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Modernising autism spectrum disorder model engineering and treatment *via* CRISPR-Cas9: A gene reprogramming approach

Role of CRISPR-Cas9 in ASD

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Abstract

Abstract

A neurological abnormality called autism spectrum disorder (AID) affects how a person perceives and interacts with others, leading to social interaction and communication issues. Limited and recurring behavioral patterns are another feature of the illness. Multiple mutations throughout development are the source of the underdevelopment disorder autism. However, a well-established model and perfect treatment for this spectrum disease is not yet discovered. The rising era of Clustered Regularly Interspaced Palindromic Repeats (CRISP)-Cas9 system can lead to streamline the complexity underlying the parthenogenesis of the AID. CRISPR-Cas9 system is a powerful genetic engineering tool used to edit genome at the targeted site in a precise manner. The major hurdle in studying the AID is the lack of appropriate animal models presenting the complex symptoms of ASD therefore, CRISPR-Cas9 is being used worldwide to mimic the ASD like pathology in various systems like *in vitro* cell lines, *in vitro* 3D organoid models and *in vivo* animal models. Apart from being used in establishing ASD models, CRISPR-Cas9 can also be used to treat the complexities of ASD. The aim of this review is to summarize and critically analyse the CRISPR–Cas9 mediated discoveries in the field of ASD established in the past years.

Key Words: Autism Spectrum Disorder; CRISPR-Cas9; cellular models; organoids; animal models; therapeutic strategies

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Core Tip: There are several reviews in the literature explaining the underlying mechanisms contributing to the pathophysiology of autism spectrum disorder by performing several pre-clinical experiments. Given the significant role of genetics (de

novo or inheritable) in the development of ASD, disease specific- models has to be established for investigating the mechanism involved. Therefore, this review has specifically focussed on the use of emerging genomic editing tool, CRISPR/Cas9 for generating different types of pre-clinical models as well as new therapeutic options, providing a novel insight about disease

INTRODUCTION

INTRODUCTION

Identifying double helix DNA structure and finding technologies to manipulate it ultimately led to an extensive investigation of gnomic structure [1].Manipulation of gnomic structure requires various gnomic editing techniques including homing-denuclearizes or mega nucleates, zinc finger nucleates (Zens),transcription activator like refectory nucleates (Talent), and clustered regularly intersected short palindromic repeats (CRISP) associated protein 9 (Cas9)[2]. Advancement in this field has permitted researchers to alter the DNA of model organism and get the model of interest. In this context, the discovery of the CRISPR-Cas9 system has greatly and enormously expanded the field of study related to the genetic underpinnings of complex and heterogeneous disorders like autism spectrum disorder (AID) [3]. From bacterial defense systems to gnomic engineering tools, CRISPR-Cas9 has been proven beneficial in providing a novel insight into a possible genetic mutation in AID[4].The spectrum of disorders that come under ASD is pervasive. Due to the complexity of this medical condition, it is challenging to determine the diagnostic threshold, making diagnosis difficult. Despite the apparent difficulties connected with identification of ASD aetiologies, intensive genetic investigations have shown that ASD has a substantial genetic basis, and genetic analysis has revealed many susceptibility genes[5]. In addition to this, ASD has been found to be associated with several other disorders such as anxiety, depression, attention-deficit hyperactivity disorder (ADHD), obsessive-compulsive disorder (OCD) [6] and with genetic syndromes like Rett syndromes, Angel man syndrome, Timothy epilepsy, and Fragile-X syndrome [7,8].Depending upon the

origin of the condition, ASD is diagnosed as syndromic if it is due to specific genetic syndromes with well-defined aetiology, such as Rett syndrome and identified as non-syndromic if ASD is diagnosed as the first diagnosis in patients having less-characterized aetiology[9,10]. There are multiple factors involved that contribute to the development of ASD in case of non-syndromic ASD[10]. Therefore, due to the several aetiologies involved, ASD is considered as heterogeneous group of highly heritable disorders [11,12] and their risk factors could be genetic as well as environmental. A significant role of genetics in the development of the disorder has been known for very long as confirmed by metanalysis of twins' studies, which stated that ASD is inherited nearly 64-91% in monozygotic twins, while around 30% in dizygotic twins [13–15]. Modeling of disease at i*n vitro, in vivo* and organoids level are major avenue of research for investigation of abnormal early brain development because several ASD-associated genes have been found to be highly expressed during prenatal brain development of patients [16]. CRISPR-Cas9 has been successfully used to generate genetically engineered models that could mimic the disorder. At the same time, gene therapies are one of the emerging fields in recent years with the aim of curing a wide range of diseases including ASD. Moreover, based on the available genetic information, novel gene therapies have also been created, which may help identify the potential ASD therapy candidates. Advent of CRISPR-Cas9 in gene therapy has been helpful in either silencing the gene using non-homologous end joining (NHEJ) or correcting the genetic mutation using non-homologous recombination [17]. These developments have given patients new hope regarding rational treatment against the disease. This article provides an overview of the potential use of CRISPR-Cas9 technology for the establishment of appropriate ASD models along with its application in therapeutic strategies at the genomic level.

