Progenitor cells derived from gene-engineered human induced pluripotent stem cells as synthetic cancer cell alternatives for in vitro pharmacology

Constanze Uhlmann1 | Ann-Christin Nickel1 | Daniel Picard2,3,4 | Andrea Rossi5 | Guanzhang Li6 | Barbara Hildebrandt7 | Gabriele Brockerhoff5 | Farina Bendt5 | Ulrike Hübenthal5 | Michael Hewera1 | Hans-Jakob Steiger1 | Dagmar Wieczorek7 | Aristoteles Perrakis8 | Wei Zhang6 | Marc Remke2,3,4 | Katharina Koch5 | Julia Tigges5 | Roland S. Croner8 | Ellen Fritsche5,9 | Ulf D. Kahlert8

1Department for Neurosurgery, Medical Faculty and University Medical Center Düsseldorf, Heinrich-Heine-University, Düsseldorf, Germany
2Division of Pediatric Neuro-Oncogenomics, German Cancer Research Center (DKFZ), Heidelberg, German Consortium for Translational Cancer Research (DKTK), partner site Essen/Düsseldorf, Düsseldorf, Germany
3Department of Pediatric Oncology, Hematology, and Clinical Immunology, Medical Faculty, University Hospital Düsseldorf, Düsseldorf, Germany
4Department of Neuropathology, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany
5Leibniz Research Institute for Environmental Medicine, Düsseldorf, Germany
6Beijing Neurosurgical Institute, Capital Medical University, Beijing, P. R. China
7Institute of Human Genetics, Medical Faculty and University Hospital Düsseldorf, Heinrich-Heine-University Düsseldorf, Germany
8Molecular and Experimental Surgery, University Clinic for General, Visceral and Vascular Surgery, University Medical Center Magdeburg and Faculty of Medicine, Otto-von-Guericke-University Magdeburg, Magdeburg, Germany
9Medical Faculty, Heinrich-Heine University, Düsseldorf, Germany

Abstract

Limitations in genetic stability and recapitulating accurate physiological disease properties challenge the utility of patient-derived (PD) cancer models for reproducible and translational research. A portfolio of isogenic human induced pluripotent stem cells (hiPSCs) with different pan-cancer relevant oncprotein signatures followed by differentiation into lineage-committed progenitor cells was genetically engineered. Characterization on molecular and biological level validated successful stable genetic alterations in pluripotency state as well as upon differentiation to prove the functionality of our approach. Meanwhile proposing core molecular networks possibly involved

Abbreviations: EC50, effective concentration 50 percent viability; EGFRvIII, epidermal growth factor receptor variant III; EV, empty vector; FACS, fluorescence activated cell sorting; Gli1, glioma-associated oncogene 1; GO, gene ontology; HDAC, histone deacetylase; hiPSC, human induced pluripotent stem cells; IPA, Ingenuity pathway analysis; MTT, thiazolyl blue tetrazolium bromide; NPC, neural progenitor cells; PBS, phosphate buffer saline; PD, patient-derived; TP53, tumor protein S3; WT, wildtype

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2022 The Authors. Biotechnology Journal published by Wiley-VCH GmbH.


1 | INTRODUCTION

In vitro tumor models present the basic fundament of early-stage cancer research. Processing of tumor tissue, derived from surgical resection, to establish chronic in vitro models presents the traditional attempt in human cancer disease-modeling.\(^1\) Historically, this approach has been proven to be successful, as a large portion of our current oncology blockbusters are based on discoveries made in such patient-derived (PD) model systems.\(^2\) With the emergence of evidence that dysregulated stem cells are responsible for cardinal aspects of initiation and progression of malignant tumors,\(^3\) stem cell technologies impact the creation of recent PD cancer models with the establishment of spheroid, organoid, or assembled culture protocols to generate cellular more complex – and therefore more physiologically relevant – test systems.\(^4\) However, the recent advancements in molecular technology revealed that genetic instability of cancer cell lines occurs which seems to be a driver of heterogeneity of results.\(^5\)\(^-\)\(^7\) Moreover, the molecular and cellular heterogeneity of the malignant tumors not only reasons why thereof-derived cell models, originating from a spatially isolated piece of the resection specimen, are incapable of sufficiently recapitulating tumor properties,\(^8\) but also makes it virtually impossible to isolate and present a functional model for the cell of origin for the entire tumor. Those fundamental disease modeling aspects have been proposed to be contributing factors to the so-called “reproducibility crisis in cancer research,” which is suggested to be a cardinal factor for the ethical and economical dilemma of low frequency of successful translation of preclinical cancer research findings into clinical use.\(^9\)\(^-\)\(^11\)

With the technological advancements in cell biology and cell engineering, the recent years documented the emergence of alternative tumor modeling procedures avoiding the use of patient material.\(^12\) As such, hiPSCs derived from healthy donors present an unlimited resource of cells to serve as receiver matrices for genetic elements encoding for cancer-relevant transformations. Such healthy donor-derived tumor models – herein referred to as hiPSC-oncogene models – manifest themselves as a sustainable alternative disease modeling strategy in the current cancer research community,\(^13\)\(^-\)\(^18\) complementing the portfolio of lab tools alongside the use of PD systems. It is speculated that iPSC models of cancer, derived through synthetic approaches or reprogramming of tumor cells, may be beneficial to homogeneously depict a cell of origin of cancers.\(^12\)\(^,\)\(^19\) Moreover, we have recently shown that hiPSC-oncogene models present a functional in vitro platform suitable for long-term storage while ensuring longitudinal reproducibility for in vitro substance testing.\(^20\)

This work presents the generation, characterization, and application of isogenic hiPSCs with overexpression of different pan-cancer relevant oncogenes including tumor protein S3 (TP53) R175H, glioma-associated oncogene 1 (GLI1), c-MYC, and epidermal growth factor receptor variant III (EGFRvIII) and their subsequent differentiation in tissue-specific progenitor cells. Given that malignant brain tumors present a disease class with clinical unmet needs, we chose to differentiate the hiPSC-oncogene models into the neural lineage (referred to as neural progenitor cells/ NPCs) as our example for application. The identification of biomarker-related differences in resistance to chemotherapies indicates the potential of our functional systems for early-stage drug development or the personalized medicine market.

2 | EXPERIMENTAL SECTION

2.1 | Cell culture

The hiPSC line IPS11 was obtained from Alstem Inc. (CA, USA) and provided with accompanied cell line characterization (early 2019). Cells were cultured as previously published.\(^21\) Details are described in the supplementary material. Single-cell splitting was performed for propagation and setup of functional assays and is described in detail in the supplementary materials. Cellular viability was measured using the thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich \#M2128, MO, USA) assay as previously described.\(^22\) Neural differentiation was conducted via 3D differentiation protocol as described elsewhere.\(^23\) The generated free-floating 3D-spheres were cultured in poly-(2-hydroxyethyl methacrylate) (polyHEMA)coated dishes with neural proliferation medium (DMEM and Ham’s F12 in a 2:1 (v:v) ratio; 2% B27; 1% Penicillin/Streptomycin Gibco, Invitrogen) containing 20 ng per ml FGF and 20 ng per ml EGF (Peprotec, Germany). Proliferating NPCs at a diameter of 300–500 µm were cut once a week into 200 × 200 µm\(^2\) using a tissue chopper to expand the culture as described before.\(^23\)
2.2 Cloning

Each vector with our genes of interest (GOI; GLI1, c-MYC, TP53R175H, and EGFRvIII) was cloned as previously described. A detailed protocol can be found in the supplementary material.

