High-Throughput Screening to Identify Compounds That Increase Fragile X Mental Retardation Protein Expression in Neural Stem Cells Differentiated From Fragile X Syndrome Patient-Derived Induced Pluripotent Stem Cells

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ABSTRACT

Fragile X syndrome (FXS), the most common form of inherited cognitive disability, is caused by a deficiency of the fragile X mental retardation protein (FMRP). In most patients, the absence of FMRP is due to an aberrant transcriptional silencing of the fragile X mental retardation 1 (FMR1) gene. FXS has no cure, and the available treatments only provide symptomatic relief. Given that FMR1 gene silencing in FXS patient cells can be partially reversed by treatment with compounds that target repressive epigenetic marks, restoring FMRP expression could be one approach for the treatment of FXS. We describe a homogeneous and highly sensitive time-resolved fluorescence resonance energy transfer assay for FMRP detection in a 1,536-well plate format. Using neural stem cells differentiated from an FXS patient-derived induced pluripotent stem cell (iPSC) line that does not express any FMRP, we screened a collection of approximately 5,000 known tool compounds and approved drugs using this FMRP assay and identified 6 compounds that modestly increase FMRP expression in FXS cells. Although none of these compounds resulted in clinically relevant levels of FMRP, our data provide proof of principle that this assay combined with FXS patient-derived neural stem cells can be used in a high-throughput format to identify better lead compounds for FXS drug development.

SIGNIFICANCE

In this study, a specific and sensitive fluorescence resonance energy transfer-based assay for fragile X mental retardation protein detection was developed and optimized for high-throughput screening (HTS) of compound libraries using fragile X syndrome (FXS) patient-derived neural stem cells. The data suggest that this HTS format will be useful for the identification of better lead compounds for developing new therapeutics for FXS. This assay can also be adapted for FMRP detection in clinical and research settings.

INTRODUCTION

Fragile X syndrome (FXS) is a neurodevelopmental disorder characterized by mild to severe intellectual disability and behavioral problems, such as attention deficit, anxiety, and depression [1]. FXS is also the most common known monogenic cause of autism, with 43%–67% of the male patients fulfilling the criteria of the autism spectrum disorder [2]. At the genetic level, most patients with FXS have >200 CGG-repeats (known as full mutation [FM]) in the 5′-untranslated region of the fragile X mental retardation 1 (FMR1) gene [3, 4]. Such alleles undergo CGG-repeat-mediated gene silencing [5, 6], which results in the absence of the FMR1 gene product, FMRP. FMRP is an RNA-binding protein that regulates the transport and translation of many mRNAs in the brain and plays an important role in learning and memory [7–10]. However, many FMRP-target mRNAs also have no clear relationship to neuronal development and synaptogenesis [11], suggesting its role in additional pathways. Increased FMRP expression is seen in response to cellular stress, indicating potential roles in cell survival under both normal and stress conditions [12]. FMRP has also been implicated in cancer metastasis [13] and in the DNA damage response [14, 15]. FMRP shuttles between the nucleus and cytoplasm [10, 16], and its
localization depends on the cellular context. This further supports the idea that the cellular function of FMRP might be much broader than previously thought.

FXS has no cure, and currently available treatments provide only symptomatic relief. Drug development to date for FXS has focused on targeting dysregulated signaling pathways downstream of FMRP that were identified in the studies with Fmr1 knockout (KO) mice [17]. A few compounds are currently in clinical trials for FXS treatment; however, none of these approaches directly address the fundamental issue of FMRP deficiency in patient cells. Restoring FMRP expression in FXS patients could be an ideal therapeutic approach because the expanded CGG-repeats are not a part of the open reading frame of the FMRI gene, which is otherwise normal. In addition, patients who exhibit mosaicism of either the CGG repeat size or DNA methylation levels on the FMRI gene are able to make some FMRP and present with milder clinical symptoms [18]. Furthermore, restoring FMRP expression specifically in adult neural stem cells (NSCs) rescues hippocampus-dependent learning deficits in FMRP-deficient KO mice [19]. These observations suggest that partial restoration of FMRP even after birth could be clinically beneficial for patients with FXS. It has been reported that FMR1 gene silencing could be partially reversed in FXS patient cells by compounds that target repressive chromatin modifications. These include small molecules that inhibit the activity of DNA methyltransferases [5, 20] and the protein deacetylase SIRT1 [21]. However, currently available inhibitors might not be suitable for use in patients with FXS because of their potential toxicity and/or limited efficacy. Therefore, the identification of new lead compounds capable of reversing gene silencing is necessary for drug development for the treatment of FXS.

