Cellular and molecular characterization of multiplex autism in human induced pluripotent

stem cell-derived neurons

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Abstract

Background: Autism spectrum disorder (ASD) is a neurodevelopmental disorder with pronounced heritability in the general population. This is largely attributable to effects of polygenic susceptibility, with inherited liability exhibiting distinct sex differences in phenotypic expression. Attempts to model ASD in human cellular systems have principally involved rare *de novo* mutations associated with ASD phenocopies. However, by definition, these models are not representative of polygenic liability, which accounts for the vast share of population-attributable risk.

Methods: Here, we performed what is, to our knowledge, the first attempt to model multiplex autism using patient-derived induced pluripotent stem cells (iPSCs) in a family manifesting incremental degrees of phenotypic expression of inherited liability (absent, intermediate, severe). The family members share an inherited variant of unknown significance in *GPD2*, a gene that was previously associated with developmental disability but here is insufficient by itself to cause ASD. iPSCs from three first-degree relatives and an unrelated control were differentiated into both cortical excitatory (cExN) and cortical inhibitory (cIN) neurons, and cellular phenotyping and transcriptomic analysis were conducted.

Results: cExN neurospheres from the two affected individuals were reduced in size, compared to those derived from unaffected related and unrelated individuals. This reduction was, at least in part, due to increased apoptosis of cells from affected individuals upon initiation of cExN neural induction. Likewise, cIN neural progenitor cells from affected individuals exhibited increased apoptosis, compared to both unaffected individuals. Transcriptomic analysis of both cExN and cIN neural progenitor cells revealed distinct molecular signatures associated with affectation, including misregulation of suites of genes associated with neural development, neuronal function, and behavior, as well as altered expression of ASD risk-associated genes.

Conclusions: We have provided evidence of morphological, physiological, and transcriptomic

signatures of polygenic liability to ASD from an analysis of cellular models derived from a

multiplex autism family. ASD is commonly inherited on the basis of additive genetic liability.

Therefore, identifying convergent cellular and molecular phenotypes resulting from polygenic

and monogenic susceptibility may provide a critical bridge for determining which of the disparate

effects of rare highly deleterious mutations might also apply to common autistic syndromes.

Keywords: Multiplex autism, iPSC modeling, neurodevelopment, cortical excitatory neurons,

cortical inhibitory neurons, transcriptomics, gene networks.

Background

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with a complex and poorly understood etiology (1-3). Behavioral and imaging studies have been valuable for defining deficits in affected individuals and characterizing alterations at the level of the brain. However, we are extremely limited in our ability to acquire or experimentally manipulate human brain tissue from living patients or post-mortem brain slices. This has hampered efforts to study cellular and molecular abnormalities that accompany ASD, both during and after fetal and post-natal development. Notably, both the relative integrity of brain structures in affected individuals and the diversity of ASD genetics suggest that convergent mechanisms that contribute to affectation in ASD may operate at the level of the cell (3, 4). These may be identifiable in experimental models derived from affected individuals. In particular, ASD appears to frequently involve abnormal development and/or function of two major classes of neurons in the cerebral cortex, glutamatergic excitatory projection neurons (cExNs) and GABAergic inhibitory interneurons (cINs) (3, 5, 6). *In vitro* differentiation-based models of these neuronal cell types can identify cellular and molecular deficits associated with ASD, and provide a tractable platform to screen for pharmacologic agents that can rescue these deficits.

In recent years, such cellular models of ASD have been generated either by deriving induced pluripotent stem cell lines (iPSCs) from affected individuals or by using CRISPR/Cas9-based gene editing to engineer ASD-associated mutations into wild type PSCs (7-19). Most of these studies have focused on syndromic forms of ASD, or on monogenic, *de novo* cases, where causality is attributed to mutation of a single ASD-linked gene (10, 11, 13-16). These forms of ASD are attractive for cellular modeling, as they streamline study design and reduce many potential confounding variables. Other studies have included individuals with an unknown genetic cause of ASD, but with subject selection based upon a shared phenotypic characteristic, such as macrocephaly (18-20). Together, these models have been informative, revealing both

cellular and molecular alterations associated with affectation. These include shared and model-

specific disruptions of gene expression in ASD-derived neurons, frequently involving altered

expression of genes in key developmental signaling pathways, and genes that control cellular

proliferation and growth (7, 8, 13-20). In addition, differences were observed in neural precursor

cell (NPC) proliferation and differentiation (9, 19), neurogenesis, (9, 11, 18, 20), synaptogenesis

(8, 10, 17, 18), or functional neuronal activity (7-9, 11, 16, 17). Altered expression of ASD

genes, in which a mutation is linked to ASD causation or risk, is also frequently observed (7, 8,

13-16, 18-20).

These cellular modeling studies have revealed potential contributors to affectation and,

in some cases, have identified targets amenable to pharmacological rescue *in vitro* (10, 18).

However, they do not encompass the range of contributors to ASD burden in the general

population: no single gene mutation accounts for more than 1% of overall ASD cases with

predicted monogenic causality (21), while the majority of genetic risk appears to be polygenic or

idiopathic (2, 22-28). Polygenic ASD risk can involve both common and rare variation in protein-

coding and non-coding regions of the genome, which may act in a combinatorial manner (2, 27,

29, 30). Furthermore, ASD exhibits pronounced heritability in families (estimated at 50-90%

(31)), none of which can be accounted for by *de novo* (germline) events. Even within each

multiplex ASD family, there is often a considerable range in the extent of affectation among

individuals, and in most multiplex autism families, a single causative gene mutation usually

cannot be identified (31).

While ASD burden in the general population predominantly involves polygenic or

idiopathic risk, heritability, and variable affectation (2, 21, 27, 29-31), these genetically complex

forms of the disorder have been largely neglected in cellular modeling studies. Therefore, we

deemed it important to determine whether cellular modeling of these complex but prevalent

forms of ASD could also reveal affectation-related deficits. To test this, we focused on a

multiplex ASD family with variable affectation among family members. We generated iPSC lines

from three first degree relatives (a male and a female with differing degrees of affectation and

their unaffected mother) as well as an unrelated unaffected female. We used these lines to

perform differentiation into both cExN and cIN neural progenitors and/or neurons. Models from

the affected individuals exhibited compromised cellular responses to differentiation cues and

had disrupted gene expression profiles. This included altered expression of many ASD genes,

genes with roles related to behavior, cognition, and learning, and genes involved in nervous

system development and function, including cell adhesion molecules and ion channels.

This is, to our knowledge, the first cellular modeling study of multiplex ASD, including

graded affectation among family members. We demonstrated that even genetically complex

forms of ASD have discernable cellular and molecular abnormalities that track with affectation,

some of which overlap those identified in prior modeling of syndromic, monogenic, and *de novo*

forms of ASD. Therefore, this novel study design highlights the potential for cellular modeling to

identify convergent hallmarks across the broad diversity and genetic complexity of pathways to

affectation.