GENETIC ARCHITECTURE OF ASD

Genetic background contributing to autism etiology involves copy number variations (CNS), somatic mutations, *e nova* mutations, single nucleotide variation (SN), insertions,

deletions and chromosomal abnormalities[18,19]. These factors interfere with the protein-coding genes involved in neuronal development and several other AID candidate genes related to critical processes like DNA binding, transcription, postsynaptic density and protectionist[20]. Any alteration in well-known AD-associated genes can ultimately result in impaired working of brain areas responsible for cognitive functions[21,22].Forehead box protein 1 (*FOXP1*)and fragile X messenger oligonucleotide 1 (*FMR1*) are transcription factors and regulating gene and others like methyl Cpd binding protein 2 (*MECP2*),tuberous sclerosis 1(*TSC1*),SH3- and multiple hankering repeats protein 1 (*SHANK1*),ubiquity protein ligate E3A(*UBEA3A*), and contradistinction protein-like 2 (*CNTNAP2*),are involved in a wide range of functions like chromatin remodeling, cell proliferation, maintaining synaptic activity, protein inquisitional, cell adhesion, respectively. Moreover, mutations in *MECP2* and *FMR1* are related to genetic syndromes such as Sett syndrome and Fragile X syndrome, respectively[3].Also, latest advancements in the development of next-generation sequencing (N'S) have offered opportunities for genetic analysis to elucidate the underlying genetic mechanisms of AID[23]. Whole exhume sequencing (EWES) has revealed some bimetallic mutations in proximal assembly proteins (*PEX7*),phenobarbital hydrolyses(*PAH*),introspect repeat containing nuclear envelope protein 1(*SYNE1*)associated with familial AID [24] and these genes also include those that are known to control or be controlled by synaptic activity (e.g., *MECP2, SYNE1*). While genetic analysis using whole genome sequencing (WIGS)has investigated that CNS and Sn Vs result in missed mutations with an overall increase in missed variants, including some AID risk genes [25].In addition to this, genome-wide association studies (SWAG) have been able to identify a few potential variants being implicated in the parthenogenesis of AID [26].Altogether, mutations in specific genes,known to regulate the important biological pathways, neuronal networks, synaptic activity and plasticity, *etc.*,contribute to developmental of AID and associated clinical symptoms (Figure 1).

STRUCTURE AND FUNCTION OF CRISPR-CAS9

CRISPR-Cas9 is used to cut DNA at predetermined target locations. Although the method has already been revolutionised as a gene editing tool, researchers are constantly exploring new applications. Since being discovered as a bacterial immune system against invading viruses, CRISPR-Cas9 has been adapted as an overpowered tool in genomic research. Repeat elements in CRISPR were initially noticed in *Escherichia coli* by Dr Nakata's group [27]. Contrary to conventional tandem repeats in the genome, the CRISPR repeat clusters were interestingly separated by non-repeating DNA sequences known as spacers. Complete genome sequencing of bacteria and archaea led researchers to know that these CRISPR elements are adjacent to well-conserved CRISPR-associated gene (Cas) [28]. This whole structure including palindromic repeats, spacer DNA and Cas gene, is known as the CRISPR array. After a decade of research, scientists have finally discovered that the spacer DNA sequences belong to viruses [29,30]. The study by Horvath and colleagues provided the most significant experimental data about the potential utility of CRISPR systems for bacteria. The concept that bacteria's Cas9 enzymes can be reprogrammed to target a specific DNA sequence has been the key discovery, which signalled the beginning of CRISPR as a biotechnological gene-editing tool [31,32]. CRSPR RNA (crRNA) and trans- activating CRISPR RNA (tracrRNA) are both vital parts of guide RNA (gRNA), and are required for the functioning of the CRISPR system. Notably, Jinek *et al* demonstrated that CRISPR-Cas9 could also be guided by single guide RNA (sgRNA), a chimeric RNA created by joining tracrRNA and crRNA[31]. These studies were the reason for adopting a CRISPR-Cas9 as a gene editing tool. The ability of the CRISPR-Cas9 system to produce an autism model and its therapeutic potential are the main topics of this review. In 2012, Doudna and Charpentier found that by using the appropriate template, CRISPR-Cas9 could be used to edit any desired DNA [31]. Depending on how Cas proteins act, the CRISPR-Cas9 system has been divided into type I, type II, and type III systems. Type II of these systems is the most well-studied and simplest for application in genetic engineering [33]. Cas9 protein performs the function of a genetic scissors in the type II system by producing a double-stranded break (DSB) in the DNA [34]. Cas9