In addition to FXS, CGG repeat expansion is also linked to two other disorders, fragile X associated tremor and ataxia syndrome (FXTAS, reviewed in [22]) and fragile X associated primary ovarian insufficiency (FXPOI) [23–25]. FXTAS and FXPOI are seen in the carriers of FMRI premutation (PM) alleles that have 55–200 CGG-repeats. The clinical symptoms of those with FXTAS and FXPOI are thought to arise primarily from some deleterious consequence of the expression of the PM allele [26–29]. However, PM carriers often have symptoms that are reminiscent of those seen in FXS. It is known that long CGG-repeat tracts have a negative effect on translation, thereby reducing the amount of FMRP [30] and thus potentially causing the FXS-like symptoms. Thus, compounds that increase FMRP expression might also be beneficial for such PM carriers. In addition, given that the severity of the clinical phenotype in FXS patients is inversely proportional to the FMRP levels [31], a direct and quantitative measurement of FMRP should be a better predictor of disease severity than either the number of CGG-repeats in the FMR1 gene or FMR1 mRNA levels. However, the current methods for FMRP detection such as Western blotting, immunofluorescence, and enzyme-linked immunosorbent assay (ELISA) are all limited by low sensitivity, high variability, and/or lengthy or complicated workflow.

We have developed a simple, sensitive, and quantitative assay for FMRP detection. When coupled with NSCs derived from FXS induced pluripotent stem cells (iPSCs), this assay proved to be suitable for high-throughput screening (HTS) for identification of compounds able to at least partially reactivate the silenced gene. These NSCs are particularly well suited for such screens because they carry a single, completely silenced FMR1 allele. Thus, any positive hits in our screen reflect FMR1 gene reactivation, rather than any potential effect on translation. Furthermore, NSCs grow rapidly and in a monolayer and are self-renewing, making possible the facile generation of the large homogenous cell population necessary for large screening applications. Our data suggest that this time-resolved fluorescence resonance energy transfer (TR-FRET)-based FMRP assay is suitable for use in a high-throughput format and that it could also be useful for a number of clinical and research applications.

**MATERIALS AND METHODS**

**Cell Lines and Culture Methods**

All the cell lines used in the present study are listed in Table 1. A control male fibroblast cell line (BJ) was obtained from Stemgent (Cambridge, MA, http://www.stemgent.com), and the fibroblasts from male FXS patients (C10700, C10147, and C10259) were a gift from Dr. Carl Dobkin (Department of Human Genetics, New York State Institute for Basic Research in Developmental Disabilities, New York, NY). The fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1× nonessential amino acids (NEAA), 1× GlutaMAX-1, and 1× antibiotics (all from Life Technologies, Grand Island, NY, http://www.lifetechnologies.com). Lymphoblastoid cells from control male (GM05161) and male FXS (GM032008, GM04025, GM07294, GM06897) patients were purchased from Coriell Cell Repositories (Coriell Institute for Medical Research, Camden, NJ, http://www.coriell.org) and were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal calf serum and 1× antibiotics (Life Technologies). iPSCs were derived from skin fibroblasts from an FXS patient (SC128) and were a gift of Dr. Philip Schwartz (National Human Neural Stem Cell Resource, Children’s Hospital of Orange County Children’s Research Institute, Orange CA, http://www.choc.org). The characterization of SC128 iPSCs is shown in supplemental online Figure 1A–1G. Control iPSCs (SCU16) were a gift of Dr. Barbara Mallon (Stem Cell Unit, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD, http://stemcells.nih.gov/research/niheu-research/scuunit/). FSX NSCs were generated from SC128 iPSCs by XCell Science (Novato, CA, http://www.xcell2.com). BC1 NSCs, derived from a control iPSC line using the same protocol, were purchased from XCell Science. NSCs were grown in neurobasal medium supplemented with 1× B27, 1× NEAA, 1× GlutaMAX-1 (all from Life Technologies), and 10 ng/ml recombinant human basic fibroblast growth factor (R&D Systems, Minneapolis, MN, http://www.rndsystems.com) on plates coated with Geltrex or CELStart (Life Technologies). These NSCs stained positive for the NSC markers Nestin, Pax6, and SOX2 (supplemental online Fig. 2A–2C). For neuronal differentiation, NSCs were grown on poly-l-ornithine-coated plates (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) and laminin-coated plates (Roche Applied Science, Indianapolis, IN, https://www.roche-applied-science.com) in neurobasal medium supplemented with 1× NEAA, 1× GlutaMAX-1, 1× B27, and 1× N2 (Life Technologies) for 2 weeks. These neurons stained positive for the neuronal cell markers, β-tubulin and mitogen-activated protein kinase (supplemental online Fig. 2D, 2E). The method and antibodies used for immunostaining for pluripotency and neuronal markers are described in supplemental online data.