Methods

Phenotyping of the Multiplex Family

The nuclear family consisted of working professional non-consanguineous parents, whose first

born child was a daughter with DSM-5 Autism Spectrum Disorder (ASD), Level I (requiring

support, meeting DSM-5 criteria for Asperger Syndrome) who was very high functioning and

ultimately attended college, followed by a pair of monozygotic twin boys with ASD, Level III—

one more fluently verbal than the other but both severely impaired and requiring very substantial

support (see below)—followed by a third son with very subtle autistic traits and predominantly

affected by Attention Deficit Hyperactivity Disorder which improved substantially with stimulant

138 medication treatment. Trio Exome sequencing (ES) of one of the twins and his parents revealed

139 a variant of unknown significance (VUS) in *GPD2*, which was inherited by all of the children from

140 the mother, who is of above average intelligence with no dysmorphism and no history of

141 developmental problems. All pregnancies were uncomplicated, except for the post-natal hospital

142 course of the twins.

143  The daughter was born at term with no complications or dysmorphia. Her language and

144 motor development were typical and she was able to read at an early age. By age five she was

145 reading at a fifth-grade level. She has been described as talented in writing and drawing.

146 According to her parents, she exhibited social oddities from an early age, mainly in

147 communication, and has somewhat intense/restricted interests in fantasy games. She has

148 strong language abilities, and currently attends a four-year college, but at times uses odd

149 phrases and the rhythm of her speech includes irregular pauses. She has described feeling

150 alienated and "different", and was the victim of bullying in middle school, with few close friends.

151 In late adolescence, she developed major depressive disorder with moderate severity, which

152 brought her to first psychiatric contact. She is cognizant of some degree of social awkwardness,

153 which leads to feelings of anxiety and self-consciousness. The social anxiety inhibits her from

154 activities such as eating in the cafeteria and pursuing job opportunities for which she is

155 otherwise well-qualified. She has a history of becoming emotionally dysregulated and

156 overwhelmed in times of stress, which has led to self-injurious behaviors. She has had ongoing

157 struggles with depressive decompensation and suicidal ideation. She has above average

158 intelligence but has struggled academically in college due to depression and anxiety. She is

159 medically healthy with the exception of supraventricular tachycardia secondary to

160 atrioventricular node reentry, which was treated with ablation and resulted in subsequent

161 normalization of her electrocardiogram.

The twin boys were born at 35 weeks, had breathing problems at birth, and spent ten

days in the newborn intensive care unit. Neither child has any dysmorphic features or congenital

medical abnormalities, and brain imaging studies were negative. Likewise, neither child has a

history of confirmed seizures, however, there are concerns for possible absence epilepsy. There

is no history of abnormal neurological examination or macro-/microcephaly. Development of

both siblings was delayed, but neither had appreciable regression. The more severely affected

twin (designated as the affected proband, AP, and from whom the incuced pluripotent stem cell

(iPSC) model of severe ASD affectation was acquired) began to exhibit delays in development

by nine months of age. He was speaking single words at 14 months and was ultimately

diagnosed with autism at 3.5 years old. Research confirmation of the diagnosis was obtained

using the Autism Diagnostic Observation Schedule. Compared to his twin brother, he has had

more perseverative interests on odd objects. Psychological testing at the age of nine revealed

an IQ of 65 using the Leiter International Performance Scale. Now in late adolescence, he has

the ability to engage in reciprocal and meaningful verbal exchanges, although his language is

often echolalic and repetitive. He is socially motivated and develops superficial friendships with

peers. Functionally, he is able to complete most self-care, dress himself, prepare food and feed

himself, and count money. He participates in a vocational program at school and is able to

complete rudimentary tasks assigned to him. His monozygotic twin was also diagnosed with

ASD at 3.5 years old and is less severely affected, but still requires significant support. Selected

clinical characteristics of the family members studied are summarized in Table 1.

Genotyping of the Multiplex Family

Trio ES was performed by GeneDx for the unaffected mother (UM), the AP, and the unaffected

father. Briefly, sequencing was performed on an Illumina platform and reads were aligned to

human genome build GRCh37/hg19 and analyzed using Xome Analyzer, as described (32).

Mutation-specific testing was also performed by GeneDx on the intermediate phenotype sister

(IS) and the third trait-affected brother (TB) to confirm presence of the identified *GPD2* variant in

these individuals.

iPSC generation

iPSC lines were generated by the Genome Engineering and iPSC Center at Washington

University. Briefly, renal epithelial cells were isolated and cultured from fresh urine samples and

were reprogrammed using a CytoTune-iPS 2.0 Sendai Reprogramming kit (Thermo Fisher

Scientific), following the manufacturer's instructions. iPSC clones were then picked, expanded,

and characterized for pluripotency by immunocytochemistry (ICC) and RT-qPCR. At least three

clonal iPSC lines were derived for each subject and two different clones were used for

experimentation.

iPSC maintenance and differentiation

iPSC lines were grown under feeder-free conditions on Matrigel (Corning) in mTeSR1

(STEMCELL Technologies). cExN and cIN differentiation of iPSCs was performed using

previously described protocols (33). Briefly, for cExN differentiation, iPSCs were dissociated to

single cells with Accutase (Life Technologies) and 40,000 cells were seeded in V-bottom 96-well

non-adherent plates (Corning). Plates were spun at 200xg for five minutes to generate embryoid

bodies (EBs) and were incubated in 5% CO_2 at 37°C in cExN differentiation medium with 10µM

Y-27632 (Tocris Biosciences). cExN differentiation medium components include Neurobasal-A

(Life Technologies), 1X B-27 supplement (without Vitamin A) (Life Technologies), 10µM SB-

431542 (Tocris Biosciences), 100nM LDN-193189 (Tocris Biosciences). On day four of

differentiation, EBs were transferred from V-bottom plates to Poly-L-Ornithine- (20µg/ml) and

laminin- (10µg/ml) coated plates. Media (without Y-27632) was replenished every other day,

and on day 12 Neural Rosette Selection reagent (STEMCELL Technologies) was used to select

neural progenitor cells (NPCs) from within neural rosettes, per the manufacturer's instructions.

cExN NPCs were grown as a monolayer using cExN differentiation media for up to 15 passages. cIN differentiation media contained the same components as cExN differentiation media, while also including 1µM Purmorphamine (Calbiochem) and 2µM XAV-939 (Tocris Biosciences). EBs were generated as described for cExNs. At day four of differentiation, the EBs were transferred to non-adherent plates and were placed on an orbital shaker (80 rpm) in an incubator with 5% CO_2 at 37°C. The media was replenished every other day and, at day ten, EBs were transferred to Matrigel- and laminin- (5µg/ml) coated plates. Y-27632 was included in media until day eight of differentiation. On day 12 of differentiation, NPCs were dissociated with Accutase and maintained as a monolayer for up to 15 passages. For both cIN and cExN NPC growth analysis, an equal number of cells were seeded on Matrigel- and laminin- (5µg/ml) coated plates and total cells were counted four days later when the cells reached 70-80% confluence.

For differentiation of cExN NPCs into neurons for maturation, 40,000 cells per well were seeded in V-bottom 96-well non-adherent plates. Plates were spun at 200xg for five minutes and incubated in 5% CO_2 at 37°C in maturation medium with Y-27632. Maturation medium components include Neurobasal-A, 1X B-27 supplement (without Vitamin A), 200µM cAMP (Sigma), 200µM Ascorbic acid (Sigma), and 20ng BDNF (Tocris Biosciences). After two days, EBs were transferred to Matrigel- and laminin- (5µg/ml) coated plates and media was replenished every other day (without Y-27632). On day 12 of neuronal differentiation and maturation, cells were dissociated with Accutase and seeded in an eight-well chamber for ICC.

