Meeting Report: Using Stem Cells for Biological and Therapeutics Discovery in Mental Illness, April 2012

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ABSTRACT

This report synthesizes the discussions during a workshop convened April 24–25, 2012, by the National Institute of Mental Health and the Foundation for the NIH in Bethesda, Maryland, that focused on progress and challenges in the use of patient-derived reprogrammed cells for basic biological discovery, target identification, screening, and drug development for mental illnesses such as schizophrenia, bipolar disorder, and autism spectrum disorders. The workshop revealed that the greatest progress has been made in reprogramming methods and agreed-upon standards for validating the resulting induced pluripotent stem cell lines. However, challenges remain in several areas, including efficiently generating and validating specific neural cell types with respect to regional identity, establishing assays with predictive validity to mental illness pathophysiology, and generating sufficient statistical power and data reproducibility across laboratories. A brainstorming session yielded a number of suggestions, including calls to (a) facilitate the replication of results by standardizing protocols and samples used across laboratories; (b) improve technology by generating cheaper/faster targeting methods, reporters, and assays; and (c) improve resource sharing and standardizing protocols and samples used across laboratories; (b) improve technology by generating cheaper/faster targeting methods, reporters, and assays; and (c) improve resource sharing and possibly incorporated into a public-private partnership. The meeting provided an important venue for academic, government, and private sector scientists to address potential opportunities for translational and clinical applications of reprogrammed cell research. A number of activities since the workshop have reflected the feedback from meeting participants.

INTRODUCTION

On April 24–25, 2012, the National Institute of Mental Health (NIMH) and the Foundation for the NIH convened a meeting in Bethesda, Maryland, of scientists representing academic research, industry, government, and funding organizations to discuss the latest technological advances in using patient-derived reprogrammed cells, such as induced pluripotent stem cells (iPSCs) and induced neuronal cells (iNCS), to identify molecular, cellular, and developmental alterations relevant to psychiatric disorders. The meeting was cosponsored by Janssen Pharmaceuticals, Eli Lilly and Company, and One Mind for Research and was cochaired by Drs. Stephen Haggarty of Harvard Medical School and the Broad Institute, Kalpana Merchant of Eli Lilly and Company, and Jeffrey Nye of Janssen Pharmaceuticals. The major goals of the meeting were to address challenges and strategies for using these cells in basic biological discovery, target identification, and drug development to treat mental illnesses.

The meeting opened with the recognition that the heterogeneity of mental illnesses, uncertain etiology, and the recently publicized failures of data replication in preclinical research [1] necessitated a cultural shift in the way science is done toward a more collaborative effort rather than the “silos” typical of traditional academia. With the increasing popularity of these tools, it is critical to quickly establish rigorous, transparent, and reproducible methods to avoid a proliferation of poorly designed and executed studies. With these caveats, patient cell-based assays can potentially link genotype with molecular and cellular pathophysiology and lead toward precision medicine-oriented diagnostics and drug design.

PATIENT SELECTION CRITERIA: CLINICAL PHENOTYPING AND GENETICS

The first session focused on a critical first step, which is determining the criteria used for selecting and stratifying patients for cell-based analysis. Schizophrenia shares clinical features with other disorders, so diagnosis is often made by ruling out other diseases. However, patient drug response, physiological measures such as eye
movement, cognitive tasks, and imaging abnormalities in white matter along with other biomarkers, risk factors such as infection, and heritable traits may be used to categorize patients for cell-based studies. Bipolar disorder likewise has clinical overlap with schizophrenia, major depressive disorder, panic disorder, and attention deficit-hyperactivity disorder [2]. This heterogeneity is reflected in the difficulty in replicating genome-wide association studies, suggesting that there are mechanistically distinct forms of the illness; classification of the disorder by subphenotypes (e.g., rapid cycling, alcoholism, and suicidal ideation) may be a more fruitful approach. Efficacy of lithium treatment response is still the most robust way of stratifying bipolar patients and is being used in some of the iPSC studies. Autism spectrum disorder (ASD) is associated with four genomic regions [3], only two of which have moderate significance; there are likely hundreds of genes with relatively small effects on ASD risk. Mono- genic disorders, such as Fragile X syndrome and Rett syndrome, are more tractable disorders for which substantial iPSC progress has been made [4–11] and that may not differ substantively from other ASDs at a cellular level. However, it was noted there still should be parallel research on polygenic models, with patient recruitment based on gene variants, biomarkers, and symptomatology. Two opinions emerged at the meeting. The first suggested that the best approach would be to focus on highly penetrant single-gene disorders, along with copy number variants, with a focus on early-onset cases, affected families, and/or twin studies. Others thought that it may too soon to search for disorder specificity; reducing heterogeneity will require large sample sizes and an approach targeting treatment response and common molecular or developmental pathways rather than specific genes. However, all agreed that patients should receive comprehensive genomic characterization and clinical assessment to go along with cellular characterization.

