Stem Cell Models of Autism Spectrum Disorders: An Entree into Gene-Environment Interactions

Ricardo Dolmetsch Ph.D.
Department of Neurobiology
Stanford University
Are human stem cells a useful tool to study the environmental contributions to autism?
“He seems self satisfied and has no apparent affection when petted....He seems to almost draw into his shell and live within himself.”

“Words to him had a literal inflexible meaning. He seemed unable to generalize”

“He wandered about making stereotyped movements with his fingers...He spun with great pleasure anything he could seize upon to spin.”
Autism is a diverse spectrum of disorders
Autism

Prevalence in 2009: 1/100

Autism per 10,000 Births

- Autism
  - Prevalence in 2009: 1/100

Monozygotic: 60-90%
Dizygotic: 0-6%
Siblings: 4-5%

Birth Year


Autism per 10,000 Births

- 0
- 10
- 20
- 30
- 40
SNP - single nucleotide polymorphisms
RM - rare mutations
CNV - copy number variation
Autism is highly heritable

SNP - single nucleotide polymorphisms
RM - rare mutations
CNV - copy number variation
What about environmental agents?

- Valproic acid during pregnancy
- Influenza or Rubella infection during pregnancy
- Auto-immune disease?
- Environmental Toxins?
How do we find how a mutation causes autism?
How do genetic mutations lead to changes in thoughts and behavior?
You make a mouse
But....

Autism involves many genes and it is hard to replicate a human genetic background in a mouse

And...

mice are not humans
Harvest skin cells from patients

Reprogram skin cells into pluripotent stem cells

Convert stem cells into neurons

Phenotype neurons
Which patients

- Timothy Syndrome
- Ch22q13 DS
- Williams Beuren Syndrome
- Craniovelofacial Syndrome (Ch22q11 DS)
- Other copy number variations (Ch16p11.2)
- Idiopathic autism
Harvest skin cells from patients

Reprogram skin cells into pluripotent stem cells

Convert stem cells into neurons

Phenotype neurons
Making stem cells from the skin cells of ASD patients

+ Oct3/4
Sox-2
Klf-4

Kazutoshi Takahashi... Shinya
Yamanaka Cell 2007
Harvest skin cells from patients → Reprogram skin cells into pluripotent stem cells → Convert stem cells into neurons → Phenotype neurons
Recapitulating human neural development in vitro

<table>
<thead>
<tr>
<th>Blastocyst</th>
<th>Neurulation</th>
<th>Differentiation and Migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>FoxG1 NCad</td>
<td>Nestin NCadh Hoechst</td>
<td>Pax6</td>
</tr>
<tr>
<td>Pax6 NCad</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.5 Neurogenesis, Gliogenesis and Migration

The CNS uses several strategies to generate distinct classes of neurons during development (Jacobson 1991; McConnell 1995; Nieuwenhuys 1998b): dorso-ventral polarization in the spinal cord, segmentation in the brain stem and lamination in the cerebral cortex. Most types of neurons are generated in the primary proliferative compartment (the ventricular zone), but several cell types arise from secondary proliferative compartments, including the subventricular zone and the external granular or germinal layer.