protein contains two structural lobes, one that aids in recognition (REC) and the other that aids in nuclease (NUC) activity. The REC lobe consists of REC1 and REC2, which are involved in the recognition of gRNA.NUC also has a protospacer adjacent motif (PAM) interacting domain responsible for the binding of Cas9 to targeted DNA. The gRNA is used to target viral DNA in prokaryotes, but when utilised as a gene-editing tool, it can be synthetically constructed to target virtually any gene that needs to be changed. The three phases of the CRISPR-Cas9 genome editing system are recognition, cleavage, and repair [35]. sgRNA binds to a complementary area on the targeted DNA to begin the recognition process.PAM is a 2–5 base pairs sequence that has "NGG" pattern, where "N" stands for any nucleotide followed by two guanine nucleotides. Once PAM site is identified, double stranded DNA starts melting at target site followed by RNA-DNA hybrid formation. Now, the Cas9 protein is ready to make DSB at targeted DNA at 3 base pair upstream to PAM [36]. In the last step, the double stranded blunt ended breaks are repaired by NHEJ and homology directed repair system (HDR) by cellular machinery [34,37,38]. By inserting a donor DNA template with sequence homology at the anticipated DSB site, HDR carries out the precise gene insertion or replacement [39].The property of CRISPR-Cas9 to either activate genes or to repress genes has been utilised to regulate the transcriptional level of gene expression.

CRISPR-CAS9 MEDIATED GENETIC ENGINEERING OF ASD

Most cases with ASD are idiopathic, with illusive aetiology[40]. The heterogeneous molecular nature of ASD makes it really difficult to understand the associated risk factors and the underlying mechanisms. Modelling ASD is notably challenging due to its multigenic aetiology. Only pertinent and validated disease-specific models could be helpful in discovering novel biomarkers and related therapeutic targets [41].

CRISPR-Cas9 engineered cellular models of ASD

Numerous neurodevelopmental diseases, including ASD are studied using cellular models because ofthe short experimental period, no ethical concerns and are less expensive.Researchers can create early human brain development, alterations in ASD or

any other neurological disorders using *in vitro* models. Induced pluripotent stem cells (iPSCs), which can grow indefinitely *in vitro*, can be created by reprogramming somatic cells. Patient-derived cellular models have proven to be extremely validated and realistic while preserving the genetic makeup of the donor and are an effective tool for deciphering the pathophysiology of ASD.The emergence of the genomic editing tool,CRISPR-Cas9 is helpful in facilitating more efficient *in vitro* models of ASD considering its genetic background. Using this technique, the researcher can edit primary cultured neural cells or isogenic cell lines by either introducing mutations or correcting them, derived from ASD patients.Moreover, this technology reduces genetic background variationand directly correlates the observed symptoms and the associated mutation [42], which further provides information about the role of the particular ASD risk gene in neurodevelopment.