For neurosphere size measurement analysis, p-values: *$P<0.05$, **$P<0.01$, ***$P<0.001$ were determined by a two-tailed Student's t-test.

Sanger Sequencing

DNA was isolated from cell lines using the PureLink Genomic DNA Kit (Invitrogen). Primers

were designed to amplify a 248 base pair region of *GPD2* flanking the identified point mutation

(forward primer: AAGCAGCAGACTGCATTTCA, reverse primer:

CACCATGGCACACACTTACC). Sanger sequencing was performed on this PCR amplified

fragment using either the forward or reverse primer. CodonCode Aligner software was used to

analyze sequencing results.

Immunocytochemistry (ICC) and Immunoblotting

For ICC, cells were plated on eight-well chamber slides coated with Matrigel and laminin (5

μg/mL). After one day, cells were washed once with PBS without calcium and magnesium (PBS

- Ca^{2+}/Mg^{2+}) and fixed in 4% Paraformaldehyde for 20 minutes, followed by washing with PBS +

Ca^{2+}/Mg^{2+}. Cells were blocked with blocking buffer (10% donkey serum, 1% BSA, and 0.1%

TritonX-100 in PBS + Ca^{2+}/Mg^{2+}) for at least one hour and incubated with primary antibodies

overnight (Additional file 1: Table S1) in antibody dilution buffer (1% donkey serum, 1% BSA,

and 0.1% TritonX-100 in PBS + Ca^{2+}/Mg^{2+}). After overnight incubation, cells were washed three

times with wash buffer (0.1% Triton X-100 in PBS + Ca^{2+}/Mg^{2+}). Cells were incubated with

corresponding secondary antibodies (Additional file 1: Table S1), along with DAPI (1mg/mL;

ThermoFisher Scientific), diluted in antibody dilution buffer for one hour. Following secondary

antibody incubation, cells were washed twice with wash buffer and once with PBS + Ca^{2+}/Mg^{2+}.

Slides were mounted with Prolong Gold anti-fade agent (Life Technologies). Images were

obtained using a spinning-disk confocal microscope (Quorum) with MetaMorph software and

were processed using ImageJ. For immunoblotting, cell lysate was extracted and 30μg of

protein was used per lane. Antibodies used are listed in Additional file 1: Table S1.

FACS analysis

For each experiment, approximately one million cells were pelleted, washed with PBS –

Ca^{2+}/Mg^{2+}, resuspended in PBS – Ca^{2+}/Mg^{2+} and fixed by adding 70% ice-cold ethanol dropwise

while vortexing. Cells were stained with 10ug/mL propidium iodide (Sigma) and 200ug/mL

RNase A (Fisher Scientific) in FACS buffer (PBS – Ca^{2+}/Mg^{2+}, 0.2% BSA, 1mM EDTA). FACS

was performed on single-cell suspensions and the cell cycle analysis function of FlowJo was

used to analyze cell cycle composition for each sample, based on propidium iodide staining to

detect DNA content in each cell. p-values: *$P<0.05$, **$P<0.01$, ***$P<0.001$ were determined by a

two-tailed Student's t-test.

RNA-Seq and RT-qPCR

Total RNA was collected from iPSC-derived day 12 cExN and cIN NPCs using the NucleoSpin

RNA II kit (Takara) per the manufacturer's instructions. RNA was quantified using a NanoDrop

ND-1000 spectrophotometer (Thermo Scientific), and the integrity of RNA was confirmed with

an Agilent Bioanalyzer 2100 to ensure a RIN value above eight. RNA-sequencing (RNA-seq)

library preparation and Illumina sequencing were performed by the Genome Technology Access

Center at Washington University. For RT-qPCR, 1μg total RNA was reverse transcribed using

iScript Reverse Transcription Supermix (Bio-Rad). Equal quantities of cDNA were used as a

template for RT-qPCR, using the Applied Biosystems Fast Real-Time quantitative PCR system.

RPL30 mRNA levels were used as endogenous controls for normalization. p-values: *$P<0.05$,

**$P<0.01$, ***$P<0.001$ were determined by a two-tailed Student's t-test.

Bioinformatics and IPA analyses

RNA-seq data analysis was performed as described in (33) to curate differentially expressed

gene (DEG) lists. To uncover the biological significance of DEGs, network analysis was

performed with the data interpretation tool Ingenuity Pathway Analysis (IPA) (Qiagen). IPA's

Ingenuity Knowledge Base uses network-eligible DEGs to generate networks and to define

connections between one or more networks. Based on the number of eligible DEGs, IPA

defines network scores as inversely proportional to the probability of finding the network and

defines significant networks (p≤0.001). Within each network, red symbols indicate upregulated

genes and green symbols indicate downregulated genes, where the color intensity represents

relative degree of differential expression.

Results

Phenotyping and genotyping of the multiplex family

The multiplex Autism Spectrum Disorder (ASD) pedigree selected for study (Fig. 1; Table 1)

underwent clinical phenotyping and genotyping (see Methods). From this pedigree, the

individuals selected for iPSC line derivation and modeling included the affected proband (AP),

his sister, who has an intermediate phenotype (IS), and their unaffected mother (UM) (indicated

in Fig. 1). As described above, a non-synonymous single nucleotide variant in the *GPD2* gene

was identified in the UM and all of the children (chr2:157352686 (hg19) G>A, NM_001083112.2

c.233G>A, p.G78E). This variant is not present in the father. The variant is in exon three of the

GPD2 gene, within the region encoding the flavin adenine dinucleotide (FAD)-binding domain of

the GPD2 protein (Additional file 2: Fig. S1A). The GeneDx interpretation of this variant states

that it was not observed in approximately 6,500 individuals of European and African American

ancestry in the NHLBI Exome Sequencing Project and that it is evolutionarily conserved. In

addition, *in silico* analysis predicts that this variant is probably damaging to the protein structure

and function. Overall, GeneDx designated this as a variant of uncertain significance (VUS)

following the American College of Medical Genetics criteria. Subsequent mutation-specific

testing and Sanger sequencing identified this same variant in the AP, IS and trait-affected

brother (TB), while absent in an unrelated, unaffected control (UC) (Additional file 2: Fig. S1B).

Given the differential affectation of members of this pedigree carrying this variant, it was

apparent that this inherited VUS was insufficient to cause ASD by itself, but may have

contributed to polygenic risk/liability in this family.

Generation of subject-derived iPSC models and directed differentiation into cortical

excitatory neurons

Multiple clonal iPSC lines were derived from the UM, IS, and AP, and were characterized in

parallel with a single, clonal iPSC line derived from the UC. When grown in stem cell

maintenance media, there were no observable differences in expression of pluripotency

markers between these iPSC lines, as assessed by RT-qPCR (Additional file 2: Fig. S1C) and

immunocytochemistry (ICC) (Additional file 2: Fig. S1D). In addition, no differences in GPD2

protein levels were detected in iPSCs by ICC (Additional file 2: Fig. S1D) or Western blotting

(Additional file 2: Fig. S1E). Finally, we used FACS analysis of propidium iodide (PI)-stained

iPSCs to assess cell cycle progression and detected no observable differences between these

iPSC lines, which had similar percentages of cells in each stage of the cell cycle (Additional file

2: Fig. S1F-G).