**STREAMLINING CELL REPROGRAMMING AND VALIDATION FOR LARGER-SCALE STUDIES**

Great progress has been made in optimizing reprogramming methods, as indicated by several presentations, and the starting material for reprogramming received a great deal of attention. Although fibroblasts are well-established and expandable source cells, patients may not consent to the invasive skin biopsy procedure. An alternative source is an extensive library of blood stocks that has already been banked, providing an existing supply of source cells and addressing the issue of recruitment (though reconsent may be required). Additionally, blood cells are likely oligoclonal rather than polyclonal, with the potential for less heterogeneity than fibroblasts. Their disadvantage is the finite volume of source material per patient and the comparatively limited track record of reprogramming from blood.

One high-risk assumption of patient-derived cell reprogramming based studies is that causal abnormalities are intrinsic to individual cells and are present and retained in the specific cells being isolated and manipulated. Although introduction of genetic mutations during the reprogramming process is an ongoing concern, this may be less a consequence of the reprogramming process and more a consequence of varying frequency genomic polymorphisms among source cells (e.g., fibroblasts), indicating that the human donor material itself is genetically mosaic [12, 13]. This can yield reprogrammed cells with substantially different properties [5]. This property can be exploited when the more common molecular or developmental pathways rather than specific differences in patterns or efficiency of differentiation.

A trend toward larger-scale studies will require development of current good manufacturing practice (cGMP), including elimination of variation in the iPSC derivation and validation process. Many groups have optimized nonintegrating (e.g., episomal and Sendai virus) reprogramming methods and replacement of undefined components such as Sendai medium with a fully defined growth medium. One group found that a comparison of blood-based reprogramming yielded better efficiency with CD34-positive cells than B cells. Automation of the cell culture process has allowed parallel analysis of iPSC-derived cells, including cardiomyocytes, from up to 250 subjects using replicate lines. Work was also presented on alternative direct reprogramming technologies to bypass the pluripotent state (e.g., iPCs); a modified protocol uses FOXG1 and SOX2 to reprogram fibroblasts to induced neural progenitor cells (iNCs), whereas BRN2 is additionally required to confer oligodendrocyte potential and tripotency to these cells [14]. Like iPSCs, iNCs have the advantage of being a renewable resource.

Several speakers commented that conventional validation assays, focusing on limited marker polymerase chain reaction, immunocytochemistry, germ-layer generation, and teratoma assays, are being replaced by genome-wide analysis. One “big data” approach to phenotypic comparison of iPSC lines is the Stem Cell Matrix (http://www.stemcellmatrix.org/) [15], which provides a collection of 8,000 samples consisting of fetal/adult tissues and cell lines and can be augmented by data sets from other laboratories to avoid sampling bias. PluriTest (http://www.pluritest.org/) [16] can replace older methods of pluripotency validation, such as teratoma assays, which are time/resource-intensive and provide poor discrimination of line-to-line variation. This discrimination is critical, since it was observed that genomic changes occur over time in iPSC culture, with a particular tendency toward duplication in certain chromosomal regions. This may result in selection of abnormal cells in expansion and/or changes in patterns or efficiency of differentiation.

