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REPORT

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A drug screening platform for protein expression levels in neurological disorders

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ABSTRACT

Neurological and psychiatric diseases and disorders affect more than half of the population. Many of these diseases are caused by the malfunctioning of protein synthesis, where too little or too much production of a protein harms a cell and its functions within the brain. We developed a drug screening platform to identify compounds that target the primary cause of these diseases, namely protein expression amounts. This cellular assay monitors protein expression of a target disease gene along with the protein expression of a control gene using the Protein Quantitation Ratioing (PQR) technique. PQR tracks protein concentration using fluorescence. We used human cells and CRISPR-Cas9 genome editing to insert the *Protein Quantitation Reporter* into target genes. These cells are used in high-throughput drug screening measuring the fluorescence as the assay. Drug hits can be validated using the same PQR technique or animal models of the disease.

METHOD SUMMARY

Inserting a *Protein Quantitation Reporter (PQR)* into a gene allows for measuring endogenous protein amounts in single living cells over time. Using protein quantitation as a readout, a drug screening assay targeting diseases caused by abnormal protein levels can identify compounds capable of restoring these proteins to their normal concentrations. Human cell lines that express high levels of a target disease gene can be chosen for *PQR* knock-in to facilitate the evaluation of CRISPR-Cas9 guide RNAs and the DNA repair template containing the *PQR*. Genome-editing induced pluripotent stem cells to knock in a *PQR* provides an unlimited supply of differentiated cells, such as neurons. High-throughput drug screening can be performed using PQR knock-in cells to quantify the target protein in cells before, during, and after drug administration.

HIGHLIGHTS

1. The assay can identify drugs that directly address the molecular cause of a disease.
2. The Protein Quantitation Ratioing (PQR) technique allows for tracking and measuring protein amounts over time in single living cells before, during, and after drug administration.
3. Genome editing to insert the *PQR* into the target gene allows tracking of endogenous protein expression.
4. Using human cell lines allow for faster production of knock-in cells.
5. Patient mutations can be replicated using genome editing during the knock-in step.
6. Using induced pluripotent stem cells allow for an unlimited supply of genome edited differentiated cells such as neurons with the *PQR* knock-in reporter.

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1. Introduction

Protein synthesis within cells is a tightly regulated process where too little or too much of a single protein can cause diseases and disorders such as cancer, neurodegenerative diseases, and psychiatric disorders. Despite having two copies of the genome, there are more than 1,000 disorders and diseases caused by loss of only

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one copy of a gene, called haploinsufficiency, where the expression of the remaining unaltered gene is insufficient to prevent the disorder. More than 20% of haploinsufficiencies result in neurological disorders [1,2].

Tracking protein expression over time in cells is a useful tool to interrogate the mechanisms of these genetic diseases. The Protein Quantitation Ratioing (PQR) technique measures protein production of any gene over time in single cells *in vivo* [3]. The PQR technique works by inserting the *Protein Quantitation Reporter* DNA sequence into any gene of interest. During protein synthesis, the PQR peptide sequence produces one molecule of a fluorescent protein reporter (e.g., GFP) for every one molecule of protein of interest (Figure 1a). The GFP molecule is produced at the protein translation step and is separated from the protein of interest, and so does not interfere with the endogenous protein's activity. Because the GFP is produced in a 1:1 equimolar ratio with the protein of interest, the GFP fluorescence intensity (i.e., brightness) of the cell is used as a readout for protein abundance. Thus, the brighter the cell, the more protein is expressed of the gene of interest [3].

Using CRISPR-Cas9 genome editing, the *PQR* can be inserted into any gene of interest to report endogenous protein production, including in *Drosophila melanogaster* [4], zebrafish [5], mouse [6,7], and human genomes [3,6,8]. For example, we have used PQR to compare mRNA versus protein amounts in single cells [6], or to measure the protein expression dynamics for both parental alleles simultaneously in cells over time in the living animal [4]. Here, we demonstrate the use of PQR to quantify protein expression of disease-associated genes such as *GBA1*, *SHANK3*, and *SLC6A15* in human cells. These cells are then used in high-throughput drug screens based on their PQR fluorescence to identify compounds that can increase the target gene's protein amounts.

2. Materials and methods

2.1. Cell culture

HEP-G2 and HEK293 cells were cultured at 37 °C under 5% CO_2 in Dulbecco's Modified Eagle Medium (Wisent, St-Bruno, QC). All media were supplemented with 10% fetal bovine serum (FBS) (Wisent), and 100 units/mL penicillin (Life Technologies, Carlsbad, CA) and 100 µg/mL streptomycin (Life Technologies).

iPSCs were cultured in growth media mTESR plus, or mTESR1. The monolayer neural induction protocol (STEMCELL Technologies) was used to obtain NPCs. Briefly, iPSCs were seeded at 3×10^6 cells/mL on 6-well plates coated with Matrigel (Corning) containing Neural induction media with SMADi supplemented with 10 µM Y-27632 (STEMCELL Technologies) on day 0. From day 1 to day 6, media changes were performed with Neural induction media and SMADi alone. Cells were passaged after at least 6 days for 3 rounds.

