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The human model: changing focus on autism research

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Abstract

The lack of live human brain cells for research has slowed progress toward understanding the mechanisms underlying autism spectrum disorders (ASDs). A human model using reprogrammed patient somatic cells offers an attractive alternative as it captures a patient's genome in relevant cell types. Despite the current limitations, the disease-in-a-dish approach allows for progressive time-course analyses of target cells, offering a unique opportunity to investigate the cellular and molecular alterations before symptomatic onset. Understanding the current drawbacks of this model is essential for the correct data interpretation and extrapolation of conclusions applicable to the human brain. Innovative strategies for collecting biological material and clinical information from large patient cohorts are important for increasing the statistical power that will allow for the extraction of information from the noise resulting from the variability introduced by reprogramming and differentiation methods. Working with large patient cohorts is also important for understanding how brain cells derived from diverse human genetic backgrounds respond to specific drugs, creating the possibility of personalized medicine for ASD.

Keywords

human-specific disease modeling; human induced pluripotent stem cells; human neurons; brain; drug screening; autism spectrum disorders

Introduction

Autism Spectrum Disorder (ASD) is a lifelong developmental disability that is mainly characterized by difficulties in social communication and the presence of focused repetitive or stereotyped behaviors and appears within the first three years of life(1). Because different etiologies can generate a similar behavioral outcome, many disorders with autistic features are grouped under the ASD umbrella.

Family history and twin studies suggest that these disorders share genetic roots in at least some cases (2, 3). The mounting evidence suggests that heritable and *de novo* genetic variations play a significant role, but these studies have also reported striking genetic

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heterogeneity(4-6). Disorders such as Fragile X, Rett syndrome (RTT) and Timothy syndrome are caused by specific genetic alterations that also present neurodevelopmental and speech delays, resulting in an autistic phenotype. Although these syndromic forms are no longer clinically grouped under ASD, these disorders have provided useful insights into sporadic or idiopathic (non-syndromic) forms of autism. Current genomic efforts to discover novel causative variants have focused on small chromosomal deletions or duplications in the form of copy number variations (CNVs) measured by the genotyping of large numbers of individuals(7, 8). CNVs were found in cadherins and protocadherins, implicating the neuronal cell adhesion pathway in ASD or the ubiquitin-proteasome system, which regulates synaptic attributes such as neurotransmitter release and synaptic vesicle recycling(9). Other studies have found that genes with rare CNV defects interfere with neurodevelopmental pathways by affecting the maturation and function of glutamatergic synapses that may be disrupted in ASD(7, 10). Recent studies integrating ASD candidate genes with spatiotemporal coexpression networks have demonstrated that ASD genes converge on the transcriptional regulation in pyramidal (glutamatergic) cortical neurons during mid-fetal human development(11, 12).

Neuropathological imaging has also provided important insights into ASD. Macrocephaly and altered brain development trajectories with early overgrowth and later normalization have been reported in some ASD patients(13). This increase in brain size during the first three years of life was shown to precede the first clinical manifestation(14-19). Several pieces of evidence suggest that accelerated brain growth in this ASD population begins prenatally and continues during the first few years of life(14, 17, 20-22). Some MRI findings correlate directly with a post-mortem analysis, such as a weight (size) increase in ASD brains at early ages, describing the neurons as more packed and a reduced number of Purkinje cells in the cerebellum(23). Subsequent studies have focused on identifying cellular abnormalities, such as increases in the number of neurons(24) and the glial density(25) in the prefrontal cortex.

The prevalence rate of ASD has dramatically risen over the years. The exact reasons for this increase remain unclear; however, the improvement in and availability of diagnosis and a legitimate increase in the rate of affected newborns may be contributing factors(26, 27). According to the CDC's 2014 Autism and Developmental Disabilities Monitoring (ADDM) Network, approximately 1 in 68 children has been identified with an ASD in the United States. ASD is almost five times more common among males than females.

There is no cure for ASD. ASD treatment requires a strong collaboration among multiple professionals, and the cornerstone of treatment involves individualized educational interventions, including early and intensive behavioral strategies and therapies for better clinical outcomes(28, 29). The cost for this type of personalized treatment can be quite high(30). As these children mature into autistic adults, the majority do not live independently(31). Thus, the need for early diagnosis and better treatment of ASD is not only an increasing concern among scientists and physicians but also an increasing concern from an economic perspective(32). However, the human nature of ASD, with its intrinsic heterogeneity and large spectrum of clinical symptoms among patients, is a major challenge for studying ASD.