It was shown that female iPSCs undergo a gradual erosion of X chromosome inactivation during culture, characterized by a loss of XIST gene expression and foci of H3-K27 trimethylation, as well as an irreversible transcriptional derepression of genes on the previously inactive X [17]. This phenomenon can confound analysis of some disorders and provides a cautionary tale about the need for repeated validation of the epigenetic status of cells in any iPSC-based study. It was suggested there should be a quick method of functionally validating a reprogrammed cell line in the absence of extensive or complex differentiation protocols; this may involve rescue with a wild-type gene variant when the genetic lesion is known and highly penetrant (e.g., Fragile X and Rett syndromes). Participants agreed that these issues should be addressed in any cGMP for reprogrammed cell line derivation and validation.

**GETTING THE “RIGHT” CELL: THE IMPORTANCE OF NEURAL DIFFERENTIATION AND REPORTING PROTOCOLS**

A major pitfall of in vitro studies is the assumption that limited characterization of differentiated cell types is sufficient for researchers to validate that they are working with a cell type as it
exists in vivo. Although meeting participants agreed that neurons with a glutamatergic phenotype could be readily generated from iPSCs, these neurons could express a variety of markers, suggesting a poorly defined regional identity and not, for example, cortical laminar-specified pyramidal neurons. This fact puts the onus on the researcher to verify that conditions are optimized for the regional specification and maturation state appropriate to the disorder being studied. One group has acknowledged this challenge by systematically defining the cortical GABAergic interneuron phenotype in embryonic stem cells and iPSCs, using reporters as a complement to cell surface marker-based selection [18]. These inhibitory neurons are implicated in a wide variety of psychiatric and neurological disorders, emphasizing the need for well-validated approaches for generating and isolating this cell type. Nkx2.1 reporters are being developed to identify progenitors for parvalbumin- and somatostatin-expressing interneurons, and Lhx6 reporters are being developed to identify progenitors for striatal and cortical interneurons. Participants debated the relative merits of bacterial artificial chromosome-based approaches, which are susceptible to insertion effects but are free of potential haploinsufficiency that can plague knockin-based approaches. Site-directed integration of transgenes into iPSCs (e.g., via zinc-finger nuclease or transcription activator-like effector nuclease [TALEN]-based approaches) reduces variability between lines, thus allowing a more robust comparison of control versus disease states.

It was noted that there are currently insufficient cell surface markers to efficiently purify many neuronal subpopulations and that such a project would require the production of a broad repertoire of fluorescent reporter lines for regional or cell type specification. The application of emerging single cell analysis technologies (e.g., microfluidics) to iPSC studies was viewed as a potentially powerful way to study multiple neuronal subtypes at once, possibly obviating the need for extensive purification techniques. However, the improvement in the speed and efficiency of differentiation techniques for certain cell types (e.g., dopaminergic neurons and motor neurons) suggests that a concerted effort can and should be made for improving neuronal and glial differentiation and purification protocols across the board.

**Assays in Two and Three Dimensions**

A second high-risk assumption of patient-derived cell reprogramming based studies is that causal abnormalities can be resolved using assays that reconstitute some molecular or cellular process that is predictive of the system-level pathophysiology. Work on degenerative disorders provided an example of progress in this area. The Amyotrophic Lateral Sclerosis iPSC Consortium is supported by the National Institute of Neurological Disorders and Stroke and focuses on familial mutations such as disease-associated gene variants in mouse or human iPSCs [21]. Recent progress has come from studies of Timothy syndrome (TS), caused by a point mutation in the voltage-gated calcium channel CACNA1C, which results in ASD symptoms in 80% of patients. Analysis of a Fluidigm array yielded a bar-coded readout of approximately 48 genes used to classify a few hundred iPSC-derived neurons [19]. Compared with controls, neurons from TS patients assumed upper cortical layer fates at the expense of lower cortical layer fates. The remaining lower layer cells were more frequently corticospinal, with fewer corticocortical neurons. This was similar to results found in CACNA1C mutant mice, supporting the link between the mutation and specific developmental changes. These studies also found dopamine-related changes that are the focus of further investigation.

A potentially powerful approach is a three-dimensional culture paradigm involving self-organizing cell aggregates that recapitulate features of the developing forebrain, including a neural tube-like organization with apical-basal polarity, localization of proliferative zones to the apical/luminal surface, the presence of radial glia, and evidence of cortical layer-specific neuronal markers [20]. Differentiated GABAergic neurons appear in these structures, as do neurons displaying immature synapses and glutamate transporters. Gene expression profiling indicated a downregulation of pluripotency genes and an upregulation of genes involved in neural differentiation, maturation, and synaptogenesis. This assay system may provide a more physiologically relevant system to assay aberrant developmental processes ranging from specification to proliferation, migration, and differentiation.

