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Modeling the complex genetic architectures of brain disease.

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Abstract

The genetic architecture of each individual is comprised of common and rare variants acting alone and in combination to confer risk for disease. The cell-type-specific and/or context-dependent functional consequences of the risk variants linked to brain disease needs to be resolved. Coupling human induced pluripotent stem cell (hiPSC)-based technology with CRISPR-based genome engineering facilitates precise isogenic comparisons of variants across genetic backgrounds. Although functional validation studies are still typically performed of one variant in isolation, and in one cell type at a time, complex genetic diseases require multiplexed gene perturbations to interrogate combinations of genes and resolve physiologically relevant disease biology. Our aim is to discuss advances at the intersection of genomics, hiPSCs and CRISPR. A better understanding of the molecular mechanisms underlying disease risk will improve genetic diagnosis, drive phenotypic drug discovery, and pave the way towards precision medicine.

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AUTHOR CONTRIBUTIONS

M.B.F., T.A. and K.J.B. wrote the manuscript.

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Keywords

Human induced pluripotent stem cells; neurons; genetics; psychiatric disease; neurodegenerative disease; CRISPR-Cas; disease modeling

INTRODUCTION

Although genetic studies have identified hundreds of common and rare variants significantly associated with neurodegenerative and psychiatric diseases (reviewed [1]), large-scale genetic approaches have often failed to deliver actionable results or new guidance for clinical or translational research [2]. Genetic, epigenetic and transcriptomic datasets can inform the search for putative causal variants, but novel experimental paradigms are urgently needed to explore the phenotypic impact of hundreds of disease associated loci (reviewed in [1]). Moreover, because risk loci for brain diseases are enriched at defined developmental windows [3] and in specific cell types [4,5], genetic variants must be evaluated in the relevant neural cell types at the appropriate stage of maturity.

Human induced pluripotent stem cells (hiPSCs)-based models represent patient-specific platforms with which to study neurodegenerative and psychiatric disease. First, we discuss how hiPSCs can now be used to generate all of the major cell types in the brain, which fortuitously, generally resemble fetal brain tissue[6-10], making them particularly well-suited for the functional evaluation of genetic variants linked to psychiatric disease risk (as opposed to the study of processes observed late in disease-state). Second, we consider how combining hiPSC-based models with advanced applications of Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-based systems for (epi)genome and transcriptome engineering makes possible the study of combinatorial disease-relevant perturbations in cell-type specific isogenic systems (as highlighted by [11]). Third, we consider how genotype-based diagnosis and treatment makes possible a future whereby patients are identified and treated prior to symptom onset, dramatically expanding the therapeutic window of intervention, and making possible the prevention rather than treatment of disease [12,13].

hiPSC-BASED PLATFORMS TO EVALUATE GENOMIC HYPOTHESES

Today, hiPSC studies can be applied to defined and scalable two-dimensional (2D) cultures and/or more complex and physiologically relevant three-dimensional (3D) organoid systems (Fig. 1). While 2D neuron cultures require weeks to achieve electrophysiological maturity, organoids typically require months to demonstrate comparable properties[8]. In either case, the immaturity of hiPSC-derived neurons and glia relative to the human brain, generally resembling fetal brain tissue[6-10], makes them particularly well-suited for the functional evaluation of genetic variants linked to psychiatric disease risk. The strengths and limitations of both approaches, discussed below, are well-counter balanced. We see great utility in applying these approaches together to screen, discover and validate the physiological relevance of disease-specific risk variants.

Today, it is possible to rapidly generate highly pure populations of glutamatergic [14], GABAergic [15], dopaminergic [16], serotonergic [17] and motor neurons [18], as well as astrocytes [19] and oligodendrocytes [20] through transcription factor-based induction methods. Defined "co-cultures" of two or more cell types further permits the analysis of neuron-glia [21] and neuron-neuron [22] interactions, and so can better recapitulate a more physiologically relevant context. While the advantages of 2D platforms include scalability and ease of manipulation, limitations reflect technical constraints on the length of time that cells can adhere to plastic, which limit extended time courses of maturation, coupled with unnatural physical restrictions on cellular dynamics (e.g., migration of neural progenitor cells) [23]. Conversely, organoids improve the physiological relevance of hiPSC experiments by making possible complex 3D interactions between neurons, astrocytes [24], oligodendrocytes [25], vascular [26,27] and microglia [28]. Furthermore, directed differentiation results in region-specific patterned organoids (e.g. excitatory [29-31], inhibitory[29-31], and dorsal thalamic[32]) that can be fused to yield "assembloids" for the study of cell-cell interactions and non-cell autonomous effects in a more physiologically relevant context [31-33]. While early (undirected) 3D approaches yielded highly variable populations within and between organoids, differentiations, and donors [34], new methods that apply patterning signals to achieve improved reproducibility, particular across extended differentiation timelines [35,36], make possible controlled differentiation for disease modeling.