As it is known that, aberrant neurogenesis and synaptogenesis lead to functional impairments in brain networks in ASD[41]. Therefore, early molecular events during ASD development can be replicated in a model system in neurogenin 2 (NGN2)-directed induced iPSCs (for excitatory neurons) that are further differentiated into forebrain glutamatergic neurons. Using that information and based on the WES results of some selected ASD-associated risk genes, CRISPR-Cas9 approach was used to generate knockout (KO) iPSCs for the functional studies of genes (anosmin 1(*ANOS1*), fragile X messenger ribonucleoprotein 2 (*FMR2*),calcium voltage-gated channel subunit alpha1 C(*CACNA1C*),astrotactin 2(*ASTN2*), alpha-thalassemia/mental retardation, X-linked(*ATRX*),chromodomain helicase DNA binding protein 8(*CHD8*),disks large-associated protein 2(*DLGAP2*), teneurintransmembrane protein 1(*TENM1*), potassium voltage-gated channel subfamily q member 2(*KCNQ2*), and sodium voltage-gated channel alpha subunit 2(*SCN2A*)). They revealed that given ASD genes could result in similar electrophysiological phenotypes and transcriptional rewiring in the human iPSC-derived excitatory neurons model system[43]. Apart from the role of the sh3- and multiple ankyrin repeats protein 3(*SHANK3)*gene in synaptogenesis,one of the other consequences ofits haploinsufficiency is hyperpolarization-activated cation

(Ih)channelopathywhich contributes to ASD pathogenesis. This impairment was analysed by generating*SHANK3*deletion by CRISPR in human embryonic stem cells[44].Findings also highlighted that iPSCs-derived glutamatergic neurons deficient in at least one allele of contactin 5/ euchromatic histone lysine methyltransferase 2 (*CNTN5 /EHMT2*) resulting in ASD-associated phenotypes presented the increased synaptic activity of excitatory neurons *in vitro*[45]. In addition-to this, CRISPR mediated inactivation of *EHMT1* in human neurons is directly associated with n-methyl-D-aspartate receptor (NMDAR) hyperfunction, which is implicated in ASD pathophysiology[46].The major obstacle in the way of treatment of ASD is testing different drug candidates because of its etiological heterogeneity. So, *in vitro* study has been done using CRISPR tool for introducing mutations in activity-dependent neuroprotective protein (*ADNP*),dead-box helicase 3 X-Linked(*DDX3X*) and *FOXP1* genes to create a relevant ASD model[47].Similarly, hemizygous *CHD8* (*CHD8*+/−) iPSC lines were designed to investigate the role of *CHD8* in embryo development at the molecular and cellular levels. According to transcriptomic profiling, *CHD8* regulates several other genes connected to the development of ASD[48]. In addition to ASD-associated genes, the role of long non-coding RNAs (lncRNAs), such as patched domain containing 1-antisense RNA(*PTCHD1-AS*)[49]and molybdenum cofactor sulfurase (*COSMOC/MOCOS*)[50]in ASD development was also studied using CRISPR technology inhuman induced pluripotent stem cells (hiPSCs).Cellular models are briefly summarized in table 1.These aforementioned findings indicate that these ASD associated genes may be a therapeutic target for the treatment of ASD.

CRISPR-Cas9 engineered organoids of ASD

The lack of suitable ASD models has always been a hindrance in ASD research because neither 2D cell culture nor animal models can accurately mimic the aetiology of ASD. Therefore, 3D *in vitro* models like organoids, have recently been emerged in the field of research. They have been shown to reproduce the gene expression profile, transcriptome, epigenome, and disease dynamics of both idiopathic and syndromic ASDs[51].Like other cellular models, iPSC-derived organoids are being used because of

no ethical concerns and are preferred over 2D culture and animal models as they can generate more disease-specific models. This methodology has become even more reliable due to the integration of CRISPR-Cas9 to produce isogenic controls, significantly reducing genetic background differences. Idiopathic ASD has been connected to abnormalities in several genes, and genetic research has found multiple mutations that are linked to this condition [52].Enhanced neurogenesis in idiopathic ASD has been studied through CRISPR engineered organoid model to create mutations in histone methyltransferase*SUV420H1*, the tumor suppressor phosphatase and TENsin homolog(*PTEN*)[53], *CHD8*, the GTPase-encoding RAS-related protein Rab-39B (*RAB39b*)[54]. These genes are linked to macrocephalic ASD and CRISPR-mediated deletion resulted in larger haploinsufficient cerebral organoids in comparison to isogenic control due to over-activation of P13K-AKT-mTOR pathway [54]. Modelling of syndromic ASD is also being achieved using cerebral organoids to investigate the underlying genetic mechanism. One of the important ASD-associated genes *MECP2* is considered critical for early brain development, but its loss-of-function mutations is common underlying aetiology of Rett syndrome[55], causing severe impairment in human interneurons and ultimately neurogenesis. Human *MECP2*-KO neurons and cortical organoids were used using CRISPR to investigate its neuropathological function[56,57]. Mutation (deletion) in *UBE3A* is also related to the pathology of syndromic ASD and organoid model derived from human iPSCs demonstrated hyper excitability in brains contributing to network dysfunction[58]. Similarly, cerebral organoids are used for studying other syndromic ASD such as a mutation in *TSC1/TSC2* genes in CRISPR-engineered human cortical spheroid model [59] causing synaptic imbalances, with an increase in γ-aminobutyric acid (GABA)ergic synapses [60]. Human cortico-striatal organoids were studied using CRISPR-generated *SHANK3* gene deletion for modelling autism[61].hiPSCs-derived brain organoids with CRISPR-Cas9 induced *FMR1*KO which is causing abnormal increase in astrocytes number, was utilized to model FXS, a syndromic ASD[62]. Various organoid models of syndromic as well as idiopathic ASD is summarized in table 1.

