INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs) have been established as the first-line treatment for major depression since their introduction in the early 1980s.1–3 These drugs are known to bind with high affinity and inhibit the activity of the presynaptic serotonin transporter (SERT), the specific SSRIs drug target. The biogenic amine hypothesis of depression, albeit controversial, argues that SSRI drugs relieve depression primarily via their SERT blocking action that in turn increases the central nervous system serotonin availability, in particular in the thalamo–prefrontal cortex pathway.4,5 However, a key unresolved issue is that, although maximal SERT inhibition is achieved within few days of starting SSRI drug medication, it takes around 4 weeks for the onset of remission of depression.1–3 Recovery from depression was proposed to require synaptogenesis as well as neurogenesis5–9 combined with migration of hippocampal neuronal stem cells,7,10–12 which may account for this long delay. However, the mechanisms by which SSRIs-mediated SERT blockade leads to the brain synaptogenesis/neurogenesis or neuronal rewiring remain enigmatic. Animal studies have shown downregulation of the brain SERT expression by chronic SSRI medication,13–15 however, human Positron emission tomography imaging studies yielded ambiguous findings.16–18

We set out to study this question by using the unbiased genome-wide approach, rather than examining changes in expression levels of presumed relevant genes. We chose to study human cells for the genome-wide search, rather than using animal models for depression, which have some limitations.19,20 Moreover, we compared human cell lines from four unrelated donors for improved power: we profiled the expression of genes and microRNAs (miRNAs) of human lymphoblastoid cell lines (LCLs) chronically exposed to 1 μM paroxetine. LCLs have been instrumental in pharmacogenomic discovery.21–23 We recently utilized LCLs from unrelated healthy individuals for conducting a genome-wide transcriptomic microarray based search for SSRI sensitivity biomarkers and reported several genes as tentative SSRI response biomarkers.24,25 Among these, CHL1, coding for a cell adhesion protein implicated in neurogenesis and synaptogenesis,26–28 seems a promising biomarker, as it is essential for correct neuronal wiring from the thalamus to the prefrontal cortex,29 a neuronal pathway crucial for mood control. Moreover, CHL1 was reported as a potential SSRI sensitivity biomarker in a
large study in major depression patients. The identification of CHL1 as a potential SSRI response biomarker was further supported by our genome-wide miRNA expression studies, where levels of miR-151-3p, which targets CHL1, were related to SSRI responsiveness of LCLs.

Here we describe our genome-wide transcriptomic findings from microarray expression profiling experiments in human LCLs chronically treated with the SSRI paroxetine. We report that the expression of ITGB3 (integrin beta-3) as well as miR-221 and miR-222, which target ITGB3, exhibited the most consistent expression level changes following chronic paroxetine exposure in LCLs representing four unrelated male individuals. Our genome-wide expression profiling observations highlight the importance of neurogenesis and synaptogenesis in the mode of action of SSRI antidepressant drugs and further support a key role for CHL1 in determining SSRI sensitivity. These findings further point to a key role of cell adhesion proteins such as CHL1 and ITGB3 in remission from depression.

MATERIALS AND METHODS
Human LCLs and chronic paroxetine treatment
Human LCLs were obtained from the National Laboratory for the Genetics of Israeli Populations (NLGIP) at Tel-Aviv University as described. The cell lines were immortalized from the peripheral blood lymphocytes of healthy adult male donors of Ashkenazi Jewish ancestry. Four NLGIP cell lines were used, coded 1126, 1131, 1235 and 1371. Cells were maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum and antibiotics (100 U ml⁻¹ penicillin; 100 µg ml⁻¹ streptomycin) and kept at a temperature of 37 °C, with 6% CO₂ and 100% humidity. Paroxetine was purchased from Sigma-Aldrich (St Louis, MO, USA) and solutions were prepared in phosphate-buffered saline. For chronic treatment, cell lines in logarithmic growth were exposed to 1 µM paroxetine for 21 days. Fresh paroxetine (from a 1000-fold stock solution) was added on each feeding the cell cultures according to added medium volume (every 2 to 3 days). Control cultures (grown in parallel) received similar volume of phosphate-buffered saline on each feeding.

