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TITLE: Testing Brain Overgrowth and Synaptic Models of Autism Using NPCs and Neurons from Patient-Derived iPS Cells

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**14. ABSTRACT**
Autism and autism spectrum disorders (ASD) are complex neurodevelopmental diseases that affect about 1% of children in the United States. Such disorders are characterized by deficits in verbal communication, impaired social interaction, and limited and repetitive interests and behavior. Recent studies have led to two major hypotheses for autism pathogenesis: early brain overgrowth and synaptogenesis defects. The goal of this project is to produce human cellular models of non-syndromic ASD. We used cellular reprogramming to develop iPSCs from ASD patients (and non-autistic controls) for the production of patient-derived neural progenitors (NPCs) and neurons to study cellular phenotypes that directly test whether brain overgrowth and/or synaptogenesis mechanisms are found in ASD NPCs and neurons. Patient-derived NPCs and neurons from these ASD and control individuals will be used for the functional characterization of iPSCs-derived NPCs and neurons from ASD and control individuals for potential autism-specific defects in proliferation, neural development and synaptogenesis, and for gene expression studies. We have made significant progress on these aims in the first year.

**15. SUBJECT TERMS**
Autism, induced pluripotent stem cells, gene expression.
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1. INTRODUCTION:
Autism spectrum disorders (ASD) are complex neurodevelopmental diseases that affect more than 1% of children in the United States. Neuropathological imaging and genetic studies have led to several models for autism pathogenesis, including excess neural proliferation, cell cycle dysregulation, altered brain growth or developmental disconnection leading to dysfunctional long-range neuronal networks. However, the major impediment to testing these hypotheses is the lack of consistent brain pathological abnormality that can be modeled in relevant animal and cell models. Recently, reprogramming of human somatic cells to a pluripotent state (induced pluripotent stem cells or iPSCs) has provided a timely opportunity to produce relevant cellular models of human complex neurogenetic diseases. The goal of this project is to produce human cellular models of non-syndromic ASD. We used cellular reprogramming to develop iPSCs from ASD patients (and non-autistic controls) for the production of patient-derived neural progenitors (NPCs) and neurons to study cellular phenotypes that directly test whether brain overgrowth and/or synaptogenesis mechanisms are found in ASD NPCs and neurons.

2. KEYWORDS:
Autism spectrum disorder, ASD, neurodevelopmental disease, disease modeling, induced pluripotent stem cell, iPSSs, human neurons, brain overgrowth.

3. OVERALL PROJECT SUMMARY
In the past year of funding, we have made significant progress. We generated and characterized iPSC lines from 8 sporadic ASD patients with early brain overgrowth and 5 age/gender-matched control lines. These cells were used to derive neural progenitor cells and neurons in culture. We uncovered molecular and phenotypic evidence that both cell proliferation and synaptogenesis are affected in this model system. Specifically, we detected altered cell cycle and altered levels of excitatory and inhibitory neurotransmitters in neural cells at early stages of differentiation. We have written up this work and it is nearly ready for submission for publication (Marchetto et al., in preparation). This work demonstrates, for the first time, that in heterogeneous conditions such as ASD, selection of subjects based on endophenotypes can improve the power to detect biologically relevant pathway disruption that may help guide the development of novel therapies.

During the first year of the research grant we focused on the functional characterization of the iPSC-derived neuroprogenitors (NPCs) and neurons from ASD patients and age/gender matched neurotypical individuals. We have screened individuals for potential autism-specific defects in proliferation, neural development and synaptogenesis as proposed in the Statement of Work (SOW), Task 1.

**Task 1: Functional characterization of iPSCs-derived NPCs and neurons from ASD and control individuals for defects in proliferation, neural development and synaptogenesis (months 1-16).**

**Subtask 1a.** Characterize ASD-iPSCs from 7 ASD patients and 6 controls (at least 2 clones for each individual) for pluripotency and neural differentiation potential (months 1-6).