To obtain dopaminergic neurons, we used the STEM™ differentiation and maturation kit (STEMCELL technologies). Briefly, NPCs were plated onto 6-well plates coated with PLO/laminin and resuspended in 2 mL dopaminergic differentiation media. Media change was performed every day until cells reached 90% confluency. Cells were passaged and seeded at a density of 6×10^4 cells/mL and resuspended in dopaminergic maturation media 1 (STEMCELL Technologies) for 4 days. Media changes were performed every day. On day 5, we changed to Dopaminergic Media 2 (STEMCELL Technologies) for another 5 days.

2.2. CRISPR/Cas9 genome editing

The genomic sequences of each of the target genes, *Apolipoprotein E* (*APOE*), *Euchromatic Histone-lysine N-methyltransferase 1* (*EHMT1*), *β-Glucocerebrosidase* (*GBA1*), *SH3 and multiple ankyrin repeat domains 3* (*SHANK3*), and *Sodium-dependent neutral amino acid transporter B(0)AT2* (*SLC6A15*), and *Ribosomal protein L13A* (*RPL13A*) were PCR amplified from the human cell lines. The *Protein Quantitation Reporters* (*PQRs*) used were as described for mammalian genomes [3]. The target gene fragments with the *PQR* and fluorescent protein gene were assembled into cassettes and placed between the homology arms using In-Fusion Cloning (TAKARA Bio) into a pUC19 vector. Molecular assembly strategies were generated using the automated Molecular Assembly feature in GeneDig.org [9], primarily using Gibson assembly and restriction enzyme digest and ligation. The homology arms did not include the endogenous promoter, thus preventing the expression of the transgene until the in-frame genomic integration at the correct locus occurred during genome editing. Sizes of homology arms were ~1 kb.