In the cortex, as a result of their abnormal development, imbalances in glutamatergic

excitatory neurons (cExN) and GABAergic inhibitory interneurons (cINs) are thought to

contribute to neurodevelopmental disorders including ASD (3, 5, 34). We therefore differentiated

iPSCs derived from the UC, and from three family members (the UM, IS and AP), in parallel into

either cExN or cIN neural progenitor cells (NPCs) and/or neurons, to determine if we observed

any alterations in the *in vitro* development of either or both of these neural cell types. We

performed 12 days of cExN differentiation (four days as embryoid bodies (EBs) in V-bottom

plates, followed by eight days with the EBs plated for two-dimensional (2-D) culture; Fig. 2A). At

all time points assessed during this differentiation, the IS and AP lines generated significantly

smaller neurospheres than the UC and UM. The UM neurospheres were also slightly smaller

than those of the UC (Fig. 2B-C).

To identify whether an increase in apoptosis and/or a decrease in proliferation could be

contributing to these differences in neurosphere size, we performed FACS analysis of PI-

stained cells at day four of differentiation and found that the IS and AP neurospheres had a

significantly higher fraction of sub-G1 (apoptotic) cells, compared to neurospheres derived from

the UM and UC lines (<2N DNA content; Fig. 2D-E, G). There was a corresponding decrease in

the percentage of cells in the G1 phase of the cell cycle in the IS and AP neurospheres (2N

DNA content; Fig. 2D, F-G). However, neurospheres from all lines had similar percentages of

cells in the S and G2/M phases of the cell cycle, suggesting that their cell cycle characteristics

and rates of progression were otherwise similar (S phase and 4N DNA content; Fig. 2D, G). To

determine whether induction of neural differentiation was a stressor that was contributing to this

increase in apoptosis in the IS and AP line-derived neurospheres, we compared sphere size

after culturing spheres from each line either in stem cell maintenance media (mTeSR) or in

neural induction media. In general, sphere size was larger for all cell lines when kept in mTeSR

media rather than neural induction media, while the differences in sphere size for the IS and AP

versus the UC and UM was much less pronounced in mTeSR relative to differences seen under

neural induction conditions (Fig. 2H-I). These data suggest that, by comparison with the UC and

UM, the IS and AP lines have a slightly elevated propensity to undergo apoptosis upon

dissociation and sphere formation, while this is exacerbated by induction of neural

differentiation.

We next maintained these four lines as NPCs after neural rosette selection at day 12

and then subjected them to PI staining and FACS analysis. Unlike the results from earlier time

points, the cExN NPCs showed no significant differences in cell cycle across the four lines

(Additional file 2: Fig. S2A-B), nor in the rate of growth/death over the course of culture for four

days (Additional file 2: Fig. S2C). However, morphological analysis by bright field imaging

indicated a possible adhesion defect in the IS NPCs, as indicated by uneven growth on the cell

culture plate surface (Additional file 2: Fig. S2D). At the NPC stage, GPD2 protein levels

remained similar across the four lines, as was shown for iPSCs (Additional file 2: Fig. S2E).

Finally, to determine if the NPCs derived from the affected individuals exhibited an

altered capacity to differentiate into cExN neurons, NPCs from the four lines were further

differentiated for 12 days as shown (Additional file 2: Fig. S3A) and subjected to ICC. No

apparent differences between the four lines were observed in the expression of NPC markers

(PAX6, NESTIN, and SOX1) or markers of immature (TUJ1) and mature cExN neurons

(VGLUT, MAP2) (Additional file 2: Fig. S3B). Furthermore, there were no observable differences

between the lines in the fraction of cells expressing Ki-67, a marker of cell proliferation, or

cleaved Caspase-3, a marker of apoptosis (Additional file 2: Fig. S3B).

Differentiation of subject-derived iPSCs into cortical interneuron progenitors

We also characterized cellular phenotypes of these four lines during differentiation into cIN

NPCs, to determine any differences between the development of this neural cell type in lines

derived from affected versus unaffected individuals. The differentiation scheme to produce cIN

NPCs is outlined in Figure 3A. On day five of differentiation in this scheme, neurospheres

derived from the IS line were smaller than those of the UM. Conversely, the AP line-derived

neurospheres were slightly larger than the UM line neurospheres (Fig. 3B-C).

After dissociation on day 12 of differentiation, we assessed the cell cycle of the cIN

NPCs using FACS of PI-stained cells. The IS and AP cIN NPCs had an increased sub-G1 cell

population, compared to the UC and UM NPCs, an indication of increased apoptosis in the cells

from the affected individuals (Fig. 3D). Correspondingly, there was a decrease in the proportion

of cells in the G1 phase of the cell cycle (Fig. 3E-F). However, no differences were observed in

frequencies of cells in the S and G2 phases of the cell cycle between lines, suggesting that

these lines had similar proliferation rates (Additional file 2: Fig. S2F). This result was supported

by analysis of NPC cell counts after four days of growth, which revealed a significant reduction

in the number of AP NPCs, as well as a slight reduction in the number of IS NPCs, compared to

the UM NPCs (Additional file 2: Fig. S2G). These reductions in NPC number may result from the

increased NPC apoptosis detected in our PI FACS analysis. The AP NPCs also exhibited

altered morphology that could indicate impaired adhesion capacity relative to the control UM/UC

lines, which could also contribute to the reduction in the number of AP NPCs persisting in the

culture after four days of growth (Additional file 2: Fig. S2H).

Transcriptomic differences in neural progenitor cells derived from affected individuals

versus controls

To investigate which classes of genes could be differentially expressed in neural cells from the

affected individuals, by comparison with the unaffected controls, we performed RNA-seq

analysis on both cExN and cIN NPCs at day 12 of differentiation for all four subject-derived

lines. Four biological replicates were analyzed for each sample type and were clustered by

principal component analysis (PCA) of processed reads (Additional file 2: Fig. S4A-B). We

defined genes that were significantly differentially expressed genes (DEGs) in pairwise

comparisons of these four sample types for either cExN or cIN NPCs, selecting DEGs with a p-

value of <0.05 and a fold difference between sample types of >2 (Additional file 3: Table S2). In

a within-family comparison of the UM, IS, and AP samples, greater numbers of DEGs were

obtained in the cIN NPC pairwise comparisons, versus the numbers of DEGs obtained for cExN

NPC pairwise comparisons (Additional file 2: Fig. S4C-D). These data indicate that the cIN

samples from the affected individuals (IS/AP) exhibit more transcriptomic differences from the

UM control than the affected individual-derived cExN samples.

We focused first on identifying classes of genes that were differentially expressed in

NPCs derived from the affected individuals, by comparison with unaffected controls. To do this,

we defined the subset of DEGs that were similarly expressed in samples from both affected

individuals (AP/IS) but that differed in expression by comparison with the unaffected mother

(UM) sample. Relative expression is also shown for the UC, for a full cross-sample comparison.

