Stem Cells as a Good Tool to Investigate Dysregulated Biological Systems in Autism Spectrum Disorders

Karina Griesi-Oliveira, Daniele Yumi Sunaga, Lucas Alvizi, Estevão Vadasz, and Maria Rita Passos-Bueno

Identification of the causes of autism spectrum disorders (ASDs) is hampered by their genetic heterogeneity; however, the different genetic alterations leading to ASD seem to be implicated in the disturbance of common molecular pathways or biological processes. In this scenario, the search for differentially expressed genes (DEGs) between ASD patients and controls is a good alternative to identify the molecular etiology of such disorders. Here, we employed genome-wide expression analysis to compare the transcriptome of stem cells of human exfoliated deciduous teeth (SHEDs) of idiopathic autistic patients \((n = 7)\) and control samples \((n = 6)\). Nearly half of the 683 identified DEGs are expressed in the brain \((P = 0.003)\), and a significant number of them are involved in mechanisms previously associated with ASD such as protein synthesis, cytoskeleton regulation, cellular adhesion and alternative splicing, which validate the use of SHEDs to disentangle the causes of autism. Autistic patients also presented overexpression of genes regulated by androgen receptor (AR), and AR itself, which in turn interacts with \(CHD8\) (chromodomain helicase DNA binding protein 8), a gene recently shown to be associated with autism and found to be upregulated in some patients tested here. These data provide a rationale for the mechanisms through which \(CHD8\) leads to these diseases. In summary, our results suggest that ASD share deregulated pathways and revealed that SHEDs represent an alternative cell source to be used in the understanding of the biological mechanisms involved in the etiology of ASD. Autism Res 2013, ••: ••–••. © 2013 International Society for Autism Research, Wiley Periodicals, Inc.

**Keywords:** expression studies; androgen signaling; CHD8; stem cells of human exfoliated deciduous teeth

**Introduction**

Autism spectrum disorders (ASDs), characterized by impairments in communication and social skills and stereotyped behavior, have a strong genetic component. Their genetic architecture is still debated with clear evidence of allelic and locus heterogeneity [Betancur, 2011; El-Fishawy & State, 2010].

Transcriptome analysis in ASD has shown deregulation of genes and pathways related to neurological functions and central nervous system development [Baron, Liu, Hicks, & Gregg, 2006; Gregg et al., 2008; Hu, Frank, Heine, Lee, & Quackenbush, 2006; Hu et al., 2009a,b; Seno et al., 2010; Voineagu et al., 2011]. Most of these studies were carried out using lymphoblasts, lymphoblastoid cell lines (LCLs) or brain tissue. Lymphoblasts and LCL have shown several limitations due to their embryonic origin and cell differentiation degree. On the other hand, expression studies using postmortem brain tissue are hampered mainly by the potential biases from post-mortem effects and the relatively small sample sizes. Therefore, it is of value the identification of other cell sources to study ASD.

Stem cells of human exfoliated deciduous teeth (SHEDs) are an easily accessible cell source, with an ectodermic origin and expression of neuronal markers upon differentiation, which makes them functionally and embryonically more related to nervous tissue cells than the previously used lymphocytes and LCL [d’Aquino et al., 2009; Miura et al., 2003]. To investigate the applicability of SHEDs to study the pathogenesis of ASD, we have asked if ASD candidate genes and biological processes/pathways involved in neuronal development and function were enriched upon functional annotation analysis of the differentially expressed genes identified. Next, we detected deregulation of the androgen signaling pathway. The relationship between androgen signaling and chromodomain helicase DNA binding protein 8 (\(CHD8\)), a candidate gene recently identified in ASD exome sequencing studies [Neale et al., 2012; O’Roak et al., 2012a,b; Sanders et al., 2012; Talkowski et al., 2012], is discussed.
Material and Methods

Patients Ascertainment

We have so far evaluated 600 families with ASD following previous standardized criteria [Orabona et al., 2009]. All probands were diagnosed according to Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria (Institute of Psychiatry, Hospital das Clinicas, University of Sao Paulo, IPq-USP) and using an interview based on Autism Diagnostic Interview-Revised. Of the seven patients included in the study, four were high-functioning and three low-functioning autistic individuals. Blood samples were collected for DNA analysis. All the seven patients were male, Fragile-X negative and negative for the copy number variations on 15q11-13, 16p11 and 22q13 (tested by SALSA MLPA P343 Autism-1 probe-mix, MRC-Holland, Amsterdam, the Netherlands). Medical and family history was also obtained. SHEDs lineages used as controls were obtained from deciduous teeth of healthy volunteers (six males for the first analysis (DS1) and four males and two females—the available samples at the time of the experiments—for the second analysis (DS2)). This project has been approved by the Ethics Committee of the Institutes where the study was conducted. After a complete description of the study, written informed consent was signed by the parents.