**Validating the Pathophysiological Relevance of Cell Phenotypes**

Important questions remain as to how easily iPSC- and iNC-based results can be validated against other assays of pathophysiology, such as disease-associated gene variants in mouse or human post-mortem studies. The truncated DISC1 mutation presents an obvious opportunity for side-by-side comparisons, because various DISC1 mutant mice and human iPSCs [21] have been generated. Post-mortem analysis has the advantage of utilizing actual patient tissue but is disadvantaged by its late stage in the disease trajectory and by variable tissue quality. Nonetheless, where post-mortem findings supported iPSC-based results, it would be seen as at least partial validation of pathophysiological relevance. Clinical measures such as behavioral assessment, drug response, or structural/functional imaging could be compared with cell data, particularly where measurements provide mechanistic insight (e.g., myelin tracts). Engraftment assays are valuable for studying human cells in the context of circuits, but proper integration of human cells in mouse remains a methodological hurdle. Some attempts have been limited by incomplete maturation of human neurons into the mouse cortex. Initial success has occurred with hippocampal engraftment of human DISC1 mutant neurons in mouse, suggesting that adult neurogenic regions may provide a more conducive environment for human neuron maturation than other regions.
Molecular Discovery, Target Identification, and Screening

Although the challenges are acknowledged, efforts have begun to identify novel molecular targets and adapt cellular assays for high-throughput screening. Transcriptional profiling of control subject glutamatergic neurons has shown that the differentiation process causes large changes in a number of genes associated with schizophrenia, bipolar disorder, and ASD, including transcription factors, chromatin modifiers, splice isoforms, and long noncoding RNAs; neurons showed higher expression of genes known to have allele-biased expression, many of which are also known to be associated with mental illnesses [22]. This analysis is now extending to schizophrenia patients with and without 22q11 deletions, to determine whether the expression trajectories are altered for any of the genes.

Studies of bipolar disorder patients include quantitative phosphoproteomic profiling of iPSCs and glutamatergic-neuron derivatives. Comparison of control subjects and lithium-responsive bipolar disorder patients yielded a set of proteins that are differentially regulated during lithium treatment. Both lithium and other forms of glycogen synthase kinase-3β (GSK3β) inhibition prevented phosphorylation at specific amino acid residues for a promising candidate protein involved in neurite extension. Studies will extend to patients with CACNA1C, ODZ4, and other gene variants implicated by ongoing human genetic studies of bipolar disorder. Scalable assays using iPSC-derived neural progenitors in a 384-well format are being used to report on WNT and GSK3β signaling (implicated in therapeutic lithium response and neurogenesis) and measures of neural differentiation and survival. Pilot screening thus far has shown a novel series of compounds, including riluzole, that potentiate WNT signaling and may target neuropathic pathways [23].

Another scalable assay is the multiwell automated neurotransmission assay (MANTRA), which analyzes neurons plated in 96 wells in parallel via multielectrode tips for stimulation and a high-resolution 96-lens microscopy array for imaging [24]. A pH-sensitive green fluorescent protein-tagged reporter called SynaptophysinPJEur is used to track synaptic vesicle cycling. The platform is being utilized for analysis of human iPSC-derived neurons as part of an integrated approach for probe discovery with rodent model systems and human clinical phenotyping. By measuring kinetics of synaptic transmission, the MANTRA assay represents an interesting high-throughput screening alternative to standard electrophysiology.