CRISPR-MEDIATED FUNCTIONAL VALIDATION OF DISEASE RISK

By precisely targeting a growing diversity of CRISPR proteins and effectors to defined genomic loci via a synthetically delivered guide RNA (gRNA), CRISPR-based technologies have transformed our ability to reengineer the human genome, epigenome and transcriptome (reviewed in [37]). Today, the integration of CRISPR technology with patient-specific hiPSC-based studies makes possible the functional validation of putative causal variants and genes in a cell-type-specific and donor-dependent manner.[38] Of course, complex genetic diseases do not arise from unidirectional differences in gene network expression (e.g. some common variants are predicted to up-regulate cis-genes, others to down-regulate), and so we also discuss future strategies to engineer large-scale bidirectional gene network perturbations via combinatorial effectors and systems.

Prioritization of Risk Variants for CRISPR-based Perturbation

Most loci identified in genome-wide association studies (GWAS) are poor candidates for CRISPR editing. Fine mapping analysis [39] only rarely identifies one single-nucleotide polymorphism (SNP) that is an excellent candidate for CRISPR editing, particularly if this SNP is expected to overlap with putative promoters or enhancers. More commonly, post-mortem expression and GWAS data are integrated to test for colocalization of expression quantitative trait loci (eQTL) and GWAS associations (e.g. COLOC[40,41]) and to calculate predicted differential expression in the brain (e.g. prediXcan[42]). Such multi-SNP approaches infer the magnitude and directionality of gene expression perturbations with tissue-level precision, and so instead prioritize genes for CRISPRa- or CRISPRi-based studies.

CRISPR Engineering the Genome and Transcriptome

The Cas9 nuclease achieves genomic editing via double stranded DNA breaks to induce insertions and deletions at gRNA targeted sites [43], with its greatest limitations being the efficiency of editing achieved and the frequency of off-target effects. Even as new CRISPR systems are identified and applied to genome editing, including Cpf1 [44] with increased activity and targeting ranges [45], and CasX [46] with increased editing specificities, research and clinical applications of genome editors remain constrained by the occurrence of off-target effects [47]. “Prime editing”, which relies upon the fusion of Cas9 to an engineered reverse transcriptase, enables efficient genome editing without double stranded DNA breaks, dramatically reducing off-target effects and promising wider applications of genome base editing [48].

Alternative applications of CRISPR platforms function via an enzymatically dead nuclease (dCas9) fused to a variety of effectors, which can be targeted to specific regions of the genome or transcriptome[49]. For example, CRISPRi/a use fusions of dCas9 to a Krüppel-associated box (KRAB) repressor domain for inactivation [50], or tripartite activator VP64-p65-Rta (VPR) [51] for activation, among others (Table 1). While the ability to edit single genes with relative ease is useful, the power of CRISPR systems is greatly enhanced by the ability to manipulate large numbers of genes simultaneously using pooled or multiplexable platforms.

Large-Scale Network Engineering

We recently applied combinatorial perturbation of four schizophrenia (SZ)-associated risk genes [52]. Our results suggested that the downstream effects of combinatorial perturbation exceeded what would be expected from the additive effect of individually perturbed genes. Observed synergistic genes converged on synaptic function, and linked rare and common variant genes implicated in psychiatric disease risk, emphasizing the importance of considering the polygenic nature of SZ and other neuropsychiatric disorders. Future studies must investigate the impact of combinatorial perturbations of dozens to hundreds of risk variants, each in the appropriate disease-relevant direction (some up, others down).

Although dCas9-based CRISPRa and CRISPRi cannot be used in conjunction due to their common Cas protein, a combination of non-complementary CRISPR systems from different bacterial classes would theoretically enable simultaneous, bidirectional manipulation of gene networks (Fig. 2a). Not only do Cpf1 [53] and CasRx [54] represent compatible Cas proteins for such bidirectional perturbations, but both also possess the ability to self-process pre-gRNAs, simplifying multiplexed genome engineering through the expression of a single CRISPR array.

