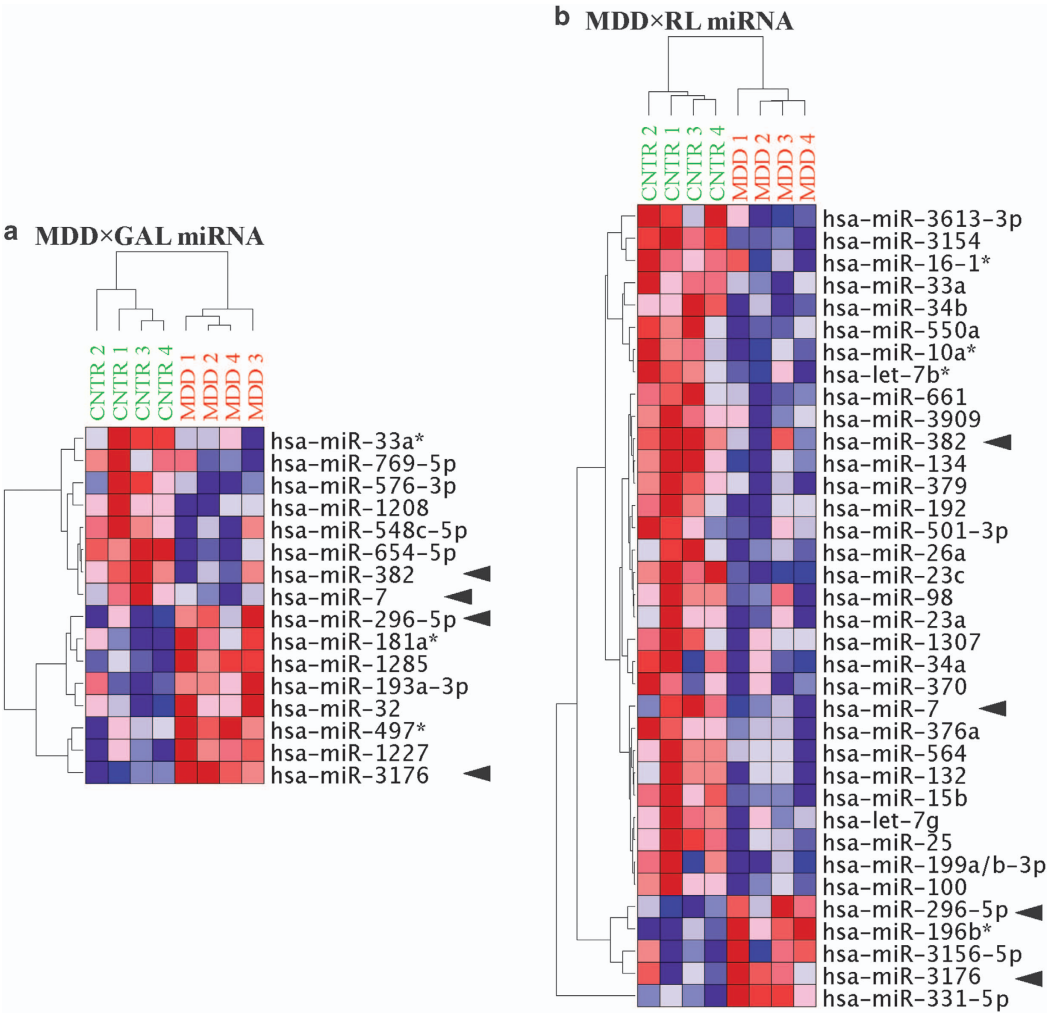

Figure 4. Disease × stressor interaction miRNA signatures. (**a**) MDD × GAL and (**b**) MDD × RL. The ΔΔCts ($\Delta\Delta Ct_{GAL} = \Delta Ct_{GAL} - \Delta Ct_{STD}$; $\Delta\Delta Ct_{RL} = \Delta Ct_{RL} - \Delta Ct_{STD}$) of the miRNAs with significant disease × challenge interaction were subjected to unsupervised hierarchical clustering. The colored squares represent the increase (red) or decrease (blue) of each ΔΔCt from the mean. Increased ΔΔCt represents reduced level of miRNA in metabolic stress compared with STD conditions. Clear separation of MDD and CNTR groups was observed. Four miRNAs (that is, miR-7, miR-382, miR-296-5p and miR-3176), denoted by arrows, are commonly present in both signatures. CNTR, control; GAL, galactose; MDD, major depressive disorder; miRNA, microRNA; RL, reduced lipid; STD, standard.