Isolation of Human Dental Pulp Stem Cells and RNA Extraction

SHEDs lineages were obtained according to previously published protocols [Costa et al., 2008]. RNA samples were extracted using the NucleoSpin RNA II (Macherey-Nagel, Düren, Germany) extraction kit. Sample concentration and quality were evaluated by Nanodrop 1000 (Nanodrop products, Wilmington, DE, USA) and gel electrophoresis. All the samples were obtained from subconfluent cultures (80–90% of confluence) between passages three and five.

Microarray Studies

For microarray experiments, 100 ng of RNA was reverted to cDNA, amplified, labeled and hybridized to the Affymetrix Human Gene 1.0ST chip (Affymetrix, Santa Clara, CA, USA), following the manufacturer’s protocol. The chips were scanned by GeneChip® Scanner 3000 7G System (Affymetrix, Santa Clara, CA, USA), and a quality control was processed by Affymetrix® Expression Console Software (Affymetrix, Santa Clara, CA, USA). Normalization was carried out using the Robust Multi-array Average method [Irizarry et al., 2003]. A subset of genes was selected using interquartile range as filtering criteria [von Heydebreck, Huber, & Gentleman, 2004].

Genes that did not show an expression variation over all samples greater than 0.5 were removed (10,474 genes were retained).

Differentially expressed genes were selected with the RankProd method, considering a P-value cutoff of 0.05 adjusted for false discovery rate (FDR) [Benjamini & Hochberg, 1995]. A gene selected by RankProd may exhibit high expression levels (high rankings) in only a subgroup of patients, which is a good alternative for the study of complex and heterogeneous phenotypes such as ASD [Breitling, Armengaud, Amtmann, & Herzyk, 2004; Bueno et al., 2011; Raj, Rifkin, Andersen, & van Oudenaarden, 2010].

Functional annotation analysis was conducted using Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com/), and Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/). The lists of androgen receptor (AR)-regulated genes (obtained from IPA database), autism candidate genes [extracted from Xu et al., 2012], differentially expressed genes (DEGs) on ASD lymphocyte/LCL [compiled from Baron et al., 2006; Gregg et al., 2008; Hu et al., 2006, 2009a,b; Nishimura et al., 2007], DEGs on ASD brain [Voineagu et al., 2011] and mutated genes found by exome studies [compiled from Chahrour et al., 2012; Iossifov et al., 2012; Neale et al., 2012; O’Roak et al., 2011; 2012a,b; Sanders et al., 2012; Talkowski et al., 2012] were created in IPA in order to be used as reference sets for enrichment analysis of our DEGs data sets. Fisher’s exact test was used for all the enrichment analysis.

Results

We identified 683 DEGs (data set 1—DS1; Table S1) by comparing seven patients and six controls. We found that a significant percentage of DEGs are expressed in brain (42.8%; \(P = 0.003\)) and fetal brain (5.56%; \(P = 0.01\)) (Table S1). Gene ontology enrichment analysis revealed that downregulated genes are enriched by functional categories related to protein synthesis and mRNA processing (Fig. 1a and Table S1). Upregulated genes are enriched by genes that undergo alternative splicing, involved in neurogenesis, cell adhesion and cytoskeleton assembly, among other relevant biological systems involved with ASD (Fig. 1b and Table S1). Further, DEGs specifically related to nervous system development are mainly involved in neurite formation and growth, and glial cell biology (Fig. 1c).

Consistent with the biological functions mentioned earlier, we highlight two main groups of relevant pathways among the 68 canonical pathways (Table S1) enriched by the DEGs: a group of pathways involved in protein synthesis (mTOR signaling, PTEN signaling, EIF2
signaling and regulation of eIF4 and p70S6K signaling) and a group involved in cytoskeleton organization/neurite formation (axonal guidance signaling, FAK signaling, RhoGDI signaling, integrin signaling and regulation of actin-based motility by Rho) (Fig. 1d).