2.3 Lentivirus production

Generation of lentiviral particles was done with a 3rd generation lentiviral packaging system as described previously and in the supplementary material.

2.4 Transduction of hiPSCs

Lentiviral transduction of hiPSCs was performed as previously published and in the supplementary material.

2.5 Western blot

Proteins were extracted from cells with a cold protein lysis buffer and a proteinase inhibitor mixture (Roche Applied Science #11697498001, Basel, Switzerland). Protein concentration was determined with the DC Protein Assay Kit (Bio-Rad Laboratories Inc., CA, USA). Protein lysates were separated on a precast gradient SDS-PAGE gel (Bio-Rad Laboratories Inc., #4561083, CA, USA) and transferred on a nitrocellulose membrane. Proteins were blocked using 5% milk (TP53R175H) or 5% bovine serum albumin (GLI1, EGFRvIII, c-MYC) in TBS-T. Primary antibodies were incubated in blocking solution on a vertical shaker at 4°C overnight. Primary antibodies were GLI1 (Cell Signaling Technologies #2643S, 1:1,000, MA, USA), c-MYC (Invitrogen #13-2500, 1:1,000, CA, USA), EGFR (Cell Signaling Technologies #2524S, 1:1,000, MA, USA), and the housekeeping gene GAPDH (ProteinTech Group Inc., #60004-1, 1:5,000, IL, USA). The next day, secondary anti-mouse and anti-rabbit antibodies conjugated with a fluorophore (LI-COR Biosciences #926-68072; #926-32211, NE, USA) or peroxidase (Jackson ImmunoResearch Laboratories Inc. #115-035-144; #115-035-003, PA, USA) were diluted 1:10,000 in the blocking reagent and incubated for 1 h at room temperature (RT). PageRuler Prestained Protein Ladder (ThermoFisher #26616) was used to verify band sizes. Proteins of transgenes run 29 kDa higher due to the EGFP-tag expression encoded for the pSin backbone. Signal was detected for fluorescence with the LI-COR Odyssey CLX Imager (LI-COR Biosciences, NE, USA) or film-based system for peroxidase-coupled antibodies to detect the chemiluminescent signal with SuperSignal West Pico (Thermo Fisher Scientific, MA, USA). The film-based system was used to detect the protein expression of c-MYC, all other proteins were detected via the fluorescence signal.

2.6 DNA methylation analysis

DNA was extracted according to the manufacturers’ instructions (DNA Blood Tissue Kit, Qiagen, Germany). Samples were diluted to a concentration of 25 ng µl⁻¹ and sent in for analysis to the German Cancer Research Center (DKFZ, Heidelberg, Germany). DNA was bisulfite converted and applied to the Infinium MethylationEPIC Array (Illumina Inc., CA, USA). Molecular subgrouping, copy number profiling, and beta-methylation were performed as described before. Principle component analysis (PCA) was conducted using Partek Genomics Suite (Partek Incorporated, MO, USA) using the covariance option, where genes with a higher variance have more influence on the clustering.

Ingenuity pathway analysis (IPA; Qiagen, Germany) was conducted using genes with probes that were unique to each sample and had differential expression (<0.3582 unmethylated and ≥0.3582 methylated) except for the TP53R175H/EGFRvIII where genes present in the single stable lines could also be in the double stable line. IPA was run with default settings except for the knowledge base where high-confidence predictions were added. The significance cut-off for IPA was set to p-value < 0.05 for identification of canonical pathways and upstream regulators. Additionally, for upstream regulators, biological drugs, all chemical and miRNA entries, were filtered out. Upstream targets were filtered to include at least three target genes to be considered for further analysis. According to instructions of application specialists of the next generation molecular cancer diagnostic platform (www.molecularneuropathology.org), one biological replicate/condition was submitted for DNA-methylation assessment. The relevant was uploaded in NCBI GEO with timestamped metadata description and could be made available upon request.

2.7 Gene expression analysis

Total RNA of three biological replicates was extracted using the RNA Spin Column Extraction Kit (Macherey-Nagel, Germany) and transferred to the Biological–Medical Research Center (BMFZ) of the Heinrich–Heine-University Düsseldorf, Germany. RNA was prepared according to the manufacturer’s instructions using the VAHTS Stranded mRNA-Seq Library Prep Kit for Illumina V2 (Vazyme, China). A 300 ng of total RNA was used for the preparation and library amplification. For sequencing, the HiSeq 3000 platform (Illumina Inc., CA, USA) was used. Each sample was sequenced with 2 × 151 base pairs (bp) paired-end read and with at least 50 million reads. Results were analyzed using R software. All samples were analyzed compared to the empty vector (EV) control using the unpaired student’s t-test. Additionally, the fold change was calculated with the ratio of the average gene expression. Significantly increased genes were described with a p-value < 0.05 in t-test and fold change values > 2. Decreased genes had a p-value of < 0.05 and fold changes < 0.05. The results of the differentially expressed genes were displayed in volcano plots drawn in R. The significantly increased or decreased genes obtained from the volcano plot analysis were analyzed using the online tool DAVID.
Bioinformatics Resources 6.8. If Homo sapiens was selected as background. The obtained gene ontology (GO) terms were sorted in ascending order of p-value. The relevant was uploaded in NCBI GEO with timestamped metadata description and can be made available upon request.

2.8 | Flow cytometry analysis

Stem cell marker expression on the cell was evaluated using the BD Stemflow Human Pluripotent Stem Cell Transcription Factor Analysis Kit (Becton, Dickinson and Company #560589, CA, USA). The detailed protocol could be found in the supplementary material. For NPC, spheres were chopped to 0.1 mm and singularized using Accutase. In brief, 5 × 10^5 cells were stained each using the following antibodies: PerCP-Cy 5.5 Mouse anti-Oct3/4 (BD, 51-9006267), PE Mouse anti-Human Pax-6 (BD, 561552), Alexa Fluor 647 Mouse Anti-Nestin (560341), PerCP-Cy 5.5 Mouse IgG1, K Isotype Control (BD, 51-9006267), PE Mouse IgG2a, k Isotype Control (BD, 558595), and Alexa Fluor 647 Mouse IgG1 K Isotype Control (BD, 557732). Assessment of cell viability included live staining of the cells using the fixable viability stain (Fvs) 510 (#564406; BD, USA). Briefly: Cells were stained with Fvs 510 for 15 min at room temperature. Then, cells were washed using BD Staining buffer (#554656). After washing, cells were fixed in BD Cytofix fixation buffer (#554655) for 20 min at room temperature. Then, per cells were permeabilized using cold BD Phosflow Perm Buffer III (#558050) for 30 min on ice, before staining for Oct3/4-PerCP, Pax6-PE, and Nestin-Alexa 647 and respective isotype controls for 30 min at room temperature in BD Stain buffer. Cells were washed and resuspended in staining buffer and analyzed using a BD FACS CantoTM II system using BD FACS Diva Software Version 6.1.3.