RNA extraction
Total RNA purification was achieved using phenol-chloroform extraction; cells were centrifuged and then lysed using Tri-reagent (T9424, Sigma-Aldrich). Following RNA extraction, Affymetrix GeneChip Human Gene 1.0 ST arrays or Affymetrix GeneChip miRNA 2.0 arrays were used for detecting the expression levels of genes and miRNAs, respectively, according to the manufacturer’s protocol (see Methods). Data were analyzed by Partek Genomics Suite and normalized as described in Materials and methods. Tables 1 and 2 present genes and miRNAs, respectively, whose expression levels showed P < 0.001 in preparations from LCL cultures chronically exposed to paroxetine compared with parallel controls. As shown, ITGB3 (coding for ITGB3; also known as platelet glycoprotein IIb and CD61) exhibited the most statistically significant change in expression levels following 21 days paroxetine exposure, (1.92-fold increased expression; P = 7.50 × 10⁻⁸) for the four LCLs. Four genes (MAL, HECW2, ITGB3 and KLHL24) and two miRNAs (miR-221 and miR-222) from Tables 1 and 2 were selected for validation with real-time PCR in each of the four individual LCLs (Figures 1 and 2), confirming the effects of paroxetine on their expression levels. The choice of genes and miRNAs was made as they are known to be expressed in the brain tissues and implicated in neurogenesis. As shown in Figures 1 and 2, the altered expression levels of these selected genes and miRNAs following chronic paroxetine exposure were closely similar in the LCLs of four unrelated donors.

DISCUSSION
Transcriptomic changes following chronic paroxetine exposure
The expression levels of 14 genes were changed by > 1.5-fold and P < 0.001 following chronic paroxetine exposure in the four tested human LCLs from unrelated male donors (Table 1). Among them ITGB3 exhibited the most statistically significant change; its expression increased on average by 1.92-fold (P = 7.50 × 10⁻⁸) as confirmed by real-time PCR (Table 1, Figure 1). This observation is unexpected, as none of these 14 genes are related to established serotonin signaling or metabolism. ITGB3 has neither been previously implicated in the etiology of major depression nor in the mode of action of SSRI antidepressant drugs. Of note, this identification is supported by our genome-wide array based identification of decreased expression levels of two miRNAs, miR-221 and miR-222, both predicted to target ITGB3, and as confirmed by real-time PCR, using the same LCLs and experimental protocol (Table 2, Figure 2). The targeting of ITGB3 by both miR-221 and miR-222 is predicted by each of the following software: TargetScan.
Moreover, paroxetine was employed in our recently published LCLs studies on tentative SSRI response biomarkers, so that combining our previous and current studies using the same drug allows us to integrate the data and to propose a new model for SSRI antidepressants mode of action (below).

The identification of elevated ITGB3 expression following chronic paroxetine exposure is intriguing. Integrins, including ITGB3, are implicated in cell adhesion and connectivity and are essential for synaptogenesis. For example, ITGB3 regulates excitatory synaptic strength and hippocampal AMPA receptors expression. Remission from depression is presumed to be dependent upon establishing new neuronal connections, in particular in the prefrontal cortex.

MAL (T-cell differentiation protein), whose expression was also increased by chronic paroxetine in the same LCLs (2.316-fold; Table 1, Figure 1) is implicated in myelin formation. Notably, its expression was reduced in post-mortem temporal cortex of major depression patients. Other genes listed in Table 1 were not implicated in major depression to our knowledge. Notably, TSPAN12 (tetraspanin 12; Table 1) was implicated in cleavage of the amyloid precursor protein and its deletion was reported in autism, a disorder associated with incorrect brain circuitry. In addition, DTX1 (deltex homolog 1; Table 1) was implicated in neurogenesis and in gliogenic specification of mouse mesencephalic neural crest cells. Lastly, PLOD2 (procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2; Table 1) was detected in a genome-wide search for major genes implicated in axonal regeneration. Together, our genome-wide expression profiling observations in paroxetine-treated LCLs point to SSRI-mediated upregulation of genes implicated in synaptogenesis/neurogenesis, supporting the concept that SSRI drugs relieve depression by promoting these processes.