**TR-FRET-Based FMRP Assay**

Identification of the best pair of anti-FMRP antibodies for detecting cellular FMRP levels using the TR-FRET assay was contracted to Cisbio US (Bedford, MA, http://www.cisbio.com). For this purpose, five different antibodies against FMRP were tested. Each antibody was labeled with two different fluorophores, europium-cryptate donor...
(Ab-K) and the d2 acceptor dye (Ab-d2) using N-hydroxysuccinimide chemistry on primary amines. Each labeled antibody was given an identification number for easier handling of the pairs (supplemental online Table 1; supplemental online Fig. 3). All the labeled antibody pairs were assessed for their ability to produce a signal difference between the positive (control) and negative (FXS) cell lysates. The assay was done in 20-μl final volume in 384-well low-volume, solid white plates (Greiner Bio One, Monroe, NC, http://www.greinerbioone.com). Next, 10 μl of cell lysate were incubated with 5 μl of each of the donor- and acceptor-labeled antibodies at room temperature overnight and read on a compatible plate reader (Envision Plate Reader, PerkinElmer Life and Analytical Sciences, Waltham, MA, http://www.perkinelmer.com). The negative control well contained lysis buffer with Ab-K and Ab-d2. This well reflected the background of the assay and was used to calculate the specific signal to background ratio, which was calculated as follows: \[
\text{Ratio}_{665/615} = \frac{\text{Ratio}_{665/615} \text{(sample)} - \text{Ratio}_{665/615} \text{(negative control)}}{\text{Ratio}_{665/615} \text{(negative control)}}
\]

The best pair of antibodies for the FMRP assay was identified as anti-FMRP (clone 2D4)-K (Ab-7-K) and anti-FMRP (D14F4)-d2 (Ab-2-d2). The optimal antibody concentration was then determined by testing 3 different concentrations of the Ab-2-d2 against 3 different concentrations of Ab-7-K. Anti-FMRP antibodies labeled with d2 [anti-FMRP (D14F4)-d2] and K [anti-FMRP (clone 2D4)-K] and detection reagents for TR-FRET assay were purchased from Cisbio US. The antibodies were diluted 50-fold before use in the lysis protocol in a total assay volume of 20 μl in a 384-well plate.

To optimize the assay for use in HTS, we seeded the different cell types in low-volume, solid white 384-well (catalog no. 784075; Greiner Bio One) and 1,536-well (catalog no. 789173-F; Greiner Bio One) plates using a MultiDrop Combi Dispenser (Thermo Scientific, Waltham, MA, http://www.thermofisher.com). For the 384-well plates, 8 μl of cells, and for the 1,536-well plates, 4 μl of cells were plated and allowed to attach overnight in a 37°C humidified chamber. After overnight incubation, 23 μl of 10 mM compound stock solution in dimethyl sulfoxide (DMSO) was added, and the cells were stimulated for 24 or 48 hours. Then, 4 μl or 2 μl of 4× lysis buffer (FMRP Assay Kit; Cisbio US) was dispersed in each well of a 384- or 1,536-well plate, respectively, using a BioRAPTR FRD Dispenser (Beckman Coulter, Brea, CA, http://www.beckmancoulter.com), and the cells were incubated at different temperatures (4°C, room temperature, or 37°C) to promote lysis, followed by an addition of 2 μl (for the 384-well plate) or 1 μl (for the 1,536-well plate) of each of anti-FMRP (clone 2D4)-K and anti-FMRP (D14F4)-d2, diluted 1:25 in the detection buffer supplied in the assay kit (Cisbio US). The plates were sealed to reduce evaporation and incubated overnight at 4°C. The fluorescence signals in assay plates were detected using the Envision Plate Reader (PerkinElmer). TR-FRET ratio was calculated as the emission of acceptor fluorophore at 665 nm over the emission of donor fluorophore at 615 nm (gain, 2,300; integration time, 400 microseconds; integration start time, 60 microseconds; positioning delay, 0.2 second; measurement start time, 0 second; number of flashes per well, 200). The final assay steps are listed in Table 2.

**RNA Isolation and Quantitative Reverse Transcription-Polymerase Chain Reaction**

Total RNA was isolated from fibroblasts, lymphoblastoid cells, NSCs, and neurons using TRIzol (Life Technologies) and quantified on NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, http://www.nanodrop.com). Next, 300 ng of total RNA was reverse transcribed in a 20-μl final volume using VILO master mix (Life Technologies), according to the manufacturer’s recommendations. Real-time polymerase chain reaction (PCR) was performed in triplicate using 2 μl of the cDNA, FAM-labeled FMR1 and VIC-labeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-glucuronidase probe primers (Life Technologies) and TaqMan FAST universal PCR master mix (Life Technologies) on StepOnePlus Real-Time PCR system (Life Technologies).