2.9 | Immunocytochemistry

Immunocytochemical (ICC) staining of hiPSC was performed using the stem cell markers Alexa Fluor 555 OCT3/4 (BD Bioscience #560306, CA, USA) and Alexa Fluor 647 TRA-1-60 (BD Bioscience #561552, CA, USA) in a dilution of 1:50. Each hiPSC model, reaching 80%–90% confluency in a 6 well plate, was split in a 1:10 ratio in 96 wells of a 96-well plate. Cells were cultivated with daily medium change and fixed with 4% paraformaldehyde (PFA) at 37°C for 30 min when distinguished colonies were still visible (≥40%–50% confluency). Fixed cells were stored in DPBS −/− at 4°C in the dark until the staining was started. At first, cells were washed three times using 100 µl per well phosphate buffer saline (PBS) and permeabilized using 0.1% Triton X-100 diluted in PBS. After blocking with 10% goat-serum (Merck #G9023, Germany) in PBS, conjugated antibodies against OCT3/4 and TRA-1-60 and 1% Hoechst33258 (100 µl per well; Sigma Aldrich, Germany) were incubated overnight at 4°C. The next morning, antibodies were discarded and cells were washed three times with 100 µl PBS per well. Marker expression was detected in the Cellomics ArrayScan VTI (Thermo Scientific, MA, USA) with the Photometrics X1 camera (Thermo Scientific, MA, USA). Pictures were merged and brightness was adapted in ImageJ.

2.10 | Cytogenetic analysis of generated hiPSC-oncogene models

Karyotype analysis of wildtype (WT) and generated hiPSC-oncogene models was performed at the Institute of Human Genetics at the University Hospital Düsseldorf, Germany as described in Tigges et al. 21

2.11 | In vitro pharmacology

Prominent FDA-approved drugs used in cancer therapy were selected (Table S2) to validate the utility of the hiPSC-oncogene models and thereof derived NPCs to have utility in functional assays. Most drugs function as inhibitors to prevent signal transduction or cell proliferation. As a positive control, staurosporine was used and paracetamol, a well-known pain medication, was used as a negative control. All drugs were applied in serial dilution yielding the following concentrations: 20 µM, 2 µM, 200 nM, 20 nM, 2 nM, 200 pM, and 2 pM. Cell viability of technical triplicates in three biological replicates was assessed, similar as conducted before. 24

For hiPSC: Viability was measured after 48 h of incubation time using MTT as described above. Normalization of results was done in reference to DMSO-treated hiPSCs and the two lowest concentrations. 28 Drugs were analyzed for their efficiency based on the effective concentration 50 (EC50) using the GraphPad Prism 8 software (GraphPad Software, CA, USA). The upper threshold was set to the value of 100 for subsequent curve-fitting calculations. The evaluation of the in vitro drug response experiments resulted in the stratification of the substances according to their average efficacy over all models, namely good, medium, and low efficacy (Table S2).

For NPCs: Spheres were dissociated using TrypLE Express (Gibco, Thermo Fischer, Germany). Briefly, the spheres were washed once with PBS and dissociated using 500 µl TrypLE for up to 3 minutes. Cells were washed with a 5 ml neural proliferation medium and quantified using Trypan blue exclusion assay. To perform the drug screen, 5000 cells per well were seeded in 30 µl of the aforementioned medium into 384 well plates and treated with drugs based on the results in the hiPSC-screen (analysis was focussed on the substances that showed altered effectivity when introducing oncogene overexpression). Substance effects on cell viability were assessed after 72 h drug exposure using luminescence-based CellTiterGlo (Promega, Germany) as previously described. 29

2.12 | RT-qPCR

The analysis of gene expression was conducted using an SYBR green-based method on BioRad instrumentation. Details on the method, comprising RNA extraction, cDNA synthesis, and PCR were described in
FIGURE 1  Verification of lentiviral transduction via Western blot. Protein lysates were analyzed for expression of GLI1 (A+D), c-MYC (B+E), as well as TP53 and EGFRvIII (C+F). Expression was validated in comparison to the protein expression of GAPDH for GLI1 (D), c-MYC (E), and TP53R175H/EGFRvIII (F). Representative pictures of the Western blots are shown which were also used for the quantification. WT, wild type; EV, empty vector; GLI1, glioma-associated oncogene 1; TP53, tumor protein 53; EGFRvIII, epidermal growth factor receptor variant III.

more detail before. Primers used can be found in Table S3. The fold change in gene expression was calculated using the delta-delta method \((-\Delta\Delta Ct)\) and statistical analysis was performed using GraphPad Prism 8 software and Microsoft Excel. As a statistical test, one-way ANOVA was performed.

3 | RESULTS

3.1 | Generation of hiPSC lines with stable oncogene overexpression

Vectors for the 3rd generation lentiviral packaging system were cloned and Sanger sequencing verified successful integration of the GOI. Positive sequenced vectors were used for the lentivirus production and transduction of hiPSCs. Successful integration was verified by protein expression of the target genes via western blot which confirmed overexpression of GLI1, c-MYC, TP53, and EGFRvIII (Figure 1A–C).

Expression of the respective oncoproteins was quantified in comparison to the housekeeping protein GAPDH and normalized to the expression in the empty vector (EV) control. Quantification revealed a three- and five-fold increase for the expression of GLI1 and the GLI1 isoform 1 (respectively, Figure 1A and D). Overexpression of c-MYC (Figure 1B) was 38-fold compared to IPS11 EV (Figure 1E). The created single mutation lines TP53R175H and EGFRvIII as well as the double mutation line of TP53R175H/EGFRvIII displayed overexpression of both, TP53 and EGFRvIII (Figure 1C and F). For the single mutation, the increase of TP53 was two-fold, while expression of TP53 in the double mutation was nearly 7.5-times as high as in the TP53R175H model alone (Figure 1F). Expression of EGFRvIII in the single mutation is about 20-fold increase, in the double mutation we reached up to 350-fold increase, (Figure 1F). Viability assessment over 4 days in culture with all models compared to WT and EV control revealed no significant changes in viability (Figure S1). Figure 1 shows representative western blots of our assessments.

3.2 | Expression of stem cell markers by hiPSC-oncogene models

To exclude an effect of the introduced oncogenes on hiPSCs pluripotency, respective stem cell marker expression was analyzed using FACS analysis and ICC staining. For FACS analysis we used the common stem cell markers NANOG, SOX2, and OCT3/4, all of which are transcription factors that are expressed in hiPSCs and have been termed the “core” pluripotency factors in human embryonic stem cells. This combination of markers has been widely used to characterize iPSCs.

All cell lines expressed the analyzed markers over the threshold of 70%, in detail: IPS11 c-MYC and IPS11 TP53R175H/EGFRvIII had the lowest expression of NANOG/SOX2 with 78.16% and 75.78%, respectively (Table 1). For the gate of SOX2/OCT3/4, the lowest expression was detected in the double mutation with TP53R175H/EGFRvIII with 84.22%. All other models reached an expression of over 92% (Table 1).

