Supplementary information for

Induced pluripotent stem cell-derived astrocytes from patients with schizophrenia exhibit an inflammatory phenotype that affects vascularization

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Cell lines

Four control iPSC cell lines were used: one from the Coriell Institute Biobank and the other three from subjects whose cells were reprogrammed at the D'Or Institute for Research and Education. To decrease genetic heterogeneity among SCZ cell lines, two of the SCZ patients were siblings (Patients 1 & 2); their cells were purchased from the Coriell Institute Biobank. The third SCZ cell line was reprogrammed at the D'Or Institute for Research and Education (Patient 3). The identities and properties of the cell lines used are summarized in Supplementary Table 1.

Supplementary Table 1. Summary of hiPSC characteristics

Cell ID	Cell Line	Gender	Age of Collection	Cell Source	Institution	Previous Publications	Use in experiments
GM23279A	CTR1	F	36y	Dermal fibroblasts	Coriell Biobank	Casas et al. (2018), Ledur et al. (2020), Trindade et al. (2020)	Figures 2-6 and Supp. Figure S1
CF1	CTR2	M	37y	Dermal fibroblasts	D'Or Institute*	Casas et al. (2018), Ledur et al. (2020), Trindade et al. (2020)	Figures 2-6 and Supp. Figure S1
CF2	CTR3	M	31y	Dermal fibroblasts	D'Or Institute*	Casas et al. (2018), Ledur et al. (2020), Trindade et al. (2020)	Figures 2-6 and Supp. Figure S1
C15	CTR4	F	16y	Urine cells	D'Or Institute*	Ledur et al. (2020), Trindade et al. (2020)	Figures 3-6 and Supp. Figure S1
GM23760B	SCZ1	M	26y	Dermal fibroblasts	Coriell Biobank	Casas et al. (2018)	Figures 2-6 and Supp. Figure S1
GM23761B	SCZ2	F	27y	Dermal fibroblasts	Coriell Biobank	Casas et al. (2018)	Figures 2-6 and Supp. Figure S1
EZQ4	SCZ3	M	42y	Dermal fibroblasts	D'Or Institute*	Casas et al. (2018)	Figures 2-6 and Supp. Figure S1

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Immunocytochemistry

For immunolabelling, hiPSC-derived astrocyte cultures were plated in treated glass coverslips, or 96-multiwell cell-carrier plates were used (PerkinElmer, USA). On both, cultures were fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.3% Triton X-100, and exposed to a blocking solution containing 2% bovine serum albumin or 5% normal goat serum (all from Sigma Aldrich, USA). Fixed cells were incubated overnight at 4°C with primary antibodies (Supplementary Table 2). Subsequently, the samples were incubated with secondary antibodies. Cell nuclei were stained with 0.5 µg/ml 4′-6-diamino-2-phenylindole for 5 min.

Supplementary Table 2. Antibodies' information

Antibody	Catalog #	Dilution	Manufacturer
Primary antibody			
mouse antiGFAP	MO1505	1:200 IF	Neuromics, Minneapolis, MN, USA
mouse anti-vimentin	MO22179	1:500 IF	Neuromics, Minneapolis, MN, USA
rabbit anti ALDH1L1	ab190298	1:1000 IF	Abcam, Cambridge, UK
rabbit anti-EAAT1	ab416	1:100 IF	Abcam, Cambridge, UK
rabbit anti-EAAT2	ab41621	1:200 IF	Abcam, Cambridge, UK
rabbit anti-connexin 43	3512	1:100 IF	Cell Signaling, Danvers, MA, USA
mouse anti-NF-κB p65	sc-8008	1:100 IF	Santa Cruz Biotechnology, Dallas, TX, USA
Primary antibody			
goat anti-mouse Alexa Fluor 488 IgG	A-11001	1:400 IF	Thermo Fisher Scientific, Waltham, MA USA
goat anti-rabbit Alexa Fluor 594 IgG	A-11037	1:400 IF	Thermo Fisher Scientific, Waltham, MA USA
goat anti-mouse Alexa Fluor 594 IgG	A-11032	1:400 IF	Thermo Fisher Scientific, Waltham, MA USA