**Compound Libraries and Screening**

The library of pharmaco logically active compounds (LOPAC1280) containing 1,280 compounds was purchased from Sigma-Aldrich (St. Louis, MO). The library of U.S. Food and Drug Administration (FDA)-approved drugs was set up internally [32]. The compounds were stimulated for 30 minutes using a MultiDrop Combi Dispenser (Thermo Scientific, Waltham, MA, http://www.thermofisher.com). The final concentration was 50 μM in 10 μl of the cDNA, FAM-labeled FMR1 and VIC-labeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-glucuronidase probe primers (Life Technologies) and TaqMan FAST universal PCR master mix (Life Technologies) on StepOnePlus Real-Time PCR system (Life Technologies).
were dissolved in 100% DMSO as 10 mM stock solutions and were further diluted in 384-well plates to 7 concentrations at a 1:5 ratio. A CyBi-Well dispensing station with a 384-well head (Cybio Inc., Woburn, MA, http://www.cybio-ag.com) was used to reformat compounds in 384- to 1,536-well plates. Screening experiments were performed in 1,536-well plates. In brief, 2,500 cells were plated in 8 μl of growth medium per well using the Multidrop Combi Dispenser (Thermo Scientific) and cultured for 24 hours. The compound libraries were transferred in a volume of 23 nl per well using the NX-TR Pintool (Wako Scientific Solutions, San Diego, CA, http://www.wakoautomation.com). The cells were grown for 24 or 48 hours at 37°C. The FMRP assay was then performed as described above. The untreated cells remained viable and healthy in 1,536-well plates for at least 72 hours as assessed by their morphology and ability to remain adherent using light microscopy.

Data Analysis
The 100% signal was defined from control wells devoid of compounds, and the basal signal was obtained from the FXS patient wells. The primary screen data were analyzed using customized software developed internally [33] that attempts to normalize the data and correct for screening artifacts using a combination of control wells within and between the screening plates. Plate statistics, including signal-to-background ratio and Z factor, were calculated comparing the responses of DMSO vehicle-treated healthy control and FXS patient cells. The signal-to-background ratio was calculated as a comparison between the healthy control and FXS patient cells treated with DMSO.

### RESULTS

#### Identification of a Pair of Anti-FMRP Antibodies for Cell-Based FMRP Assay

We applied the TR-FRET assay technology to develop a sensitive and homogenous assay for quantitation of the FMRP levels in cells that could be used for HTS. The assay principle is illustrated in Figure 1A (inset). It uses two anti-FMRP antibodies, labeled with a pair of fluorophores that consist of a donor (europium cryptate, K) linked to the antibody-1 and an acceptor (d2) coupled to the antibody-2. On binding of the two antibodies to FMRP, the fluorescence resonance energy is transferred from the donor to the acceptor fluorophore owing to their close proximity, thus eliminating background signals.

First, five different anti-FMRP antibodies were each labeled with K and d2 and tested with lysates prepared from control and FXS fibroblasts (supplemental online Table 1) to identify the antibody pair with a higher signal-to-basal (S/B) ratio. Six antibody pairs from the 10 combinations tested were able to detect the difference in the FMRP levels between the healthy control (high FMRP) and the FXS (low FMRP level) cell lysates. The best pair was identified as anti-FMRP (clone 2D4)-K and anti-FMRP (D14F4)-d2 (supplemental online Fig. 3). We further evaluated five antibody pairs in a cell-based assay format using healthy control and FXS patient-derived fibroblasts for which the cells were lysed in the same wells during the FMRP detection phase. The anti-FMRP (clone 2D4)-K and anti-FMRP (D14F4)-d2 pair was also the best pair of antibodies for the cell-based FMRP assay (Fig. 1A). Using a purified recombinant FMRP preparation (OriGene

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Table 2. TR-FRET-based FMRP assay protocol in 384-well and 1,536-well plate formats

| Plate and step | Parameter | Value                        | Description |
|---------------|-----------|------------------------------|-------------|
| 384-well plate | Seed cells | 8 μl per well | 8000 Cells per well; white, solid bottom |
| 2             | Centrifuge | 1 minute, 1,000 rpm | Settle cell suspension in well |
| 3             | Incubate   | Overnight                  | 37°C, 5% CO₂, 95% RH |
| 4             | Pin compound | 23 nl              | Compound in DMSO |
| 5             | Incubate   | 24 hours                  | 37°C, 5% CO₂, 95% RH |
| 6             | Lysis, 4×   | 4 μl per well             | Incubate 30 minutes at 4°C |
| 7             | Antibody   | 2 μl per well of each     | 25-Fold dilution of anti-FMRP-K and anti-FMRP-d2 in detection buffer |
| 8             | Incubate   | Overnight                  | 4°C |
| 9             | Read       | Envision                  | Ex = 320 nm, Em1 = 615 nm, Em2 = 665 nm |