Comparing our data set with DEGs identified by other global expression studies using lymphoblasts/LCL, we found an overlap of only 10 genes \( (P = 0.3) \) (Table 2). On the other hand, 20 genes identified in our analysis were also found as deregulated in ASD brain \( (P = 0.01) \). A significant number of DEGs from DS1 (listed in Table 1) are ASD candidate genes \( (P = 2 \times 10^{-3}) \), and 12 DEGs were found to harbor de novo mutations in ASD patients screened by O’Roak and colleagues \( [2012b] \). As CHD8 seems to play a role in AR-mediated transcriptional regulation \( [Menon, Yates, & Bochar, 2010] \), we have looked for AR and its downstream regulated genes among our DEGs. We observed that AR and 17 AR-regulated genes are also overexpressed in ASD patients \( (P < 10^{-4}; \) Fig. 2). Four (cut-like homeobox 1, glutathione S-transferase mu 1, hepatocyte growth factor (hepapoietin A; scatter factor) and insulin-like growth factor 2 (somatomedin A)) of these 17 AR-regulated genes had already been identified as ASD candidate genes \( (P = 0.009; \) Table 2).

To verify if our data were replicable, we compared the same patients against a new set of controls \( (n = 6) \), and we found 701 DEGs (DS2; Table S3). We compared DS2 with DS1 and found an overlap of 206 genes \( (P = 0.3) \) that were differentially expressed in the same direction in both analyses. The most relevant biological categories found in the functional analysis of DS1 were also enriched in DS2 and DS3 (Table 3). Noteworthy, all the canonical pathways related to protein synthesis cited earlier, in addition to axonal guidance signaling and regulation of actin-based motility by Rho, were also significantly enriched by DEGs in DS3, showing that the genes in common between the two analyses are those that are the most functionally relevant for the studied disease. We verified that DS2 and DS3 DEG lists also have a significant overlap with ASD candidate genes (27 DEGs, \( P = 9 \times 10^{-4} \) and 9 DEGs, \( P = 0.03 \), respectively) [Xu et al., 2012]. AR is also upregulated in DS2, and the number of DEGs that are regulated by AR is significant both in DS2 and DS3 (15 DEGs, \( P < 10^{-3} \) and 6 DEGs, \( P = 0.02 \), respectively).

Figure 1. Function annotation analysis of DS1. (A) Biological function categories enriched by downregulated genes. (B) Biological function categories enriched by upregulated genes. (C) Biological function categories enriched specifically among the differentially expressed genes (DEGs) involved in nervous system development and function. (D) Canonical pathways enriched by the DEGs. All the charts show only the most functionally relevant categories found. The numbers at right of each bar refers to the number of genes within each category.
Finally, we compared the transcriptome of six patients affected by a nonneurological disorder (nonsyndromic cleft lip/palate—NSCLP) against the first set of control samples. We verified that only 40 genes out of 230 NSCLP DEGs are also present in the list of ASD DEGs (DS1). Neither the relevant functional categories nor the canonical pathways found in the analysis of ASD patients were detected as enriched in the functional annotation analysis of NSCLP patients DEGs, except for cell adhesion. These analyses thus reinforce the confidence of our results.

Discussion

In this study, we compared the expression profile of SHEDs from idiopathic autistic patients with those of nonaffected controls. We showed that the group of DEGs is enriched by genes expressed in brain, ASD candidate genes, genes that harbor mutations identified by exome studies and DEGs found in ASD brain. We also found a great overlap with the ontological categories found in previous expression studies on ASD lymphocytes/LCL [Abrahams & Geschwind, 2008], such as GTPase regulator activity, protochaderin genes and alternative splicing. Moreover, functional annotation analysis in the present data set revealed enrichment of a considerable number of other biological functions and signaling pathways that were already related to ASD, namely: pathways involved in cytoskeleton dynamics, such as axonal guidance signaling and regulation of actin-based motility by Rho [Anitha et al., 2008; Hu et al., 2009a; Melin et al., 2006; Sbacchi et al., 2010]; the protein synthesis-related pathways mTOR and PTEN signaling [Cuscó et al., 2009; Gkogkas et al., 2013; Kelleher & Bear, 2008; Neves-Pereira et al., 2009]; RNA editing and alternatively spliced genes [Abrahams & Geschwind, 2008; Smith & Sadee, 2011; Talebizadeh et al., 2006; Voineagu et al., 2011]; and cell adhesion molecules [Betancur, Sakurai, & Buxbaum, 2011]. Therefore, SHEDs’ transcriptome revealed deregulation of several candidate genes, pathways and biological systems previously pointed out as associated with ASD, suggesting that these cells are a good alternative to study ASD. Although our second set of controls were composed by males and females, which can be considered a limitation of this study.
study, specially considering the sexual bias in ASD, the results found in DS2 analysis were consistent to those found in DS1.