Real-time quantitative polymerase chain reaction (RT-qPCR)

Cultures of hiPSC-derived astrocytes (2 × 104 cells/cm2 in T-75 culture flasks) were harvested 4-days after plating, with Accutase (Merck, Darmstadt, Germany) and centrifuged at 300 × g for 5 min. The supernatant was discarded, and the pellet was submitted to RNA extraction. Total RNA was isolated from the pellet with TRIzol™ (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. RNA samples were resuspended (final volume of 12 µl

nuclease-free water) and quantified by absorbance 260 nm in a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific). One microgram of total DNase-treated RNA was reverse-transcribed in a 20-µl reaction volume using M-MLV (Thermo Fisher Scientific). The resulting cDNA was diluted five-fold and RT-qPCR was performed with Eva™ Green PCR Master Mix (Biotium, Fremont, CA, USA) in a StepOnePlus™ Real-Time PCR Platform (Applied Biosystems, Waltham, MA, USA) to detect transcripts of *NFKB1, GAPDH*, and *HPRT1* (sequences are displayed in Supplementary Table 3). The primers used in this study were validated by serial dilution for efficiency calculation Efficiencies from all tested primers ranged from 90% to 110%, not separated by >10%. RT-qPCR data were calculated by the $2^{-\Delta\Delta Ct}$ method using the mean values of *HPRT1* and *GAPDH* for normalization of the results.

Supplementary Table 3. Primers' Sequences

Gene	Gene Name	Forward primer	Reverse primer
NFKB1	Nuclear Factor Kappa B Subunit 1	CCC ACG AGC TTG TAG GAA AGG	GGA TTC CCA GGT TCT GGA AAC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GCC CTC AAC GAC CAC TTT G	CCA CCA CCC TGT TGC TGT AG
HPRT1	hypoxanthine phosphoribosyl transferase 1	CGT CGT GAT TAG TGA TGA ACC	AGA GGG CTA CAA TGT GAT GGC

Multiplex analysis of cytokines

ProcartaPlex™ bead-based multiplex immunoassays were used to evaluate the astrocyte conditioned media (ACM) of 10 ng/ml TNFα or vehicle-stimulated astrocytes. The platform MAGPIX™ was used for simultaneous detection of interleukins and chemokines IL-1β, IL-2, IL-4, IL-6, IL-8, TNF-α, IL-10, IL-13, interferon (INF)-γ, and brain-derived neurotrophic factor (BDNF). Briefly, 50 µl of coated magnetic beads solution (Luminex™ Corporation) was added to each well of a 96-well plate. The loaded plates were placed onto a magnetic plate washer; the beads were allowed to accumulate on the well bottoms and then washed. We added 50-µl ACM sample aliquots or standards to the wells and left them to incubate for 2 h at room temperature

(RT) on a Capp 18-X shaking platform (500 rpm). The plates were washed and the detection antibody mixture was added and incubated with the beads at RT for 30 min on the shaking platform (500 rpm). Streptavidin solution was added to each well and incubated for 30 min (500 rpm) at RT. After washing, the reading buffer was added to the wells and incubated for 5 min (500 rpm) at RT. Data were acquired with MAGPIX™.

Sample preparation for proteomic analyses

Whole-cell content and astrocytes conditioned media (ACM) samples of hiPSC-derived astrocytes from control subjects and patients with SCZ were prepared for proteomic analyses.