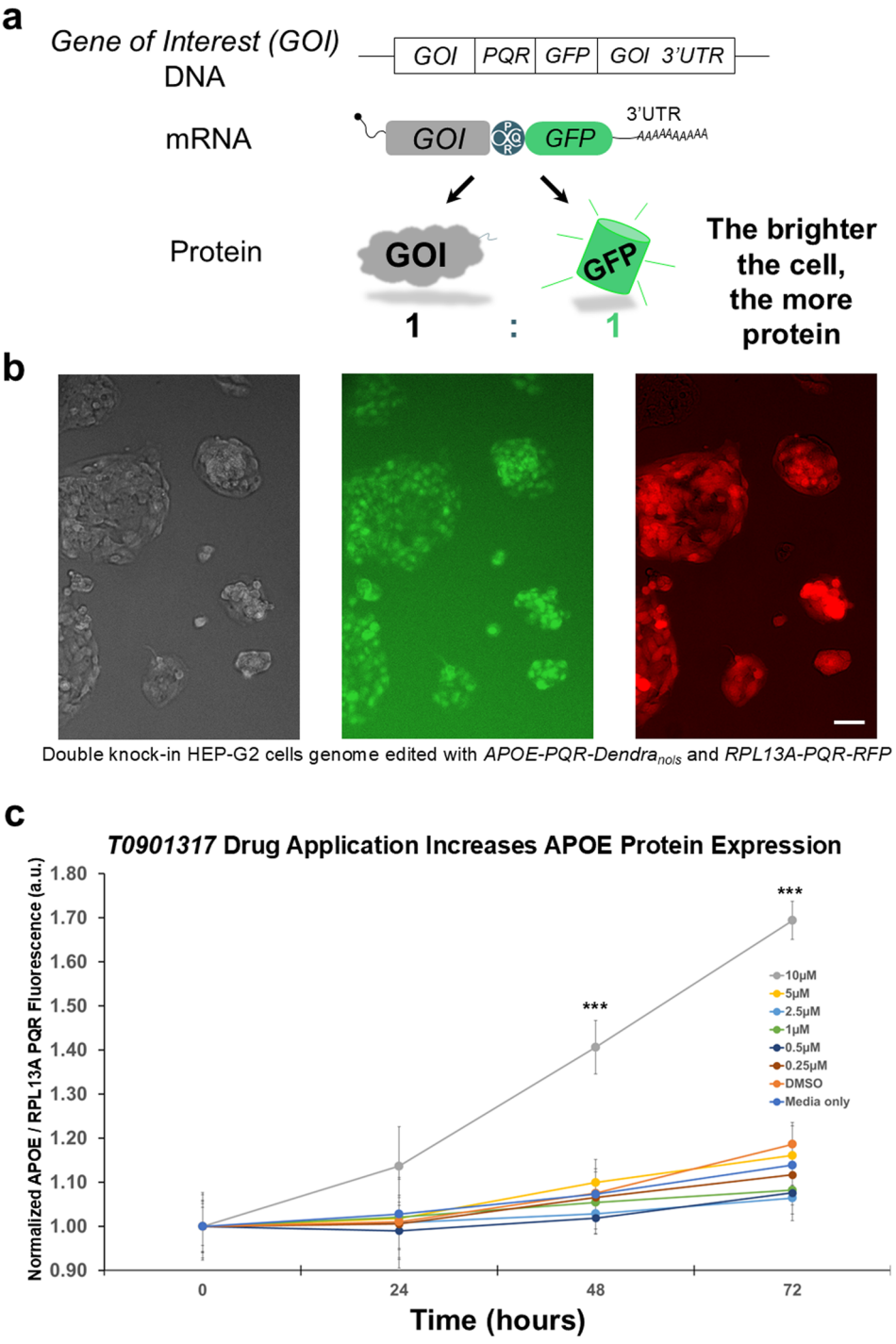

Figure 1. Protein Quantitation Ratioing (PQR) can measure endogenous protein expression over time in single living cells. (a) Knock-in of a *Protein Quantitation Reporter* (*PQR*) into a gene of interest (GOI) using genome editing can quantify the protein levels using the amount of GFP expressed in a cell (i.e., brightness). The *PQR* creates a polycistronic mRNA for co-transcription and co-translation of GFP and the gene of interest. The *PQR* construct allows for one molecule of GFP to be synthesized for every one molecule of the protein of interest synthesized. The fluorescence intensity of a cell can then be used to quantitate its concentration. (b) Representative images of human HEP-G2 hepatocytes expressing APOE-PQR-Dendra$_{nols}$ and RPL13A-PQR-RFP. The brightness of the Dendra in the green channel quantifies the steady state amount of APOE for each cell, and in the red channel, the steady state amount of RPL13A protein for each cell. Scale bar is 25 μm. (c) 10 μM T0901317 significantly increased endogenous APOE protein expression after two days in HEP-G2 cells ($p < 0.001$). APOE fluorescence in the green channel was normalized to the control RPL13A protein amounts in the red channel, expressed in arbitrary units (a.u.). Error bars are standard deviation.

Four different single guide RNAs (sgRNAs) were designed to guide the Cas9 nuclease to make a double strand break at the end of the coding region of each gene. They were designed in a 20 bp DNA oligonucleotide format. Individual sgRNAs were cloned into a dual promoter plasmid, such that both the sgRNA and Cas9 were expressed from the same vector. Transfection was achieved using Lipofectamine 3000 (ThermoFisher) or electroporation using a NEPA21 (NepaGene). 800 ng of *CRISPR-Cas9* plasmid DNA were co-transfected with 800 ng of repair template circular plasmid in 12-well plates. Efficiency of sgRNA/Cas9 was evaluated five days after transfection by cellular fluorescence and genotyping. Editing efficiency was between 1% – 5% based on cellular fluorescence, so colonies were isolated by mouth pipetting and then enriched until they reached >99% purity [3,6,8].

Genotyping experiments were performed in experimental duplicate. Integration of *PQR* into the endogenous genomic locus was validated by genomic DNA extraction six days post-transfection and genotyping using primers outside of and within the homology arms of the repair template (Supplemental Figures 1–6). The 5′ (upstream) and 3′ (downstream) ends of the insertion were probed with two sets of primers and the endogenous locus was PCR amplified. Restriction digests were then performed on PCR products at sites specific for *PQR*. All genomes were sequenced to identify the *PQR* and genomic junctions.

2.3. Drug screening

T0901317 (Abcam) was dissolved in DMSO and applied at 250 nM to 10 µM concentrations. Fluorescence images of >100 cells/well in 384-well plates were taken using a Perkin-Elmer Opera Phenix High Content Imager. Liquid handling was performed using a Beckman Biomek Integrated Robotic System, and drug compounds were applied using a Labcyte Echo 555 Acoustic Droplet liquid handler.

2.4. Image analysis and statistical analysis

Images were analyzed using the Perkin-Elmer Opera Harmony software or ImageJ and selected on the basis of identification of single cells with normal morphology. Images were adjusted for contrast and brightness only. Average pixel intensities were calculated for each well using the automated cell detection software (Supplemental Figure 7). We verified that the RPL13A-PQR control channel fluorescence did not increase or decrease significantly throughout the time-lapse imaging. Student's *t*-tests were performed to test the null hypothesis that the average fluorescence values from the different conditions were the same, from two-tailed distributions of similar variances ($p < 0.05$).