A consistent result in our analysis was the differentially expression of AR-regulated genes, which was found in all data sets tested. It is also of note that a significant number of such DEGs had been previously suggested to be ASD candidate genes. Alteration in AR signaling supports the “extreme male brain” theory for ASD [Baron-Cohen, Knickmeyer, & Belmonte, 2005] and could explain the sexual bias seen in these disorders. Moreover, alteration in androgen metabolism and signaling in ASD patients have been suggested by other global expression studies [Hu et al., 2009a,b; Sarachana, Zhou, Chen, Manji, & Hu, 2010]. However, none of these studies presented any deregulated upstream molecule possibly interacting with this pathway. Analysis of DS1 suggests that CHD8, which is known to interact with AR to mediate its transcriptional regulation activity, is a possible upstream regulator of such pathway. Involvement of CHD8 in ASD pathology is becoming evident with the recent findings of exome studies [Neale et al., 2012; O’Roak et al., 2012a,b; Sanders et al., 2012; Talkowski et al., 2012]. We believe that investigation of CHD8 and AR interaction should be further explored to uncover the functional implications of CHD8 mutations in ASD etiology.

In summary, our results suggest that despite our lack of knowledge about the mutational mechanism in the studied ASD patients, their altered genomes lead to expression deregulation in shared pathways that could be detected even with a small sample size. Our work also showed that SHEDs are an alternative cell type to explore deregulation of biological systems in ASD patients. We do not expect that SHEDs will substitute the use of neuronal-derived stem cells, such as from induced pluripotent stem cells, for functional analysis. However, as SHEDs are obtained noninvasively and require less manipulation, they represent a good option to identify new pathways and gene interactions in ASD.

Acknowledgments

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Table 3. Functional Annotation Analysis Results for DS2 and DS3 lists Compared with the Relevant Categories Highlighted in the Analysis of DS1

<table>
<thead>
<tr>
<th>Categories</th>
<th>DS2</th>
<th>DS3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-value</td>
<td>Number of genes</td>
</tr>
<tr>
<td><strong>Biological functions enriched by upregulated genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>1.2E-03</td>
<td>19</td>
</tr>
<tr>
<td>Alternative splicing</td>
<td>2.5E-03</td>
<td>181</td>
</tr>
<tr>
<td>Actin binding</td>
<td>5.1E-03</td>
<td>12</td>
</tr>
<tr>
<td>Neurogenesis</td>
<td>8.7E-04</td>
<td>10</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>2.3E-02</td>
<td>21</td>
</tr>
<tr>
<td><strong>Biological functions enriched by downregulated genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribonucleoprotein</td>
<td>4.3E-04</td>
<td>11</td>
</tr>
<tr>
<td>Ribosomal protein</td>
<td>6.5E-03</td>
<td>7</td>
</tr>
<tr>
<td>Ribosome</td>
<td>1.6E-03</td>
<td>5</td>
</tr>
<tr>
<td>Protein biosynthesis</td>
<td>6.5E-03</td>
<td>7</td>
</tr>
<tr>
<td>Spliceosome</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>mRNA splicing</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>mRNA processing</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Canonical pathways</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTEN signaling</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EIF2 signaling</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FAK signaling</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Regulation of eIF4 and p70S6K signaling</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Axonal guidance signaling</td>
<td>2.2E-03</td>
<td>24</td>
</tr>
<tr>
<td>RhoGDI signaling</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>mTOR signaling</td>
<td>7.2E-02</td>
<td>10</td>
</tr>
<tr>
<td>Regulation of actin-based motility by Rho</td>
<td>5.4E-02</td>
<td>6</td>
</tr>
</tbody>
</table>

EIF2, eukaryotic translation initiation factor 2; eIF4, eukaryotic translation initiation factor 4; FAK, focal adhesion kinase; mTOR, mammalian target of rapamycin; p70S6K, ribosomal protein S6 kinase, 70kDa; PTEN, phosphatase and tensin homolog; RhoGDI, Rho GDP dissociation inhibitor (GDI).

References


ENGRAILED2 autism spectrum disorder-associated haplotype function. Human Molecular Genetics, 21, 1566–1580.


**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Table S1** Canonical pathways enriched by differentially expressed genes (DEGs) from DS1

**Table S2** Genes also found as differentially expressed by other expression studies

**Table S3** Canonical pathways enriched by the differentially expressed genes (DEGs) from DS3