

CRISPR/Cas-Based Approaches to Study Schizophrenia

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Genomic association studies have revealed the complex genetic architecture of schizophrenia (SZ) and other neurodevelopmental disorders (NDDs). High-throughput models, such as cells and their derivatives, are needed to decipher the molecular basis of SZ pathology. The time is coming for high-throughput genetic technologies based on CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat)/Cas systems to manipulate multiple genomic targets. CRISPR/Cas tools make it possible to find and explore the complex relationship between genotype and phenotype of neuronal cells.

CRISPR/Cas system

genome editing

epigenome editing

schizophrenia

1. Introduction

Major psychiatric disorders, such as autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), bipolar disorder (BD), depression, and schizophrenia (SZ), are characterized by complex etiopathogenetic mechanisms involving neuroanatomic abnormalities, biochemical imbalances, genetic and epigenetic changes ^[1]. Some the disorders, such as SZ, ASD, and BD, have a genetic component, with heritability typically estimated by twin studies to be 40% to 80%, with much of it due to common risk alleles ^[2]. Large-scale genetic studies have convincingly shown that distinct psychiatric disorders are likely to share common genetic risk variants ^[3].

One way to assess the functionality of genetic risk variants is to identify and investigate their relationship with gene function and phenotypes at the cellular level. Thus, modeling neurodevelopmental disorders (NDDs) in different cell types provides mechanistic insights into the connections between genetic risk variants and the pathogenesis of NDDs. Cellular models can also provide information about potential therapeutic strategies because they have the predictability to change the aberrant phenotype to a normal level by genetic intervention or drug administration.

Since induced pluripotent stem cells (iPSCs) differentiate into neurons following the same trajectory as in the developing embryo, these cells are a convenient tool for studying NDDs such as SZ and ASD. Neurobiologists can compare iPSC-derived neurons from patients and control groups to try to identify the genetic and molecular basis underlying abnormal brain development and function. The observed neurodevelopmental changes may include an altered rate of cell proliferation and ability to migrate in neuronal progenitor cells (NPCs), abnormalities in neurite morphology, as well as disturbing expression dynamics of neuronal genes and pathways and electrophysiological properties of neurons. ^[4]. However, iPSCs obtained from different donors have large genetic and epigenetic

differences, which also affects their ability to differentiate even when using the same protocol [5]. Isogenic iPSC-derived cell models can help overcome the limitations of intersubject cell models. Isogenic cells can be created from healthy donor cells by introducing potentially causative variants or from patient cells by curing pathogenic alleles. The original and mutated iPSC lines or their differentiated derivatives can then be compared to study the effects of the introduced mutations. Despite their low throughput, single-gene cellular models still have their advantages. They are useful for proving causality, performing mechanistic studies, and assessing the relative contribution of specific pathogenic variants or risk genes to the severity of NDD. Single-gene cell models are adequate for studying monogenic forms of mental disorders, such as Timothy syndrome, a monogenic form of ASD caused by loss-of-function mutations in *CACNA1C* (Calcium Voltage-Gated Channel Subunit Alpha1 C) [6]. In addition to iPSCs and their derivatives, neuropsychiatric studies have also used neuronal cell lines such as human neuroblastoma SH-SY5Y [7].

Genome editing technologies, generally the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat)/Cas systems, are used to produce isogenic cell lines and have contributed greatly to the functional analysis of prioritized risk variants associated with NDDs (e.g., [8]). The principle behind the CRISPR/Cas9 genome editing technology is as follows. The Cas9 endonuclease, after binding its single guide RNA (sgRNA), searches for genomic targets that are complementary to the sgRNA spacer and have a short NGG motif adjacent to the right side of the target sequence. When binding to the target, the spacer forms a heteroduplex with the complementary strand of the target, displacing the non-target DNA strand. Once the spacer:target annealing process is complete, the nuclease activity of the two Cas9 nickase domains are activated, resulting in a double-strand break (DSB) with blunt ends. This DSB can be repaired by the cell using the error-prone nonhomologous end-joining pathway or the homologous-directed repair pathway if a donor DNA fragment homologous to the edited site of the genome is provided [9].