Current ASD models

Studies using several experimental models have improved our understanding of ASD. The best models consider the sophistication of the human brain within the constraints of cost and practicality. The inaccessibility to live neurons, either from post-mortem brains or living individuals, has hindered the investigation of the mechanisms underlying ASDs. Other issues associated with post-mortem analyses are similar to those associated with live imaging, such as the sample size, gender, age and heterogeneity of the disorder itself. All of the above-mentioned issues are added to the possible lack of information on the medical or drug-use history of the individuals whose brains are being studied and the differences in the methodology or statistical analyses used among the research groups. Nonetheless, useful information on the ASD pathology has been extracted from gene expression studies using postmortem brain tissues(33, 34). Furthermore, we have also gained a significant amount of knowledge of the genetics of ASD by performing genomic analyses on blood samples from affected and non-affected individuals(8, 35, 36). However, these are not ideal cell types for neuroscience experimentation because blood cells do not exhibit several of the specialized structures (for example, the synaptic machinery) found in neurons. Fetal primary human progenitor/stem cells represent an acceptable experimental ASD model, but the intrinsic difficulties in their manipulation, expansion and accessibility restrict their use(37, 38).

Finally, the inherent differences between the mouse and human genetic backgrounds(39), immune systems(40) and brain circuits(41) contribute to the challenges of using rodent models of ASD(42). With a relatively shorter evolutionary distance and a more heterogeneous genetic background than inbred laboratory mice, non-human primate models have also been used to study ASD(43). Recent efforts have also focused on the targeted genetic manipulation of non-human primates to carry alterations found in syndromic forms of ASD, but mechanistic insights from these models have not yet been reported(44, 45) and may be inaccurate because of the uniqueness of the brain transcriptional networks in modern humans(34)(46). Thus, a new human model with unlimited access to relevant cellular material could nicely complement the efforts from previous approaches.

A human pluripotent experimental model for ASD

Despite some ethical controversy, human embryonic stem cell (hESC) research has become a promising area in developmental research. For the first time, researchers can potentially explore the early stages of human development *in vitro* using this powerful tool to gain insights into human neurodevelopment(47). However, the progress toward understanding neurodevelopmental diseases has been hampered by the scarce availability of disease-specific hESCs carrying ASD genetic alterations in the genome. The generation of induced pluripotent stem cells (iPSCs) using human cells has been accomplished(48, 49). This breakthrough involves a relatively simple approach that uses a set of transcription factors to jump-start and reprogram the entire genetic network landscape to a pluripotent stage. In addition to overcoming ethical issues, this new technique has gained attention for its potential to generate disease-specific pluripotent stem cells with unprecedented simplicity. Human iPSCs are tempting models for understanding complex disorders with heritable and

sporadic conditions, which allow researchers to recapitulate an individual's development in the laboratory(50).

Given the uniqueness of human cognition and behavior, an *in vitro* human neurodevelopmental model capable of recapitulating the early stages of development could reveal specific biochemical and cellular features of our species that are difficult to reproduce in other models(37, 51). The iPSC technology also presents the potential opportunity to manipulate phenotypic alterations with candidate drugs, paving the way for future drug-screening platforms.(52).

Major roadblocks to establishing an iPSC ASD human model

The introduction of iPSCs has been a breakthrough for studying human diseases. However, understanding the limitations of this approach is important to extract the most relevant data from this model. As with all *in vitro* models, cells in culture are not in the same environment as the living organism. Additionally, the current culture conditions are not yet fully optimized for deriving enriched populations of disease-relevant neuronal subtypes(53-60) or glial cells(61) from human pluripotent stem cells despite recent efforts.

A recent study comparing primary human fetal progenitor cells, which were isolated directly from the developing brain, with differentiating iPSCs found that although both of these *in vitro* models recapitulated certain aspects of human corticogenesis and the transcriptional network related to ASD, the iPSC-derived neurons exhibited relatively lower transcriptional overlap with *in vivo* human development(38). The high variability reported for these experiments clearly demonstrates that different experimental protocols may affect the degree of cortical maturity that iPSC-derived neurons can achieve in culture.