1,536-well plate

| Plate and step | Parameter | Value                        | Description |
|---------------|-----------|------------------------------|-------------|
| 1             | Seed cells | 4 μl per well | 2,500 cells per well; white, solid bottom |
| 2             | Centrifuge | 1 minute, 1,000 rpm | Settle cell suspension in well |
| 3             | Incubate   | Overnight                  | 37°C, 5% CO₂, 95% RH |
| 4             | Pin compound | 23 nl              | Compound in DMSO |
| 5             | Incubate   | 24 hours                  | 37°C, 5% CO₂, 95% RH |
| 6             | Lysis, 4×   | 2 μl per well             | Incubate 30 minutes at 4°C |
| 7             | Antibody   | 1 μl per well of each     | 25-Fold dilution of anti-FMRP-K and anti-FMRP-d2 in detection buffer |
| 8             | Incubate   | Overnight                  | 4°C |
| 9             | Read       | Envision                  | Ex = 320 nm, Em1 = 615 nm, Em2 = 665 nm |

Abbreviations: d2, d2 acceptor dye; DMSO, dimethyl sulfoxide; Em, emission; Ex, excitation; FMRP, fragile X mental retardation protein; K, europium-cryptate donor; RH, relative humidity; TR-FRET, time-resolved fluorescence resonance energy transfer.
Validation of the FMRP Assay for HTS

To evaluate the assay robustness and sensitivity, we initially determined the TR-FRET signals using healthy control (BJ) and FXS patient fibroblasts (C10700) using a DMSO plate, which is a solvent control for compound libraries. The final concentration of DMSO in the assay plate was 0.5%, which is commonly used in cell-based assays. The total signal (S) was defined as the FMRP level in the control fibroblasts (BJ), and the basal signal (B) was defined as the FMRP level in the FXS fibroblasts (C10700). We determined the S/B ratio, coefficient of variation (CV), and Z factor in the FXS fibroblasts, which demonstrated that this FMRP assay is robust for high throughput screening in the 1,536-well plate format (Fig. 3). We then performed a test screen using the LOPAC1280 compound library with this FMRP assay and FXS patient-derived fibroblasts. The S/B, CV, and Z factor values were 4.3-fold, 4.3%, and 0.70, respectively. We identified one hit compound, protoporphyrin IX, in the LOPAC1280 screen using FXS fibroblasts that was further validated in the secondary assays (see below). This confirmed the suitability of the FMRP assay for HTS.

Optimization of the Cell-Based FMRP Assay

To develop a sensitive and reproducible assay for HTS, we first optimized the cell density in 384- and 1,536-well plates using control and FXS fibroblasts (Fig. 2A, 2B) and lymphoblastoid cells (supplemental online Fig. 4). We found that a strong signal with good discrimination between the control and FXS patient-derived cells could be obtained with 8,000 cells per well in the 384-well plates and 2,500 cells per well in the 1,536-well plates. We also determined that the optimal temperature for cell lysis and antibody incubation was 4°C for 16 hours or overnight. Next, we confirmed that the best pair of anti-FMRP antibodies was also suitable for quantification of the endogenous levels of FMRP in NSCs and neurons grown in 1,536-well plates (Fig. 2C, 2D). We concluded that this FMRP assay is suitable for detecting differential levels of FMRP in a variety of human cell types.

Evaluation of the Suitability of FXS NSCs for Use in HTS With FMRP Assay

Although the TR-FRET-based FMRP assay works well with many cell types, most fibroblast cell lines derived from FXS patients are mosaic for FM and PM length alleles. They thus express some FMR1 mRNA and FMRP protein from the PM alleles that are not methylated. Therefore, it is difficult to distinguish whether any increase in FMRP expression is due to the effect on reactivation of the silenced FM allele or improved translation from the active PM allele. Furthermore, fibroblasts grow slowly and have a finite lifespan. FX IPCSC-derived NSCs offer a number of advantages for HTS, because in addition to being able to rapidly generate large numbers of cells, this cell line carries a single completely silenced FX allele. That the FMR1 gene is completely silenced is useful, because any positive hits in this assay would indicate a compound able to reactivate the gene. To confirm whether these NSCs were indeed suitable for use in HTS with this TR-FRET-based FMRP assay, we treated these NSCs with 1 μM 5-aza-2′-deoxycytidine (AZA) to reactivate the FMR1 gene. The FMR1 mRNA levels after AZA treatment increased to 1% of GAPDH mRNA. In contrast, in the control BC1 NSCs, FMR1 mRNA was present at approximately 10% of GAPDH mRNA. We were able to detect FMRP in AZA-treated FXS NSCs using both the TR-FRET FMRP assay and Western blot analysis (supplemental online Fig. 5).