We recruited 8 ASD Individuals with quantitative MRI-validated early brain enlargement ranging from mild brain enlargement to macroencephaly and 5 age/gender-matched control individuals for skin biopsies and phenotypic characterization. These patients were recruited with Dr. Eric Courchesne (UCSD School of Medicine). The ASD donors displayed larger brain size compared to the normal average brain size of typically developing control subjects at any given age (Figure 1A, B). Copy Number Variation analysis using DNA extracted from the donors’ whole blood did not show the presence of any rare structural variant known to be associated with ASD of unknown etiology. A small duplication involving intron 2 of the NLGN1 gene was detected in one ASD case; however, this event has also been reported in typically developing controls (http://dgv.tcag.ca/). Exome sequencing of DNA from the 8 ASD fibroblasts revealed 1,059 total mutations in all 8 libraries (1011 SNVs and 48 stop-gains). No mutations in genes known to result in abnormal brain development were detected, and no mutations were found in two alleles of a single gene in any patient. However, 46 mutations on known autistic genes were found, of which about half were found likely to be damaging by both SIFT and PolyPhen programs.
Figure 1. Derivation of NPCs from ASD and control subjects. (A) Left panel, scatterplot of Total Brain Volume (TBV) across ages. Open black circles indicate brain size of typically developing subjects. Black solid dots represent control donors. Red solid dots represent ASD donors. (B) Three-dimensional reconstructions of the brain from one control donor and one ASD donor. (C) iPSCs from ASD and control were differentiated to NPCs. From passages 2 to 6 cells were plated at the same density and population-doubling time at each passage was calculated. Results of 5-6 lines (2 clones per line) are presented as mean±SEM (*repeated measurements p=0.02, post-hoc p<0.04). (D) Adherent monolayer NPCs from control and ASD iPSCs were dissociated, counted for calculation of population doubling time and prepared for cell cycle analysis. Results are presented as the time spent in each cell cycle stage (n≥4, mean±SEM, ANOVA p<0.04, post-hoc p<0.04 for comparing the time spent in G1 phase in the ASD NPCs with those of the control NPCs, respectively). (E) Control and ASD NPCs were immunostained with DAPI (Blue), anti-pHH3 (Green) and anti-ki67 (Red) (Scale bar: 200mm), Representative images of the staining are shown. (F) Quantification of the percentage of Ki67+ and Ki67+pHH3+ labeled cells are presented as mean±SEM (n≥5; *p<0.03 for comparing the results of the ASD with those of the control NPCs). (G) Representative images of the NPC staining for Sox1 (Scale bar: 200mm). (H) Quantification of the percentage of Pax6+, OTX2+, Sox1+ and Vimentin+ labeled cells is presented as mean±SEM (n≥5; *p<0.006 for comparing the results of the ASD with those of the control NPCs). (I) Representative immunoblot of control and ASD-derived NPCs that were lysed and immunoblot for Pax6, OTX2, Sox1, Vimentin and GAPDH. (J) The levels of Pax6, OTX2, Sox1 and Vimentin, which were normalized to GAPDH levels, were quantified and results are presented as mean±SEM (n≥5; *p<0.007 for comparing the results of the ASD with those of the control NPCs). (K) RNA of control and autistic NPCs was extracted and RT-PCR was performed. The levels of Pax6, OTX2, Sox1, which were normalized to HPRT1, are presented as mean±SEM (n≥3; *p<0.04 for comparing the results of the ASD with those of the control NPCs).
Of note, stop-gains in the canonical Wnt pathway genes CTNNB1 (coding for β-catenin, Arch) and FZD6 (coding for Frizzled 6, in Acai), as well as a missense mutation in DVL2 (coding for Dishevelled-2, in Apex). These findings are notable considering that Dishevelled mouse mutants display social behavior deficits and embryonic brain growth abnormalities that result from dysregulation of a β-catenin/Brn2/Tbr2 transcriptional cascade (Belinson et al. manuscript in preparation). However, when genes containing all SNVs and stop-gain mutations were subjected to gene-ontogeny (GO) analysis, no GO term or pathway reached statistical significance, although Wnt and Notch signaling pathways were suggestive for significance.