Further, stem cell marker expression of TRA-1-60 and OCT3/4 was analyzed by ICC staining. For all analyzed hiPSC lines, WT, and generated models, we detected an expression of both stem cell markers, although in the EV and EGFRvIII models the staining was less intense (Figure 2). Overall, based on the FACS and ICC staining results, oncogene overexpression does not seem to influence the stemness characteristics of the generated hiPSC-oncogene models. In addition, we confirmed a normal male karyotype of all models using classical G-banding.
TABLE 1  Stem cell marker expression of hiPSC-oncogene models

| model          | NANOG/SOX2 | SOX2/OCT3/4 |
|----------------|------------|-------------|
| iPS11-EV       | 94.45      | 93.84       |
| iPS11-c-MYC    | 78.16      | 92.06       |
| iPS11-GLI1     | 90.98      | 94.38       |
| iPS11-TP53R175H| 91.37      | 94.97       |
| iPS11-EGFRvIII | 86.39      | 92.36       |
| iPS11-TP53R175H/EGFRvIII | 75.78 | 84.22 |

All models were stained against NANOG, SOX2, and OCT3/4. Gates were set for NANOG/SOX2 and SOX2/OCT3/4. Expression of markers was measured via flow cytometry and indicates the pluripotency of the hiPSC models. All values are listed in % of viable cells. Exemplary dot blots of the data acquisition are presented in Supplementary files.

thereby confirming the chromosomal integrity of the models (Figure S2). FACS dot blots of example rounds of staining of hiPSC-oncogene models are provided in Figure S8.

3.3 DNA-methylation analysis of hiPSC models

To further characterize the generated hiPSC-oncogene models, we conducted methylome analyses. The methylome profiles of all models were filtered to identify uniquely methylated (<0.3582) and unmethylated (≥0.3582) areas. In the hierarchical clustering, each of the hiPSC-oncogene models revealed a unique methylation profile (Figure 3A). The controls, WT and EV, had a different methylation profile than the generated hiPSC-oncogene models (Figure 3A). Models with the expression of TP53R175H and EGFRvIII were clustered together as well and displayed the highest difference toward the control models (Figure 3A).

In accordance with the hierarchical cluster, small variations were identified between the TP53R175H, EGFRvIII, and TP53R175H/EGFRvIII models in the PCA (Figure 3B). This analysis was conducted to identify epigenetic variations between the hiPSC-oncogene models. The EV control had the highest epigenetic variation and clustered the furthest away from all other models (Figure 3B). In addition, we performed the Qiagen IPA to identify upstream targets of the respective hiPSC-oncogene models and canonical pathways using the genes with uniquely (un)methylated probes. For the TP53R175H, EGFRvIII, and TP53R175H/EGFRvIII models we did not apply the filter of uniquely regulated genes as genes of the single stable lines could also be expressed in the double stable line. Upstream targets were analyzed for each hiPSC-oncogene model and genes which interact with our introduced oncogenes were detected (Figure 3C). Details of strongest dysregulated signaling pathways and associated upstream gene targets, as well as overrepresented gene ontologies upon individual oncogene activation, can be found in Figures S3–S5.

3.4 Transcriptome analysis of hiPSC models

Compared to EV control, all hiPSC-oncogene models had ≈30–40 genes that are either up- or downregulated except for the EGFRvIII model, in which we identified around 60–80 differentially regulated genes (Figure 4A). For each of the GLI1, c-MYC, and EGFRvIII models we detected one highly significantly up-regulated gene, which was the expression of TP53R175H and EGFRvIII were clustered together as well and displayed the highest difference toward the control models (Figure 3A).

In accordance with the hierarchical cluster, small variations were identified between the TP53R175H, EGFRvIII, and TP53R175H/EGFRvIII models in the PCA (Figure 3B). This analysis was conducted to identify epigenetic variations between the hiPSC-oncogene models. The EV control had the highest epigenetic variation and clustered the furthest away from all other models (Figure 3B). In addition, we performed the Qiagen IPA to identify upstream targets of the respective hiPSC-oncogene models and canonical pathways using the genes with uniquely (un)methylated probes. For the TP53R175H, EGFRvIII, and TP53R175H/EGFRvIII models we did not apply the filter of uniquely regulated genes as genes of the single stable lines could also be expressed in the double stable line. Upstream targets were analyzed for each hiPSC-oncogene model and genes which interact with our introduced oncogenes were detected (Figure 3C). Details of strongest dysregulated signaling pathways and associated upstream gene targets, as well as overrepresented gene ontologies upon individual oncogene activation, can be found in Figures S3–S5.

FIGURE 2 Immunocytochemical staining of hiPSC-oncogene models. Generated hiPSC models were stained with stem cell markers to verify their stemness properties using antibodies against TRA-1-60 (red) and OCT3/4 (green), nuclei were counterstained with Hoechst 33258 (blue). Six technical replicates were stained for each model. One representative hiPSC colony is displayed for each model. The scale bar is 250 µm. Pictures were evaluated with ImageJ.
FIGURE 3  Hierarchical and PCA clustering and identification of upstream targets. Methylation profiles of the samples were compared to each other for uniquely regulated genes and visualized in a hierarchical cluster using Partek Genomics Suite (Partek Incorporated, MO, USA; (A). Uniquely regulated genes were identified by comparison of the generated models to the iPS11 EV line. TP53R175H, EGFRvIII, and TP53R175H/EGFRvIII were analyzed together. Further, principal component analysis (PCA) was performed and the generated hiPSC-oncogene models were clustered using Partek Genomics Suite (Partek Incorporated, MO, USA; (B). Methylation and PCA clustering were analyzed using the ingenuity pathway analysis (IPA; Qiagen, Germany) to identify upstream targets (C). TP53R175H, EGFRvIII, and TP53R175H/EGFRvIII were analyzed together as the genes were introduced in a single- and double-mutation cell line. For each model, we display the three upstream targets based on the number of target genes. The significance cut-off value for IPA was set at $p < 0.05$. One biological replicate was analyzed for each model.

introduced oncogene of the respective model, indicating a successful gene integration and regulation (as defined by negative log $p$-value $> 4$ as compared to EV control model; Figure 4A). The double construct TP53R175H/EGFRvIII was the only model which had one gene (complement C7) highly significantly down-regulated (as defined by negative log $p$-value $> 4$ as compared to EV control model). We further evaluated the significantly regulated genes using GO enrichment analysis (Figure 4B). Transcriptomic changes in our hiPSC-oncogene models feature expression changes that have been described before in association with the introduced oncogenes. We did not detect any significantly enriched GO terms in the WT cells compared to the EV control.

3.5  | Lineage differentiation

A central verification for the suitability of our disease modeling attempt is the ability of hiPSC oncogene models to differentiate into lineage-committed progenitors, as oncogenes acquire their tumorigenic potential in the context of tissues and somatic cells. Table 2 shows the reduction of pluripotency marker and induction of NPC markers upon application of differentiation protocol verifying the maintenance of differentiation potential of our engineered models. FACS dot blots of example rounds of staining NPCs are provided in Figure S8.
FIGURE 4  Volcano plot and enrichment analysis for transcriptome analysis in hiPSC-oncogene models. A volcano plot was done for all models and results of individual models were compared to the empty vector (EV) control (A). Based on the RNA transcriptome sequencing, we analyzed the significance (unpaired student’s $t$-test, $p$-value < 0.05) and fold change. Genes are either significantly increased (fold change > 2, red), decreased (fold change < 0.5, blue) or not regulated (< 2 and > 0.5, gray). The $y$-axis indicates the significance of the gene regulation. A volcano plot was created with R. Arrows are used to mark the respective highest specific upregulated genes. Gene ontology (GO) terms were analyzed for the significantly regulated genes from the volcano plot. GO terms were analyzed by using the online tool DAVID Bioinformatics Resources 6.8 (B). Results were sorted in ascending order and the first three significant GO terms for each hiPSC-oncogene model are displayed. No significant GO term could be identified for the WT in comparison to the empty vector control (EV). The gene count is visualized using the columns and the $p$-values using dots.
TABLE 2  Expression of stem cell marker OCT3/4 and neural progenitor markers PAX6 and Nestin on lineage-committed progenitor cells (NPCs) derived from oncogene overexpressing hiPSCs

| model               | PAX6 | OCT3/4 | Nestin |
|---------------------|------|--------|--------|
| iNSP-EV             | 50.8 | 7.9    | 78.4   |
| NPC-c-Myc           | 68.8 | 7.6    | 89.2   |
| NPC-Gli1            | 26.5 | 74.2   | 97.4   |
| NPC-TP53R175H       | 62   | 58.2   | 94.5   |
| NPC-EGFRvIII        | 50.5 | 15.3   | 91.8   |
| NPC-EGFRvIII/TP53R175H | 47.3 | 33.8   | 88.1   |