3. Results

3.1. T0901317 drug application increases APOE protein expression

We first sought to verify that PQR can measure drug-induced changes in protein amounts in human cells over time. Because there are currently no therapeutic drugs that can increase the specific protein expression of any neurological disease-associated genes, it is difficult to find an appropriate positive control drug. We chose the drug T0901317, which increases Apolipoprotein E (APOE) protein and mRNA via activation of liver X receptor alpha [10–13]. We used CRISPR-Cas9 genome editing to insert a *PQR* into the *Apolipoprotein E* gene, whose variants are the most common genetic risk factor for Alzheimer's disease, but this is not due to altered APOE protein expression. We generated *APOE-PQR-Dendra2*$_{nols}$ and *RPL13A-PQR-RFP*, double knock-in human HEP-G2 cells (Figure 1b). Dendra2 is a photoconvertible green to red fluorophore (Chudakov *et al.*, 2007), and the nucleolar localization signal (nols) is used to sequester the fluorophore into the nucleolus for ease of imaging and analysis [3,4]. We use the *RPL13A* gene as a control [3,4,6,8] in a second imaging channel for changes in imaging conditions, imaging artifacts from the plate, cell health, and drugs that non-selectively alter protein expression rather than just the target gene.

Previous studies have used concentrations of T0901317 ranging from 2 nM and 10 µM over timescales of 24 hours to 7 days in different cell types ranging from human THP-1 macrophage-like cell lines to HEP-G2 cell lines to mice *in vivo* [10–16]. These studies found that after T0901317 administration, *APOE* mRNA levels

increased between zero to 3-fold, and APOE protein increased between zero to 2-fold, depending on the cell type and conditions. The wide range of these previous results is likely due to the differences in cell types used in the studies, and the differences in liver X receptor alpha expression and activation.

Using the *APOE-PQR-Dendra2$_{nols}$*; *RPL13A-PQR-RFP* double knock-in human HEP-G2 cells, we imaged APOE and RPL13A protein levels over 3 days in multi-well plates using a high-throughput imager with liquid handling. The drug T0901317 was administered at concentrations ranging from 250 nM to 10 µM in a 384 well plate, as well as the negative controls dimethyl sulfoxide (DMSO) or media alone. Application of 10 µM T0901317 significantly increased APOE protein by 40% after 2 days and 60% on day 3, without affecting the control protein RPL13A ($p < 0.001$, $n > 100$ cells per well, 28 wells, *t*-test) (Figure 1c). Concentrations lower than 10 µM did not significantly alter APOE nor RPL13A protein levels. These results confirm the increase of APOE protein expression by a known drug T0901317 and validate that PQR can measure drug-induced changes in protein amounts in human cells over time.

3.2. High-throughput drug screening for protein expression changes

Using CRISPR-Cas9 genome editing, we inserted a *PQR* into four other genes, *EHMT1*, *GBA1*, *SHANK3*, and *SLC6A15* in HEK293T human cells. *EHMT1* is a haploinsufficient gene that causes Kleefstra syndrome, a complex disorder of developmental delay and intellectual disability [17]. *GBA1* is the most frequent genetic risk factor for Parkinson's disease [18,19], and in many of these cases there is not enough of the GBA1 protein being made [19–22]. *SHANK3* is a haploinsufficient gene that causes Phelan-McDermid syndrome but whose changes in protein levels are also associated with autism and obsessive-compulsive disorder [23]. *SLC6A15* is a gene implicated in stress susceptibility and depression [24]. Thus, these are each examples of neurological diseases and disorders whose etiology is insufficient protein expression from a single gene (i.e., the molecular cause is specifically low protein amounts of the disease-associated gene).

We generated *EHMT1-PQR-GFP*, *GBA1-PQR-GFP$_{nols}$*, *SHANK3-PQR-RFP$_{nols}$*, or *SLC6A15-PQR-RFP$_{nols}$* HEK293T cells (Figure 2a). We also demonstrated that patient mutations can be replicated during the *PQR* insertion genome editing step. For example, we recreated the *GBA1* patient mutation L444P (Figure 2a) that is one of the most common mutations in the GBA1 protein [25].

Next, we screened through thousands of drug compounds using the *GBA1-PQR-GFP$_{nols}$*; *RPL13A-PQR-RFP* HEK293T cells, and the *SLC6A15-PQR-GFP$_{nols}$*; *RPL13A-PQR-RFP* HEK293T cells (Figure 2b). Cells were seeded at 2,000 cells per well in a 384-well plate and imaged using a high content imager with integrated liquid handling, and drug compounds were applied using an acoustic droplet liquid handler. The RPL13A-PQR control channel was monitored to exclude drugs that affected RPL13A as well as the target gene, and then the target gene PQR fluorescence values were normalized to the RPL13A levels. Other internal negative controls included randomly interspersed DMSO or media only controls in wells. However, artifacts near the edges of the 384-well plates were still sometimes observable, that the internal controls could not overcome. These "edge effects" are common in high-throughput screening [26] and are caused by several factors, such as evaporation and temperature differences.

After a first-round drug screen, the top drug hits can be selected for follow-up based on criteria such as significant difference from the standard deviation of the mean, fold change, or time course. We performed a validation screen of the top hits (Figure 2c), which we defined as those compounds that increased target protein expression greater than three standard deviations above the average signal at either 24, 48, or 72 hours. The top drug results from this follow-up screen can be verified (see Discussion) *in vivo* using wildtype animals or disease models, or *in vitro* using human primary cells.