Another relevant issue concerns the use of proper cellular controls. Based on previous experiences with mouse models, isogenic cell lines may represent the ultimate control. Genome-editing allows for rigorous study designs that could substantially alleviate concerns regarding potential off-target effects or other confounding effects caused by clonal sequence variation(62-65). Additionally, genome-editing technologies can be used to add reporters, biosensors and tagged proteins to easily assay biological endpoints in a more homogenous cellular population. Random X chromosome silencing during the reprogramming of female cell lines is another strategy for generating isogenic control cell lines when the target gene is located on the X chromosome(5, 66). However, the eventual loss of X chromosome inactivation that could artificially "rescue" cellular phenotypes is a concern(67). In complex diseases, in which the genetic contribution comes from multiple genetic variants, it may be advantageous to coordinate collaborative initiatives to share control lines(68).

Finally, confirming that the *in vitro* cellular phenotypes are relevant to the disease is currently another major obstacle for investigators in this field. Because several cellular and molecular phenotypes in a dish may not be recapitulated in postmortem brain tissues or animal models for the reasons previously discussed, confirmation in another model would definitely increase the confidence in disease relevance.

Undoubtedly, the iPSC technology still requires a series of optimization steps, which are necessary for not only the study of ASDs but also the study of any neurological disorder. Despite all these caveats and limitations, the study of ASD-derived brain cell types is already generating novel insights that should be considered with an open-mind for novel perspectives(69).

Modeling syndromic ASDs using iPSCs

We have previously used iPSCs to study Rett syndrome (RTT), a syndromic type of ASD(5) caused by alterations in the methyl-CpG-binding protein-2 (MeCP2) gene. RTT-derived cortical glutamatergic neurons exhibited several morphometric alterations and reduced excitatory synapses, leading to significant functional deficits in the RTT neuronal networks. Interestingly, the morphometric observations mirrored the data from RTT postmortem brain tissues to some extent(70). Moreover, treating the RTT-derived neurons with insulin growth factor 1 (IGF1) increased the number of synapses, rescuing the neuronal defects. IGF1 is now in clinical trials for treating RTT and potentially other ASDs(71). This outcome suggests that the iPSC-derived neurons may be helpful in the screening of future candidate drugs. Our methods have been subsequently validated and improved upon by other research groups, suggesting that this platform can be quite robust despite the potential caveats previously discussed(66, 72, 73). Since this first report, several other scientific articles showing that iPSCs can be used to model other syndromic types of autism have been published.

Fragile X syndrome (FXS) is caused by a CGG repeat expansion in the promoter region of the fragile X mental retardation 1 (FMR1) gene, which leads to hypermethylation and gene silencing(74). Initial developmental studies on FXS using human embryonic stem cells derived from blastocysts revealed that the FMR1 promoter was unmethylated and thus expressed at the pluripotent stage(75). In contrast, initial studies using FXS iPSCs showed that FMR1 was inactive because it retained the epigenetic silent status(76). A follow-up study showed that the FMR1 silent status actually varied among several clonal iPSC lines(77). Moreover, the lines with reduced FRM1 expression generated aberrant neuronal differentiation(77). Another recently reported study showed that the FXS-derived cortical neurons were aberrant neurons with reduced neuritogenesis(78). Thus, despite the initial hurdles, these reports demonstrate the potential of iPSCs to generate FXS models in a dish.

Timothy Syndrome (TS) is another rare ASD that is caused by mutations in the *CACNA1C* gene(79). A human iPSC model for TS has helped identify several abnormalities in the derived neurons, including defects in calcium signaling, reductions in cortical and callosal projection markers, an increased number of neurons expressing tyrosine hydroxylase and an activity-dependent dendritic retraction (80, 81). Interestingly, the increase in tyrosine hydroxylase was rescued after treatment with roscovitine, an L-type channel blocker, emphasizing the potential for a drug-screening platform using TS-derived neurons. Neuroligins, a class of genes previously implicated in ASD, were also targeted for disease modeling using reprogramming techniques(53, 82).

Finally, syndromic non-monogenetic ASDs were also modeled using human reprogramming techniques. Phelan-McDermid syndrome (PMDS), also known as 22q13.3 deletion syndrome, is caused by a loss of genes located in the 22q13 chromosomal region, which typically includes deleting *SHANK3*, a gene previously implicated in autism(83). The neurons generated from two PMDS patients demonstrated an altered excitatory electrophysiology and fewer synapses compared with controls. These neuronal defects were rescued by the ectopic expression of *SHANK3*, further supporting the causative role of *SHANK3* in this syndrome(84). Maternally inherited duplications of the 15q11-q13.1 chromosomal region are also associated with ASD(85). This region includes the ubiquitin protein ligase E3A (*UBE3A*), which is disrupted in another neurodevelopmental disorder called Angelman syndrome(86). The transcriptional analysis of iPSC-derived neurons from five patients with CNVs in 15q11-q13.1 revealed that the chromatin structure can influence the gene expression across this region, suggesting that common molecular pathways may be disrupted in both Angelman and the duplication syndrome(87). A similar gene expression strategy was used to model CNVs in chromosome 7q11.23, a region that contains approximately 25 genes. Deletions in this region cause Williams Syndrome, whereas duplications are generally associated with ASDs(88). The authors showed that transcriptional dose-dependent dysregulation can even be detected at the iPSC stage, but it is enhanced in the differentiated cell types(89). The CNV models described here strongly suggest that the general approach is sound and reliable, thereby allowing for the discovery of important molecular pathways related to ASD pathogenesis.