The Cas9 endonuclease can be modified by inactivating one of its nickase domains and fusing with a deamination enzyme. Usually, cytosine or adenine deaminase produces a new type of genome editor called base editors (cytosine base editor, CBE, and adenine base editor, ABE, respectively) [10][11]. Cytosine is desaminated to deoxyuracil, which reads as thymine during DNA replication, and adenine is desaminated to inosine, which reads as guanine. Thus, CBE action converts C•G into a T•A base pair, and ABE action converts A•T to G•C base pair without toxic DSBs. Basic editors enable the creation and study of putative causal single nucleotide polymorphisms (SNPs) [12][13].

The complete inactivation of Cas9 nuclease domains (called dead Cas9 nucleases, dCas9) and its fusion with functional domains that act to modify DNA or histones results in epigenetic editors [14]. The fusion of dCas9 with transcription activation or repression domains creates artificial activators or repressors of transcription. Epigenetic editors change the expression of genes without changing their sequences and can be used to investigate genetic variants located in the *cis*- or *trans*- regulatory regions of NDDs risk genes.

CRISPR-based technologies have clear advantages over other genome editing technologies. In particular, they can be used to relatively rapidly create isogenic cell models with small changes, such as SNPs or deletions of large

genomic regions. A major advantage of CRISPR/Cas systems is the possibility of simultaneous modification of multiple targets. This is important for the study of complex multigenic psychiatric disorders. In addition, CRISPR/Cas systems allow screening studies to rapidly identify multiple causal risk variants in a single experiment [15].

2. Recent Insights into the Genetic Architecture of SZ and Other NDDs

It has been well-known that heritability plays an important role in the development of NDDs such as SZ and ASD. Genome-wide association studies (GWAS) are a powerful hypothesis-free approach to searching for genetic NDDs risk loci. Risk loci mapped to genes, and genomic features can provide clues to unravel the pathogenic mechanism and provide the right choice of cell model and CRISPR/Cas tool for further validation and research.

In 2022, Trubetsky et al. conducted the largest GWAS to date on SZ [16]. The study identified 342 independent SNPs mapped to 287 distinct genomic loci that may increase the risk of SZ [16]. Using a combination of fine mapping, transcriptomic analysis and functional genomic annotations three groups of genes were identified. One group comprises the genes having at least one non-synonymous or untranslated region variant. This group includes genes such as *SLC39A8*, *IRF3*, *KLF6*, *THAP8*, *WSCD2*, *PJA1* and *CUL9*. The second group comprises genes that are reliably explained by variants that affect gene expression. Examples of genes are *ACE*, *DCLK3*, *SNAP91*. The third group includes genes encoding proteins localized in synapses and functioning in them. Synaptic dysfunction is considered to be a central component of the pathophysiology of SZ [17]. Genes in this group encode voltage-gated calcium and chloride channels (*CACNA1C* and *CLCN3*), metabotropic receptors (glutamate (*GRM1*) and gamma-aminobutyric acid (*GABBR2*)) and the ligand-binding subunit of the N-methyl-d-aspartate (NMDA) receptor (*GRIN2A*).

Heritability (h^2) estimated from family and twin studies is 0.81, 0.80, and 0.75 for SZ, BD, and ASD, respectively [18]. However, SNP-based heritability (h^2_{SNP}) estimated by GWAS is 0.23, 0.25, and 0.17 for SZ, BD, and ASD, respectively [19]. The difference between h^2 and h^2_{SNP} can serve as an estimate of missing heritability. It has previously been suggested that missing heritability is a hidden heritability that cannot be evaluated because of the drawbacks of the genetic mapping approaches [18]. Thus, one way to identify the genetic components of missing heritability is to develop new approaches for obtaining and analyzing genetic data. For example, additional SZ and BD risk genes have been identified by collecting SNPs into large groups and accounting for their non-additive interaction effects [20]. This approach makes it possible to examine SNPs that have not reached genome-wide significance. Another way to identify components of missing heritability is to analyze rare genetic variants.