4. Discussion

Traditional drug screening assays use cellular pathology or cellular phenotypes associated with the disease [27–30]. Here, we show that drug screens for genetic disorders caused by abnormal protein expression can be performed using a *PQR* genetic tag and simple fluorescence microscopy. We used HEP-G2 and HEK293T cell lines as an easier model system for culturing, genome editing, and live imaging. We first confirmed the mRNA expression levels of the target gene within the cell line using proteinatlas.org [31]. However, high-throughput drug screens are not limited to cell lines and can also be performed on cells differentiated from pluripotent

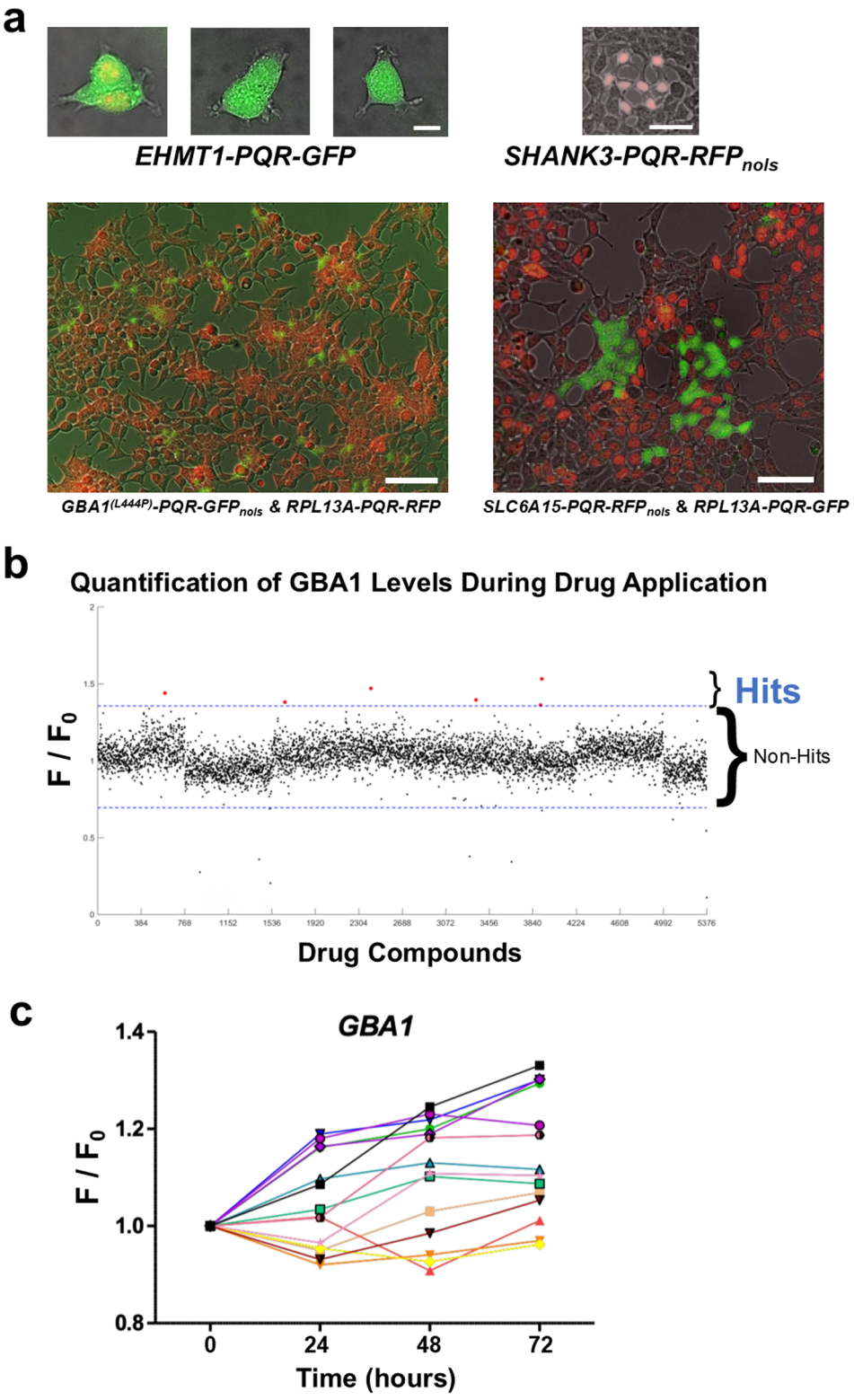

Figure 2. PQR can be used to screen drugs that increase endogenous protein levels.
(a) Representative images of human HEK293T cells that have been genome-edited to insert a *PQR* and a fluorophore gene into four different target genes, *EHMT1*, *GBA1*, *SHANK3*, and *SLC6A15*. Scale bars are 10 µm (upper panels) and 50 µm (lower panels). (b) Representative example of a drug screen of >5,000 drug compounds using the *GBA1-PQR-GFP*; *RPL13A-PQR-RFP$_{nols}$* HEK293T cells at 24 hours after drug administration. Six drug compounds (red dots) significantly increased GBA1 levels normalized to RPL13A levels greater than three standard deviations (blue dotted lines). (c) A secondary screen of the top hits from the first screen can identify false positives. A representative example of a validation screen of the drug hits (each colored symbol/line represents a single drug) with GBA1 protein expression over days is shown.