miR-7)[44] in response to RL, whereas MDD fibroblasts down-regulated them. This particular difference in the MDD response compared with CNTR further supports the notion of mitochondrial dysfunction in MDD. The RL challenge was also very informative in uncovering abnormalities in the process of cell migration; 39% of the miRNAs in the MDD × RL miRNA signature were regulators of metastasis. Also, MDD responded to RL with enrichment of ECM (extracellular matrix) pathway and TFF (Trefoil factors initiate mucosal healing) pathway; both important players in the processes of cell migration. The deficit of MDD in cell migration was not as apparent during adaptation to GAL, but was observed in a previous study in STD conditions.[32]

An important question is whether the changes we detect in peripheral tissues are relevant to a disease process that takes place primarily in the brain. Genetic factors associated with MDD are likely to be present throughout the entire body and not just be specific to the brain. The adaptive deficits detected in fibroblast cell lines can be linked back to the function of neurons and the brain, with multiple examples found in the mRNA and miRNA challenge-response signatures and in the altered molecular pathways. On mRNA level, such examples are presented by genes important for neurite outgrowth, neurodevelopment and synaptic plasticity: Actin-related protein 2/3 complex, subunit 5 (ARPC5),[45] ArfGAP with coiled-coil, ankyrin repeat and PH domains 2 (ACAP2)[46] and Beta-1,3-glucuronyltransferase 2 (glucuronosyltransferase S, B3GAT2).[47] Multiple important roles in the nervous system are associated with miRNA challenge-response signatures as well. Generally, 31% (GAL) and 47% (RL) of the miRNAs from the miRNA signatures are either regulators of molecular events in the brain or biological markers associated with psychiatric disorders in the periphery. In particular, we identified miRNAs with roles in neurite outgrowth and synapse formation: miR-7,[48] miR-26a,[49] miR-34a,[50] miR-134 (ref. 51) and miR-132;[52,53] cell cycle progression, apoptosis and specification of neurons: miR-25,[54] miR-34a,[55] miR-497 (ref. 56) and miR-376a;[57] brain morphogenesis: miR-7,[58] miR-370 (ref. 59) and myelination in the central nervous system: miR-23a.[60] Furthermore, our study identified miRNAs implicated in the pathological development of schizophrenia, such as miR-132 (ref. 61) and miR-382;[62] and of Alzheimer's, such as miR-98.[63] In addition, several of the miRNAs with aberrant challenge-response in MDD have been described as potential peripheral biomarkers for psychiatric or neurological diseases: miR-564 (ref. 64) and miR-132 for schizophrenia;[65] hsa-let-7g and miR-15b for Alzheimer's;[66] and miR-1285, miR-34a and miR-34b for Huntington's

Table 2. Experimentally established involvement of the miRNAs from MDD × GAL and MDD × RL signatures in cellular processes (on the basis of PubMed search)