We also performed test screens using the LOPAC1280 compound library with this FMRP assay and FXS NSCs and neurons that we generated from these NSCs. The S/B, CV, and Z factor...
values were 5.1-fold, 7.6%, and 0.42 for the NSCs and 3.9-fold, 6.3%, and 0.37 for the neurons, respectively. Protoporphyrin IX (PPIX) and SB216763 were identified as hit compounds and were positive in the quantitative reverse transcription (qRT)-PCR assay for FMR1 mRNA and were further confirmed by the dose-response experiment using the TR-FRET FMRP assay (see below). Our results demonstrated the suitability of FXS NSCs in HTS using this FMRP assay for the identification of compounds that can reverse FMR1 gene silencing.

Drug Repurposing Screen Using FXS NSCs
We then used the FXS NSCs to perform a larger screen of an FDA-approved drug library that contained ~4,000 compounds using the FMRP assay in 1,536-well plate format. The compounds were prioritized for confirmation according to the increases in FMRP levels. The top 20 compounds (supplemental online Table 2) were then chosen and retested in a secondary assay using qRT-PCR to quantify the FMR1 mRNA levels and were further confirmed by the dose-response experiment using the TR-FRET FMRP assay (see below). Our results demonstrated the suitability of FXS NSCs in HTS using this FMRP assay for the identification of compounds that can reverse FMR1 gene silencing.

Validation of Hit Compounds by Secondary Assays
To confirm the 6 hits identified from the primary screens (Table 3), we determined the dose response of these compounds in the FMRP assay with compound concentrations ranging from 15 to 60 μM. Of the 6 compounds identified in the LOPAC1280 and drug repurposing screens, 4 were found to increase the FMRP levels in the FXS NSCs in a dose-dependent manner (Fig. 4A, 4B). At the highest concentrations tested (60 μM), SB216763 and PPIX produced FMRP signals that corresponded to 0.5–1.6 times that seen in control cells.

Figure 2. Optimization of the FMRP assay for HTS in 384-well and 1,536-well plates. (A, B): Indicated number of control and FXS (C10700, C10259, and C10147) fibroblasts were seeded in 384-well and 1,536-well plates, and the FMRP assay was performed as described in Materials and Methods. (C): Different numbers of control (BC1) and FXS (SC128) NSCs were plated in 1,536-well plate, and the FMRP levels were measured using the time-resolved fluorescence resonance energy transfer (TR-FRET) assay. (D): Neurons derived from BC1 and SC128 NSCs were seeded at 2,500 cells per well in 1,536-well plates, and the FMRP levels were measured using the TR-FRET assay. The y-axis shows the FMRP levels as the 665/615 nm TR-FRET ratio obtained in FXS cells over that observed in the control cells. Abbreviations: FMRP, fragile X mental retardation protein; FXS, fragile X syndrome; NSCs, neural stem cells.

Figure 3. Scatter plot for signal-to-basal ratio and Z score in the time-resolved fluorescence resonance energy transfer (TR-FRET) FMRP assay. The TR-FRET assay was conducted with dimethyl sulfoxide (DMSO) plates in three different cell types [fibroblast (A), NSCs (B), and neurons (C)] to measure the performance parameters of this assay. Columns 1 and 2 (A, C) and column 1 (B) contained control cells indicating a positive response, and the remainder of the columns contained FXS cells with 0% response. All wells had an equal amount of DMSO added (0.5% final). The S/B ratio, Z score, and CV for each cell type are shown. Abbreviations: CV, coefficient of variation; FMRP, fragile X mental retardation protein; FXS, fragile X syndrome; NSCs, neural stem cells; S/B, signal-to-basal.
represent SD. In control NSCs, the FMR1 mRNA was an average from three independent drug treatments; error bars expressed as a percentage of GAPDH mRNA. Results shown were treated with SB216763 for 72 hours and tibrofan for 24 hours pounds with qRT-PCR for FMR1 mRNA levels in FXS neurons. The cells were treated with PPIX and SB216763 for 72 hours or geliomycin and tibrofan for qRT-PCR for FMR1 mRNA levels in FXS NSCs. Cells were treated with SB216763 or geliomycin and tibrofan for 24 hours and analyzed using the FMRP assay. The FMRP levels are shown as the percentage of those observed in the control cells.