In order to identify rare genetic variants associated with SZ, the Schizophrenia Exome Sequencing Meta-Analysis (SCHEMA) consortium sequenced and analyzed the exomes of 24,248 people with SZ and 97,322 healthy controls, the largest data set to date on SZ exomes [21]. As a result, the authors identified 244 candidate genes carrying two types of disruptive ultra-rare coding variants. The first type includes truncated protein variants (PTVs), defined as variants with stop, frameshift, or essential splice donor or acceptor variants. The second type represents

damaging missense variants. Of these, ten genes with the highest SZ risk were identified. They were annotated for the following functions: ion transport (*CACNA1G*, *GRIN2A*, and *GRIA3* (Glutamate Ionotropic Receptor AMPA Type Subunit 3)), neuronal migration and growth (*TRIO*), regulation of transcription (*SP4*, *RB1CC1*, and *SETD1A*), nuclear transport (*XPO7*) and ubiquitin ligation (*CUL1* and *HERC1*). Note that *CACNA1G*, *GRIN2A*, and *CUL1* are also identified in the GWAS mentioned above [16]. The overlap between GWAS and exome sequencing results supports the emerging consensus that rare and common genetic risk factors converge in the same molecular mechanisms of NDDs.

3. Applications of CRISPR-Based Genome Editing Technologies to Study SZ and Other NDDs

The identification of rare loss-of-function coding variants, such as PTVs, provides the most direct biologically interpretable links between gene function and the pathogenesis of a given mental illness. Loss-of-function gene mutations can be easily modeled by genetic disruption in cellular or animal models. Moreover, these mutations can be studied in both homozygous and heterozygous states. The latter is more relevant to human diseases because patients often have only one of their alleles disrupted. Genome editing with CRISPR-Cas systems facilitates the creation of isogenic models for subsequent molecular and phenotypic characterization to deepen the understanding of the mechanism of action of the mutation of interest.

Cellular models are highly convenient for introducing desired genetic changes and studying the associated molecular mechanisms. Even non-neuronal cells, such as HEK293 and their derivatives, can be used to study the molecular mechanisms associated with mutations in genes associated with a high risk of SZ and other NDDs. HEK293, unlike neuronal cells, does not require specific culturing conditions and can be efficiently transfected. However, not all SZ risk genes can be adequately studied in HEK293. For example, CRISPR/Cas9-mediated deletion of two downstream *FOXP2* enhancers in the SK-N-MC neuroblastoma cell line leads to impaired expression of *FOXP2* and its target genes, whereas deletion of the same enhancers in HEK293 has no effect [22]. This example shows that the data obtained in HEK293 lines need further verification in neuronal cell lines. Moreover, HEK293 lacks the constrictive validity characteristic for neuronal cell lines and therefore is not suitable for the electrophysiological, morphological, and other functional characterization of gene knockouts or their alleles related to the pathophysiology of the NDDs. Neurons differentiated from CRISPR/Cas9-edited human embryonic stem cells (hESCs) or human iPSCs (hiPSCs) represent more relevant cellular models for studying pathogenic molecular mechanisms associated with SZ. The use of patient-derived iPSCs offers great opportunities for the study of human neurodegenerative and psychiatric diseases [23]. iPSCs were initially used to model diseases with highly pervasive genetic variants with a large phenotypic effect. By now, their application has expanded to the field of modeling psychiatric diseases and generating patient-specific organoids. The ability of iPSC-derived neurons to reproduce fundamental neuronal functions, including conducting action potentials and releasing neurotransmitters, has led to the development of functional analysis of variants associated with SZ.

Cellular models and neural networks helped to reveal many important molecular and cellular mechanisms of NDD. However, they cannot be used to study higher levels of organization characteristic of brain structure as well as its

development. This necessitates the creation of another type of model that would mimic the brain features and development. Cerebral organoids derived from hiPSCs have become such a model. They have an advanced three-dimensional structure (forebrain, midbrain, and hindbrain) and a complex organization similar to the human fetal brain [24].

Assembloids represent the next level of 3D brain models with increased cellular composition and structural complexity [25]. A recently developed protocol for creating cortico-striatal assembloids [26] should help investigate corticostriatal connections that are affected in neuropsychiatric diseases, including ASD [27] and SZ [28].