ASD and non-affected control fibroblasts were transduced with 4 retroviral reprogramming vectors (Sox2, Oct4, c-Myc and Klf4), as described elsewhere (Takahashi et al., 2007). Following 2 to 3 weeks of culture in human embryonic stem cells (hESC)-supporting conditions, compact refractile ESC-like colonies emerged from a background of fibroblasts. iPSC colonies were then manually picked and cultured under feeder-free conditions. Cells were mechanically expanded for at least 10 passages and tested for the expression of pluripotent markers. We obtained several clones that continuously expressed pluripotent markers, such as Nanog, Lin28, Tra-1-81 and Sox2 from each control wild-type (WT)-iPSCs and ASD-iPSCs. We excluded all karyotypically unstable clones from further experiments.

The production of iPSCs described in Task 1a was done in Dr. Gage’s laboratory, Salk Institute, La Jolla, CA, while the exome sequencing was performed in the lab of Dr. Wynshaw-Boris’s laboratory, UCSF, San Francisco, CA/Case Western Reserve University, Cleveland, OH.

Subask 1b. Perform NPC differentiation using the iPSCs clones characterized in Subtask 1a and access cell proliferation using the methods described below (months 6-12).

Our recent analyses showed that iPSC-derived neural cell types could recapitulate human mid-fetal cortical development and thus could be used to model this developmental stage in ASD (Stein et al., 2014). We used our previous published protocol to generate NPCs from iPSCs (Marchetto et al., 2010) in the presence of Noggin. Briefly, we initiated neural differentiation by plating 1-week-old embryoid bodies (EBs) treated with Noggin. After a week in culture, EB-derived rosettes became apparent in the dish. Rosettes were then manually collected, dissociated and re-plated. The NPCs derived from rosettes formed a homogeneous population after a few passages from ASD Individuals and controls, and continued to proliferate in the presence of FGF2 as adherent monolayers.

We hypothesized that an alteration of the rates of NPC proliferation could result in early brain overgrowth. Proliferation was measured by calculating the population doubling time from plating at passages 3 to 7 (P3-7) in continuous culture. From P4, the population doubling time decreased in ASD NPCs from all 8 Individuals compared to NPCs from all 5 controls, reaching statistical significance at P6 (Figure 1C). Cell cycle analysis at P6 revealed that shortening of G1 and S phases was the main reason for the decrease in the population doubling time; with no change of G2-M phase length (Figure 1D). Double labeling for Ki67 and pH3 revealed an increased percentage of Ki67+ (cycling cells) in ASD relative to control NPCs, whereas the percentage of pH3+Ki67+ (G2-M phase mitotic cells) was unaffected in autistic NPCs (Figure 1E). These findings demonstrate that iPSC-derived NPCs from ASD Individuals with macrencephaly proliferated faster than those derived from controls. Next, we characterized the expression of forebrain and midbrain markers in the ASD-derived NPCs and those from control individuals. SOX1 (a marker for maintenance of radial glial NPCs identity), and Vimentin (the intermediate filament protein of NPCs) were up-regulated in the ASD NPCs compared to control NPCs (Figure 1G-K). SOX1 and Vimentin were previously shown to correlate with induced proliferation of NPCs (Alonso, 2001; Elkouris et al., 2011), consistent with the increased rate of proliferation of NPCs derived from the ASD individuals. The expression of PAX6 (a marker of forebrain neural-ectoderm) and OTX2 (a marker of the midbrain and forebrain), measured by immunocytochemistry, Western blot and qPCR, were unchanged in ASD and control NPCs (Figure 1G-K). In addition, the expression of the intermediate NPCs marker TBR2 was low and did not differ between ASD and control NPCs (data not shown).

