The neurotrophic hypothesis of depression suggests an association between effects on neuroplasticity and clinical response to antidepressant drug therapy. We studied individual variability in antidepressant drug effects on cell proliferation in lymphoblastoid cell lines (LCLs) from \( n = 25 \) therapy-resistant patients versus \( n = 25 \) first-line therapy responders from the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study. Furthermore, the variability in gene expression of genes associated with cell proliferation was analyzed for tentative candidate genes for prediction of individual LCL donor’s treatment response. Cell proliferation was quantified by EdU (5-ethyl-2′-deoxyuridine) assays after 21-day incubation of LCLs with fluoxetine (0.5 ng \( \mu \)g \(^{-1} \)) and citalopram (0.3 ng \( \mu \)g \(^{-1} \)) as developed and described earlier. Gene expression of a panel of candidate genes derived from genome-wide expression analyses of antidepressant effects on cell proliferation of LCLs from the Munich Antidepressant Response Signature (MARS) study was analyzed by real-time PCR. Significant differences in \textit{in vitro} cell proliferation effects were detected between the group of LCLs from first-line therapy responders and LCLs from treatment-resistant patients. Gene expression analysis of the candidate gene panel revealed and confirmed influence of the candidate genes ABCB1, FZD7 and WNT2B on antidepressant drug resistance. The potential of these genes as tentative biomarkers for antidepressant drug resistance was confirmed. \textit{In vitro} cell proliferation testing may serve as functional biomarker for individual neuroplasticity effects of antidepressants.

**INTRODUCTION**

Depressive disorders are among the leading causes of disability worldwide\(^1\) with a lifetime prevalence of more than 16%.\(^2\) Depression contributes to decreased quality of life including morbidity, loss of productivity and suicidal thoughts.\(^3\) The neurotrophic hypothesis of depression suggests a chronic hyperactivity of the hypothalamic–pituitary–adrenal axis leading to a lowered growth factor expression and trophic changes in the brain.\(^4\) The hippocampus, a cerebral structure involved in emotion processing and stress response seems to be the most affected area in depression-associated neurotrophic changes.\(^5\) A recent meta-analysis considering 8927 samples from 15 different magnetic resonance imaging studies confirmed significant lower hippocampal volumes in depressed patients compared with healthy controls.\(^6\) Antidepressant treatment has been shown to be associated with reversing hippocampal atrophy by the enhancement of neuronal proliferation and synaptic plasticity.\(^7\) Furthermore, treatment efficacy in patients with smaller hippocampus volume has been observed to be delayed over the time of 3–4 weeks,\(^8\) which is usually the earliest time for reliable evaluation of treatment efficacy.\(^9\) This phenomenon, together with the usually low (<30%) response rates to first-line antidepressant medications,\(^10,11\) point to a need to identify response or non-response biomarkers for the prediction of individual antidepressant treatment effects.

Here, we want to identify possible functional biomarkers on neuroplasticity effects of antidepressants for associations with treatment response and resistance in patient-derived lymphoblastoid cell lines (LCLs) from the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study. LCLs are emerging tools in the field of personalized medicine research to study individual drug effects \textit{ex vivo} (for example, reviewed in refs 12,13). In addition, recently identified tentative gene expression biomarkers for neuroplasticity in antidepressant drug response will be studied for confirmation in this independent cohort from the STAR*D study: In a genome-wide approach using patient-derived LCLs from the Munich Antidepressant Response Signature (MARS) study, we identified five potential gene expression biomarkers that have been associated with cell proliferative effects of antidepressants (\textit{ex vivo}) or with LCL donor’s clinical response/remission in antidepressant drug therapy: transcription factor 7-like 2 (TCF7L2), frizzled class receptor 7 (FZD7), wingless-type MMTV integration site family member 2B (WNT2B), p-glycoprotein (P-GP, ABCB1) and sulfotransferase 4A1 (SULT4A1).