The functional genomic approaches we have described so far are ‘genotype-to-phenotype’ approaches, applying prior knowledge in a hypothesis-driven manner to test the causal role of specific genes. By contrast, forward genetic screens are ‘phenotype-to-genotype’ approaches, broadly manipulating many genes and then characterizing the mutations that resulted in selected phenotypic changes. Recent advances in CRISPR-based approaches have opened new opportunities to conduct forward genetic screens in an unbiased manner [55-57].

Notably, approaches such as Perturb-seq[58], CRISPR-seq[59], CROP-seq[60] and ECCITE-seq[61] are promising new avenues to conduct large scale genetic screens that couple CRISPR-based perturbations to single-cell RNA sequencing for analytical readouts. Reciprocally, massively parallel reporter assays (MPRAs) can test the activity of regulatory sequences specifically in neuronal cell types [62], functionally validating the impact of non-coding variants at a massive scale. Such large-scale screens may also be extended into high-throughput phenotypic drug screens (Fig. 2b). Thus, the systematic targeting of multiple loci via combinatorial systems can provide a powerful platform to model complex genetic disease risk, through the interrogation of entire gene networks within genetic architectures, and subsequent evaluation of functional deficits resulting from these combined biological processes.

Combinatorial perturbations still face certain limitations: (i) the directional expression of some causal genetic variants are yet unknown, (ii) the magnitude of individual and combinatorial CRISPR-based perturbation may not recapitulate physiologically relevant effect sizes, and (iii) combinatorial perturbations do not recapitulate the entirety of an individual's genetic architecture. More broadly, CRISPR-based perturbations still cannot mimic megabase-sized copy number variations (CNVs) found in rare genetic disorders. Overall, as large-scale applications of CRISPR-based methods are increasingly feasible, such strategies will help to functionally elucidate the impact of causal genetic variants linked to neurodegenerative and psychiatric disease. Ultimately, the combination of hiPSC- and CRISPR-based platforms will help elucidate downstream phenotypic and functional deficits that genomic approaches alone cannot resolve (Table 1**).**

A PATHWAY TO PRECISION MEDICINE

Perhaps the greatest promise of hiPSC-based disease models is the potential to discover drugs capable of ameliorating observed *in vitro* phenotypes, with the hope that these drugs might represent novel clinical therapeutics that could be tailored to patients with greater precision. This potential is based on three critical biological premises: i) patient-specific drug response is predictable based either on patient genotype or the *in vitro* response of patient-specific hiPSCs-derived neural cells, ii) drug responsiveness is life-time stable, iii) clinical drug responsiveness is correlated to target engagement in disease relevant cell types, rather than arising as an indirect result of side effect tolerance of a potentially limitless number of non-disease-relevant cell types. Today, numerous hiPSC-based studies have shown that established and novel drugs can ameliorate key cellular and molecular disease phenotypes [63].

Given the complex polygenic nature of neurodegenerative and psychiatric disease, it seems unreasonable to expect that the effectiveness of any new drug might hold true for all patients. Although not true for all patients, defined subsets of SZ cases show aberrant pathway, transcriptomic or functional deficits downstream of GWAS-identified variants (e.g. C4A [64], miR-9 [65]). Moving forward, it is imperative that clinical treatment strategies include better ways to stratify patients, perhaps through a combination of genetics and *in vitro* testing, matching them to the most appropriate drug. Integrating hiPSC-based models with CRISPR engineering could yield a platform to conduct drug screens and predict clinical response.