For the whole-cell content, hiPSC-derived astrocyte cultures (2×10^4 cells/cm^2 in 75-cm^2 culture flasks) were harvested four days after plating with Accutase (Merck, Darmstadt, Germany) and centrifuged at 300 ×*g* for 5 min. The supernatant was discarded. The pellet was washed with PBS and stored at -80°C until used. Cell pellets were homogenized in lysis buffer (7 M urea, 2 M thiourea, 1% CHAPS, 70 mM DTT, and Roche cOmplete™ EDTA-free protease inhibitor cocktail; Sigma Aldrich, USA) for 2 h in ice, lysates were centrifuged at 10,000 ×*g* at 4°C, and supernatant samples were collected. Initial protein content was quantified using the BCA method according to the manufacturer's protocol (Bio-Rad Laboratories, USA). Samples (50 µg) were then subjected to SDS–polyacrylamide gel electrophoresis, and gel slicing followed by *in-gel* reduction, alkylation, and overnight trypsin digestion (1:100, w/w) at 37°C. The peptides obtained were extracted with 50 mM AmBic buffer and dried with a SpeedVac (Thermo Fisher Scientific, USA), and stored at −80°C prior to quantitative and qualitative analyses by shotgun mass spectrometry (MS). Biological triplicates of both control and SCZ hiPSC-derived astrocytes were analyzed.

For ACM sample preparation, as described for the Multiplex analyses, hiPSC-derived astrocyte cultures (2×10^4 cells/cm^2 in 75-cm^2 culture flasks) were serum-deprived for 24h, four days after plating. Next, 10 ng/ml TNF-α or vehicle was added for an additional 24 h. The conditioned

media were collected, aliquoted, and stored at -80°C until use. For proteomics analyses, ACM was 10-fold concentrated with AmiconUltra centrifugal filters 3,000 MWCO (Merck-Millipore, Germany). The recovered protein was quantified with the BCA method, and 100-µg samples were subjected to gel electrophoresis and protein digestion as described above. At least five biological replicates were analyzed per group (vehicle and TNF-α stimulated A_{CTR}CM and A_{SCZ}CM).

Liquid chromatography (LC)-MS

Proteomic analyses were performed using a reverse-phase LC Acquity UPLC M-Class System coupled to a Synapt G2-Si mass spectrometer (both from Waters Corporation, Milford MA, USA). Data were acquired by DIA with ion-mobility separation and high-definition data-independent MS. Whole-cell peptide loads were separated in first-dimension chromatography on an M-Class BEH C18 column (130 Å, 5 µm, 300 µm × 50 mm, Waters). Three discontinuous fractionation steps were performed (13%, 18%, and 50% acetonitrile). Peptide loads were directed, after each step, to second-dimension chromatography on a nanoACQUITY UPLC HSS T3 column (1.8 µm, 75 µm × 150 mm; Waters). Peptides were eluted directly into a Synapt G2-Si MS platform with a 36-min acetonitrile gradient (7–40%, v/v, flow rate of 0.4 µl/min). For ACM, the first dimension was skipped, and peptide loads were directed to the second-dimension column (as specified above) and eluted with a 95-min acetonitrile gradient (7–40%, v/v, flow rate 0.4 µl/min). MS/MS analyses were performed by nano-electrospray ionization in positive ion mode nanoESI (+) and a NanoLock Spray (Waters Corporation, Manchester, UK) ionization source. The lock mass channel was sampled every 30 s. Glu1-Fibrinopeptide B human solution was used to calibrate the mass spectrometer with an MS/MS spectrum reference using the NanoLock Spray source. Samples were run in biological replicates.

Data processing and quantification

High-definition data-independent MS raw files were processed for label-free identification and quantification in Progenesis® QI for proteomics version 4.0, including software Apex3D, Peptide 3D, and ion accounting informatics programs (Waters). Starting with LC-MS dataset loading, the software performed the alignment and peak detection, providing a list of peptide ions (peptides) to be explored with multivariate statistical methods in Peptide Ion Stats. Finally, proteins were identified with dedicated algorithms and cross-matched with Uniprot human proteome database (version 2018/09); default parameters for ion accounting and relative quantitation with the Hi-N (3) method of peptide comparison were used [1,2]. Reversed database queries were used to remove false positives. Protein/peptide level FDR was 1%. Digestion by trypsin allowed a limit of one missed cleavage. Methionine-oxidations and carbamidomethylation were considered variable and fixed modifications, respectively. Identifications not satisfying these criteria were rejected. The proteomic search data are provided in Supplementary Tables 4 and 5.