**MATERIALS AND METHODS**

Cell lines and study population

Lymphoblastoid cell lines from the STAR*D project were purchased from the NIMH Center for Collaborative Genetic Studies, Rodgers repository (Bethesda, MD, USA). The STAR*D study (ClinicalTrials.gov Identifier: NCT00021528) is an open label, randomized, multicenter, controlled clinical study that aimed to study effective subsequent treatment strategies after a first unsuccessful antidepressant therapy.\(^14\) The study consisted of four treatment levels. After each level, responders were allocated to a 12-month follow-up period during which the patients were further monitored and treated with the beneficial treatment regimen. Patients who experienced non-response or intolerable side effects entered the subsequent treatment level.

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All patients were first-line treated with a citalopram monotherapy at the first level of this study. In level 2, they had to choose between adding another antidepressant (bupropion or buspirone) and switching to different medication (with random assignment to sertraline, bupropion or venlafaxine). In a similar way, level 3 consisted of either add-on (lithium or triiodothyronine) or switch (mirtazapine or nortriptyline). During the final level, all previous medications were taken off and patients were randomly assigned to one of two treatment strategies: monotherapy with tranylcypromine versus combination of venlafaxine and mirtazapine.

A total of 50 cell lines were obtained (Table 1), derived from \( n = 25 \) responders to the first level (citalopram) of treatment and from \( n = 25 \) treatment-resistant patients who did not show response after undergoing the whole treatment algorithm (level 4). All patients were patients of Caucasian origin, for first-line treatment (level 1) all have been treated with citalopram monotherapy in defined doses ranging from 5 to 40 mg. Depressive symptoms were rated by Quick Inventory of Depressive Symptomatology (QIDS)\(^1\) over the course of up to 14 weeks. The complete clinical data are listed in the Supplementary Information. LCLs were ordered to cover all previous medications were taken off and patients were indicated as \( \Delta \Delta CT \) values, gene expression fold changes were calculated by \( \Delta \Delta CT \) method using GAPDH as reference gene.\(^2,3\)

### Statistical analyses

To test for differences between antidepressant-treated and -untreated proliferation rates in the same cell lines, the paired \( t \)-test was used. Between the groups of responders and treatment-resistant patients, the proliferation rates were compared with Student’s \( t \)-tests. To measure the strength of the relationship between the proliferation rates of citalopram- and fluoxetine-incubated cells, Spearman’s correlation coefficient (\( \rho \)) was calculated. In dependence of the nature of the data types of the clinical covariates (gender, anxiety status, menopausal status), either parametric (Student’s \( t \)-tests, Spearman correlation) or nonparametric tests (Wilcoxon–Mann–Whitney rank-sum test, Spearman’s rank correlation) were used when analyzed with respect to the proliferation rates and gene expression data. As expression levels between various treatment conditions and cell lines were largely different, nonparametric tests (Wilcoxon–Mann–Whitney rank-sum test) were given preference to check for statistically significant group differences concerning fold changes after the in vitro treatment with antidepressants. The statistical power amounts to 93.4% for EdU phenotyping experiments and to 99.9% for RT-PCR validation experiments with effect sizes of \( r = 2 \) and significant levels of \( \alpha = 0.05 \) each. All the \( P \)-values are reported as nominal \( P \)-values and are unadjusted for multiple testing. The statistical analyses were carried out using IBM SPSS Statistics 21 (Ehningen, Germany).