Among the miRNAs identified by our microarray based genome-wide transcriptomic profiling study miR-221 and miR-222 exhibited 1.525- and 1.416-fold lower expression, respectively, following chronic paroxetine exposure of the same LCLs (Table 2). These findings were confirmed by real-time PCR (Figure 2). As both these miRNAs are predicted to target ITGB3 with conserved binding sites (Supplementary Table S1), the observations on their reduced expression complement the observed higher expression levels of ITGB3 following chronic paroxetine. Of note, a genome-wide miRNA microarray study found that miR-221 was downregulated in the hippocampi of rats chronically treated with lithium, a mood-stabilizer drug also employed for augmenting antidepressant therapy.

Strengths and constraints

An obvious strength of our study is the hypothesis-free nature of genome-wide expression studies. Moreover, we examined gene and miRNA expression in LCLs from four unrelated male donors, so that confounders caused by the presence of unique polymorphic DNA sequence alleles or epigenomic modifications are unlikely to have contributed to our observations. Nevertheless, care is called for when interpreting genome-wide data and our observations should thus be considered as tentative until independently confirmed. Our study has several additional limitations as discussed below.

First, we utilized LCLs from healthy unrelated donors for studying the transcriptional effects of chronic paroxetine exposure. Although these immortalized cell lines capture the natural variation of the human genome and epigenome, one has to keep in mind that transcriptional drug effects may differ in the brain compared with the blood-born cells. In particular, compared with neurons, LCLs exhibit low methylation signatures so that epigenomic effects on transcription may not faithfully represent the situation in neurons. Yet, Zhang et al. have proposed that, although the methylene and the transcriptome are modified during the establishment of LCLs, the cells maintain a relatively

Table 1. Genes whose expression was affected by chronic paroxetine exposure of LCLs

| Gene    | Fold-change | P value |
|---------|-------------|---------|
| ITGB3   | 1.925       | 7.50 × 10⁻⁶ |
| HECD2   | 1.812       | 4.69 × 10⁻⁶ |
| OVOS    | –1.517      | 7.31 × 10⁻⁶ |
| CD109   | 1.586       | 1.21 × 10⁻⁵ |
| RNF144A | 1.590       | 4.81 × 10⁻⁵ |
| MAL     | 2.316       | 5.55 × 10⁻⁵ |
| KLHL24  | 1.50        | 7.89 × 10⁻⁵ |
| TSPAN12 | 1.54        | 1.77 × 10⁻⁷ |
| CCL28   | 1.51        | 2.21 × 10⁻⁷ |
| CD180   | –1.51       | 2.52 × 10⁻⁴ |
| PLOD2   | 1.71        | 5.52 × 10⁻⁴ |
| CD244   | 1.87        | 8.77 × 10⁻⁴ |
| DTX1    | 1.50        | 9.36 × 10⁻⁴ |
| DNNSE1L3| 1.96        | 9.44 × 10⁻⁴ |

Abbreviation: LCLs, lymphoblastoid cell lines. The 14 listed genes exhibited >1.5-fold difference and P < 0.001 in expression levels in four LCLs following treatment for 21 days with 1 μM paroxetine. *Fold-change represents expression levels following paroxetine exposure compared with controls grown and studied by microarrays in parallel. As shown, half of these top miRNAs were upregulated by chronic paroxetine exposure. Expression levels were determined with whole-genome expression microarrays and genes are arranged by increasing P-values. The expression differences for four selected genes (in bold font) were confirmed by real-time PCR experiments (Figure 1). Note: two transcripts with no assigned genes (LOC100289612; C1orf186) were excluded.