Participants debated what elements of patient-derived reprogrammed cells made them superior to previous assays in the drug development pipeline. One of the key arguments in favor of iPSC- and INC-based screening assays is that they utilize human neurons/glia and thus recapitulate gene expression, signaling isoforms, and cellular processes that may be species- and tissue-specific. Furthermore, there is a shortage of good cellular phenotypes for disease pathophysiology; approaches that utilize unbiased profiling of thoroughly validated cell types (e.g., ventral midbrain dopaminergic neurons and callosal projection neurons) may yield novel hypothesis-generating data sets that can be used to generate screening assays. One such platform is the NIH Library of Integrated Network-based Cellular Signatures (LINCS) project, which generates gene signatures through high-throughput screening of cellular responses to various perturbagens. The characterization of cells from patients with especially strong drug responsiveness (e.g., lithium and clozapine) may provide a unique opportunity to generate a robust cellular signature of disease-relevant pathways.

Other participants countered that the utility of any screening assay for psychiatric disorders depended critically on its validation in a more circuit-based model system (e.g., in vivo). They suggested that an appropriate strategy would integrate high-throughput primary screening assays (e.g., signaling, synaptic vesicle cycling, and neurotransmission) with secondary validation assays involving human cell engrafment and/or human disease-associated gene alleles in mouse.

A tantalizing example of how rapidly progress from biological to therapeutics discovery can be made is with familial dysautonomia, a rare autosomal recessive disorder caused by a point mutation in the transcriptional elongation complex gene IKBkap, causing autonomic nervous system degeneration. Analysis of iPSCs showed selective expression of the splicing defect in neural crest stem cells and a reduced neurogenic propensity for MASH1 neurons. The phenotype lent itself to high-throughput screening of 7,000 Food and Drug Administration-approved and known bioactive compounds, yielding 43 hits based on the ability to rescue the splicing defect. These were further characterized for dose response, cytotoxicity, and functional rescue of autonomic neurogenesis [25]. Although mental illnesses are molecularly more heterogeneous, a key lesson from this fruitful screening approach is the importance of testing a causal cellular pathway in a well-validated cell type with relevance to patient pathophysiology.

Coordinating Resources and Future Efforts

The final session incorporated ideas from a series of small brainstorming groups who addressed the question “How can we move more rapidly towards new patient treatments through the application of cell reprogramming technologies?” Much discussion focused on a need for greater collaboration and interdisciplinary training, particularly in skills that spanned academic and industry research. Suggestions to facilitate technology were mainly in the context of generating standardized, scalable, and shared resources such as less costly, more efficient cell differentiation, gene targeting, and reporter techniques. Scientific cultures in other areas such as genetics and clinical studies could serve as a blueprint for efforts in patient cell reprogramming. All agreed that cellular analysis should be sufficiently powered and must be integrated with whole genome sequencing and careful clinical phenotyping of patients, which should incorporate measures that can validate cellular phenotypes. Although some advocated for a new large-scale collection of iPSCs for different psychiatric diseases, others confirmed that a number of these studies were ongoing, and a collaborative culture would begin with transparent sharing of cell lines and protocols to contain costs and enhance reproducibility. Centralized collection of biomaterials and reconsenting for more comprehensive genetic analysis and clinical phenotyping, where needed, could provide leverage for more collaborative future efforts. It was noted that similar large public-private collaborations, such as the Innovative Medicines Initiative in Europe, are under way and provide contingencies for international coordination; several participants advocated for joining efforts across national borders.

Among the hurdles facing such collaborations are the differing imperatives articulated by members of the private, academic, and government spheres at the brainstorming session. Since private sector scientists (excluding nonprofits) are engaged...
in research and development of different proprietary and revenue-generating technologies, products, or services, a partnership that is considered precompetitive from the viewpoint of a compound-centric pharmaceutical company would likely involve the sharing of methods and assays that would be considered proprietary by smaller biotechnology firms. The more utilitarian focus of industry includes workflow optimization, standardization and quality control (e.g., sample preservation and handling and neural differentiation protocols optimized for efficiency and correct specification state), assay miniaturization, stabilization, and lab-to-industrial scaling, which is not within the scope of most basic academic research. As a result, new technologies such as iPSCs are slow to be adapted to drug discovery, in part because method and assay reproducibility has yet to be established, and there is a limited history of reliable vendors, data set generation, and evidence that the paradigms are worth displacing a long established history with more conventional cell lines and model organisms. What a successful public-private partnership would do is distribute risk among multiple parties and create a more favorable environment for high-risk tool development and discovery science. Although academic scientists have more freedom from the profit imperative to engage in innovative basic research, there is little incentive currently for academic scientists to invest time and resources in the kind of method optimization and data reproducibility needed to bridge that gap, when these efforts are unlikely to receive financial support or facilitate career development. Additionally, there is a tension between requiring continuous funding/resources and uncertainty about negotiating intellectual property issues with industry groups. The challenge within all spheres is to provide an environment conducive to methodological rigor rather than perpetual novelty and to provide something of significant value to the academic scientist in exchange for access to novel tools and data. Finally, government scientists engage in work that is in the public domain, particularly those in research funding or oversight. They share with private nonprofit foundations and advocacy groups a core mission of disease elucidation, prevention, and treatment. From this perspective of finite resources relative to a wide range of needs, an ideal partnership is one that maximizes the availability of newly generated resources and data to the broadest range of stakeholders, one that integrates multidisciplinary research programs so that basic research tools and assays can be seamlessly handed off for optimization in the therapeutics discovery pipeline. Since broad sharing and transparency is sometimes seen as anathema to the more competitive enterprises in the private sector and academia, the challenge is to shift the cost/benefit ratio in favor of sharing.