452 and 437 DEGs for the cExN and cIN NPC samples met these criteria, respectively.

Hierarchical clustering and visualization of the relative expression of these DEGs across the

four sample types is shown for the cExN NPCs (Fig. 4A, Additional file 4: Table S3). We next

used Ingenuity Pathway Analysis (IPA) to assess the potential biological significance of these

genes. For the 452 DEGs in the cExN NPCs described above, the most significant function- and

disease-related gene ontology (GO) terms included 'behavior', 'neurological disease', and

'embryonic development' (Fig. 4B). Network analysis using IPA revealed several interesting

networks of DEGs related to these GO terms, including networks related to 'locomotion' (from

DEGs within the 'behavior' GO term) and 'behavior and developmental disorder' (Fig. 4C-D).

Within the 'locomotion' network, most genes were upregulated in the affected individuals

compared to the controls, including genes relating to neural adhesion and ion channels (Fig. 4C

and Additional file 5: Table S4). Genes with known roles in NPCs or neurons, as well as stress-

related genes were present in the larger 'behavior and developmental disorder' network (Fig. 4D

and Additional file 5: Table S4). Interestingly, another network comprising genes from the GO

term 'neurological disease' is related to 'inflammation of central nervous system' (Additional file

2: Fig. S5A and Additional file 5: Table S4).

IPA analysis of the cIN DEGs also revealed several interesting classes of genes that

were differentially expressed between the affected participants and unaffected control NPC-

derived samples. Hierarchical clustering and visualization of the relative expression of DEGs

across the four sample types for the cIN NPCs is shown in Fig. 4E (Additional file 4: Table S3).

The top GO terms included 'developmental disorder', 'behavior', 'nervous system development

and function', 'psychological disorders', and 'neurological disease' (Fig. 4F). Within the term

'behavior', a network that includes 'learning'-, 'cognition'-, and 'behavior'-related genes was

identified (Fig. 4G, Additional file 5: Table S4). The network related to the GO term

'psychological disorder' includes genes related to 'anxiety disorders', 'mood disorders', and

'depressive disorder' (Fig. 4H). The 'nervous system development and function' network

includes genes involved in the 'quantity of neurons' and 'quantity of synapse', as well as cell

adhesion genes (Additional file 2: Fig. S5B and Additional file 5: Table S4). Finally, a

'neurological' network included a number of genes also present in the other networks (Additional

file 2: Fig. S5C). Taken together, this analysis of DEGs in both cExN and cIN NPCs shows

evidence of altered expression of a number of neurological and psychological disease-relevant

gene classes in the AP- and IS-derived lines, relative to lines derived from the UM and/or UC.

Within-family comparison identifies a transcriptome signature specific to neural

progenitor cells derived from the ASD-affected proband

As differences in genetic background can confound differential gene expression analysis (35),

we also performed a pairwise, within-family data comparison of DEGs that distinguish the UM-,

IS-, and AP-derived samples, focusing on DEGs specific to the AP that could contribute to the

greater degree of affectation observed. Using pairwise comparisons of DEGs, we defined 190

genes which were uniquely differentially expressed in cExN NPCs from the AP (Fig. 5A). The

top GO terms associated with these AP-specific DEGs included 'psychological disorders',

'behavior', 'nervous system development and function', 'developmental disorder', and

'neurological disease' terms (Fig. 5B). Within the 'behavior' term, network analysis showed

genes related to 'memory' and 'learning' to be dysregulated (Fig. 5C and Additional file 5: Table

S4). Within the 'nervous system development and function' term, a network of dysregulated

genes related to 'differentiation of neurons' was identified (Fig. 5D, Table S4).

Similar analysis was performed on the cIN samples, revealing 384 DEGs unique to the

AP samples in the within-family comparison (Fig. 5E). IPA analysis identified classes of DEGs

related to the GO terms 'psychological disorders', 'developmental disorder', 'neurological

disease', 'behavior', and 'nervous system development and function' (Fig. 5F). Within the

'behavior' disease term, a network of genes related to 'behavior' and 'cognition' was identified

(Fig. 5G and Additional file 5: Table S4). Within the 'nervous system development and function'

term, a network of genes related to 'development of neurons' and 'synaptic transmission' had

altered expression in the AP versus the IS/UM-derived samples (Fig. 5H and Additional file 5:

Table S4). Together, this analysis identifies AP-unique DEGs in both cExN and cIN NPCs,

many of which are broadly related to neural development, as well as to specific aspects of ASD,

such as behavioral alterations. These gene expression changes therefore correlate with and

may contribute to, the severity of affectation in the AP.

Comparison of differentially expressed genes with ASD-associated genes and validation

The Simons Foundation Autism Research Initiative (SFARI) (36) maintains a database of genes

that are mutated to cause, or contribute to, ASD risk. We compared our DEGs to these ASD

genes, to assess whether their dysregulated expression could contribute to affectation in these

individuals. Of 452 DEGs in the cExN differentiation scheme that had similar expression in the

AP and IS that differed from that seen in the UM, 30 (6.6%) were ASD genes (Fig. 6A). For the

corresponding cIN NPC comparison, 46 of 437 DEGs (10.5%) were ASD genes in the SFARI

Gene database (Fig. 6B). Based upon the 1019 genes present in the SFARI Gene database

(36) and the total of 27,731 genes with >0.1 RPKM average expression across all cExN and cIN

samples, the number of AP- and IS-specific DEGs that are AD genes is significantly greater

than would be expected by chance (hypergeometric distribution, p=9.35x10^{-5} and 1.95X10^{-12} for

cExN and cIN data, respectively). Therefore, it is possible that misregulated expression and

consequently function of ASD genes contributed to disruption of neural development and/or

continues to contribute to altered neurological function in the IS and AP.

We validated the differential expression of a subset of the DEGs described above by RT-

qPCR analysis, isolating RNA from NPCs derived from a second set of iPSC clones that differed

from those used for the RNA-seq experiments. Expression changes of DEGs selected from the

cExN NPC RNA-seq data (Fig. 7A) were robustly recapitulated in these experiments (Fig. 7B).

Genes from the 'behavior and developmental disorder', from other identified networks, and

genes involved in neurodevelopment were evaluated (Additional file 5: Table S4). We also

derived cIN NPC RNA from a second set of iPSC clones and validated the corresponding RNA-

seq data for a subset of the DEGs (Fig. 7C). Differential expression was assessed for SFARI

ASD genes (36), and for genes encoding transcription factors, ion channels, and cell adhesion

molecules (Fig. 7D-E and Additional file 5: Table S4). In addition, we validated a subset of

DEGs in cINs which also had differential expression in cExN NPCs (Fig. 7E). Together, these

analyses revealed that, relative to unaffected individuals, samples from affected individuals

exhibited altered expression of classes of genes involved in behavior, learning, cognition, mood

disorders, and neurodevelopment, including perturbed ASD gene expression, suggesting that

these differences could contribute to aberrant neural development or function in the affected

individuals.