2.5.1 Neurogenesis: Primary and Secondary Proliferative Compartments

The neural plate and early neural tube consist of a single layer of columnar cells, the neuroepithelium. Through thickening, this layer gradually forms a pseudostratified epithelium, i.e. its nuclei become arranged in more and more layers, but all elements remain in contact with the outer and inner surfaces (Fig. 2.17). Mitotic figures are only found along the ventricular surface (Fig. 2.18). Early students of the developing neural tube, like His (1889), thought that these mitoses belong to cells which form a ventricular layer of germinal cells (Keimzellen), and that the more peripherally located cells represent spongioblasts, primordial glial cells, forming a syncytial meshwork (Markgerüst). Neuroblasts arising from the germinal cells were supposed to migrate peripherally in the intercellular spaces of this meshwork. Although Schaper (1897a, b) already challenged His's concept of neurogenesis, it was Sauer (1935a, b) who proved that the neural tube is composed of discrete cells that do not form a syncytium. In fact, His's radially arranged columnar cells (spongioblasts) and the rounded cells near the lumen, passing through mitosis, are not two types of cells, but are the interkinetic and mitotic stages of the same cell (Figs. 2.18a, 2.21). Thus, the early neural tube is composed of a single type of epithelial cell in various stages of the mitotic cycle: the resting cells reside in the outer part of the wall, and the nuclei of the cells that are going to divide are moving towards the ventricular surface. At the end of this migration phase, the peripheral processes lose their contacts with the outer surface and retract. The cells round up and divide into two daughter cells each. Each daughter cell produces a new peripheral process, and their nuclei move away from the ventricle. Sauer's cytological studies were confirmed by numerous studies using \[ ^3 \text{H} \text{H}\]thymidine autoradiography (Fujita 1963, 1966), and electron microscopy (Hinds and Ruffett 1971; Meller and Tetzlaff 1975).

At a certain developmental stage the nuclei of the elongated neuroepithelial cells withdraw from the most superficial layer of the neural tube (Fig. 2.19). The outer, anuclear zone, or marginal layer, first consists of the external processes of the neuroepithelial cells, but is soon invaded by the axonal processes of maturing neuroblasts. The inner zone is known as the matrix layer (Kahle 1951; Fujita 1963, 1966; Keyser...
Neurogenesis and Gliogenesis

Neurons

Glia

Catecholaminergic

Cholinergic
Neurons fire action potentials and form synapses.
Neurons have calcium elevations
Phenotype neurons

Convert stem cells into neurons

Reprogram skin cells into pluripotent stem cells

Harvest skin cells from patients
Experiment One

Muscle total RNA was prepared using a 10-fold serial dilution to produce samples of 10 ng, 1 ng, 0.1 ng, 0.01 ng, and 0.001 ng concentrations (the lower concentrations resembling the total RNA contents of a single-cell). Each preparation was setup as triplicate reactions for each protocol, against 48 assays. Replicate assays were performed to enhance data reliability. The data was collected on the BioMark system.

Comparison of Two Protocols for Single-Cell Gene Expression on Dynamic Arrays

The following is a supplement to the application note BioMark Dynamic Arrays for Single-Cell Gene Expression Analysis (MRKT00075a) and the BioMark Advanced Development Protocol 5. The supplement describes experiments performed with dynamic arrays to compare two protocols for single cell analysis — one recently developed by Fluidigm, the other a traditional two-step Reverse Transcription (RT) preamplification (PreAmp) protocol. Both experiments were performed with RNA standards, instead of real cells, to control for the reproducibility of sample concentration.

Figure 1. Standard Curves for CSNK2B and GAPDH assays.

Table 1. Average $C_{\text{t}}$ and Sigma $C_{\text{t}}$ values.

<table>
<thead>
<tr>
<th>Target RNA Concentration (ng)</th>
<th>Average $C_{\text{t}}$ (single-cell GE protocol)</th>
<th>Average $C_{\text{t}}$ (2-step RT-PreAmp)</th>
<th>Sigma $C_{\text{t}}$ (single-cell GE protocol)</th>
<th>Sigma $C_{\text{t}}$ (2-step RT-PreAmp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>14.7</td>
<td>16.2</td>
<td>0.14</td>
<td>0.09</td>
</tr>
<tr>
<td>1</td>
<td>18.3</td>
<td>19.5</td>
<td>0.18</td>
<td>0.20</td>
</tr>
<tr>
<td>0.1</td>
<td>21.7</td>
<td>23.2</td>
<td>0.29</td>
<td>0.75</td>
</tr>
<tr>
<td>0.01</td>
<td>25.5</td>
<td>25.7</td>
<td>1.30</td>
<td>1.02</td>
</tr>
<tr>
<td>0.001</td>
<td>31.0</td>
<td>n/a</td>
<td>1.66</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Muscle total RNA Sample and CSNK2B Assay
3 preps * 4 assay replicates * 3 sample replicates = 36 data points per concentration (Error bars reflect +/- 1 standard deviation.)