Modeling non-syndromic ASDs using iPSCs

Based on the examples of syndromic ASD, it can be tentatively concluded that the iPSC-derived neurons of idiopathic or non-syndromic ASD patients can serve as an important tool for studying rare ASD variants(88, 90-93)(94, 95). However, demonstrating the causal role for rare variants will be particularly challenging because it may require an unattainable large sample size for proper statistical power(96-98).

Based on this caveat, our group has focused on the reprogramming of human dental pulp stem cells from ASD children using the deciduous tooth as a source of somatic cells. The “Tooth-Fairy Project”(99) has dramatically increased over the past years because of the use of social networks to connect with ASD families. We have been collecting dental pulp from ASD patients from Brazil and the USA, and we have a cellular and genetic ASD bank that represents part of the heterogeneity of the ASD population. Currently, we have over 3,500 ASD families listed and approximately 300 samples collected. The bank will cross-reference all the genotypes and cellular phenotypes with detailed clinical information. The bank will be useful for modeling the differences in the ASDs patients with distinct clinical outcomes using reprogramming strategies. Moreover, we believe that iPSCs together with genomic analyses, such as whole-genome sequencing, will help categorize ASDs and clarify some of the molecular pathways leading to the autistic behavior.

Recently, we have also made progress in modeling non-syndromic autism. Starting with the dental pulp cells isolated from one of the ASD subjects enrolled on the “Tooth-Fairy Project,” we characterized the breakpoints of a *de novo* balanced translocation t(3;11)

(p21;q22) that disrupts the *TRPC6* gene. *TRPC6*, a gene not previously implicated in ASD, encodes the canonical transient receptor potential 6 channel, a voltage-independent Ca²⁺-permeable cation channel involved in dendritic spine and excitatory synapse formation(100, 101). The biological impact of the genetic alteration in the index case and its functional relationship to the ASD etiology was evaluated in several analyses using cortical neurons derived from iPSCs. We demonstrated that a *TRPC6* reduction or haploinsufficiency leads to altered neuronal development, morphology, and function(54). The observed neuronal phenotypes could then be rescued by *TRPC6* complementation and by treatment with IGF1 or hyperforin, a TRPC6-specific agonist, suggesting that ASD individuals with alterations in this pathway may benefit from these drugs. We also demonstrated that the MeCP2 level affects *TRPC6* expression, revealing common pathways among ASDs (**Figure 1**). The genetic sequencing of *TRPC6* in 1041 ASD individuals and 2872 controls revealed that there are significantly more nonsynonymous mutations in the ASD population and identified loss-of-function mutations with incomplete penetrance in two patients(54). Taken together, these findings suggest that *TRPC6* is a novel predisposing gene for ASD that may act in a multiple-hit model. This was the first study to use iPSC-derived human neurons to model non-syndromic ASD and illustrate the potential of modeling genetically complex sporadic diseases using these types of cells.

A drug-screening platform using iPSC-derived brain cell types

The lack of pharmacological treatments for the core symptoms of ASD may be a consequence of the lack of a human model. ASD pre-clinical assays are mostly based on transformed cell lines and animal models that cannot fully recapitulate the relevant cellular function because of obvious interspecies differences and differences in their pharmacokinetic properties(102, 103). Drug-screening platforms require suitable cellular phenotypes for the high-throughput instrumentation screening and a large amount of the target cell types. The current differentiation strategies can be further developed for use in scale-up methods in the near future. Morphometric measurements can be easily taken using high-content imaging software. Gene expression differences and biochemical assays are also valuable alternatives. However, functional electrophysiology studies may not be practical due to the long maturation time required *in vitro*. Direct cell fate conversion may be an alternative(104, 105), but the ability to scale up production and the skipping of important neurodevelopmental milestones may be a challenge(106).