The studies discussed above investigate individual genes. However, SZ and other NDDs are complex multigenic disorders, so studying individual targets limits the ability to identify causative variants and decreases the depth of the understanding of the complex mechanisms characteristic of NDDs. The CRISPR knockout (KO) screening technology allows multiple gene targeting in a single experiment and greatly expands the possibilities of identifying causal variants and investigating multigenic molecular mechanisms. Since neurons are non-dividing and hard to transfect cells, the CRISPR KO screenings are applied to iPSC-derived cellular models. Moreover, Cas9 doxycycline controllable systems are used [29][30] to overcome Cas9 toxicity to iPSCs [31]. CRISPR KO screenings are usually performed for a functionally related group of genes, for example, kinases [29] or high-risk NDDs genes [30], to exclude genes irrelevant to the study, decrease the number of false-positive hits, decrease the loss of edited cells with lowered competitive fitness and thereby increase the sensitivity of the analysis. So, CRISPR KO screening of 425 genes associated with the risk of ASD and other NDDs was performed in human forebrain assembloids (hFAs) to search for genes involved in the development and migration of cortical interneurons [30]. hFAs were derived from hiPSCs derived from human subpallial organoids (hSO) and human cortical organoids. As a result, loss of *SMAD4* (SMAD Family Member 2) or *CSDE1* (Cold Shock Domain Containing E1) disturbs subpallium differentiation and decreases hSO size. Loss of *TERF2* (Telomeric Repeat Binding Factor 2) and *LNPK* (Lunapark, ER Junction Formation Factor) impairs interneuron migration but does not affect subpallium differentiation. The study also showed that not all high-ranked hits of the primary screening could then be validated. Thus, it should be noted that CRISPR KO screening identifies candidate genes that need further validation in single-gene models.

4. Epigenetics of SZ and Other NDDs

Some authors believe that the genetic component cannot explain the entire heritability of SZ and other NDDs. Since all psychiatric concordance rates are well below 100% for monozygotic twins [32], it has been suggested that another important component of missed heritability is epigenetic inheritance [33]. Recent advances in functional genomics show that genetic variations and epigenetic dysregulation of transcriptional networks are associated with neuropsychiatric disorders [34].

A set of transcriptional programs controls the selective expression of neuronal identity genes during brain development. Gene expression programs are coordinated in part by basic epigenetic mechanisms such as DNA methylation/hydroxymethylation, posttranslational modifications of histone proteins, nucleosome remodeling/re-

positioning, and regulation of non-coding RNAs [35]. To uncover the role of epigenetic factors in psychiatric disorders, researchers conduct epigenome-wide association studies (EWAS) [36]. It has been shown previously that genetic variants can affect the level of DNA methylation at genomic CpG-rich loci. These genetic variants are called quantitative methylation trait loci (mQTLs) [37]. Most EWAS studies examine the relationship between DNA methylation and NDDs and use whole-genome bisulfite sequencing (WGBS) to map methylated cytosines with single-base resolution [38]. Because autism-related genes are often involved in chromatin remodeling and transcriptional regulation, their dysfunction during development may contribute to disease pathogenesis [39]. The critical role of chromatin remodeling and histone modification mechanisms is also observed in neurons both during development and in adulthood in response to external stimuli. Through epigenetic regulation of neuronal gene expression, environmental stimuli are transferred to neurobiological substrates capable of controlling behavior both in health and disease. Disease-related changes in the local chromatin structure at specific gene promoters can induce transcriptional changes that are directly related to the underlying etiology or secondary events in the pathophysiology of the disease.

In the adult brain, specific gene expression programs are altered by neuronal activity and behavioral experience, and these changes are crucial for adaptive behavior [40]. Dysregulation of gene expression programs both during development and in the adult brain is associated with numerous neuropsychiatric diseases such as addiction [41], depression [42], and SZ [43]. In recent years, epigenetic studies in the postmortem brain in SZ have mainly focused on identifying differentially methylated sites and genes in the cortex and other brain regions [44].