### RESULTS

Phenotyping of LCL proliferation via EdU-incorporation assays

After incubation of the LCLs with therapeutic concentrations of citalopram (0.3 ng \( \mu \)l\(^{-1}\)) or fluoxetine (0.5 ng \( \mu \)l\(^{-1}\)) for 3 weeks, EdU-based proliferation phenotyping experiments revealed strong interindividual differences between single cell lines (Figure 1). The relative LCL proliferation rates ranged from 0.0 to 428.4%. Averaged over all 50 cell lines, the overall proliferative effects were reported compared with MOCK-treated controls (set to 100%): fluoxetine mean 130.34% ± 56.32 (\( P = 0.006 \)) and citalopram mean 127.59% ± 61.00 (\( P = 0.026 \)). A significant correlation between the fluoxetine- and citalopram-mediated (both selective serotonin reuptake inhibitors (SSRIs)) LCL proliferation rates was detected (\( P = 0.875, P < 0.001 \); Figure 2). The differences in relative LCL proliferation rates between the two groups of first-line responders versus treatment-resistant patients were investigated. The effects in cell lines from the first-line responding patients showed significantly increased LCL proliferation after in vitro treatment with fluoxetine and citalopram, whereas the cell lines derived from treatment-resistant patients showed low and even decreased LCL proliferation after incubation with fluoxetine and experiments were carried out as already published.\(^2,3\) QuantiTect and custom-made primers were purchased from Qiagen (Hilden, Germany) and Eurofins Genomics (Ebersberg, Germany), respectively (Table 2) These primers allow the measurement of gene expression levels of five candidate biomarker genes (Table 2) that have been recently identified by whole genome gene expression experiments of LCLs derived from depressed patients participating in the MARS study.\(^2,3\) The basal gene expression was indicated as \( \Delta CT \) values, gene expression fold changes were calculated by \( \Delta CT \) method using GAPDH as reference gene.\(^2,3\)

### Table 1. Characteristics of the STAR*D LCL study cohort

| Gender | Responder | Treatment-resistant patients |
|--------|-----------|-----------------------------|
| Male   | \( n = 10 \) | \( n = 14 \) |
| Female | \( n = 15 \) | \( n = 11 \) |
| Age    | 48.12 ± 13.8 | 48.96 ± 9.5 |
| QIDS   | Week 0    | 17.3 ± 3.2 |
|        | Week 14   | 2.6 ± 1.9 |

Abbreviations: LCL, lymphoblastoid cell line; QIDS, Quick Inventory of Depressive Symptomatology; STAR*D, Sequenced Treatment Alternatives to Relieve Depression.

### Table 2. Primers used for real-time PCR experiments

| Gene   | Assay name or sequence | Recent findings in MARS LCLs |
|--------|------------------------|-----------------------------|
| WNT28  | Hs_WNT28_va1.1_SG      | Association of fold changes by fluoxetine with LCL donor's clinical remission |
| SULT4A1| Hs_SULT4A1_1.1_SG      | Association of basal gene expression with LCL donor's clinical response |
| ABCB1  | Hs_ABCB1_1.1_SG        | Association of basal gene expression with LCL proliferation |
| TCF7L2 | Hs_TCF7L2.1_SG         | Association of basal gene expression and fold changes by fluoxetine with LCL proliferation |
| FZD7   | Fwd: 5′-CCTTCCCCTCTTCATGCC-3′ Rev: 5′-CAGCCGGACAGGAAGATGAT-3′ | Association of fold changes by fluoxetine with LCL proliferation |
| GAPDH  | Hs_CACNA2D3.1.1_SG     | Housekeeping gene |

Abbreviations: LCL, lymphoblastoid cell line; MARS, Munich Antidepressant Response Signature.
citalopram, respectively (Figure 3). A positive correlation between percentage QIDS reduction and LCL proliferation was detected by Spearman’s correlation analysis for both citalopram- \( (\rho = 0.310, P = 0.028) \) and fluoxetine-treated \( (\rho = 0.287, P = 0.043) \) cell lines (Figure 4). The covariates analyses revealed no significant associations between LCL proliferation and either gender \( (P_{\text{Fluoxetine}} = 0.142, P_{\text{Citalopram}} = 0.052) \), age \( (P_{\text{Fluoxetine}} = -0.802, P_{\text{Citalopram}} = 0.581; P_{\text{Citalopram}} = 0.054, P_{\text{Citalopram}} = 0.710) \), menopausal status \( (P_{\text{Fluoxetine}} = 0.731, P_{\text{Citalopram}} = 0.416) \) or anxiety status (anxious versus non-anxious depression; \( P_{\text{Fluoxetine}} = 0.771, P_{\text{Citalopram}} = 0.330 \)).