Table 2. MicroRNAs whose expression was affected by chronic paroxetine exposure of LCLs

| MiRNA | Fold-change | P value |
|-------|-------------|---------|
| miR-3195 | 1.527       | 8.90 × 10⁻⁸ |
| miR-1246 | 2.406       | 7.01 × 10⁻⁶ |
| miR-221 | –1.525      | 7.22 × 10⁻⁶ |
| miR-1290 | 4.001       | 1.42 × 10⁻⁹ |
| miR-1263 | –1.411      | 8.36 × 10⁻⁵ |
| miR-550-star | –1.428    | 8.63 × 10⁻⁵ |
| miR-29c-star | 1.640      | 2.68 × 10⁻⁴ |
| miR-3178 | 1.440       | 3.95 × 10⁻⁴ |
| miR-222 | –1.416      | 5.07 × 10⁻⁴ |
| miR-664 | –1.615      | 5.19 × 10⁻⁴ |

Abbreviations: LCLs, lymphoblastoid cell lines; miRNA, micro RNA. The 10 listed miRNAs exhibited >1.4-fold difference and P < 0.001 in expression levels in four LCLs following treatment for 21 days with 1 μM paroxetine. *Fold-change represents expression levels following paroxetine exposure compared with controls grown and studied by microarrays in parallel. As shown, one half of these top miRNAs were upregulated by chronic paroxetine exposure. Expression levels were determined with whole-genome expression microarrays and miRNAs are arranged by increasing P-values. The expression differences for two selected miRNAs (in bold font) were confirmed by real-time PCR experiments (Figure 2). According to PubMed search (27 May 2013) the only miRNAs in this list reportedly were confirmed by real-time PCR experiments based on preliminary real-time experiments (details about these miRNA target prediction tools).

Diana, TargetRank and PITA (Supplementary Table S1; see ref. 25 for details about these miRNA target prediction tools). We chose the period of 21 days for the genome-wide microarray experiments based on preliminary real-time experiments (not shown) and as this period reflects the onset of recovery from depressive symptoms in SSRI-mediated patients. Paroxetine was selected for these experiments as it has the highest affinity for SERT, the SSRI drug target, among approved SSRI drugs. This choice minimizes the probability for off-target drug effects.

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stable status during cell culturing. Furthermore, the alterations are not random events, so LCLs generated separately from the same donor will have more similar methylation and transcription patterns than those generated from different donors. Thus, the different transcriptomic profiles of LCLs may in part reflect different donors’ methylation status.

Second, we utilized a concentration of paroxetine (1 mM), which was about three- to fivefold higher than its therapeutic plasma concentrations in paroxetine-treated major depression patients. This concentration was chosen for the microarray experiments based on preliminary real-time PCR findings (not shown). Moreover, we did not test the stability of paroxetine with our experimental protocol and its concentrations might have been reduced by binding to albumin in our serum-containing medium (however, fresh paroxetine was added whenever feeding the cells, see Materials and Methods). Of note, LCLs do not express CYP2D6, the major phase-I drug metabolizing enzyme of paroxetine, hence the observed transcriptomic changes were unlikely mediated by a paroxetine metabolite.

Lastly, the statistical significance of the changes of genes and miRNAs expression levels is low for a genome-wide study, reflecting our small sample size (eight microarrays, two for each of four human LCLs). Indeed, only the expression of one gene, ITGB3, exhibited genome-wide significance. However, genome-wide expression profiling arrays are less likely to generate false hits compared with single nucleotide polymorphism arrays which typically contain nearly one million single nucleotide polymorphism probes. In addition, we selected four genes and two miRNAs for validation by real-time PCR experiments and found good agreement with our array data. Moreover, two of the miRNAs most notably affected by chronic paroxetine exposure, miR-221 and...
miR-222, showed decreased expression levels corresponding with the observed upregulation of their target gene ITGB3. These observations support the novelty of the increased expression of ITGB3 following chronic paroxetine (further examples for inverse correlations of miRNAs levels with their target genes are shown in Supplementary Table S1).