5. Application of CRISPR-Based Epigenetic Editors to Study SZ and Other NDDs

The development of nuclease-free Cas9 derivatives opens up a series of CRISPR/Cas tools aimed at manipulating epigenetics, i.e., DNA and histone modifications, and creating artificial transcription factors. CRISPR/dCas9 epigenetic editors allow manipulation of neuron-specific transcriptional programs to identify epigenetic hallmarks of NDDs and link them to genetic risk loci. Currently, the development of more efficient epigenetic CRISPR/Cas editors, for example, CRISPR/Cas9 repressors, is ongoing. The most commonly used dCas9-KRAB repressor contains the KRAB domain from KOX1 (ZNF10, Zinc Finger Protein 10) [45]. De novo DNA methyltransferases Dnmt3a and Dnmt3L fused to dCas9 can be used to establish long-term and long-range methylation of DNA loci [46]. Another study showed that some loci could be silenced by the histone methyltransferase EZH2 but not by the KRAB methyltransferase fused to dCas9 [47]. The highest levels of epigenetic silencing of target genes can be achieved when histone and DNA methyltransferase activities are combined as separate chimeric proteins, such as dCas9-Dnmt3a-Dnmt3L + dCas9-Ezh2 or dCas9-Dnmt3a-Dnmt3L + dCas9-KRAB) [48] or as a single KRAB-dCas9-Dnmt3a-Dnmt3L fusion protein [49].

Artificial CRISPR/Cas9 transcription factors are created by fusing transactivation domains to dCas9. Examples of transactivation domains used are the transactivating subunit of nuclear factor- κ B (p65), the VP16 activation domain of herpes simplex virus (VP16), and four repeats of the VP16 activation domain (VP64). The strength of CRISPR activators can be increased by using a combination of transactivation domains such as VPR (consisting of VP64,

p65, and RTa) or arrays of activation domains in the SunTag system (VP64 array recruited to dCas9) [50]. Other examples of epigenetic CRISPR editors are fusions of dCas9 with the catalytic domains of methylcytosine dioxygenase Tet1 (Tet Methylcytosine Dioxygenase 1) or human histone acetyltransferases p300 or LSD-1 [51].

The applications of epigenetic editors to understand the epigenetic mechanisms involved in SZ and other NDDs can be divided into two directions of research. The first direction is the application of CRISPR/Cas9-based tools to manipulate the activity of genome-encoded DNA methyltransferases and other natural epigenetic mechanisms to elucidate their role in the regulation of risk genes for SZ and other NDDs. The second direction is the use of artificial epigenetic editors based on CRISPR/Cas system to directly influence the expression of target genes.

CRISPR-based interference/activation screenings (CRISPR i/a) can be a valuable tool in epigenetic studies of polygenic NDDs. Like CRISPR KO screening, they allow the search for causative variants and risk genes within a single experiment. Since the CRISPR i/a systems do not damage DNA and do not affect neuronal differentiation and activity nonspecifically [52], they are an adequate alternative for CRISPR KO screenings.

CRISPRi screens assess genes and pathways for their disruption. In contrast, some genetic variants lead to the upregulation of genes and pathways. CRISPRa screens are more suitable for studying the functional significance of such variants. CRISPRa screening was applied to NGN2 (Neurogenin 2)-induced glutamatergic neurons to investigate the molecular mechanisms associated with the overexpression of twelve upregulated high-risk genes of SZ (*CALN1*, *CLCN3*, *FES*, *INO80E*, *NAGA*, *NEK4*, *PLCL1*, *SF3B1*, *TMEM219*, *UBE2Q2L*, *ZNF823*, and *ZNF804A*) [53]. In order to track the developmental pathways affected by the overexpression of these genes; transcriptomic studies were performed at two different time points. As a result, the authors showed that the common effects converge on developmental pathways involved in patterning, regionalization and growth, neuroactive ligand-receptor signaling, and voltage-gated ion channel activity. Moreover, in silico modeling studies have shown that convergence increases with increasing polygenicity, confirming the polygenic additive model of SZ.

6. Conclusions and Perspectives

The collective efforts of international consortia and laboratory teams have made it possible to identify a large number of priority genes and variants responsible for the significant risk of the development of SZ and other NDDs. The identification of causative variants can be performed using various cellular models obtained by applying CRISPR-based genetic and epigenetic editors. CRISPR screening technology combined with transcriptomic studies can reveal pathways associated with disrupted high-risk NDDs genes leading to characteristic disease phenotypes. It can be believed that the combined use of CRISPR/Cas strategies to manipulate genome architecture, genome sequence, and epigenome has great potential to decipher complex gene regulatory networks in neuronal circuits and discover links between the complex genetic architecture of mental disorders and their phenotypes. To deepen the mechanistic understanding of the network dysregulation underlying the major symptoms of mental disorders, the existing models need to be further developed.

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