CRISPR-Cas9 engineered animal models of ASD

Despite the capabilities of *in vitro* models to recapitulate the basic aetiology of ASD, animal models are preferred as a more fundamental tool to fully understand the complexity involved in diseases. Animal models basically allow a researcher to investigate behavioural and developmental features in addition to molecular parameters. However, generating an ASD animal model is a time-consuming procedure and also involves ethical concerns, but it is helpful in studying neurodevelopmental disorders. Moreover, in the case of ASD, it is helpful in validating the implication of critical genes in the development of ASD. The emerging CRISPR-Cas9 approach has been a great help in creating various genetic animal models (KO, Knock-in, over expression and point mutation)to study various ASD-associated genes identified in an individual with ASD condition.ASD models can be studied on multiple species like rodents including mice and rats, monkeys, fruit flies and zebrafish, depending upon the requirement and purpose of the experiment[63].CRISPR-mediated generation of mutation in the *SHANK3*geneby targeting and making insertion and deletions (indels) in exon 21 has developed an ASD model in monkeysand their F1 offspring, showing atypical autistic phenotypes like increased repetitive behaviour along with socialand learning deficits[64].Studieshave reported that CRISPR-mediated mutation inASD-associated genes such as AT-Rich Interaction Domain 1B (*ARID1B*)[65], *CHD8*[66], and ASH1 Like histone lysine methyltransferase(*ASH1L*)[67]showed ASD-like symptoms in mice.To investigate genes implicated in ASD such as cytoplasmic FMR1 interacting protein(*CYFIP1*)[68], transcription factor 4 (*TCF4*)[69],and *UBE3A*[70] in a rat modelled with CRISPR engineered technology was studied, which showed autistic phenotypes likealteration in behavioural flexibility, learning ability and memory difficulties. Similarly, a zebrafish model of ASD using the CRISPR strategy has been used to study the functional role of genes in the development of ASD such as *CHD8*[71],*FMR1*[72],nuclear receptor subfamily 3 group c member 2 (*NR3C2)* [73],and *SHANK3*[74]. Major ASD-linked phenotypes observed in these zebra fish models are

macrocephaly, hyperactivity, anxiety, impaired social behaviour, sleep disturbances, and altered neuronal development (summarized in table 1).

CRISPR-CAS9-BASED THERAPEUTIC STRATEGIES AND POTENTIAL TARGETS

Over the years, the CRISPR-Cas9 genome editing tool has evolved as a specific delivery tool for delivering genes to the target cells including neural and brain cells. One such benchmark was set by Staahl *et al*, where the engineered variants of Cas9 ribonucleoprotein complex were delivered to the mice hippocampus, striatum and cortex region and demonstrated the *in vivo* neuronal gene editing [75]. The advances in the genome editing tool have opened the door for eradicating the genetic mutations underlying severe neurological diseases like ASD. Several genes that are linked to ASD can be targeted for correction using the CRISPR-Cas9 approach to reduce the disease burden (summarized in Figure 1). The genes that undergo mutations in ASD and ASD-associated monogenic syndromes include *CACNA1C, FOXP1/2,*wingless-related integration site-2(*WNT-2*), *CHD8,*homeobox B1(*HOXB1*),reelin(*RELN*), inner mitochondrial membrane peptidase subunit 2(*IMMP2L*),oxytocin receptor gene(*OXTR*),methylenetetrahydrofolate reductase(*MTHFR*),sh3- and multiple ankyrin repeats protein 2/3(*SHANK2/3),*γ-Aminobutyric acid type A(*GABAA*)receptor subunit,homeobox A1(*HOXA1*), *UBE3A,*NCK Associated Protein 1(*NCKAP1*),human serotonin transporter gene *(SLC6A4),*POU class 3 homeobox 2(*POU3F2*), reduced arabinose yariv1/suppression Of tumorigenicity 8(*RAY1/ST8*), *FMR1, MECP2, TSC1, PTK7, SCN3A, CNTNAP2* [5,26,76–82]. Some of these genes for monogenic syndromes associated with ASD are targeted using the CRISPR-Cas9 tool *in vitro* and *in vivo*; however, many others remain to be explored.