Considering these experimental design aspects, our observations should be viewed as tentative until validated by further studies on the transcriptomic effects of SSRI drugs. The implication of the genes and miRNAs reported here for the etiology and treatment of major depression should ideally be examined with the blood samples of large cohorts of major depression patients, both before and following several weeks of treatment with SSRI drugs. Comparing such transcriptomic changes between good and poor SSRI responders may contribute to the personalized treatment of major depression.

A hypothetical potential role for ITGB3 in mediating the action of SSRI antidepressants

We have previously reported that low basal expression levels of CHL1 (close homologue of L1), which codes for a cell adhesion protein implicated in neurogenesis and synaptogenesis, correlated with higher sensitivity of LCLs to growth inhibition by SSRI drugs.24 We subsequently reported that mir-151-3p, predicted to target CHL1, exhibited higher expression levels in the same LCLs,25 thereby lending support for a role for CHL1 levels in SSRI sensitivity. It was therefore surprising that the expression levels of either CHL1 or mir-151-3p were not modified by chronic paroxetine exposure in our current study. In addition, none of the genes whose expression levels were modified by chronic paroxetine exposure (Table 1) code for the SERT, serotonin receptors or serotonin metabolizing enzymes, all of which have received attention in the context of research on the mode of action of antidepressant drugs.

We postulate a tentative working hypothesis (Figure 3) for the involvement of ITGB3, whose expression was most consistently increased following chronic paroxetine, in the mode of action of SSRIs and its relation to the previously reported role of CHL1 expression levels in modulating SSRI sensitivity of LCLs.24 Our working hypothesis is built upon the observation that the integrin beta-3 subunit encoded by ITGB3 is required for the activity of the SERT, the drug target of SSRIs, as evident from the drastically reduced serotonin-uptake activity in ITGB3-knockout mice.45 Notably, these mice exhibit absence of preference for social novelty and increased grooming in novel environments, behaviors relevant for autism spectrum disorder.36 Neuroanatomical assessment of these mice indicated that many brain regions had significantly different relative volumes, including a smaller corpus callosum volume and bilateral decreases in the hippocampus, striatum and cerebellum, all relevant to autism.47 Together these findings suggest that ITGB3 is crucial for correct neuroanatomical development of the brain, and a property it shares with CHL1.29 Integrins also interact with CHL1 and are required for its cell migration, neurite outgrowth promoting action,48–50; albeit, the exact integrin subtype(s) implicated in CHL1-mediated neurite outgrowth await identification. We therefore postulate that CHL1 competes with SERT on a limited cell membrane reservoir of ITGB3. Low levels of CHL1 allow more ITGB3 to interact with SERT and support its action; thus, lower CHL1 expression correlate with higher SERT activity, hence higher SSRI responsiveness, manifested as more robust inhibition of LCLs growth by SSRIs in our previous studies.42,25 Along the lines of this model (Figure 3), cells exposed to chronic SSRI compensate for the blocked SERT by upregulating the expression of its co-activator ITGB3 (rather than the expression of SERT itself). Extrapolating our hypothesis from LCLs to the brain serotonergic neurons, the resulting higher ITGB3 levels that follow chronic SSRI treatment in turn allow more dynamic action of CHL1 for supporting synaptogenesis/neurogenesis. As such events are presumably crucial for successful remission from depression,6–12 our working hypothesis supports a role for ITGB3 in the treatment of depression. Moreover, our findings and model, suggestive of a tentative role for elevated expression of cell adhesion proteins for remission from depression, may in part explain the enigmatic slow onset of the antidepressant action of SSRI drugs. Elucidating the roles of ITGB3, CHL1 and additional cell adhesion proteins in the mode of action of SSRI antidepressants requires studies with animal models for depression and eventually clinical studies.

NOTE ADDED IN PROOF

A new study51 demonstrates that expression of ITGB3 is essential for serotonin transporter activity in mouse brain.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1. Gelenberg AJ. A review of the current guidelines for depression treatment. J Clin Psychiatry 2010; 71: e15.
2. Rayner L, Price A, Evans A, Valsraj K, Hotopf M, Higginson IJ. Antidepressants for the treatment of depression in palliative care: systematic review and meta-analysis. Palliat Med 2011; 25: 36–51.