Molecular and cellular phenotypes observed in patient hiPSC-derived neurons have been ameliorated by pharmacological treatment across a variety of disease models. For example, loxapine improved neuronal connectivity deficits in SZ hiPSC-neurons [66], insulin growth factor-1 rescued deficits in neural activity in autism spectrum disorder (ASD) hiPSC-neurons [67], and β- and γ-secretase inhibitors reduced amyloid and tau pathology in hiPSC-neurons from Alzheimer's disease (AD) patients [68]. By comparing the transcriptional responses of hiPSC-derived neural progenitor cells derived from SZ cases and controls, we reported differential regulation of neuropsychiatric disease-associated genes in a diagnosis-dependent manner [69], demonstrating the potential value of patient-specific platforms in drug discovery. It is critical to move towards predicting clinical response, either through genetic and/or hiPSC-based strategies. Neurons derived from lithium-responsive, but not non-responsive, bipolar disorder (BD) patients showed ameliorated hyperexcitability following lithium treatment [70]. A follow-up study applied to a second cohort trained a naïve Bayes classifier capable of predicting with more than 92% accuracy whether a new patient would show clinical response to lithium [71]. An important next step will be CRISPR-based functional validation of the genetic variant(s) linked to lithium responsiveness [72]. Towards this, a recent study demonstrated genotype-dependent changes in mRNA expression following treatment of hiPSC-neurons with clinically relevant dosages of valproic acid [73], indicating that precision medicine drug screening approaches might be possible. Although these studies indicate that hiPSC-based models may represent a new drug screening strategy, they fall short of demonstrating that hiPSC-based models can be used to guide precision patient-based medicine in the clinic. Improvements in the efficiency and complexity of cell-based drug screening assays are necessary to accelerate phenotypic drug discovery applications [74].

We anticipate that future genomic approaches will provide the means to stratify patients with overlapping risk combinations into "genetically defined" cohorts. This would yield specific hypotheses and drug targets to be evaluated by integrating patient-derived hiPSCs and CRISPR-engineered isogenic models. Convergent genes and gene networks significantly associated with disease risk enriched with rare and common variant disease risk represent promising targets for drug screens (Fig. 3).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Given the growing number of identified variants linked to disease risk and the extensive clinical heterogeneity between patients, neurodegenerative and psychiatric diseases are notoriously challenging to treat effectively. Nonetheless, proof-of-principle hiPSC-based models have demonstrated the efficacy of patient-derived neurons and glia to recapitulate transcriptomic and cellular features of brain disease. Today, CRISPR-based isogenic experiments in patient and control hiPSC-derived neural cells make possible more precise interrogation of variants, genes, and gene networks relevant to disease biology. However, there still is a vital need to further develop and improve these models, especially in recapitulating the complex phenotypic characterizations readily available in animal models. Our hope is that the integration of whole-genome sequencing, hiPSC-based disease modeling, CRISPR-mediated functional validation and phenotypic drug discovery in neural-

cell-based screens will enable genotype-based diagnoses and drug treatment predictions, making possible a future for precision medicine.