Expression of markers was measured via flow cytometry and indicates the lineage commitment of the models. All values are listed in % of viable cells. Exemplary dot blots of the data acquisition are presented in Supplementary files.

3.6 Molecular characterization of progenitor cells

To validate the lineage commitment we chose the current state-of-the-art DNA-methylation-based molecular cancer diagnostics used in clinical pathology. Figure 5 shows a clear separation of hiPSC models (due to their monolayer growth designated as 2D) compared to their lineage differentiated counterparts (due to their spheroidal growth designated as 3D). Hierarchical clustering (Figure 5A) and principal component analysis (PCA, Figure 5B). Figure 5C shows the continuous overexpression of the relevant oncogenes in the progenitors derived from the respective hiPSC model. This data validates the functionality of the basic concept of our method and indicates its versatility as a cell platform to generate modularly altered and tissue-specific cell systems derived from a highly homogenous population of cells of origin.

3.7 Biomarker overexpression causes specific and comparable alterations in drug resistance in pluripotent and differentiated cells

Analyses of the resulting concentration-response curves reveal that WT and EV controls in the hiPSC models exert similar responses toward the drug treatments (Table 3, Figure S6). The highest efficacy on reducing cell viability was vinblastine sulfate (EC50 values between 0.0001 and 0.0008 µM) followed by panobinostat (EC50 values between 0.02 and 0.14 µM; Table 3; Figure S6). While a similar pattern of stress resistance to the kinase inhibitor panobinostat in EV/WT models was detected, we found an about 5-fold higher resistance in the GLI1 and a 7-fold higher resistance in the TP53R175H/EGFRvIII model. Likewise, resistance to the prominent, cell cycle progression targeting cancer drug vinblastine sulfate was increased in the GLI1-

FIGURE 5 Molecular characterization of lineage differentiated progenitors. (A+ B) DNA methylation patterns of hiPSC models (grown in 2D) are compared to differentiated counterparts differentiated (neural progenitors, grown as 3D spheres). Hierarchical clustering (A) and PCA (B) of the DNA-methylome of the cells. (C) RT-qPCR-based validation of overexpression of oncogenes EGFR, p53, and Gli1 in the 3D differentiation-derived neural progenitors
### TABLE 3  In vitro pharmacology response in hiPSC-oncogene models

| Substance          | iPS11-WT | iPS11- EV | iPS11-GLI1 | iPS11-cMYC | iPS11-TP53R175H | iPS11-EGFRvIII | iPS11–EGFRvIII/TP53R175H |
|--------------------|----------|-----------|------------|------------|-----------------|----------------|--------------------------|
| Panobinostat       | 0.0228   | 0.0222    | 0.1085     | 0.0531     | 0.0207          | 0.0290         | 0.1401                   |
| Vinblastine sulfate| 0.0003   | 0.0001    | 0.0072     | 0.0006     | 0.0001          | 0.0008         | 0.0005                   |
| Apatinib mesylate  | X        | X         | X          | X          | X               | X              | X                        |
| Lomustine          | 10.16    | 16.76     | X          | 19.54      | 17.33           | 14.25          | X                        |
| Duvelisib          | 7.9080   | 5.8080    | 16.21      | 13.03      | 6.1720          | 5.3680         | 6.2790                   |
| Cariprazine        | X        | X         | X          | X          | X               | X              | X                        |
| Calcium folinate   | X        | X         | X          | X          | X               | X              | X                        |
| Rivastigmine       | X        | X         | X          | X          | X               | X              | X                        |
| Almotriptan malate | X        | X         | X          | X          | X               | X              | X                        |
| Paracetamol        | X        | X         | X          | X          | X               | X              | X                        |
| Staurosporine      | 0.3972   | 0.1621    | 0.3872     | 1.4940     | 0.1951          | 0.2240         | 0.3585                   |

All results were analyzed and effective concentrations (EC) 50 of all drugs were calculated using GraphPad Prism 8 (GraphPad Software, CA, USA). Cell viability results were normalized to DMSO treated hiPSCs and the two lowest concentrations. EC50 values are listed in µM and “X” indicates that an EC50 was not reached. WT, wild type; EV, empty vector; GLI1, glioma-associated oncogene 1; TP53, tumor protein 53; EGFRvIII, epidermal growth factor receptor variant III.

### TABLE 4  In vitro pharmacology response in NPC models

| Substance          | NPC WT | NPC -EV | NPC -GLI1 | NPC -c-MYC | NPC - TP53R175H | NPC -EGFRvIII | NPC - EGFRvIII/TP53R175H |
|--------------------|--------|---------|-----------|------------|-----------------|---------------|--------------------------|
| Panobinostat       | 0.05164| 0.2940  | 0.4568    | 0.1237     | 0.01986         | 1.367         | 0.2856                   |
| Vinblastine sulfate| 0.01518| 0.003848| 0.4806    | 0.01955    | 0.02161         | 7.166         | 3.291                    |
| Lomustine          | 13.96  | 188.8   | 2410      | 217.10     | 191.30          | 1364          | 270                      |
| Duvelisib          | 14.04  | 75.29   | 53.4      | 48.68      | 732.90          | 1062          | 322.50                   |
| Paracetamol        | X      | X       | X         | X          | X               | X             | X                        |
| Staurosporine      | 0.1118 | 0.5545  | 0.1986    | 0.2675     | 1.657           | 1.639         | 0.1599                   |

Effective concentrations (EC) 50 of all selected substances based on the indicative drug testing results in the hiPSCs-oncogene models. Drug treatment effects on cell viability were calculated using GraphPad Prism 8 (GraphPad Software, CA, USA). Cell viability results were normalized to DMSO treated NPCs and the two lowest concentrations. EC50 values are listed in µM and “X” indicates that an EC50 was not reached. WT, wild type; EV, empty vector; GLI1, glioma-associated oncogene 1; TP53, tumor protein 53; EGFRvIII, epidermal growth factor receptor variant III.