Gene expression analyses of the candidate genes
Previously derived tentative gene expression biomarkers were further investigated within this STAR*D cohort: Three of the tested...
candidate genes (FZD7, TCF7L2 and WNT2B) are substantial part of the canonical Wnt signaling pathway, which has a key role in the regulation of neurogenesis and synaptic plasticity. The transporter and drug metabolism enzyme genes ABCB1 and SULT4A1 are involved in neuroprotection and metabolism of neuroactive substances, respectively.

Within the STAR*D LCL cohort, the gene expression of those five genes was measured—including basal gene expression and gene expression after 3 weeks of in vitro incubation with therapeutical concentrations of fluoxetine (0.5 ng μl$^{-1}$) and citalopram (0.3 ng ml$^{-1}$). The association of gene expression changes and LCL donor’s clinical status was investigated. Significant differences between the responder-derived cell lines and the cell lines derived from treatment-resistant patients in basal gene expression of WNT2B ($P = 0.0001$) and ABCB1 ($P = 0.009$) were detected. Previous experiments using LCLs from the MARS study showed no associations of clinical parameters with the basal gene expression of these genes.$^{16}$ No significant differences were found for genes FZD7 ($P = 0.643$), TCF7L2 ($P = 0.355$) or SULT4A1 ($P = 0.943$; Figure 5a), which is in accordance with previous results except for gene SULT4A1 (in the MARS study, FZD7 and TCF7L2 genes only showed correlations between fluoxetine-induced fold changes and cell proliferation).$^{16}$ The basal gene expression of SULT4A1 was low and only detectable in 11 out of 50 cell lines ($n = 5$ non-responder-derived cell lines versus $n = 6$ responder-derived cells). The fold changes of WNT2B (FCFluoxetine$\_$Responder = 995.17 and FCFinal_Non-Responder = −675.69 with $P_{fluoxetine} = 0.046$, FCCitalopram$\_$Responder = 701.78 and FCCitalopram_Non-Responder = −1828.48 with $P_{citalopram} = 0.003$), FZD7 (FCFluoxetine$\_$Responder = −469.20 and FCFinal_Non-Responder = 720.03 with $P_{fluoxetine} = 0.003$, FCCitalopram$\_$Responder = −869.02 and FCCitalopram_Non-Responder = 963.93 with $P_{citalopram} = 0.002$) and ABCB1 (FCFluoxetine$\_$Responder = 175.05 and AFCitalopram$\_$Non-Responder = 18.30 with $P_{fluoxetine} = 0.009$, FCCitalopram$\_$Responder = 174.48 and FCCitalopram_Non-Responder = 20.64 with $P_{citalopram} = 0.010$) after in vitro treatment with fluoxetine and citalopram show significant associations with LCL donor’s clinical therapy resistance status (Figures 5b and c). These fold changes represent temporal changes after treatment of LCLs with fluoxetine and citalopram.

Previous experiments from the MARS cohort, only associations of WNT2B fold changes with

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**Figure 5.** Results of gene expression experiments of the candidate genes. (a) Basal gene expression indicated as difference of maximal cycle number and ΔCp values of untreated samples. Gene expression fold changes after 21-day in vitro treatment of LCLs with fluoxetine (b) or citalopram (c) (deviations are indicated as standard error; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). LCL, lymphoblastoid cell line.
Gene expression of candidate genes