References

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2. Silva JC, Gorenstein MV, Li G-Z, Vissers JPC, Geromanos SJ. Absolute Quantification of Proteins by LCMS E. Molecular & Cellular Proteomics. 2006 Jan 3;5(1):144–56.

Supplementary Legends

Supplementary Table 4. Proteomics data of whole-cell hiPSC-derived astrocytes from control and schizophrenia. (Excel file)

Supplementary Table 5. Proteomics data of conditioned media of hiPSC-derived astrocytes from control and schizophrenia stimulated or not with TNF-α. (Excel file).

Supplementary Figure

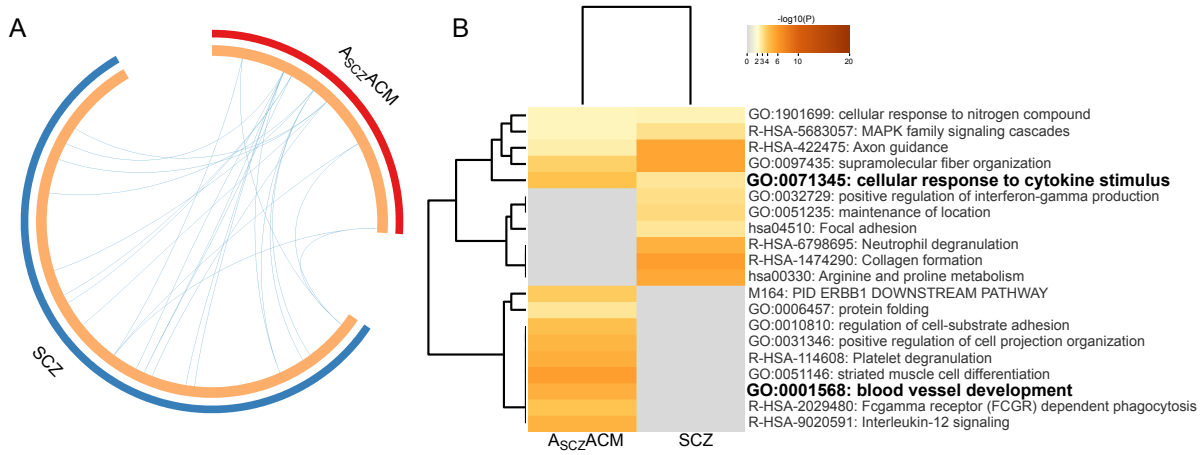

Figure S1: Compared regulation of the proteome and secretome of hiPSC-derived astrocytes in schizophrenia versus controls reveal similar cytokine influence. (A) The circus plot shows the overlap of pathways enriched by significantly regulated proteins ($p<0.05$) in SCZ versus CTR of hiPSC-derived astrocytes proteome and secretome. (B) Enrichment terms heatmap (GO/KEGG terms and canonical pathways) based on significantly regulated proteins ($p<0.05$) in Log2 Fold change of SCZ versus CTR of hiPSC-derived astrocytes respective proteome (shown as SCZ), and secretome (shown as AsczCM). For whole-cell proteome, CTR n = 3 independent cell lines and SCZ n = 3 independent cell lines were used. For secretome $A_{CTR}CM$ n = 7 CM collected, n = 3 independent CTR lines, and $A_{SCZ}CM$ n = 5 CM collected, n = 4 independent SCZ lines. Color represents cluster p-values, and white cells indicate a lack of enrichment for that term in the corresponding list.