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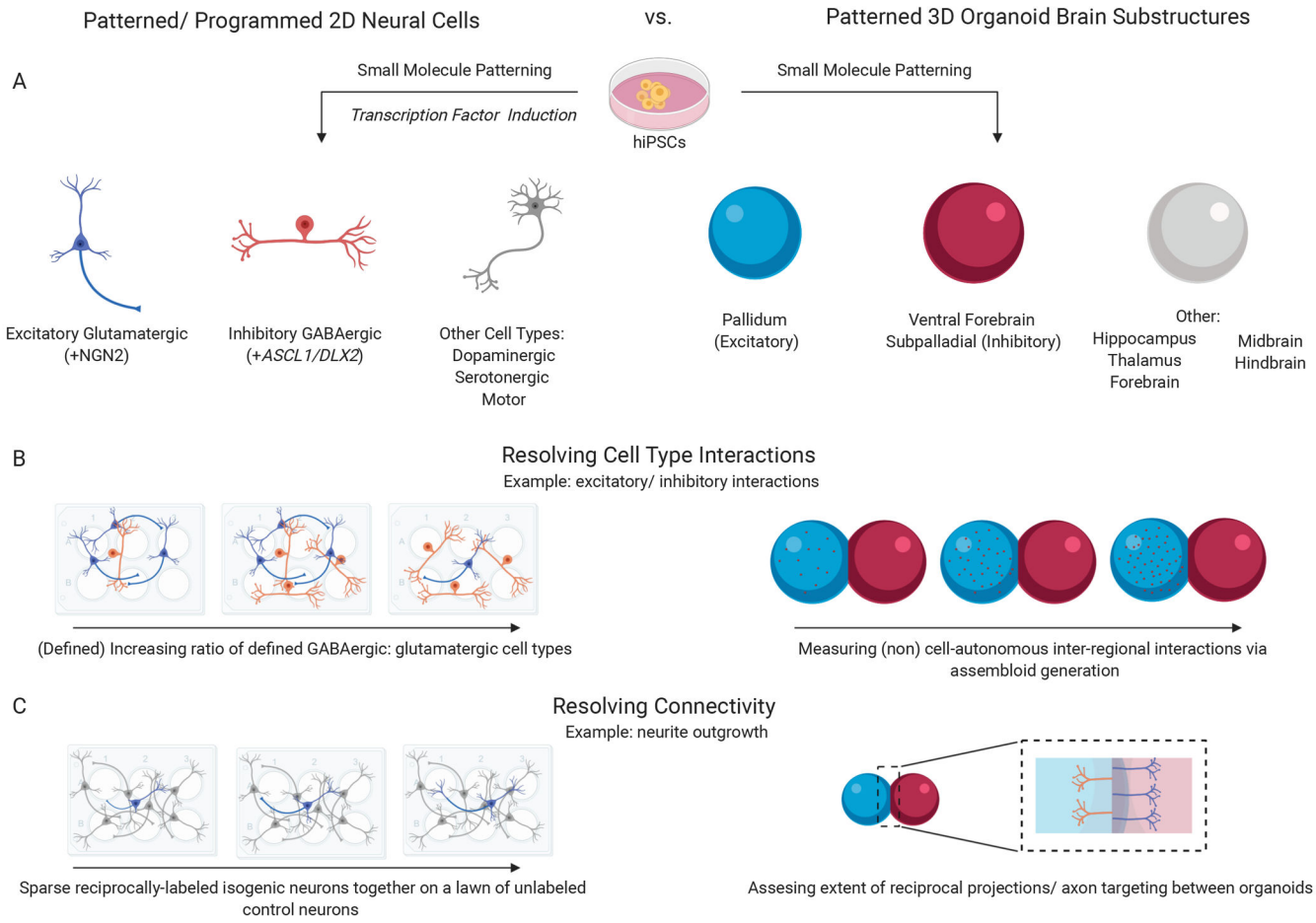

Figure 1: 2D vs. 3D culture systems to resolve cell type effects and interactions.

A, Schematic of types of 2D cell cultures and 3D substructures via small molecule patterning or transcription factor induction (2D only). **B**, Example assays to resolve cell type interactions. 2D-defined generation of glutamatergic and GABAergic cultures and 3D fusion of excitatory and inhibitory organoids (assembloid). **C.** Example assays to resolve connectivity in 2D sparsely seeded isogenic neurons on unlabeled control cells, or 3D organoid projection/axon targeting assessment. All figures were created using Biorender.com.