The most notable difference in expression levels between responder- and treatment resistance-derived LCLs were observed for WNT2B, FZD7 and ABCB1. We found significant elevated basal gene expression levels of the genes WNT2B ($\Delta$CT difference of 4.96) and ABCB1 ($\Delta$CT difference of 2.31) in the LCLs derived from patients with antidepressant treatment resistance relative to responder-derived ones. Further, in responder-derived cell lines, fold changes by SSRIs were significantly increased for the genes WNT2B (up to 2530-fold higher), FZD7 (up to 1833-fold higher) and ABCB1 (up to 157-fold higher). ABCB1 is the best studied member of the ABC transporter superfamily possessing a key role in cellular detoxification and transmembrane transport across the blood–brain barrier. The allelic spectrum is broad and includes neurotoxic agents (for example, glucocorticoids, drugs and xenobiotics) and thus, ABCB1 holds neuroprotective effects eventually resulting in an increased response to antidepressants. Periphereal glucocorticoids are stress response factors in the hypothalamic–pituitary–adrenal axis, and they normally have toxic effects on neurons and are suspected to be causative for depressions.29 Many antidepressants such as amitriptyline, citalopram, doxepin, fluoxetine or paroxetine are substrates for transport by Pgp at the blood–brain barrier influencing brain bioavailability of central nervous system (CNS) active drugs, and overexpression of Pgp has been described to be associated with treatment resistance to various antidepressant drugs.29 Carriers of defined haplotypes within the ABCB1 gene show decreased risk of developing depressions,30 and polymorphisms of the ABCB1 gene are thought to predict adverse antidepressant drug effects31 indicating a role of ABCB1 in depression. Further, a high expression of ABCB1 in the blood–brain barrier (resulting from chronic antidepressant treatment) might account for an increased clearance of neurotoxic agents (for example, glucocorticoids) from the brain. Peripheral glucocorticoids are stress factors of the HPA axis, are toxic to neurons and suspected to be risk factors for depressive disorders. These ABCB1-mediated neuroprotective effects could contribute to an increased proliferation of neuronal cells and to a modulation of neuroplasticity.

WNT2B and FZD7 are elements of the canonical WNT signaling pathway regulating neurogenesis, synaptic plasticity and dendritic arborization.32 Downstream growth factors such as FGF, BDNF and BMP are involved in depression pathogenesis as well as in the maintenance of adult neurogenesis.33–35 WNT2B is a highly conserved signal peptide and a ligand for members of the frizzled transmembrane receptor family; FZD7 belongs to this family of GPCRs. Wnt glycoproteins usually are liberated from astrocytes and show short-ranged action. An activated Wnt signaling pathway controls stem cell pluripotency and tissue regeneration,36 and regulates the expansion of CNS progenitor cells.37 Furthermore, WNT proteins support the differentiation of specific glial neuronal precursors,38 and are involved in immunological processes of microglia39—macrophage-like cells of the brain that are required for CNS homeostatic functions.40 Neurotoxic agents reduce WNT expression in developmental hippocampal neurons41 and an impaired hippocampal Wnt signaling is associated with a decreased neurogenesis, and an increase of depression-like behavior in adult rats.42 Wnt signaling is stimulated by antidepressive drugs,43 and however, no effects are recognizable in constitutively activated pathways44 indicating a role of Wnt signaling in antidepressant’s action. In accordance to these findings, we reported elevated gene expression levels of WNT2B and lowered gene expression levels of FZD7 after in vitro incubation with SSRIs in responder-derived LCLs. As FZD7 inhibits the WNT signaling while WNT2B enhances it, chronic antidepressant treatment strongly activates this pathway. This results in an increased stem cell liberation and differentiation to neurons (neurogenesis). Further, this leads to an improved maintenance of...
adult hippocampal neurogenesis, expansion of CNS progenitor cells, as well as CNS development in general. The detailed molecular mechanisms are not understood so far but it is assumed that these effects take place by integration of newborn type 2 neurons into neuronal networks. All these effects together might be responsible for an enhanced neuronal plasticity and achieving remission from depression.

CONCLUSIONS
Peripheral proliferation of LCLs derived from depressed patients with known response status revealed significant differences between cell lines derived from treatment-resistant patients and from responders. Furthermore, general proliferative effects of both fluoxetine and citalopram were detected supporting the neurotrophic hypothesis of antidepressant’s action. The low response rates as well as the high variability in antidepressant efficacy observed in clinical practice could be based on individual proliferative effects within the depressed brain and an individual susceptibility to antidepressant-mediated changes in neuroplasticity. Furthermore, candidate gene expression biomarkers that have previously been identified using MARS LCLs and that are associated with neuro-proliferation or protection were evaluated. This was supported by the fact that SSRI-mediated gene expression fold changes of WNT2B, Fzd7 and ABCB1 were correlated with proliferation rates. Significant differences between LCLs derived from responders and treatment-resistant patients for these genes were confirmed rendering them as potential temporal mediators or baseline predictors, which eventually advance the personalized treatment approach in depressions in the future.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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