Physiological process	*miRNA_ID*
MDD × GAL miRNA	
Metabolism	miR-33a*, miR-7, miR-296-5p, miR-181a*, miR-497*
Fatty acid metabolism	miR-296-5p, miR-193a-3p ×
P53	miR-1208, miR-1285, miR-7
Apoptosis/proliferation	miR-548c-5p, miR-7, miR-382, miR-296-5p, miR-181*, miR-193a-3p, miR-1285, miR-32, miR-497*
Migration/c-MET	miR-548c-5p, miR-7, miR-296-5p, miR-497*
Neurons/psychiatry	miR-7, miR-382, miR-296-5p, miR-32, miR-497*
MDD × RL miRNA	
Metabolism	miR-33a, miR-7, miR-661, miR-1307, miR-376a, miR-26a, miR-132, miR-25, miR-98, miR-34a, miR-23a, miR-199a/b-3p, miR-296-5p
Fatty acid metabolism	miR-3613-3p, miR-33a, miR-192, miR-26a, miR-34a, miR-370, miR-15b, miR-296-5p
P53	miR-34b, miR-16-1*, miR-661, miR-192, miR-7, miR-134, miR-25, miR-98, miR-34a, miR-370, miR-15b, miR-199a/b-3p
Apoptosis/proliferation	miR-33a, miR-34b, miR-16-1*, miR-661, miR-192, miR-7, miR-376a, miR-382, miR-26a, miR-134, miR-379, let-7g, miR-132, miR-25, miR-98, miR-34a, miR-370, miR-15b, miR-23a, miR-199a/b-3p, miR-100, miR-296-5p
Migration/c-MET	miR-34b, miR-661, miR-550a, miR-7, miR-376a, miR-26a, let-7g, miR-134, miR-132, miR-25,miR-98, miR-34a, miR-100, miR-296-5p
Neurons/psychiatry	miR-34b, miR-7, miR-376a, miR-382, miR-26a, miR-134, miR-564, let-7g, miR-132, miR-25, miR-98, miR-34a, miR-370, miR-15b, miR-23a, miR-199a/b-3p, miR-296-5p

Abbreviations: GAL, galactose; MDD, major depressive disorder; miRNA, microRNA; RL, reduced lipid. Metabolism/fatty acid metabolism.[36,63,74–94] Apoptosis/proliferation/p53.[37,49,54,56,58,95–118] Migration.[29,54,98,99,119–131] Neurons/psychiatry.[48–51,53,54,56–58,60,61,63–66,68–71,98,132–134]

disease.[67] This study also identified a number of miRNAs that respond to different stressors in the nervous system: miR-34b, which affects the degree of oxidative stress and survival of dopaminergic neurons;[68] miR-296-5p, which responds to oxidative stress in mouse hippocampal neuronal cultures;[69] miR-199a, which reacts to hypoxia in rat cortical pericytes;[70] and miR-98, which is elevated in the brains of newborn rats experiencing prenatal stress.[71] And last, miR-296-5p, which showed aberrant response to both RL and GAL in MDD fibroblasts, presents a strong connection to MDD; miR-296-5p has been identified as a regulator of inducible I kappa-B kinase,[72] a gene with genetic association with MDD.[73]

Taken together, this information gives confidence to the relevance of our observations in fibroblasts to the MDD disease state. This study provides a solid foundation for future experiments with human fibroblasts aimed at deciphering the systemic immune disturbances in MDD. Moreover, our results demonstrate that aberrant responses to stressors are indeed present in the peripheral tissues from MDD patients. These responses intersect multiple cellular processes associated with mitochondria, such as regulation of metabolism and energy production, cell proliferation, survival and motility; therefore, they strongly suggest that the stress-response impairments of MDD patients are most likely based on impaired mitochondrial functions.

CONFLICT OF INTEREST

RCS receives unrelated research support from Assurex Health; Bristol-Myers Squibb; Eli Lilly and Company; Elan Corporation; Euthymics Bioscience; Forest Pharmaceuticals; Janssen Pharmaceutica; Jazz Pharmaceuticals; Naurex; Novartis Pharmaceuticals; Otsuka Pharmaceuticals; Pamlab; Pfizer; Repligen Corporation; Ridge Diagnostics; St. Jude Medical; and Takeda Pharmaceuticals and consults for Bristol-Myers Squibb Company; Cerecor; Cyberonics; Eli Lilly and Company; Forest Pharmaceuticals; Janssen Pharmaceutica; Medtronic; Naurex; Pamlab; Pfizer; Ridge Diagnostics; Shire Plc; and Takeda Pharmaceuticals. The remaining authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)