In another study, we found that the genetic loss of Dishevelled 1 and 3 (Dvl1-/-3+/-) in mice lead to early embryonic brain enlargement, mediated by a deregulation of the β-catenin/Brn2/Tbr2 transcriptional cascade that is related to the social behavior deficits observed in adult Dvl mutant mice (Belinson et al., unpublished). This transient embryonic brain enlargement resulted from a transient over-proliferation of neural progenitors in the cerebral cortex, and the in vitro growth phenotype of Dvl1-/-3+/- NPCs was similar to the ASD NPCs. Thus, we hypothesized that a similar molecular mechanism might govern the aberrant proliferation in ASD-derived NPCs. To test our hypothesis, we first examined Wnt/β-catenin transcriptional activity using TOP-flash assays. β-catenin transcriptional activity was reduced in untreated ASD compared to control NPCs (Figure 2A). Activation of canonical Wnt signaling with 5 mM LiCl, which prevents GSK3-mediated degradation of β-catenin,
Figure 2. Regulation of NPC proliferation by the Wnt pathway. (A) Control and ASD NPCs were transfected with TOP-Flash and firefly renilla reporters and treated with either 5mM LiCl or 100ng/ml Wnt3A. Results are presented as mean±SEM (n≥5, *p<0.04 for comparing the luminescence ratio in the ASD NPCs with those of the control NPCs). (B) Control and ASD NPCs were fixed and immunostained for Brn2 and representative images of the staining are shown (Scale bar: 200mm). (C) Quantification of the percentage of Brn2+ labeled cells is presented as mean±SEM (n≥5; *p<0.001 for comparing the results of the ASD with those of the control NPCs). (D) Representative immunoblot of control and ASD-derived NPCs that were lysed and immunoblot for Brn2, β-catenin and GAPDH. (E) The levels of Brn2 and β-catenin, which were normalized to GAPDH levels were quantified and results are presented as mean±SEM (n≥5; *p<0.03 for comparing the results of the ASD with those of the control NPCs). (F) Representative images of pCAG-Brn2 transfected control and ASD-derived NPCs (Scale bar: 200 mm). The number of DAPI+ nuclei (G) and the percentage of Brn2+ (H) and ki67+ (I) cells were measured. Results are presented as mean±SEM (n≥4, AVOVA<0.02 *p<0.05, comparing the results of the GFP-transfected ASD NPCs with those of the control and the Brn2 transfected autistic NPCs).
elevated β-catenin transcriptional activity in ASD and control NPCs. However, β-catenin transcriptional activity was significantly reduced in LiCl-treated ASD NPCs compared to control NPCs (Figure 2A), indicating that the cause of reduced β-catenin transcriptional activity is downstream of GSK3. Activation of canonical Wnt signaling with Wnt3A resulted in a similar, marked but non-significant elevation of β-catenin transcriptional activity in both control and ASD NPCs (Figure 2A), although a similar trend of reduced β-catenin transcriptional activity in the ASD NPCs was noted. Of note, NPCs derived from the patient with the heterozygous stop-gain mutation in CTNNB1, Arch, displayed the weakest responses to LiCl and Wnt3a stimulation. To determine if this reduced activity was responsible for the growth defects displayed, we repeated the proliferation experiment in ASD-derived and control NPCs in the presence and absence of LiCl. In the absence of LiCl, all ASD-derived NPC lines displayed faster growth (reduced doubling times) than all control NPC lines. LiCl treatment slowed the growth of ASD-derived NPCs close to doubling times of control NPCs, while the growth of control NPCs was unaffected (data not shown). These findings indicate that the reduced β-catenin transcriptional activity played a functional role in the accelerated proliferation found in ASD-derived NPCs.

We found that the reduced activity in Dvl1−/−3−/− mutant mice resulted in reduced levels of the transcription factor Brn2 or Pou3f2 (Belinson et al., co-submission). To test if BRN2 levels were similarly affected in ASD NPCs, we performed immunocytochemistry for BRN2 on ASD and control NPCs. The percentage of BRN2+ cells was in fact reduced in ASD NPCs compared to controls (Figure 2B, C). Supporting this result, Western blot analysis revealed that BRN2 protein levels were also reduced in ASD NPCs compared to controls (Figure 2D, E). To examine whether exogenous BRN2 rescued the increased rate of proliferation observed in ASD NPCs, we transfected CAG-BRN2 into control and ASD NPCs and performed immunocytochemistry for BRN2 and Ki67. Transfection efficiency was similar between control and ASD NPCs (around 45%). The number of DAPI+ cells (as an index of proliferation) in ASD NPCs was similar to controls after BRN2 overexpression (Figure 2F-I). Importantly, the expression pattern of BRN2 and Ki67 in the BRN2 transfected ASD NPCs resembled that of control NPCs, providing support for the direct regulation of proliferation of autistic NPCs by BRN2. These data also suggest that partially conserved pathways regulate proliferation of NPCs in a mouse model of social behavior and ASD-derived NPCs from patients with early brain overgrowth.