Discussion

In recent years, the genetic structure of Autism Spectrum Disorder (ASD) risk in the general

population has been clarified. This work has confirmed that while, in some cases, deleterious,

single gene variants are significant contributors to ASD, the vast proportion of population

attributable risk is polygenic (2, 37). Furthermore, this risk is highly heritable, and individuals

within a multiplex family typically exhibit variable degrees of affectation (31). Here, we modeled

cellular and molecular correlates of ASD within one such multiplex family, performing cortical

neural differentiation of iPSCs derived from several family members with differential affectation.

In this family, both polygenic liability and a shared variant of unknown significance (VUS) may

contribute to risk. In cells derived from the affected individuals, we identified compromised

responses to differentiation cues and altered gene expression profiles during iPSC

differentiation into cortical excitatory (cExN) and inhibitory (cINs) neurons, compared to related

and unrelated unaffected controls. This work demonstrates that iPSC-based modeling can be

used to characterize these more genetically complex but prevalent forms of ASD, in addition to

modeling simplex and monogenic forms, which have been the focus of most studies to date.

Moreover, these data provide information on physiologic and transcriptomic signatures of

multiplex autism, with which cellular models derived from other families and other combinations

of inherited susceptibility factors can be compared in future work.

Our phenotypic analysis of these four iPSC-based models of cortical neural development

included assays conducted in the stem cells, during neural specification, in the proliferating

NPCs, and during neuronal differentiation. During cExN NPC specification and during cIN NPC

propagation, models from both affected individuals exhibited elevated fractions of cells with sub-

G1 DNA content, relative to control-derived models. These data suggest that models derived

from the affected individuals are less resistant to stressors, such as induction of differentiation,

with these stressors increasing the propensity for cells to undergo apoptosis. While the

molecular trigger for the induction of apoptosis here is unclear, expression of stress and

apoptosis-related genes, such as *CHCHD2, ANXA1, and SPATA18* are dysregulated in these

models (38-42). These findings are reminiscent of some observations made in prior work, in

which schizophrenia subject-derived iPSCs exhibited reduced neurosphere size (43) and

increased apoptosis was observed in Williams-Syndrome iPSC-derived NPCs (44).

Interestingly, few studies report cellular alterations observed prior to the NPC stage, often

focusing predominantly on phenotypes seen in NPCs and mature neurons (7-19). While this

may reflect a lack of earlier phenotypic changes in some models, our findings highlight the

importance of tracking neurodevelopmental alterations from their earliest onset. A recent report

underscores the value of using cellular modeling approaches that aim to recapitulate some

aspects of *in vivo* neurodevelopment (20). This study found that direct conversion of iPSCs into

neurons masked ASD-associated cellular phenotypes, which were observable during directed

differentiation of iPSCs (20).

In our study, transcriptomic analysis of neural progenitor cells revealed dysregulated

expression in affected individuals compared to controls of gene networks related to behavior,

psychological disorders, and neuronal development and disease. Genes encoding transcription

factors were among the neurodevelopment-related genes with reduced expression in both

affected individuals. For example, ARX is required for normal telencephalic development and is

associated with syndromic autism and other neurodevelopmental disorders (45), while EMX1

and FOXB1 also play important roles in neural development (46-48). Behavioral misregulation is

a key trait of ASD, and gene networks related to the GO term 'behavior' exhibited dysregulated

expression in both affected individuals. Genes in these networks include *COMT, ADCYAP1,*

CNR1, HTR2C, GRIK2, and *RGS4*, all of which are implicated in behavior-related phenotypes in

humans and/or mice (49-57). ASD genes were also dysregulated in these affected individuals,

relative to controls. Mutation of these genes in other individuals is implicated in autism risk or

causation. These include adhesion-related genes (*PCDHA1*, *PCHDHA6*, *PCDHGA11*, *PCDH8,*

PCDH9, PCDH10 (58-62)*, KIRREL3* (63), *CNTN3*, *CNTN*4 (64), *CNTNAP4* (65), and *THBS1*

(66)), receptor and channel genes (*CACNA2D3* and *SCN9A* (67)*, GRIK2* and *GRIK3* (68-71),

KCNJ2 (72, 73)*,* and *GRIA1* (74, 75)), and genes associated with central nervous system

development and axon guidance (*ERBB4* (76, 77), *NTNG1* (78), *TSHZ3* (79), *EBF3* (80)*,*

MYT1L (81, 82), and *ANXA1* (40-42))*.* Altered expression of ASD-associated genes has also

been observed in cellular models derived from affected individuals in other studies (8, 13-16, 19,

83). Therefore, these findings suggest that misregulated expression of suites of ASD-associated

genes may contribute to risk or affectation, and may do so by altering neurodevelopment and/or

neuronal function in these affected individuals.

A unique aspect of this study is the use of iPSC-based directed differentiation into both

cExNs and cINs, enabling us to identify neural cell type-specific alterations associated with

affectation. Although DEGs identified in affected individuals in both neural cell types were

associated with many similar functions and diseases (e.g. behavior), the specific DEGs obtained

often varied by cell type. For example, cIN DEGs included many more ASD-associated genes

and protocadherin genes, the latter of which control neuronal migration, axonal growth, and

synapse formation (60, 61). Human post-mortem cortical tissue from individuals with ASD has

been shown to exhibit disrupted expression of cIN-associated genes, evidence that this cell type

may commonly be disrupted in affected individuals *in vivo* (84). These findings suggest that

extending cellular modeling studies to multiple disease-relevant neuronal cell types, including

cINs, may reveal additional neurodevelopmental disruptions related to affectation.

To define cellular and molecular perturbations commonly related to affectation, we

compared our findings to other studies that modeled ASD by directed differentiation of iPSCs

into cExNs. We identified subsets of overlapping DEGs in comparisons with studies involving

idiopathic autism cases vs. controls (26 shared DEGs; (7)), syndromic ASD involving

macrocephaly (31 shared DEGs; (19)), and modeling of the syndromic ASD gene *CHD8 (*32

shared DEGs*;* (13)) (Additional file 6: Table S5). Data for such comparisons is limited at present

because iPSC-based models have been generated for a relatively small number of individuals

and mutations, and these almost exclusively characterize cExNs or cerebral organoids (7, 13,

18, 19).

The multiplex pedigree studied here was subjected to clinical exome sequencing, as it was

hypothesized that a single, shared, genetic contributor might mediate autism risk and differential

phenotypic expression by sex in this family. In this sequencing analysis, a thread of shared

genetic liability amongst all children was a VUS in the ASD and ID-associated gene, *GPD2* (85-

87)*,* which was inherited from their mother. However, there is variable ASD expressivity

amongst these individuals, ranging from absent, to intermediate, to severe. In addition, both

males and females in the pedigree are variably affected, indicating the presence of other

significant contributors to variation in severity of affectation within this family. This observation is

consistent with recent evidence that genetic liability for ASD is prevalently polygenic, and that,

even in multiplex pedigrees where a significant monogenic contributor has been identified,

additional polygenic risk can contribute to affectation (37). Moreover, this multiplex family was

prototypic in reflecting the most severe form of affectation occurring in a male.

We hypothesized that it might be possible to identify graded cellular phenotypes that

correlated with the level of severity of phenotypic expression. In general, we instead observed

many cellular and molecular alterations that were shared by the cellular models derived from the

affected individuals, while not being observed in those derived from the unaffected individuals.