(over 100-fold) and the EGFRvIII models (about 10-fold). Sigmoidal concentration-response curves for each hiPSC model can be found in the supplementary files (Figure S6). Importantly, a similar drug resistance pattern was observed in the differentiated NPCs (Table 4, Figure S7). Out of the experimental treatment arms, only panobinostat and vinblastine sulfate showed solid efficacy on the cells suitable for intermodel comparisons of drug resistance. Compared to EV/blank control, vinblastine resistance in Gli1 models increased about 10-fold and about 1000-fold in EGFRvIII models. Induction of Gli1 increased the resistance to panobinostat about 3-times and roughly 10-times in the EGFRvIII model. Of note, the addition of TP53R175H decreased the resistance to this drug. Taken together, this data is a proof of concept that such single cell of origin-derived, hiPSC-based cancer in vitro analogs are a suitable addition for validating biomarker-associated alterations in drug resistance identified when relying on PD cancer models only.

### DISCUSSION

PD in vitro systems represent the gold standard of functional human tumor models. Despite technical advances in culture technologies, research results relying on PD tumor models may be limited in their reproducibility and pathophysiological relevance, especially when relying on established cell lines and in the context of drug development. Together with the emergence of stem cell and genetic engineering technologies as dedicated future key innovation markets, those may be reasons why healthy donor-derived, synthetically generated in vitro cancer alternatives using stem cells penetrate the research sector as a valuable contribution to science projects. In this project, aiming toward the development of a platform technology for substance testing, we generated a collection of hiPSC-oncogene models and thereof derived lineage-committed progenitors (neural progenitor cells/ NPCs) and utilized them in identifying biomarker-associated drug resistances.
Methylome and transcriptome analyses revealed that the hiPSC-oncogene models have different expression profiles (Figures 3 and 4). Upstream target analysis of our transcriptome data identified genes, which are known to be involved in cancer development and progression. For example, TP53 was a major regulator in the models EV, EGFRvIII, TP53R175H/EGFRvIII, and TGFβ1 for EV, TP53R175H, EGFRvIII, and TP53R175H/EGFRvIII (Figure 3A). As we performed the IPA only for the significantly regulated genes and did not distinguish between up- and down-regulation, it might be possible that TP53 and TGFβ1 regulate other cellular processes in the different hiPSC-oncogene models or have different target genes. Transcriptome analysis confirmed the successful up-regulation of GLI1, c-MYC, and EGFR in our single mutation cell lines (Figure 4).

This was supported by the identification of GO terms, which are in relation to our introduced oncogenes, for example, the dorsal/ventral pattern formation (GLI1) and regulation of cell death (c-MYC and TP53R175H; Figure 4G). In summary, we can conclude that the hiPSC-oncogene models show a significant gene regulation toward the introduced oncogenes, GLI1, c-MYC, TP53R175H, and EGFRvIII. Of note, the number of significantly changed genes in the pluripotent cells models (n = 30–40, EGFRvIII models n = 40–80) is relatively low. We consider this result as a support for our approach to generate stem cell models with a limited amount of introduced “off-target” alterations, although global DNA sequencing on the cells would need to verify our hypothesis. Consequently, the synthetic models may allow functional investigation in stem cells with higher biomarker-focus as compared to similar engineered patient-derived, stem cell cultures of cancer, as they represent a cellular more heterogeneous cell matrix. However, a one-to-one comparison of transcriptomic changes in similarly engineered patient-derived cancer stem cells vs. synthetic models is needed to validate our hypothesis.

Following the confirmation transformation technology and investigating the hiPSCs molecular responses to the stimuli, we verified maintenance of stemness by functional assay of forced neural differentiation. The retrieved neural progenitors clustering distinctly stratifies the NPCs epigenome from those of their isogenic hiPSC. To pinpoint the epigenetic differences of the progenitors in dependency of the applied differentiation strategy remains the target of further investigation currently underway. These results may help to improve differentiation protocols in the future. Moreover, we cannot exclude that some of the described drug effects are a result of changes in susceptibility in response to insufficient differentiation. As such, our FACS data indicates that overexpression of GLI1 seems to reduce the differentiation capacity of hiPSCs to some extent.

The application-oriented part of our project showed the beneficial use of these models in in vitro pharmacology. We identified that GLI1 activation and TP53R175H and EGFRvIII double activation caused augmentation of therapy resistance as compared to control conditions in both the pluripotency and differentiated conditions. Most importantly, the effects were durable before and after differentiation indicating a drug discovery attempt may be valuable to conduct even before undergoing the time and resource-demanding lineage differentiation procedures. Drug resistance of stem cells in response to oncogene activation might determine the resistance of progenitor cells derived from the pluripotency stage. However, this hypothesis must be validated with repetition experiments and in the context of comparing the cell response when differentiated in different lineages respectively. In detail, decreased sensitivity of the cells was noticed to panobinostat, a histone deacetylase inhibitor market approved for the treatment of multiple myeloma, as well as for the cell cycle inhibitor vinblastine. Previously, a molecular link of vinblastine exposure and cellular transcriptional activation of TP53 has been reported, but no association to resistance in oncogenic loss of function of wild-type TP53 has been investigated. Interestingly, panobinostat was reported to block the transcription/translation of mutant TP53 in esophageal squamous cancer and preferentially reduces resistance to EGFR inhibitor treatment in lung cancer independent of EGFR mutation status. As a study in the context of colon cancer previously showed, the therapeutic potential of pharmacological HDAC inhibition (HDACi) is dependent on the TP53 mutation status of cancers and varies from the agent applied, however, no specific tests with panobinostat have been reported. Likewise, in colon cancer, GLI1-mediated resistance to pharmacological HDACi was reported. In general, little is known on the effect of the two drugs on stem cell properties of cells, especially in association with TP53/EGFR or GLI1 activation/mutation status. We previously showed that panobinostat is a lead candidate to kill neural stem cells from the fetal origin that are transformed with overexpression of dominant-negative TP53, phosphor-Akt, and c-MYC. Further drug resistance studies with GLI1/EGFRvIII knock-out stem cells, including PD tissue-specific cancer stem cells, must validate our hypothesis of those models to function as potential companion diagnostics.

We acknowledge certain technical limitations of our work, that need to be considered to critically reflect the interpretations of our results: (i) We applied a nontargeted genetic modification strategy, not appreciating the effect of random integration of our vectors in the cell genome, known to be able to cause severe undesired side effects depending on the region and quantity of integration. The use of targeted genome editing in safe harbor sites or the use of transient activation of candidate genes is desired to develop cancer cell alternatives with higher genetic control. (ii) Our proof-of-concept study ignores ethnic and gender diversity. Multiple donor-derived cell systems that underwent the same genetic modification are highly warranted to comply with current science policies and to minimize cell line-specific effects when performing experiments. Both attempts are underway in our lab.

5 | SUMMARY

We generated pluripotent isogenic human stem cells with overexpression of various pan-cancer relevant oncogenes (TP53, GLI1, c-MYC, and EGFRvIII). Those model systems allow the generation of oncogenes overexpressing tissue-specific progenitors as cancer stem cell alternatives. Utilization of our models in drug testing identified biomarker-related alterations in chemotherapy resistance. We acquired transcriptomic and epigenetic signatures that may be useful to further study the
effect of the chosen oncoproteins in the context of human stemness. Although our platform technology has several technical limitations, given its theoretic potential to be amendable in any lineage differentiation, we believe it has significant value for cancer precision medicine applications, such as the development of targeted therapies of validation of the off-target potential of drugs on noncancer stem cells. Our platform provides off-the-shelf readiness, easing possible dissemination.

ACKNOWLEDGEMENT

The authors thank T Klein, Department of Genetics, and J Gopalakrishnan, Institute of Human Genetics (all Heinrich-Heine University Düsseldorf, Germany), and J Klose (IUF Düsseldorf, Germany) for their support in this project and stimulating scientific discussions.