stem cells. Using human induced pluripotent stem cells allows for drug screens in human neurons for neurological diseases and disorders. Screening for drugs using human neurons was previously not feasible as neurons are post-mitotic and cannot be cultured and passaged indefinitely, and thus the only means of a steady supply of neurons are pluripotent stem cells. We used induced pluripotent stem cells to insert a *PQR* into the target disease genes, *GBA1*, *SHANK3*, and *SLC6A15*. We used the *GBA1-PQR-GFP$_{nols}$* knock-in stem cells to differentiate these into dopaminergic neurons (Figure 3). These PQR knock-in neurons can not only be used for drug screening in a neurodegenerative disease-relevant cell type, but can also be combined with other cellular assays such as cell survival, metabolic activity, or morphology.

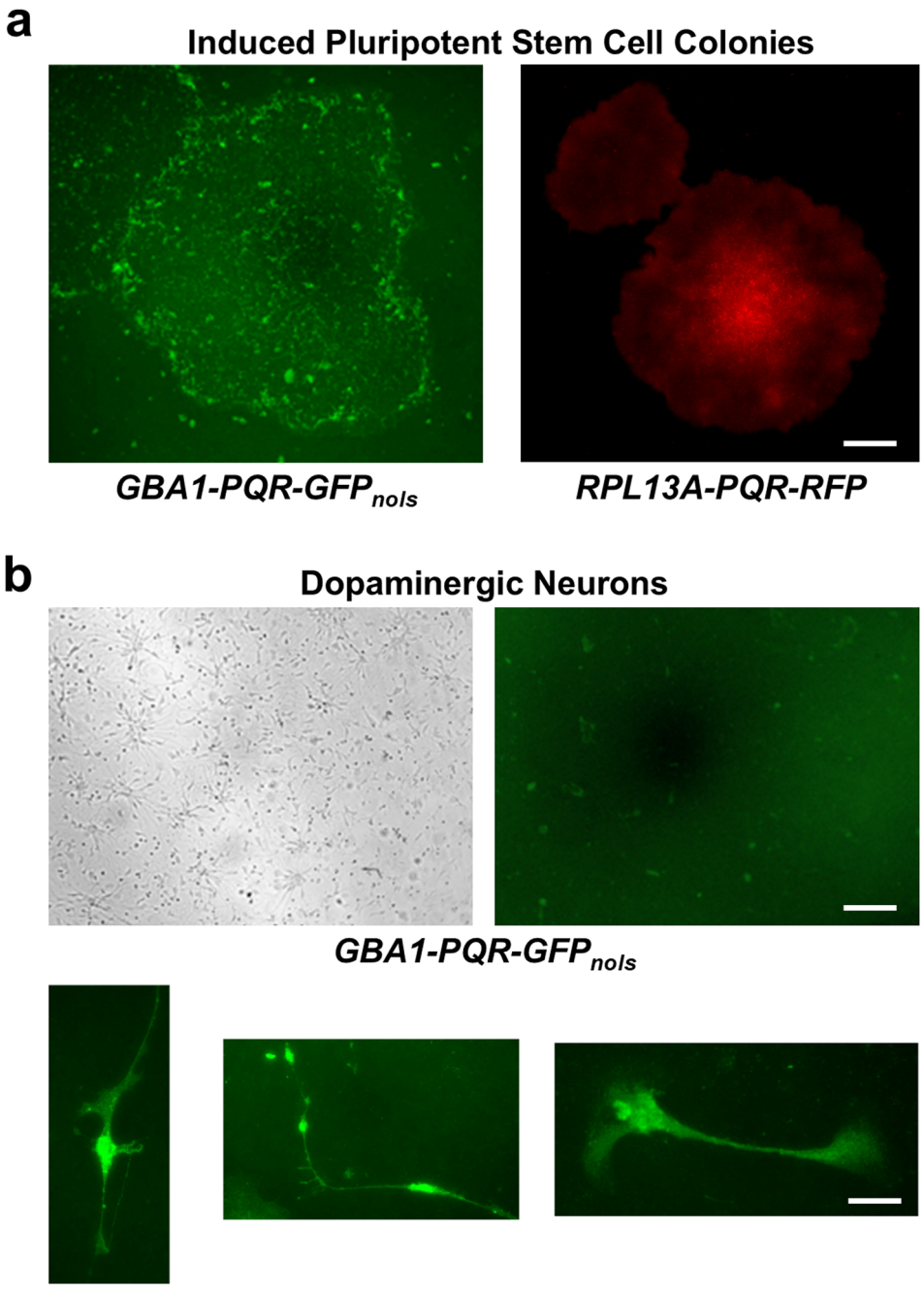

Figure 3. Human neurons can be used in drug screens for endogenous protein levels.
(a) Representative examples of knock-in human induced pluripotent stem cell colonies with *GBA1-PQR-GFP$_{nols}$* (left image) or *RPL13A-PQR-RFP* (right image). Scale bar is 50 µm. (b) Representative examples of *GBA1-PQR-GFP$_{nols}$* human dopaminergic neurons. Bottom images show single neurons stained for tyrosine hydroxylase in green. Scale bars are 50 µm in top images, 10 µm in bottom images.