Muscle total RNA Sample and GAPDH Assay
3 preps * 4 assay replicates * 3 sample replicates = 36 data points per concentration (Error bars reflect +/- 1 standard deviation.)

Results

Standard curves show that both the Fluidigm single-cell analysis protocol and the standard two-step RT-PreAmp protocol have excellent linearity and reproducibility. The $C_{\text{t}}$ and Sigma $C_{\text{t}}$ values demonstrate good sensitivity, only the Fluidigm protocol registering a $C_{\text{t}}$ value at the lowest concentration. Dynamic array heat maps show the same gene expression pattern for both protocols.
Timothy Syndrome (TS)

- QT prolongation
- Syndactyly
- Autism

Splawski et al., 2004
Are there differences in gene expression in neurons from TS patients?
Differentially Expressed Genes

Individual

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Control</th>
<th>Timothy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3</td>
<td>1 2</td>
</tr>
</tbody>
</table>

1. ARHGDIB
2. BAMBI
3. C4orf18
4. C8orf4
5. CCND2
6. CDH2
7. CDKN1A
8. CDKN2B
9. CXCR7
10. CXCR7
11. DEF8
12. EDN1
13. EFEMP1
14. EFEMP1
15. ENC1
16. EPSTI1
17. FBLN2
18. FLJ40504
19. FLNC
20. GABARAPL1
21. H19
22. HAND1
23. HAPLN1
24. HLA-A29.1
25. HLA-DMA
26. HLA-DPA1
27. HOXB5
28. HOXC6
29. IGFBP5
30. IGFBP6
31. KRT19
32. LEPR
33. MFF
34. MSX1
35. PALLD
36. PARP8
37. PCDH10
38. PITX1
39. PRDM1
40. PRSS23
41. RGMB
42. RPESP
43. RPS26L
44. S100A3
45. TBX3
46. TGFB1
47. TGM2
48. TM4SF1
49. TNNC1
Prolonged calcium responses in TS neurons

Patient 1 vs Ctl07

Patient 2 vs Ctl11

65 mM KCl
Increased numbers of TH positive neurons in TS patients
Are there differences in dendritic arborization?
Prolonged AP and cardiac arrhythmia in TS cardiomyocytes
Can we use iPS cells to study environmental triggers of autism?
Can we use iPS cells to study environmental triggers of autism?
Summary

- We have developed ways of recapitulating neuronal development in the lab using human cells
- We can generate neurons from patients with autism
- We can detect phenotypes in cells from autistic children
We are still uncovering the cellular correlates of autism.

We don’t know how much of a chemical gets to the brain of a developing child and for how long.

Some types of autism will not be cell autonomous (i.e. not for auto-immune disease).
Where are we now?

Gardner’s cycle of innovation

- Peak of Inflated Expectation
- Trough of Disillusionment
- Plateau of Productivity
- Slope of Enlightenment

- Technology Trigger
- 2008
- 2009
- 2010?
Alex Schlegovitov
Masayuki Yazawa
Sergiu Pasca
Jocelyn Krey
Susanna Wen
Karen Chan
Chan Young Park
Thomas Portmann
Georgia Panagiotakos
Masoud Sandhaghiani
Odmara Barreto Chang

Dan Geschwind, UCLA
Jon Bernstein Stanford

With support from NIGMS, NIMH, Fidelity Foundation, Simons Fund for Autism Research and the McKnight Endowment for Neuroscience, Mrs Linda Miller, Ben and Felicia Horowits and Mr and Mrs John Mcafferey