Open access funding enabled and organized by Projekt DEAL.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Upon request, the generated hiPSC-oncogene model systems together with their respective sequencing data will be available to the academic field under MTA regulations. OMICs data is stored in the NCBI GEO database as indicated in the respective method sections.

ORCID

Michael Hewera https://orcid.org/0000-0002-4918-2875

REFERENCES

1. Stringer, B. W., Day, B. W., D’souza, R. C. J., Jamieson, P. R., Ensbey, K. S., Bruce, Z. C., Lim, Y. C., Gosnade, K., Offenhauser, C., Akgül, S., Allan, S., Robertson, T., Lucas, P., Tolleson, G., Campbell, S., Winter, C., Do, H., Dobrovic, A., Inglis, P. L., ... Boyd, A. W. (2019). A reference collection of patient-derived cell line and xenograft models of proneural, classical and mesenchymal glioblastoma. Science Reports, 9, 4902.

2. Chabner, B. A. (2016). NCI-60 cell line screening: A radical departure in its time. JNCI: Journal of the National Cancer Institute, 108, djv388.

3. Atashzar, M. R., Baharouli, R., Karami, J., Abdollahi, H., Rezaei, R., Pourramezan, F., & Zoljalali Moghaddam, S. H. (2020). Cancer stem cells: A review from origin to therapeutic implications. Journal of Cellular Physiology, 235, 790–803.

4. Pyo, D. H., Hong, H. K., Lee, W. Y., & Cho, Y. B. (2020). Patient-derived cancer modeling for precision medicine in colorectal cancer: Beyond the cancer cell line. Cancer Biology & Therapy, 21, 495–502.

5. Ben-David, U., Siranosian, B., Ha, G., Tang, H., Oren, Y., Hinojara, K., Stratdhee, C. A., Dempster, J., Lyons, N. J., Burns, R., Nag, A., Kugener, G., Cimini, B., Tsvetkov, P., Maruva, Y. E., O’ourke, R., Garrity, A., Tubelli, A. A., Bandopadhayay, P., ... Golub, T. R. (2018). Genetic and transcriptional evolution alters cancer cell line drug response. Nature, 560, 325–330.

6. Ben-David, U., Ha, G., Tseng, Y.-Y., Greenwald, N. F., Oh, C., Shih, J., McFarland, J. M., Wong, B., Boehm, J. S., Beroukhim, R., & Golub, T. R. (2017). Patient-derived xenografts undergo mouse-specific tumor evolution. Nature Genetics, 49, 1567–1575.

7. Ben-David, U., Beroukhim, R., & Golub, T. R. (2019). Genomic evolution of cancer models: Perils and opportunities. Nature Reviews Cancer, 19, 97–109.

8. Yu, K., Chen, B., Aran, D., Charalel, J., Yau, C., Wolf, D. M., Van ‘T Veer, L. J., Butte, A. J., Goldstein, T., & Sierra, M. (2019). Comprehensive trancriptomic analysis of cell lines as models of primary tumors across 22 tumor types. Nature Communication, 10, 3574.

9. Freedman, L. P., Cabinj, M. I., & Simcoe, T. S. (2015). The economics of reproducibility in preclinical research. Plos Biology, 13, e1002165.

10. Haibe-Kains, B., El-Hachem, N., Birkbak, N. J., Jin, A. C., Beck, A. H., Aerts, H. J. W. L., & Quackenbush, J. (2014). Inconsistency in large pharmacogenomic studies. Nature, 504, 389–393.

11. Mak, I. W., Evaniew, N., & Ghert, M. (2014). Lost in translation: Animal models and clinical trials in cancer treatment. American Journal of Translational Research, 6, 114–118.

12. Mehrjardi, N. Z., Hänggi, D., & Kahlert, U. D. (2020). Current biomarker-associated procedures of cancer modeling—a reference in the context of IDH1 mutant glioma. Cell Death & Disease, 11, 998.

13. Hanaford, A. R., Archer, T. C., Price, A., Kahlert, U. D., Maciaczyk, J., Nikkhah, G., Kim, J. W., Ehrenberger, T., Clemens, P. A., Dančík, V., Seashore-Ludlow, B., Viswanathan, V., Stewart, M. L., Rees, M. G., Shamji, A., Schreiber, S., Fraenkel, E., Pomeroy, S. L., Mesirov, J. P., ... Raabe, E. H. (2016). DISCOVERing innovative therapies for rare tumors: Combining genetically accurate disease models with in silico analysis to identify novel therapeutic targets. Clinical Cancer Research, 22, 3903–3914.

14. Parnies, D., Zurich, M.-G., & Hartung, T. (2020). Organotypic models to study human glioblastomas: Studying the brain in its ecosystem. iScience, 23, 101633.

15. Sancho-Martinez, I., Nivet, E., Xie, Y., Hishida, T., Aguirre, A., Ocampo, A., Ma, L., Morey, R., Krause, M. N., Zembrzycki, A., Ansonge, O., Vazquez-Ferrer, E., Dubova, I., Reddy, P., Lam, D., Hishida, Y., Wu, M.-Z., Esteban, C. R., O’leary, D., ... Izpisua Belmonte, J. C. (2016). Establishment of human iPSC-based models for the study and targeting of glioma initiating cells. Nature Communication, 7, 10743.

16. Smith, R. C., & Tabar, V. (2019). Constructing and deconstructing cancers using human pluripotent stem cells and organoids. Cell Stem Cell, 24, 12–24.

17. Zhang, M., Vandana, J. J., Lacko, L., & Chen, S. (2020). Modeling cancer progression using human pluripotent stem cell-derived cells and organoids. Stem Cell Research, 49, 102063.

18. Uhlmann, C., Kuhn, L.-M., Tiggges, J., Fritsche, E., & Kahlert, U. D. (2020). Efficient modulation of TP53 expression in human induced pluripotent stem cells. Current Protocols in Stem Cell Biology, 52, e102.

19. Kim, J., Hoffman, J. P., Alpaga, R. K., Rhim, A. D., Reichert, M., Stanger, B. Z., Furth, E. E., Separvada, A. R., Yuan, C.-X., Won, K.-J., Donahue, G., Sands, J., Gumbs, A. A., & Zaret, K. S. (2013). An iPSC line from human pancreatic ductal adenocarcinoma undergoes early to invasive stages of pancreatic cancer progression. Cell Reports, 3, 2088–2099.

20. Khan, D., Nickel, A.-C., Jeising, S., Uhlmann, C., Mohammad, S., Hänggi, D., Fischer, I., & Kahlert, U. D. (2021). Testing the stability of drug resistance on cryopreserved, gene-engineered human induced pluripotent stem cells. Pharmaceuticals (Basel), 14, 919.

21. Tiggges, J., Bieleck, K., Brockerhoff, G., Hildebrandt, B., Hübenthal, U., Kap, J., Koch, K., Teichweyde, N., Wieczorek, D., Rossi, A., & Fritsche, E. (2021). Academic application of Good Cell Culture Practice for induced pluripotent stem cells. AlTEX, 38, 595–614.