The NPC lines from all patients and controls were generated at Dr. Gage’s laboratory, Salk Institute, La Jolla, CA; all the proliferation tests and β-catenin/BRN2 transcriptional activity tests, immunocytochemistry, western blot and qPCR experiments described on Task1b were performed in the lab of Dr. Wynshaw-Boris’s laboratory, UCSF, San Francisco, CA/Case Western Reserve University, Cleveland, OH.

Subtask 1c. Perform neuronal differentiation using the iPSC clones characterized in 1a and analyze neuronal maturation using the methods described below (months 8-16).

Proliferation abnormalities displayed by Dvl1−/−3−/− mouse mutants resulted in premature differentiation of deep layer cortical neurons (Belinson et al., co-submission). Therefore, we examined the earliest stages of neuronal differentiation in control and ASD NPCs following 1, 2 and 4 weeks of differentiation. Differentiated NPCs were fixed at the indicated time points and immunocytochemistry was performed for TUJ1 and Doublecortin (DCX) (Figure 3A). After 1 week of differentiation, the percentages of TUJ1 and DCX were higher in the ASD samples compared to controls (Figure 3B, C). The levels of TUJ1 protein after one week of differentiation were also elevated in ASD compared to control samples by Western blot analysis (Figure 3D, E), similar to immunocytochemistry, supporting premature differentiation of ASD NPC. However, differentiation for 2 or more weeks did not reveal differences in the percentage of differentiated cells between ASD and control samples (Figure 3A-C).

Evidence from the literature suggests an imbalance in excitatory versus inhibitory signals in developing ASD (Rubenstein, 2010; Zikopoulos and Barbas, 2013). To examine whether the ASD-NPCs led to a change in excitatory versus inhibitory cell fate, we measured Glutamatergic (NGN2) and GABAergic (MASH1, DLX2, Olig2 and NKX2.1) progenitor markers in ASD and control NPCs. A reduction in the percentage of NGN2+ NPCs in ASD NPCs compared to controls was observed (Figure 3F, G). In contrast, markers of inhibitory precursors present in the subpallium (MASH1, DLX2 and NKX2.1) were up-regulated in ASD compared to control NPCs. However, OLIG2, another subpallium marker, was unchanged between control and ASD NPCs (Figure 3G).

All the neuronal differentiation assays, immunocytochemistry, western blot and qPCR experiments described on Task1c so far were performed in the lab of Dr. Wynshaw-Boris’s laboratory, UCSF, San Francisco, CA and Case Western Reserve University, Cleveland, OH.
Figure 3. Neuronal differentiation of iPSCs. (A) Control and ASD NPCs were differentiated into neurons and fixed after 1, 2 and 4 weeks. (Scale bar: 200mm). The percentages of Tuj1+ (B) and DCX+ (C) cells were measured and results are presented as mean±SEM (n=4, AVOVA<0.02 *p<0.04, comparing the results of ASD neuron with those of the control and neurons). (D) Control and ASD NPCs were differentiated for 1 week after which cells were lysed and immunoblot for Tuj1 and GAPDH and representative blot is presented. (E) The levels of Tuj1, which were normalized to GAPDH levels, were quantified and results are presented as mean±SEM (n=4; *p<0.02 for comparing the results of the ASD with those of the control neurons). (F) Representative images of the control and ASD NPCs immunostained for Ngn2 and Mash1. (Scale bar: 200 µm). (G) Quantification of the percentage of Ngn2+, Mash1+, Olig2, Dlx2+ and Nkx2.1+ labeled cells is presented as mean±SEM (n≥5; *p<0.03 for comparing the results of the ASD with those of the control NPCs).
Task 2: Functional characterization of dysregulated pathways uncovered by gene expression from iPSC-derived NPCs and neurons (months 16-24).