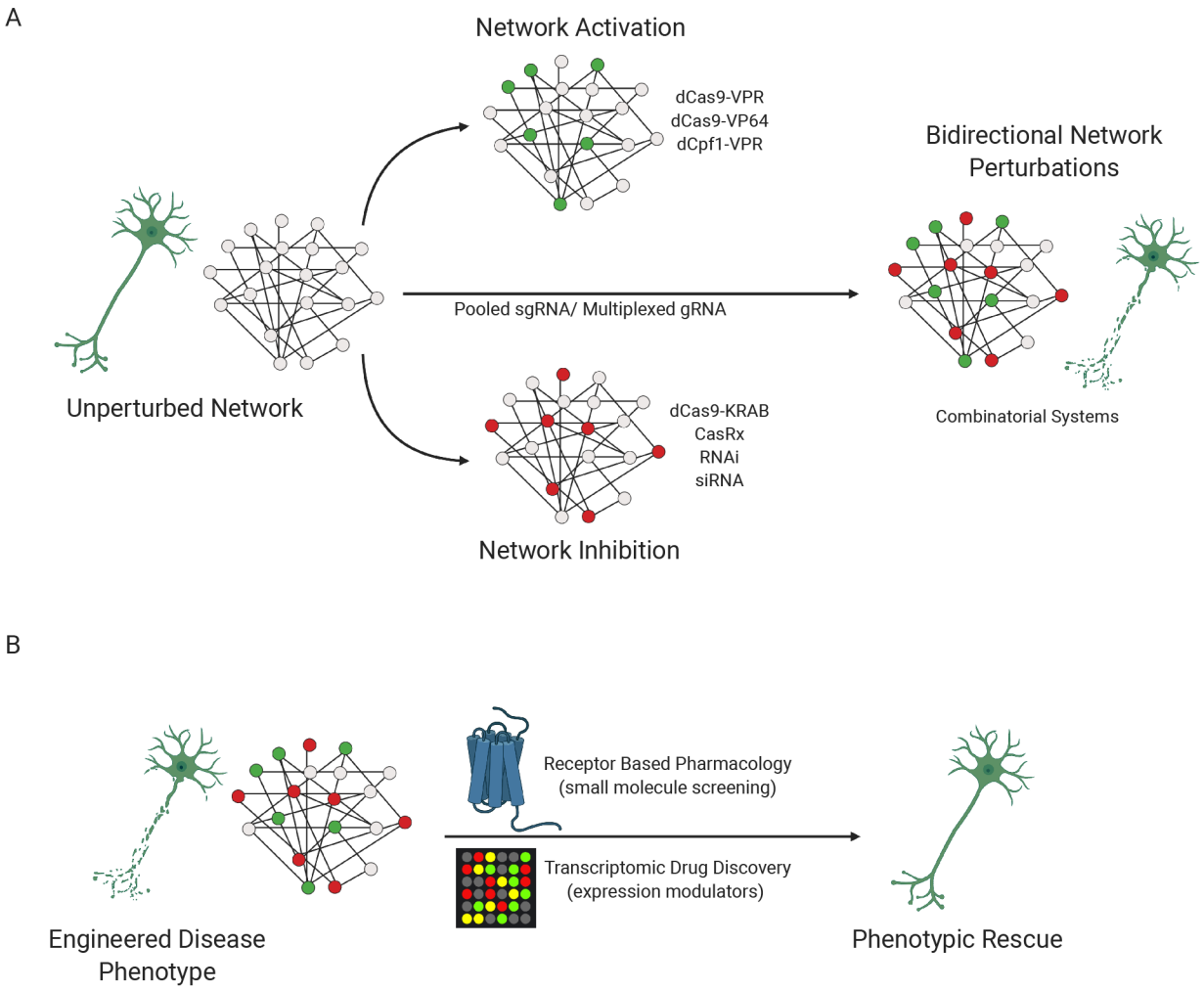

Figure 2: Unidirectional and bidirectional network perturbations.

A, The workflow schematic represents a concept of using modulatory and combinatorial CRISPR systems to perturb gene networks unidirectionally, and potentially bidirectionally. **B**, Phenotypic rescue based drug screens originating from CRISPR-engineered disease phenotypes.