Several efforts are currently under way at NIH to meet these goals through the standardization, collection, and improved distribution of resources, including biomaterials and protocols. In 2011, NIMH established the new Stem Cell Resource at the NIMH Center for Collaborative Genomic Studies of Mental Disorders, to make source cells (e.g., fibroblasts) and iPSC lines more widely accessible to the research community, to facilitate data replication, and to ensure standardization. The genetic component of this repository already houses more than 100,000 patient samples of blood, lymphoblastoid cell lines, and DNA, along with a bioinformatics infrastructure housing genetic and clinical phenotyping data. The center has supported work leading to more than 500 publications to date. The new NIH Center for Regenerative Medicine (CRM) pilots new technologies with the goal of disseminating successful procedures throughout the research community. The CRM efforts include optimizing iPSC and cell screening methods, generating reference and reporter cell lines, harmonizing informed consent language, and working with reagent vendors to enhance freedom to operate. The CRM is also coordinating with the new National Center for Advancing Translational Sciences (NCATS) to integrate iPSC technology into the therapeutic pipeline. The NIH Molecular Libraries Program (MLP), a NIH Common Fund initiative, provides access to 350,000 diverse small molecules for qualified screening projects by NIH-funded investigators; this resource is already being utilized in iPSC-based high-throughput screening projects to identify novel probes for neural activity changes relevant to psychiatric disorders.

NIMH participants noted that iPSC and iNC studies could synergize with other ongoing NIH programs, such as the public-private Biomarkers Consortium, the Psychiatric Genomics Consortium, the Research Domain Criteria project, NCATS, and NIH Common Fund Initiatives such as CRM, MLP, the Single Cell Analysis Program, and LINCs. The meeting concluded with general agreement that this discussion should serve as a foundation for collaborative relationships among public and private stakeholders to promote the translational and clinical utility of reprogrammed cell research.

**POSTSCRIPT**

Since the April 2012 meeting, a number of activities directly implemented or fit with the spirit of recommendations from workshop participants. In May 2012, Lowenthal et al. [26] urged the use of harmonized informed consent language to facilitate banking and wide availability of patient-derived cells for reprogramming, in an effort to ensure that consent language inconsistencies do not restrict potential future iPSC uses in areas such as drug discovery. In November 2012, NIMH issued a new policy (NOT-MH-13-002) that investigators using patient-derived reprogrammed cells are expected to submit these cell lines, including their source cells and associated clinical/genotypic data, to the NIMH repository. This, along with the December 2012 announcement of a new human iPSC bank to be created by Europe’s Innovative Medicines Initiative, promises to ensure availability of these resources to the research community. In September 2012, NIMH released a request for information (RFI; NOT-MH-12-034) to solicit public and detailed feedback from potentially interested parties on what form a public-private partnership involving NIMH should take. NIMH is currently assessing responses from this RFI as part of its planning efforts. The extent to which any future collaborative efforts succeed will depend on the enthusiasm of scientists in all spheres to further advance sharing, transparency, and reproducibility of resources, methods, and data.

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The author indicates no potential conflicts of interest.