However, we did define some proband-specific DEGs, not present in the less severely affected

sister, many of which relate to behavior and nervous system development. A subset of these

DEGs had graded expression, exhibiting intermediate expression levels in the intermediate

phenotype sister, between her unaffected mother and her severely affected brother. These

findings suggest that both the degree of dysregulation of expression and the number and

identity of DEGs within these networks may contribute to the level of affectation. While further

experimentation might reveal additional graded phenotypes, particularly in mature neurons, *ex*

vivo cellular modeling cannot recapitulate many aspects of fetal and post-natal

neurodevelopment that may have been perturbed to contribute to the graded affectation

observed in these individuals.

This work highlights several considerations for ongoing scientific efforts to model this

complex but prevalent form of ASD in future studies. First, since the unique characteristics of

any multiplex pedigree present challenges for cellular modeling, it is important to control for sex

and variation in affectation in subject and family selection, study design, and analysis. Related

to this point is the importance of modeling affected females in such studies. Most ASD cellular

modeling to date has been restricted to affected males (7-10, 13, 14, 16, 18, 19), given the

increased prevalence of ASD among males, and the fact that constraint to a single sex

simplifies some modeling considerations. In particular, sex chromosome dosage effects do not

need to be accounted for in male cells, while female-derived iPSC models cannot currently

recapitulate the process of random X-chromosome inactivation that occurs in developing

somatic tissues, including the brain (88, 89). Interestingly, the transcriptomic differences that we

observed here were not driven by sex-linked gene expression: very few DEGs in any potential

pairwise sample comparison (whether between same or opposite sex models) were sex

chromosome-linked potential contributors to sex-biased gene expression in the human brain

(90, 91). Therefore, this work supports the feasibility of identifying DEGs associated with

affectation by cellular model cross-comparisons, even when these models are derived from both

female and male subjects.

Another consideration for iPSC-based modeling of ASD is genetic background, which

can be a confounding variable for cross-comparisons (35). In this pedigree, ASD risk was

polygenic, such that it was not possible to engineer a correction of a single genome variant to

create pairs of isogenic mutant versus wild-type iPSC lines with an identical genetic background

for study. In such cases, modeling of first degree relatives may serve as the best control, and

modeling of multiple related individuals with varying affectation provides additional opportunities

for identifying potential contributors to these differences in affectation. Including unrelated

controls and performing comparisons with other studies can further highlight which phenotypic

and transcriptomic alterations track with affectation, even by comparison with models derived

from individuals with an unrelated genetic background.

Conclusions

In summary, this work used robust schemes for differentiation of cortical neurons from iPSCs to

model cellular and molecular signatures associated with multiplex ASD in a family reflecting

varying degrees of affectation. Even in this prevalent, complex form of ASD, involving

heritability, polygenic etiology, and variable affectation, we could identify affectation-linked

cellular and molecular alterations of neurodevelopment, some of which overlapped those

defined in other iPSC-based studies of monogenic, syndromic, and *de novo* ASD. As more

cellular models of ASD are characterized, these data can be harnessed in the search for

convergent and divergent contributors to impairment across the genetically complex and multi-

factorial pathways that give rise to ASD.

Abbreviations

ASD: Autism Spectrum Disorder; iPSCs: Induced Pluripotent Stem Cells; cExNs: Cortical

Excitatory Neurons; cINs: Cortical Inhibitory Neurons; NPCs: Neural Progenitor Cells; ES:

Exome Sequencing; UM: Unaffected Mother; AP: Affected Proband; IS: Intermediate Phenotype

Sister; UC: Unaffected Control; GTAC: Genome Technology Access Center; ICC:

Immunocytochemistry; RT-qPCR: quantitative reverse transcription PCR; EBs: Embryoid

Bodies; DEG: Differentially Expressed Gene; IPA: Ingenuity Pathway Analysis; VUS: Variant of

Unknown Significance; PI: Propidium Iodide; PCA: Principal Component Analysis; GO: Gene

Ontology; SFARI: Simons Foundation Autism Research Initiative.

Ethics approval and consent to participate

Subjects were consented for biobanking and iPSC line generation by the Washington University

Institutional Review Board of the Human Research Protection Office under human studies

protocol #201409091 (Dr. John Constantino).

Consent for publication

Consent to publish data was provided by all subjects.

Availability of data and materials

The RNA-seq data generated during the current study are available in the Gene Expression

Omnibus (GEO) repository as Series GSE129806. The ASD gene dataset analyzed during the

current study is available in the SFARI Gene database at https://gene.sfari.org.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

E.M.A.L. contributed to the study design, carried out all cExN experimentation, analyzed data,

and prepared the manuscript. K.M. contributed to the study design, carried out all cIN

experimentation, analyzed data and contributed to manuscript preparation. D.B. interpreted

clinical exome sequencing data and contributed to manuscript preparation. P.G. and B.Z.

performed RNA-seq data analysis. A.B. contributed to the study design. J.N.C. contributed to

the study design and manuscript preparation. K.L.K. contributed to the study design, data

analysis, and manuscript preparation. All authors read and approved the final manuscript.

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Figure Legends

Table 1. Selected clinical characteristics and mutational status of several individuals in the multiplex ASD pedigree.

Figure 1. **Pedigree from which samples were derived for this study.** *GPD2* mutational status (GPD2m: indicates mutational status) and degree of ASD affectation are indicated. Black shading corresponds to the affected proband (AP) and his twin brother, dark grey to the intermediate phenotype sister (IS), light grey to the trait-affected brother (TB), and white to unaffected family members, including the unaffected mother (UM). * indicates that renal epithelial cells from these individuals were used to derive multiple, clonal iPSC lines.

Figure 2. **Characterization of iPSC lines during differentiation into cExN NPCs. (A)** Differentiation scheme, including timeline and small molecules used. **(B-C)** iPSCs derived from an unrelated, unaffected control (UC), as well as the UM, IS, and AP were differentiated for 12 days to generate cExN NPCs. Neurosphere size at several time points is shown in (B) and quantified in (C) (mean±SEM; scale bar = 500µm; n=12 biological replicates, encompassing two different clonal lines from each subject, and one clonal line for the UC). **(D-G)** At day four of differentiation, cells were stained with propidium iodide and FACS analysis of DNA content was performed. (D) Representative FACS plots. In (E) <2N (sub-G1) and (F) 2N (G1) cells are quantified, with values shown for each replicate. (G) shows mean values for all cell cycle stages for each cell line (mean±SEM; n≥3 biological replicates for each subject, encompassing two different clonal lines from each subject, and one clonal line for the UC). **(H-I)** iPSCs were differentiated in either induction media or mTeSR stem cell media, and EB size was analyzed at day four of differentiation. Representative images are shown in (H) (scale bar = 500µm), with quantification in (I) (n=3 biological replicates from one clonal line for each subject). p-values:

*P<0.05, **P<0.01, ***P<0.001 were determined by a two-tailed Student's t-test and all other

pairwise comparisons had a non-signficant p-value (*P≥0.05).