The protein expression drug screening approach has several disadvantages, however. The most critical disadvantage is the lack of knowledge of drug mechanism of action. A drug may change protein expression in a variety of ways, whether entering the cell or activating a surface receptor and then altering transcription of the target gene, or transcriptional regulation, or altering translation of the target protein, or translational regulation. Even if after a drug lead has been validated *in vitro* and *in vivo* using multiple model systems and disease models, the mechanism by which it alters the target protein expression could remain a mystery. Without knowledge of how the drug acts, it will be difficult for medicinal chemistry to develop the drug further to increase the drug safety and efficacy, making it unattractive for pharmaceutical companies. One way to mitigate this disadvantage is to first determine whether the drug acts at the transcriptional level by using quantitative RT-PCR to measure changes in the mRNA in addition to changes in protein levels [4,6]. Another mitigation can include screening with drug libraries of oligonucleotides, such as antisense oligonucleotides (or antisense therapy), since nucleotides have identifiable targets. Screening using FDA-approved drug libraries may circumvent this issue, since many drugs have unknown mechanisms of action, such as modafinil, metformin, and acetaminophen/paracetamol, as well as the majority of drugs that are prescribed off-label for unapproved indications, such as ketamine for treatment-resistant depression. As long as the drug lead is extensively validated pre-clinically, then the FDA-approved drug can potentially move directly into Phase 2 clinical trials to determine efficacy, and patients may be able to use the re-purposed drug off-label.

Another disadvantage of drug screening using PQR is that it cannot detect changes post-translationally, such as changes in protein turnover, protein modifications, or protein function. Additionally, for haploinsufficiency disorders if a gene mutation is a gain of function, where the mutant protein has deleterious effects on the cell, then a drug that increases the target protein would be detrimental because this would amplify the negative effects. In these cases, the target patient population would be those with loss of function mutations. In the case of GBA1 and Parkinson's disease, for example, around 5% – 7% of Parkinson's disease patients have a known gain of function *GBA1* mutation [32–34]. Therefore, the target patient population would be those with a loss of function *GBA1* mutation, which is estimated to be more than half of GBA1-associated Parkinson's disease [32,35]. For example, in those of African ancestry with Parkinson's disease, one *GBA1* variant identified is a non-coding mutation occurring in the intron, which results in decreased GBA1 activity [36]. Even those Parkinson's disease patients without GBA1 mutations (e.g., idiopathic Parkinson's disease) have reduced GBA1 protein levels [19–22,37–40].

Finally, some genes have very tightly regulated protein expression where too little or too much can result in abnormal cellular function. *SHANK3* is one such gene, where complete loss of one copy of the gene results in Phelan–McDermid syndrome [23,41], and too much expression of SHANK3 causes manic-like behaviors [42]. Thus, any drug that alters protein abundance will require detailed dosage, pharmacodynamic, efficacy, and safety characterizations.

There are several advantages to drug screening using protein levels. Using PQR, time-lapse imaging can be performed in single cells to measure protein expression before, during, and after drug application (Figure 2c) to measure the pharmacodynamics of the drug. The PQR approach measures protein amounts, which is a more accurate reflection of the actors within a cell, rather than mRNA levels. Thus, drug-induced changes that occur post-transcription, such as protein synthesis regulation, can also be detected. The fluorophore used with the *PQR* tag can also offer several advantages, such as the use of fluorescent timer proteins and photoconvertible proteins, such as Dendra (Figure 1). Fluorescent timer proteins change their emission from blue to red over a period of hours [43,44]. Thus, the rate of protein synthesis can be examined by making repeated measurements using the blue channel, and this can be performed regularly over time scales of days of drug application. The photoconvertible fluorescent protein Dendra normally emits green fluorescence, but can be permanently photoconverted to emit red fluorescence by UV illumination [45]. The rate of protein synthesis can be measured at any arbitrary time by UV illuminating the cell to convert the existing signal to the red channel, and new protein synthesis will then occur within the green channel. Thus, fluorescent timer proteins and photoconvertible fluorescent proteins shift accumulated fluorescence into a different channel, so that new protein synthesis events can be repeatedly measured. Finally, another advantage of using PQR to measure protein levels for drug screening is the ability to use patient-derived cells, including induced pluripotent stem cells (Figure 3). The genotype of these cells can be changed using CRISPR-Cas9 to re-create (Figure 2a) or correct patient mutations in the same genome editing step as the *PQR* insertion.