Table 3. List of compounds identified in the primary screens selected for further validation in secondary assays using FXS NSCs

| Primary screen     | Compound name    | qRT-PCR | Dose response |
|--------------------|------------------|---------|---------------|
| LOPAC1280          | Protoporphyrin IX| +       | +             |
|                    | SB216763         | +       | +             |
| Repurposing Drug library | Geliomycin   | +       | +             |
|                    | Tibrofan         | +       | +             |
|                    | Sodium decanehydroxamate | +       | -             |
|                    | Deserpidine      | +       | -             |

Abbreviations: –, negative; +, positive; FXS, fragile X syndrome; NSCs, neural stem cells; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

Figure 4. Validation of hit compounds identified from screens of LOPAC and approved drug libraries using the FMRP assay and quantitative reverse transcription polymerase chain reaction (qRT-PCR) for FMR1 mRNA. (A): Structure of hit compounds evaluated in secondary assays. (B): Dose responses of hit compounds in the FMRP assay with FXS NSCs. The cells were treated with the indicated concentration of the compounds for 24 hours and analyzed using the FMRP assay. The FMRP levels are shown as the percentage of those observed in the control cells. (C): Validation of hit compounds using qRT-PCR for FMR1 mRNA levels in FXS NSCs. Cells were treated with PPIX and SB216763 for 72 hours and tibrofan for 48 hours with the indicated concentrations. (D): Validation of hit compounds with qRT-PCR for FMR1 mRNA levels in FXS neurons. The cells were treated with SB216763 for 72 hours and tibrofan for 24 hours with the indicated concentrations. (C, D): FMR1 mRNA levels are expressed as a percentage of GAPDH mRNA. Results shown are an average from three independent drug treatments; error bars represent SD. In control NSCs, the FMR1 mRNA was ~10% of GAPDH. Additional qRT-PCR data are shown in supplemental online Table 3. Abbreviations: DMSO, dimethyl sulfoxide; FMR1, fragile X mental retardation 1; FMRP, fragile X mental retardation protein; FXS, fragile X syndrome; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NSCs, neural stem cells; PPIX, protoporphyrin IX; WT, wild-type.

(Fig. 4C). Low levels of FMR1 mRNA were also seen in the FXS neurons after treatment with SB216763 but after treatment with PPIX were negative. Treatment with sodium decanehydroxamate, deserpidine, geliomycin, or tibrofan resulted in low-level reactivation of the FMR1 gene expression in FXS NSCs, but only tibrofan was positive in neurons (Fig. 4C, 4D; supplemental online Table 3).

We further evaluated the levels of FMRP in drug-treated FXS NSCs using Western blot analysis. However, no bands specific for FMRP were detected (data not shown). This could result from the low sensitivity of Western blot technique relative to the TR-FRET assay. Thus, we concluded that although these compounds resulted in some reactivation of the FMR1 gene, this effect was modest. Therefore, screening of additional compound libraries will be necessary to identify the compounds able to reactivate the gene more effectively.

**DISCUSSION**

We developed a homogeneous assay for the determination of FMRP levels in FXS patient cells using two anti-FMRP antibodies with the TR-FRET detection technology. This cell-based assay is capable of rapid quantitation of the amount of FMRP in FXS patient cells. We have shown that this assay is sensitive and robust in the 1,536-well plate format that is suitable for HTS of compound collections. A screening of a LOPAC1280 and a small drug repurposing library using this assay led to the identification of 6 hit compounds that increase FMR1 mRNA levels in FXS cells. This suggests that this cell-based FMRP assay could be useful for future screening of large compound collections to discover lead compounds for FXS drug development. Given that this FMRP assay is simple, sensitive, and quantitative, it might also be used as a clinical diagnostic tool for the assessment of patients with FXS.

Using this TR-FRET FMRP assay has several advantages compared with many of the other FMRP assays, including Western blotting and ELISA. First, the TR-FRET FMRP assay is more sensitive in that a few thousand cells cultured in the tiny wells of the 1,536-well plates are sufficient to produce a signal, even with the low level reactivation of the gene. Second, it is more robust for the quantitation of FMRP levels in FXS cells, because it has ratiometric readout from a plate reader that minimizes errors from cell numbers and liquid handling equipment. Third, it has a higher specificity for FMRP in cell lysates, because two specific anti-FMRP-antibodies are used. This also minimizes the background noise. Fourth, the experimental procedure is simple and homogeneous, with a few steps of reagent addition and incubation that do not include any cell wash steps or running protein gels. The FMRP assay is thus suitable for performing HTS to screen large compound libraries for new lead identification. While this work was in progress, another TR-FRET assay for FMRP detection was described [34]; however, how amenable that assay is to miniaturization and compound screening was not reported.