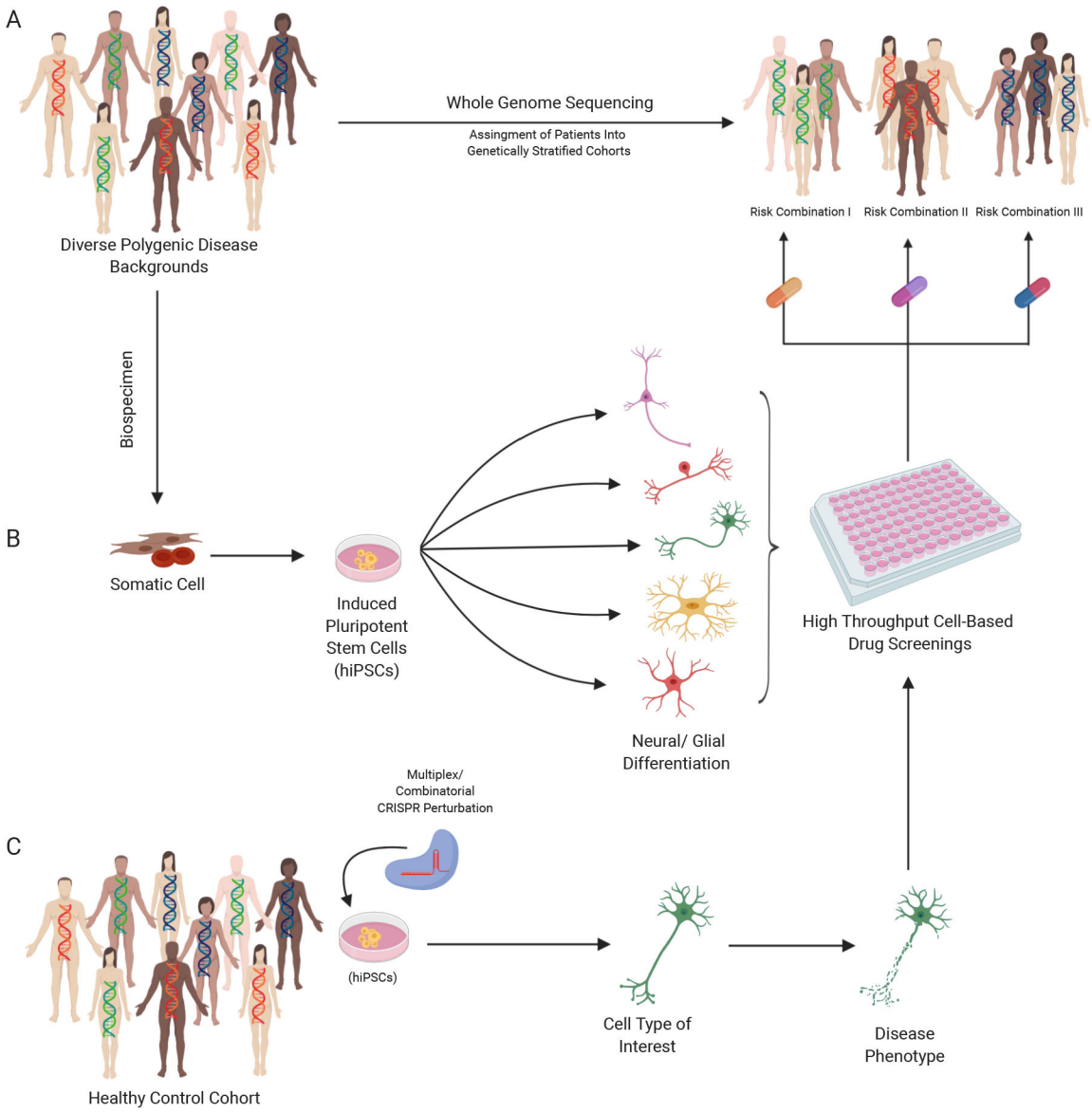

FIGURE 3: Coupling hiPSC and CRISPR platforms to accelerate functional validations of brain disease risk loci.

The rising reality of precision medicine via the integration of **A,** whole-genome sequencing to stratify patients with overlapping risk combinations into genetically defined cohorts, **B,** hiPSCs platforms to obtain patient-specific cell types and screened for phenotypic rescue, **C,** CRISPR-mediated network perturbations for engineered risk combinations and systematic cell-based drug screening, ultimately for genetically targeted therapeutics.

Table 1:

A brief list of CRISPR-based technologies with potential applicability to hiPSC-based experiments. More plasmids available at: https://www.addgene.org/crispr/.

	CRISPR Technology	Mammalian Cell Applications	hiPSC/hESC Applications	Addgene No(s).
Genome Engineering	Cas9	HEK293T, HUES9[75]	hiPSCs[76]	62988
	Cpf1	HEK293T[44]	hiPSC[77]	69982
	CasX	HEK293T[46]		
Transcriptional Regulation	dCas9-VPR (Gene Activation)	HEK293T[51]	hiPSCs[51] hiPSC-derived NPCs, neurons and astrocytes[78]	63798 99373
	dCas9-KRAB (Gene Repression)	HEK293T[50]	hiPSC-derived NPCs, neurons and astrocytes[78]	71237 99372
	dCas9-SunTag (Gene Activation)	U20S[79]	hiPSCs[80]	60904
	SAM (Gene Activation)	HEK293T[81]		75112
	dCpf1-VPR (Gene Activation)	HEK293T[82]		104567
Epigenetic Regulation	dCas9-p300 (Histone Acetylation)	HEK293T[83]		83889
	dCas9-LSD1 (Histone Demethylation)	Chicken Embryos[84]		92362
	dCas9-DNMT3A or dCas9-MQ1 (Cytosine methylation)	Mouse Embryonic Stem Cells[85]	hESCs[86]	84569
	dCas9-Tet1 (Cytosine demethylation)	Mouse Embryonic Stem Cells[85]	hiPSC-derived neurons[87]	84475
Transcriptome Engineering	Cas13a (RNA Targeting)	HEK293T[88]		91902
	Cas13b (RNA Editing)	HEK293T[89]		103854
	CasRx (Cas13d)	HEK293T[54]	hiPSCs[54]	109049

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