Although most of the research has focused on neurons, the transition to a high-throughput drug-screening platform has intrinsic challenges mainly because iPSC-neurons are dynamic cell types that require constant attention over long periods of time. It is notoriously difficult to produce a homogenous population of neuronal subtypes, and human neurons are not well adapted to the high-throughput format. Therefore, the use of other well-characterized populations of functional cell types that are easily adapted to high-throughput formats may provide a significant advantage for drug-screening initiatives. Strong evidence suggests that ASDs are diseases of the synapse, the interconnection through which electrical signals are transmitted from one neuron to the next in a circuit. Most of the research has focused on new therapeutics that target neurons and enhance the number of functional synapses in these diseases. However, a recent study has shown that glial cells may represent an exciting novel

therapeutic target, and they should be investigated in the future. New protocols for the generation of glia will allow researchers to identify the role of these cell types in ASD. Previous studies have established that astrocytes secrete signaling molecules that stimulate the formation, pruning and function of synapses throughout the brain(107, 108). Astrocyte dysfunctions are known to be associated with many mouse models of neurological diseases, but their specific contribution to the human disease pathology has yet to be determined. It is likely that some glial cells, such as astrocytes and microglia, also contribute to the disease. By mixing different cell types in co-culture experiments, it will be possible to isolate the non-cell autonomous contribution to specific neuronal parameters, such as morphology or synaptogenesis. Finally, ideal drug libraries for ASD should consist of molecules with good brain penetration. Repurposing drugs designed for other disorders for ASD is another fascinating idea that could accelerate promising pre-clinical leads to market (**Figure 2**).

The next step: mini-brains on a chip

Drug development typically begins with a screening that tests the effects of promising molecules on 2D cell cultures and then in animal models before reaching clinical trials. However, many successful pre-clinical candidates still fail in humans. Pluripotent stem cells grown in 3D cultures can self-organize into spheres that resemble a 9-week-old developing human fetus brain, forming cerebral organoids that more accurately represent the actual conditions than the 2D cultures(109, 110). Obviously, these structures are not small-scaled brains because several components are not present or appear in different places. Nonetheless, this strategy offers a panoramic picture of brain development and how the process can go wrong. An additional step would be to help organize these cells by taking advantage of tissue engineering.

Modern tissue engineering applies concepts and techniques from engineering and biology to the problems that occur when cultivating human tissues and organs in the laboratory. Typically, this process involves creating a scaffold of natural or synthetic material, seeding this scaffold with human stem cells, which are provided with specific nutrients and a specific physical environment, that will differentiate into particular cell types and populate the structure(111). Despite the success with simple tissues, such as cartilage or skin, the reconstruction of complex organs in a dish is a major challenge. Nonetheless, the generation of human neuronal microcircuits is already a reality(112). For example, scientists have designed microfluidic chambers that allow iPSC-derived hippocampal neurons to connect with cortical neurons. Multi-electrode arrays (MEA) can be incorporated to record electrical impulses or stimulate the circuitry and investigate the connectivity of functional human networks(113). As another example, human motor neurons could connect to muscle to study neuromuscular junctions. By having the different cells functioning together, these circuits will replicate the dynamic interactions that take place in the body. Perhaps this model will be the first type of *in vitro-in vivo* human correlation in the neuroscience field. The MEA recordings from these neuronal assemblies can be directly correlated with the EEG recordings from patients, for example.

Obviously, the “brain on a chip” does not precisely replicate the human brain. Generally, these are plastic devices with tiny chambers embedded with living cells that are connected

by microfluidic channels that control the flow of nutrients and oxygen in the system. However, breaking the brain into microcircuits has both financial and convenience advantages. A microscale version of the brain is easy to manipulate and the outcomes are more easily visualized, supporting multiple variables at the same time. The system has the potential to reduce or eliminate the dependency on animal models and to accelerate drug development, helping identify the ineffective or unsafe drugs earlier in the development process. Personalized medicine strategies will also gain from this technology. However, as the culture systems become more sophisticated, new strategies will emerge along with new challenges. Nonetheless, the effort and work required to generate these strategies is worth the time. In the future, doctors may no longer have to guess which of the several drugs and dosages would work best for a specific patient. They could simply test them all in a micro-model of the patient's brain and select the one with the greatest efficacy and the fewest side effects.