The lack of target specificity or the polygenic form of autism spectrum limits the use of the CRISPR-Cas9 tool as a therapeutic strategy in ASD. The CRISPR-Cas9-based therapeutic strategies that had been explored are summarized in table 2; mainly consist of the monogenic form of ASD. One of the studies by Lee *et al*, demonstrated that gold

nanoparticle delivery of CRISPR-Cas9 ribonucleoprotein rescued the exaggerated repetitive behaviours in mice caused by fragile X syndrome [83]. The study demonstrated minimal off-target effects and the editing target used was the metabotropic glutamate receptor subtype 5 (mGluR5) gene, one of the over expressed targets in ASD-associated syndromes[83–85].In another study, the CRISPR-Cas9 tool was used to correct the *MECP2* mutations responsible of ASD associated Rett syndrome *via* homology directed repair pathway in human induced pluripotent stem cells [86]. Loss of function mutation in *SHANK2* gene has been associated with monogenic ASD. CRISPR-Cas9 mediated correction of non-sense mutation on *SHANK2* was demonstrated in induced pluripotent stem cells and the positive impact on nerve cells was reported like an increase in synapse number, dendritic complexity and length [87]. In another monogenic form of ASD, the Angelman syndrome caused by deletion of the maternally inherited *UBE3A* allele, the CRISPR-Cas9 approach was used to knock out the antisense transcript of *UBE3A* in cultured human neurons and mice model. The antisense transcript of *UBE3A*, a long non-coding RNA silence the paternal copy of the *UBE3A* allele and leads to the neurodevelopmental syndrome. The CRISPR-Cas9 approach was used to terminate the long non-coding RNA termed as antisense transcript of *UBE3A*, which leads to the copy of the *UBE3A* allele available for transcription (activation of *UBE3A*) and hence rescued the anatomical and behavioural phenotypes in mice model of Angelman syndrome [88].In another study, the CRISPR-Cas9 approach was used to improve the Fragile X syndrome by knocking out the cytosine-guanine-guanine (CGG) repeats expansion, recovering *FMR1* expression *in vitro*. *FMR1* encodes fragile X mental retardation protein, which undergoes epigenetic silencing because of the addition of CGG repeats and excessive DNA methylation, thus the CRISPR-Cas9 approach was used to excise the CGG expansion in the induced pluripotent stem cells [89].A recent study used the CRISPR-Cas9 tool to activate the extracellular matrix receptor b3 integrin gene (*ITGB3*). The study at the same time also validated the involvement of *ITGB3* haploinsufficiency in the pathophysiology of ASD and ASD-associated fragile X syndrome [90].

All the CRISPR-Cas9-based therapeutic strategies established so far mainly comprise the proof of principle studies and have used conventional homology-directed repair pathway to correct the mutations in the monogenic form of ASD. However, with the advancements in CRISPR-Cas9 genome editing tools, the most recently introduced concept of base editing technique for more specific genome editing is explored in fewer studies. One such study used CRISPR-Cas9-based cytidine base editors and fourth generation of base editor(BE4)system to selectively modify disco-interacting protein 2 (*Dip2a* and *Dip2c*) genes in cell culture. Both of these genes are highly expressed in the central neuron system and known to be associated with ASD [91].In another study, the CRISPR-mediated cytidine base editor system was used to restore the impairments in social interactions and repetitive behaviours in knock-in mice model of autism. The *de novo* mutation in the gene myocyte-specific enhancer factor 2C(*MEF2C*) was introduced in the mice brain, which displayed autistic-like behaviour and further with the help of the base editing system the *MEF2C* mutation was eradicated and the reversal of symptoms was reported in mice [92].A study has demonstrated the use of CRISPR-Cas9 for correcting the mutation in gene *CNTNAP2* in organoid model derived from patients with syndromic ASD by rescuing the phenotype like organoid overgrowth. This *CNTNAP2*-organoid model provides opportunity for further mechanistic inquiry and development of new therapeutic strategies for ASD[93]. Another finding has shown the use of CRISPR activation (CRISPRa)-based approach for rescuing abnormalities in *SCN2A* haploinsufficiency-associated ASD [94].The CRISPR-Cas9 mediated base editing system is just the beginning of the era of targeted gene modification, which can bring the breakthrough in the treatment of ASD.