Because the purpose of the present pilot study was to validate the suitability of the FMRP assay for HTS, we used a wide range of drug concentrations in the primary screens to maximize the sensitivity and limit false-negative results. However, all the hit compounds we identified were only effective at very high concentrations and thus unlikely to be biologically useful without significant optimization. Even so, of the selected primary hits, six compounds were positive by qRT-PCR assay demonstrating that they were specifically acting by reactivating the silenced FMR1 gene (Fig. 4; supplemental online Table 3). Given the small size of our screen, it might not be surprising that more compounds were not identified that were effective at a lower dose.

One of the compounds we identified, SB216763, is a glycogen synthase kinase 3β (GSK3β) inhibitor. It has been previously
reported to improve hippocampus-dependent learning and to rescue neurogenesis in Fmr1 KO mice [35]. Because the KO mice cannot make any FMRP, the effects of SB216763 on learning and neurogenesis are presumably mediated via a downstream mechanism, rather than the reactivation of FMR1 gene transcription. Consistent with this hypothesis, we found that lithium chloride, another GSK3β inhibitor [36, 37], did not have the same effect as SB216763 on reactivation of FMR1 transcription in FXS cells (supplemental online Fig. 7 for fibroblasts; data not shown for NSCs and neurons because the cycle threshold value was undetermined). Thus, SB216763-like compounds could be useful at least two different ways, first by facilitating gene reactivation and second by inhibiting GSK3β.

The effects on FXS cells or Fmr1 KO mice for the other five hit compounds identified in the present study have not been reported. PPIX is an important intracellular metabolite that is a precursor to heme and cytochrome C. It is also a widely used carrier molecule for divalent cations and is known to activate guanylate cyclase [38]. Deserperpine is an antihypertherpic drug with a structure similar to reserpine that irreversibly blocks the vesicular monoamine transporter [39]. Geliomycin is an antibiotic that inhibits RNA and protein synthesis without any effect on DNA synthesis [40]. Sodium decanehydroxamate has antimicrobial activity [41, 42]; however, because many histone deacetylase (HDAC) inhibitors, such as suberoylanilide hydroxamic acid, are hydroxamic acid compounds, it is possible that some of the effect of sodium decanehydroxamate might be exerted via HDAC inhibition. Tibrofan [4,5-dibromo-N-(4-bromophenyl)thiophene-2-carboxamide] is listed as a disinfectant (available at: http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=47207792&viewopt=Deposited). The mechanism by which these compounds affect FMR1 gene reactivation needs further study.

Although all these hit compounds resulted in an increase in FMR1 transcription as assessed using qRT-PCR, none of them resulted in the production of enough FMRP to be visualized by Western blotting, perhaps because it is less sensitive than the TR-FRET assay and the effect of these compounds is relatively modest. Whether these compounds would be more effective at producing FMRP from alleles with smaller numbers of repeats and thus less of an impediment to translation remains to be examined. PPIX had the largest effect on FMRP levels in the TR-FRET-based assay, despite the low level of FMR1 mRNA produced relative to the other compounds. Thus, PPIX might increase FMRP production both by increasing transcription of the FMR1 gene and by improving translation.

Thus, our data support the idea that screening large compound collections using this assay might be useful for the identification of new lead compounds for drug development to treat patients with FXS. Furthermore, because the FMRP levels are a better predictor of disease severity than either the repeat number or methylation status [31], the TR-FRET-based FMRP assay we have described could also be useful in a clinical setting.

**CONCLUSION**

We developed a sensitive assay for the quantification of FMRP levels in FXS cells that is suitable for HTS to identify compounds able to increase FMRP production. Our initial screen of ~5,000 compounds using this FMRP assay has identified 6 hit compounds. One of these hits, PPIX, was able to both increase transcription and improve translation of the FMR1 transcript. Although most of these compounds were only able to produce low levels of FMR1 gene expression, our data have provided proof of principle that this cell-based FMRP assay can be used to screen large compound collections to discover better lead compounds for FXS drug development. In addition, because this assay allows the sensitive and specific quantification of FMRP, it might also be a useful clinical tool for the assessment of FM carriers and PM carriers, who might benefit from therapies designed for the treatment of FXS. Finally, this assay can also be used to evaluate the efficacy in clinical trials of new drugs that restore FMRP expression in patients with FXS.

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**AUTHOR CONTRIBUTIONS**

D.K.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; M.S.: collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; W.Z.: data analysis and interpretation, manuscript writing, final approval of manuscript; K.U.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

D.K., M.S., W.Z., and K.U. have a patent application pending. The other authors indicated no potential conflicts of interest.

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