Perspective

Human-derived cells are a powerful tool that nicely complements other models for studying ASDs. Although the iPSC technology is still in its early stage, it has demonstrated the potential ability to recapitulate the relevant neuronal defects of these diseases. The disease-in-a-dish approach could eventually incorporate data from other areas, such as systems biology, computational simulations, human brain imaging, and population genetics, to generate novel working hypotheses to be tested with more suitable cell types. Moreover, future advances in human-relevant models and technologies will generate a research approach that avoids the limitations of the species barrier. These types of initiatives can be combined with open-source interfaces that are user-friendly, allowing scientists to share key information to develop Adverse Outcome Pathways (AOPs) for autism spectrum disorders, an increasingly useful tool in toxicology. AOPs can be established for different autisms to accumulate evidence that supports the biological information underlying specific clinical symptoms. This strategy could be very useful for supporting the regulatory decision-making political proposals for autistic individuals. Finally, as the technology evolves, it will become applicable to personalized medicine, making predictions on the efficiency of certain drugs and doses in specific individuals.

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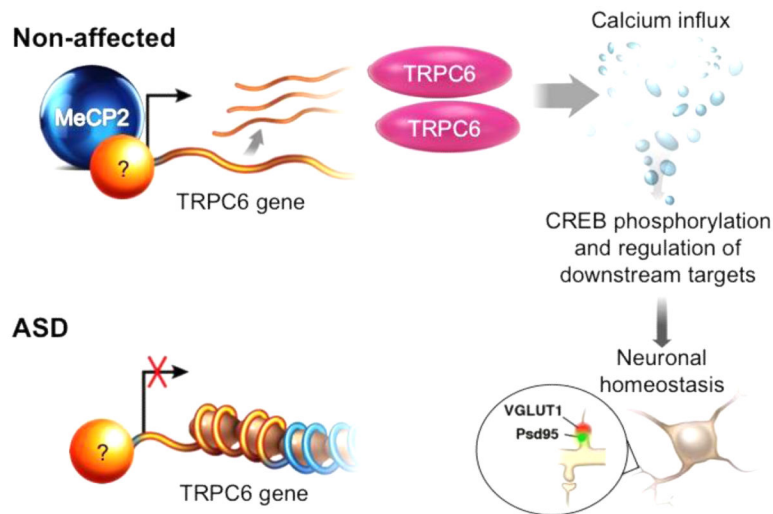


Figure 1.

The use of iPSCs to model ASD has already revealed new biological insights. A recent ASD predisposition gene (TRPC6) was shown to contribute to the formation of glutamatergic synapses in human neurons. Interestingly, TRPC6 gene expression can be controlled by MeCP2, a gene implicated in Rett syndrome, revealing the shared molecular pathways between syndromic and non-syndromic autisms. In non-affected individuals, MeCP2 is part of a currently uncharacterized activator complex in cortical neurons, stimulating the expression of TRPC6 that promotes calcium influx. The downstream activation pathway involves CREB-targets that regulate neuronal homeostasis, including the establishment of excitatory neurons. In a subset of idiopathic ASD individuals, TRPC6 is downregulated due to a reduced level of MeCP2 or mutations in the TRPC6 allele.

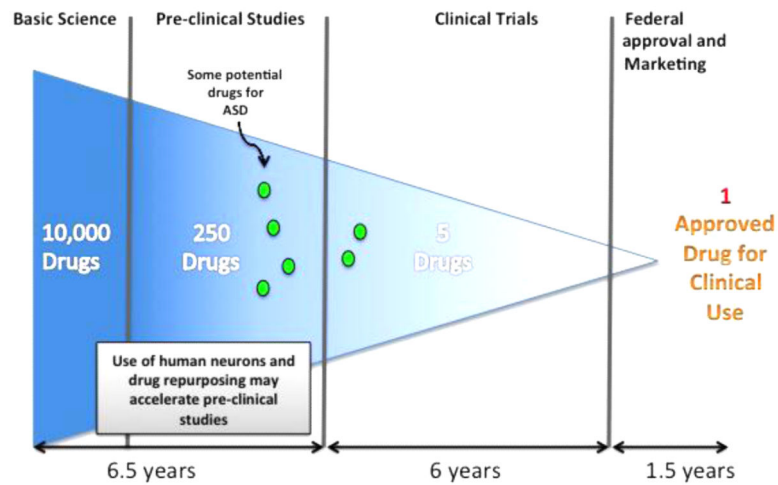


Figure 2.

Incorporation of iPSCs can accelerate drug discovery for ASDs. Adding iPSC-derived neurons can potentially impact the time frame for screening and marketing new drugs for ASDs. As the models become more sophisticated and the cost of iPSCs becomes reduced, this type of approach may also be used to categorize the ASD-responsive populations for more efficient treatment.