Figure 3. **Characterization of iPSC lines during differentiation into cIN NPCs. (A)**

Differentiation scheme, including timeline and small molecules used. **(B-C)** iPSCs were

differentiated for 12 days to obtain cIN NPCs, with differences in neurosphere size shown in (B)

and quantified in (C) (mean±SEM; scale bar = 250μm; n=3 biological replicates from one clonal

line for each subject). **(D-F)** NPCs were stained with propidium iodide and analyzed by FACS

for DNA content. In D and E, respectively, <2N (sub-G1) and 2N (G1) cells were quantified, with

values shown for each replicate (n=3 biological replicates from one clonal line for each subject).

(F) shows representative FACS plots. p-values: *P<0.05, **P<0.01, ***P<0.001 were

determined by a two-tailed Student's t-test and all other pairwise comparisons had a non-

signficant p-value (*P≥0.05).

Figure 4. **Transcriptomic analysis of genes with differential expression in affected**

subject-derived NPCs, relative to unaffected controls. (A) Hierarchical clustering of RNA-

seq data from the cExN NPCs identified differentially expressed genes (DEGs) with shared

expression in the AP and IS that differed from that in the UM. Relative expression is also shown

for the UC, for a full cross-sample comparison (p<0.05, fold-change >2; n=4 biological replicates

from one clonal line for each subject). **(B-D)** Ingenuity Pathway Analysis (IPA) of these cExN

NPC DEGs defined disease and function-associated gene ontology (GO) terms and identified

gene networks associated with the (C) 'behavior' (D) and 'behavior and developmental disorder'

GO terms. **(E)** Hierarchical clustering of RNA-seq data for the cIN NPCs identified DEGs with

shared expression in the AP and IS, that differed from that in the UM. Relative expression is

also shown for the UC, for a full cross-sample comparison (p<0.05, fold-change >2; n=4

biological replicates from one clonal line for each subject). **(F-H)** IPA analysis of these cIN NPC

DEGs defined (F) disease- and function-associated GO terms and identified gene networks

associated with (G) 'behavior' and (H) 'psychological disorder'. Within each network, red

symbols indicate upregulated genes and green symbols indicate downregulated genes, while

color intensity indicates relative degree of differential expression.

Figure 5. Within-family analysis of transcriptomic signatures specific to the affected

proband-derived samples. (A) Venn diagram for the cExN NPCs, showing the DEGs from

pairwise comparisons of different samples, including numbers of overlapping DEGs. The blue

shaded portion of the Venn diagram indicates DEGs unique to the AP, not shared by the IS or

UM. **(B-D)** Ingenuity Pathway Analysis (IPA) of the AP-unique DEGs in cExN NPCs defined

class and function-associated GO terms (B) and identified gene networks associated with

'behavior' (C) and 'nervous system development and function' (D). **(E-H)** IPA analysis of the AP-

unique DEGs in cIN NPCs determined (F) class and function-associated GO terms and

identified gene networks associated with (G) 'behavior' and (H) 'nervous system development

and function'. Within each network, red symbols indicate upregulated genes and green symbols

indicate downregulated genes, where the color intensity represents relative degree of differential

expression.

Figure 6. **Hierarchical clustering of DEGs that are also ASD genes in the SFARI autism**

gene database. **(A)** Relative gene expression for the cExN NPC samples. **(B)** Relative gene

expression for the cIN NPC samples. Data from four biological replicates are shown for each

sample type.

Figure 7. Validation of DEGs of interest identified from RNA-seq experiments by RT-

qPCR. Genes tested are related to behavior and developmental disorders, adhesion, and ion

channels. **(A-B)** Comparison of relative gene expression in cExN NPCs for the UM, IS, and AP

by (A) RNA-seq and (B) RT-qPCR, including expression analysis of genes related to 'behavior

and developmental disorders'. **(C-E)** Comparison of gene expression between the UM, IS and

AP for the cIN NPCs by (C) RNA-seq and by (D-E) RT-qPCR, both for genes that were (D)

differentially expressed only in the cIN NPCs and (E) for genes that were differentially

expressed in both cExN and cIN NPCs. p-values: *$P<0.05$, **$P<0.01$, ***$P<0.001$ were

determined by a two-tailed Student's t-test and all other pairwise comparisons had a non-

signficant p-value ($P≥0.05$). RT-qPCR data shown includes n≥3 biological replicates from one

clonal line per subject, where samples were generated for each subject by using a second

clonal iPSC line that differed from the line used for RNA-seq analysis.

Additional files

Additional file 1: Table S1. Antibodies used in immunocytochemistry and immunoblotting experiments (XLSX).

Additional file 2: Figures S1-S5. Figure legends provided in file (PDF).

Additional file 3: Table S2. DEGs from pairwise comparisons of the four sample types for cExN or cIN NPCs (XLSX).

Additional file 4: Table S3. Hierarchical clustering of the relative expression of DEGs across the four sample types for cExN and cIN NPCs was performed using ClustVis. Order of genes in the cluster and unit variance scaled relative expression values are indicated (XLSX).

Additional file 5: Table S4. Details about selected DEGs within IPA networks (PDF).

Additional file 6: Table S5. cExN DEG comparison to other studies (XLSX).

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Clinical characteristic	AP	IS	UM
Age of ASD diagnostic confirmation	3.5 years	18 years	N/A
Social Responsiveness Scale-2	83T	72T	56T (spouse-report)
Depression and Anxiety	Yes	Yes	No
Seizure history	No	No	No
Developmental delay	Yes	No	No
Eye contact	Poor	Fair	Good
Repetitive behavior	Yes	No	No
Abnormal sensory sensitivities	Yes	No	No
IQ	65	102 (Raven)	108 (Raven)
Speech delay	Yes	No	No
ASD	Severe	Moderate	No
Intellectual Disability	Yes	No	No
Mutation location	GPD2 chr2:157352686 (hg19) G>A p.G78E, c.233G>A	GPD2 chr2:157352686 (hg19) G>A p.G78E, c.233G>A	GPD2 chr2:157352686 (hg19) G>A p.G78E, c.233G>A

Fig. 1

* Cellular model developed from patient's renal epithelial cells.

Severity of ASD affectation:
- ASD-Affected Proband - AP
- Intermediate Phenotype Sister - IS
- Trait-Affected Brother - TB
- Unaffected Mother - UM

Fig. 2

Fig. 3

A Neurobasal-A medium+B-27+SB431542+LDN193189 +XAV939+Purmorphamine

| V-bottom plates | Suspension culture | 2-D Culture |

Day 0   Day 4   Day 10   Day 12

B   UC   UM   IS   AP

Day 5

Day 12

C

Neurosphere Size (μm)

UC   UM   IS   AP

D

sub-G1 population (%)

UC   UM   IS   AP

E

G1 Population (%)

UC   UM   IS   AP

F

Count

BluFL2

UC: <2N: 6.92% 2N: 56% S: 15.9% 4N: 15.1% >4N: 5.49%

UM: <2N: 6.25% 2N: 57.3% S: 15.7% 4N: 14.9% >4N: 5.88%

IS: <2N: 11.7% 2N: 47.2% S: 18.1% 4N: 14.5% >4N: 8.45%

AP: <2N: 13.5% 2N: 46.1% S: 17.4% 4N: 14.6% >4N: 8.43%

Fig. 4

Fig. 5

Fig. 6