22. Kahlert, U. D., Maciaczyk, D., Doostkam, S., Orr, B. A., Simons, B., Bogiel, T., Reithmeier, T., Prinz, M., Schubert, J., Niedermann, G., Brabletz, T., Eberhart, C. G., Nikkhah, G., & Maciaczyk, J. (2012). Activation of canonical WNT/β-catenin signaling enhances in vitro motility of glioblastoma cells by activation of ZEB1 and other activators of epithelial-to-mesenchymal transition. Cancer Letters, 325, 42–53.

23. Nimtz, L., Hofrichter, M., Kabiri, Y., Egluy, J., Theiss, S., Adjaye, J., & Fritsche E. (2016). Pluripotent stem cell-derived neurospheres as an alternative in vitro method to study neurotoxic effects on multielectrode arrays. MEA Meeting 2016 | 10th International Meeting on Substrate-Integrated Electrode Arrays.
24. Koch, K., Hartmann, R., Tsiampali, J., Uhlmann, C., Nickel, A.-C., He, X., Kamp, M. A., Sabel, M., Barker, R. A., Steiger, H.-J., Hänggi, D., Willbold, D., Maciackzyk, J., & Kahlert, U. D. (2020). A comparative pharmacometabolomic study of glutamine inhibitors in glioma stem-like cells confirms biological effectiveness but reveals differences in target-specificity. Cell Death Discovery, 6, 20.

25. Capper, D., Jones, D. T. W., Sill, M., Hovestadt, V., Schrmpf, D., Sturm, D., Koelsche, C., Sahm, F., Chavez, L., Reuss, D. E., Kratz, A., Werefs, A. K., Huang, K., Pajtler, K. W., Schweizer, L., Stichel, D., Olar, A., Engel, N. W., Lindenberg, K., ... Pfister, S. M. (2018). DNA methylation-based classification of central nervous system tumours. Nature, 555, 469–474.

26. Huang da, W., Sherman, B. T., & Lempicki, R. A. (2009). Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Research, 37, 1–13.

27. Krebs, A., Nyffeler, J., Rahenfünser, J., & Leist, M. (2018). Normalization of data for viability and relative cell function curves. Altex, 35, 268–271.

28. Nickel, A. C., Picard, D., Qin, N., Wolter, M., Kaulich, K., Hgewera, M., Pauck, D., Marquardt, V., Torga, G., Muhammad, S., Zhang, W., Schnell, O., Steiger, H.-J., Hänggi, D., Frtsche, E., Her, N.-G., Nam, D.-H., Carro, M. S., Remke, M., ... Kahlert, U. D. (2021). Longitudinal stability of molecular alterations and drug response profiles in tumor spheroid cell lines enables reproducible analyses. Biomedicine & Pharmacotherapy, 144, 112278.

29. Boyer, L. A., Lee, T. I., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., Guenther, M. G., Kumar, R. M., Murray, H. L., Jenner, R. G., Gifford, D. K., Melton, D. A., Jaenisch, R., & Young, R. A. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. Cell, 122, 947–956.

30. Pamies, D., Bal-Price, A., Simeonov, A., Tagle, D., Allen, D., Gerhold, D., Yin, D., Pistollato, F., Inutsuka, T., Sullivan, K., Stacey, G., Salem, H., Leist, M., Daneshian, M., Vemuri, M. C., McFarland, R., Coecke, S., Fitzpatrick, S. C., Lakshmiopathy, U., ... Hartung, T. (2017). Good cell culture practice for stem cells and stem-cell-derived models. Altex, 34, 95–132.

31. Kandoth, C., Mclellan, M. D., Vandin, F., Ye, K., Niu, B., Lu, C., Xie, M., Zhang, Q., Mcmichael, J. F., Wyczalkowski, M. A., Leiserson, M. D. M., Miller, C. A., Welch, J. S., Walter, M. J., Wendl, M. C., Ley, T. J., Wilson, R. K., Raphael, B. J., & Ding, L. (2013). Mutational landscape and significance across 12 major cancer types. Nature, 502, 333–339.

32. Massagué, J. (2008). TGFbeta in cancer. Cell, 134, 215–230.

33. Koga, T., Chaim, I. A., Benitez, J. A., Markmiller, S., Parisian, A. D., Hevner, R. F., Turner, K. M., Hessenauer, F. M., D’antonio, M., Nguyen, N.-P. D., Saberi, S., Ma, J., Miki, S., Boyer, A. D., Ravits, J., Frazer, K. A., Bafna, V., Chen, C. C., Mischel, P. S., ... Furnari, F. B. (2020). Longitudinal assessment of tumor development using cancer avatars derived from genetically engineered pluripotent stem cells. Nature Communication, 11, 550.

34. Koch, K., Hartmann, R., Tsiampali, J., Uhlmann, C., Nickel, A.-C., He, X., Kamp, M. A., Sabel, M., Barker, R. A., Steiger, H.-J., Hänggi, D., Willbold, D., Maciackzyk, J., & Kahlert, U. D. (2020). A comparative pharmacometabolomic study of glutamine inhibitors in glioma stem-like cells confirms biological effectiveness but reveals differences in target-specificity. Cell Death Discovery, 6, 20.

35. Tishler, R. B., Lampa, D. M., Park, S., & Price, B. D. (1995). Microtubule-active drugs taxol, vinblastine, and nocodazole increase the levels of transcriptionally active p53. Cancer Research, 55, 6021–6025.

36. Cheng, Y.-W., Liao, L.-D., Yang, Q., Chen, Y., Nie, P.-J., Zhang, X.-J., Xie, J.-J., Shan, B.-E., Zhao, L.-M., Xu, L.-Y., & Li, E.-M. (2018). The histone deacetylase inhibitor panobinostat exerts anticancer effects on esophageal squamous cell carcinoma cells by inducing cell cycle arrest. Cell Biochemistry and Function, 36, 398–407.

37. Greve, G., Schifffmann, I., Pfeifer, D., Pantic, M., Schüler, J., & Lübbert, M. (2015). The pan-HDAC inhibitor panobinostat acts as a sensitizer for erlotinib activity in EGFR-mutated and -wildtype non-small cell lung cancer cells. BMC Cancer, 15, 947.

38. Sonnemann, J., Marx, C., Becker, S., Wittig, S., Palani, C. D., Krämer, O. H., & Beck, J. F. (2014). p53-dependent and p53-independent anticancer effects of different histone deacetylase inhibitors. British Journal of Cancer, 110, 656–667.

39. Falkenberg, K. J., Newbold, A., Gould, C. M., Luu, J., Trapani, J. A., Matthews, G. M., Simpson, K. J., & Johnstone, R. W. (2016). A genome scale RNAi screen identifies GLI1 as a novel gene regulating vorinostat sensitivity. Cell Death and Differentiation, 23, 1209–1218.

40. Staunstrup, N. H., Moldt, B., Mätés, L., Villesen, P., Jakobsen, M., Ivics, Z., Izsvák, Z., & Mikkelson, J. G. (2009). Hybrid lentivirus-transposon vectors with a random integration profile in human cells. Molecular Therapy, 17, 1205–1214.

41. Woods, N.-B., Muesgg, A., Schmidt, M., Flygare, J., Olsson, K., Salmon, P., Trono, D., von Kalle, C., & Karlsson, S. (2003). Lentiviral vector transduction of NOD/SCID repopulating cells results in multiple vector integrations per transduced cell: Risk of insertional mutagenesis. Blood, 101, 1284–1289.

SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.