Progress on this task will be presented in the next period.

4. KEY RESEARCH ACCOMPLISHMENTS:

There are two key great research accomplishments in the first period of the grant, highlighted in the data presented above.

1. We demonstrated that iPSC-derived NPCs from ASD patients with macrencephaly proliferated faster than those derived from controls and that can be explained by a reduction in β-catenin transcriptional activity via regulation of the transcription factor BRN2.

2. We demonstrated that ASD neurons undergo premature differentiation, which could have important implications to proper neuronal maturation which will be further investigated.

5. CONCLUSION:

The use of iPSCs to study genetic disorders is a powerful tool to dissect molecular and cellular pathways implicated in disease pathology during early stages of human neurodevelopment. However, modeling highly complex idiopathic disorders such as ASD is challenging due to a high level of heterogeneity in the patient population. Here, we took advantage of iPSCs derived from a carefully characterized clinical cohort of ASD patients who have an anatomical phenotypic trait that occurs in about 20-30% of idiopathic ASD: an early developmental enlargement of brain volume, including macrencephaly that is frequently associated with poor prognosis. We hypothesized that the increased brain volume and neuron numbers found in ASD may result from increased rates of proliferation in neural progenitors. Consistent with this hypothesis, NPCs derived from iPSCs of such autistic patients displayed rapid rates of proliferation when compared to NPCs derived from non-affected typically developing individuals. Interestingly, all ASD-derived NPCs displayed faster proliferation than all control-derived NPCs. This cellular phenotype results from alterations in a canonical Wnt-β-catenin/BRN2 transcriptional cascade. Consistent with this iPSC-derived NPC phenotype, we found that Dvl mutants displayed adult social behavior abnormalities and transient brain overgrowth during embryonic deep layer cortical neuron formation mediated by a similar β-catenin/Brn2/Tbr2 transcriptional cascade (Belinson et al., unpublished). Transient activation of the canonical Wnt pathway in utero normalized the adult social behavior deficits in Dvl mutant mice. Under the conditions used here, we did not observe significant expression of TBR2, so we were unable to test whether TBR2 is also dysregulated in NPCs derived from ASD patients. Thus, common transcriptional mechanisms that include β-catenin and BRN2 appear to regulate NPC proliferation in a mouse model with social behavior deficits and a cellular model of human autism. Taken together, these findings indicate that increased proliferation rates found in NPCs are the likely cause of abnormal prenatal numbers of cortical neurons in the genesis of autism.

In the next year we will focus on testing the synaptogenesis and neuronal function of mature neurons (i.e. percentage of excitatory and inhibitory neurons, expression of synaptic markers and electrophysiology) and on transcription profiling (RNA expression assessment) of ASD NPCs and neurons versus control cells. We will also examine the transcriptional program regulated by b-catenin and BRN2, since these transcription factors appear to be conserved in the regulation of social behavior in mouse and human. Together, our initial results suggest that idiopathic ASD can be modeled using the iPSC technology to reveal novel cellular and molecular mechanisms underlying brain abnormalities.

6. MANUSCRIPT IN PREPARATION


7. INVENTIONS, PATENTS AND LICENSES: Nothing to report
8. REPORTABLE OUTCOMES:
Nothing to report.

9. OTHER ACHIEVEMENTS:
This award contributed to the production of control and ASD iPS cell lines from 13 individuals listed below (and 2 clones each, except for CLUE).
1-AQUA, 2-AHOY, 3-ACAI, 4-AVID, 5-ABLE, 6-AERO, 7-ARCH, 8-APEX (ASD)
9-CHAP, 10-CLAY, 11-COVE, 12-CENT, 13-CLUE (Control)

10. REFERENCES:

11. APPENDICES:
Nothing to report.