Validation of the drug hits can include experiments that would support the drug-induced increase in protein expression or address the weaknesses of the PQR assay. These could include using different *in vitro* assays such as an enzymatic functional assay, cell or neurite growth or survival assays, electrophysiological measurements, or using primary cell cultures. As mentioned above, quantitative RT-PCR experiments can be used to determine whether the drug hits act at the transcriptional level. Drugs can also be validated using different *in vivo* model organisms, such as zebrafish or *Drosophila*, to demonstrate that the drug hits act on an evolutionarily conserved mechanism and thus would be more likely to do so in clinical trials. This has been proven successful for FDA-approved compounds that have been identified in genetic model organisms first [46]. Ideally, the drug hits would be validated *in vivo* to be shown to rescue an animal model of the disease. Characterization of the drug hits themselves would include experiments to identify the half maximal effective concentration (EC50) curve, their pharmacodynamics using time-lapse imaging experiments at finer time interval acquisitions, and their blood brain barrier penetrance. Biochemical experiments to identify the drug hits' mechanism of action and binding partner(s) would address the main weakness of the PQR assay.

Further validation of drug hits can include *in vivo* mouse models. Drug candidates need to be optimized to improve pharmacokinetic and pharmacodynamic characteristics such as absorption, distribution, metabolism, and toxicity *in vivo*. Conceptually, the same protein quantification using PQR during drug application can be performed using a knock-in PQR mouse. Ideally, in each round of drug optimization the drug is continually re-validated using rapid *in vitro* and *in vivo* assays. Thus, a PQR knock-in mouse similar to the PQR knock-in human cells would be a useful whole-animal assay for several reasons. First, tracking the endogenous protein expression of a target disease gene over time in the living animal will itself be an important model for the disease. Second, imaging how the target protein expression changes within different tissues is critical for pharmacokinetics as the target protein expression may run down over time with drug duration, dosage, route, or circadian cycle. Third, imaging the target protein expression spatially across different tissues will aid in the pharmacodynamic evaluation of drug efficacy. Fourth, imaging target protein expression over time across different brain regions is important for models of neurological diseases. Fifth, imaging the target protein expression can be combined with mouse models of the disease, by crossing the mice or using chemotoxic agent models. Sixth, using near-infrared fluorescent proteins allow for nanometer to centimeter scale *in vivo* imaging (i.e., fluorescence microscopy to whole animal imaging) [47,48]. This type of PQR knock-in mouse would allow for whole animal imaging through the skin, before, during, and after drug administration to track the target protein expression throughout the body in real time. Imaging these mice through the skin, combined with drug tracing through nuclear medicine imaging, would allow for much more accurate, rapid, and cheaper pharmacology and toxicology measurements while still monitoring drug efficacy simultaneously.

5. Conclusion

Here we demonstrated a novel drug screening assay measuring protein amounts over time as the screening criteria. We used CRISPR-Cas9 genome editing to insert the *Protein Quantitation Reporter* into different target genes to allow for the monitoring of endogenous protein expression, using human cell lines for rapid production of knock-in cells. We showed that knock-in induced pluripotent stem cells allowed for an unlimited supply of differentiated cells such as neurons. Using this assay in high-throughput drug screening, we identified and validated drug compounds that increased the protein expression of target disease genes, such as GBA1 and SLC6A15.

6. Future perspective

The ideal drug discovery pipeline uses high-throughput drug screening on multiple assays simultaneously, such as time-lapse protein quantification with optical physiology, cell morphology, and molecular target activation. Combining the complementary strengths of different assays would address the weaknesses of each individual assay and provide richer data to increase the quality of drug hits, thereby increasing the chance of success in clinical trials. For neurological disorders, a simultaneous assay that assesses blood brain barrier drug penetrance would also be beneficial. Combining all of these types of assays is possible but has not yet been done. Similarly, generating animal models for *in vivo* drug validations that simultaneously combine multiple assays such as *in vivo* protein quantification (see Discussion) with behavior, optical physiology, and toxicology real-time readouts is possible and ideal, but has not

been attempted before. Generating standardized cell lines or mouse models that combine these types of assays into one line would be a useful resource for drug development.

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Ethical declaration

Human cell lines used did not require approval for use. Human stem cells were approved by the McGill University Health Center Research Ethics Board, approval 2025-3774.

Author contributions

Brian E. Chen designed the experiments and supervised the project. Farida Emran, Ibrahim Kays, Chiu-An Lo, Yueyang Li, and Brian E. Chen performed experiments and analyzed the data. Farida Emran and Brian E. Chen wrote the manuscript.

Disclosure statement

Brian E. Chen is an inventor of a patent on PQR. Brian E. Chen, Ibrahim Kays, and Chiu-An Lo are founders of a company based on the work in this manuscript. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

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Data availability statement

The datasets in the current study are available from the corresponding author on request. Correspondence and requests for materials should be addressed to brian.chen@mcgill.ca.

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