Plethora of studies is being conducted worldwide using several targets in cultured cells or in animal models, however, their extrapolation to the patients is yet to achieve. The advances in the techniques leading to improved specificity, targeted delivery and personalized therapeutics will definitely help in the bench-to-bedside conversion of these CRISPR-Cas9based therapies and help in reducing the disease burden.

CONCLUSION

CONCLUSION AND FUTURE PERSPECTIVE

Understanding brain function and its complexities have only been made possible by emerging gnomic engineering tools like TALE N, ZEN and CRISPR-Cas9 in recent years. Opportunities for manipulating the genome have created a possibility to generate models for understanding a complex neurological disorder like AID. Among these gnomic editing tools, CRISPR-Cas9 is being considered the most extensive and effective, with the advantages of low mutation rate, high target efficiency and moreover, the cost of its development is very low. CRISP has enabled the creation of models that reproduce exactly the same causal mutations identified in patients, which has made it possible to determine an appropriate and disease-specific drug therapy.

Owing to the heterogeneous nature of AID, it becomes really difficult to identify the exact cause of AID in patients as it could be genetic or environmental. No standard medication has been developed so far for treating AID, except for aripiprazole and risperidone for irritability and aggressiveness. Thus, creating a reliable model, establishing a causal factor and representing all the characteristics of the disease is difficult. *in vitro* modeling of ASD has been a great benefit to understand the underlying mechanism involved in the pathogenesis of ASD. But it does come with limitations like high heterogeneity among hiPSCslines, so reprogramming strategies are needed to be optimized. CRISPR-Cas9 potentially overcome such limitation by generating isogenic cell lines and thus increasing the reproducibility of experiments. To further investigate the pathogenesis of ASD, the genome of animals is being successfully edited to construct a validated KO, knock-in models using CRISPR. These animal models have been reported to present phenotypes including neuroanatomical, behavioural as well as morphological characteristics, caused by ASD associated genes. In that regard, such models are helpful in determining the aetiology of the condition as well as screening appropriate drugs to restore such altered phenotype. Advancement in genomic editing systems is an encouraging indication that could restore normal sequence in mutations and potentially be effective in human treatment trials. Utilization of CRISPR-Cas9 tool

is not only limited to the modelling of the ASD but also has been helpful in targeting the mutated genes and correcting them. Based on the available genetic information, ASD-associated genes have been widely explored but their therapeutic potential is limited to monogenic form of ASD and remains unexplored in polygenic form of ASD. Also, due to lack of target specificity, genetic therapy using CRISPR-Cas9 is unable to target every ASD- associated gene. Other approaches like CRISPR mediated activation of gene in which nuclease deficient Cas9 (dCas9) is fused with a transcriptional activator and CRISPR mediated base editor system in a gene therapy has been helpful in restoring and normalizing gene dosage in ASD. However, this method is not explored much in case of ASD and given it's off- target effects, optimization of this procedure is necessary before utilization in at least *in vivo.* Despite of advancements in CRISPR-Cas9 tools, there is certain number of limitations like off-targeting, delivery method, and immunogenicity, and associated risks makes it challenging to use in clinical trials. High frequency of off-targets is one of the prime concerns while using CRISPR for gene therapy which lead to further mutations in undesired genomic locations. However, emergence of bioinformatic tools have been helpful in reducing the off-target effects while predicting the off-target modifications. Another major concern is immunogenicity caused by the introduction of Cas9 protein and delivery methods using viral vectors. As Cas9 is derived from *Streptococcus pyogenes,* which has already been responsible for various human infections and most of have them already harbour pre-existing Anti-Cas9 antibodies. Therefore, when it is introduced in human body for therapy purpose, it is recognised as foreign antigen, developing an immune response and causing further degradation of Cas9 and hence preventing it to carry out any gene editing. Another safety concern arose was DSBs induced by CRISPR, which oftenly triggers the apoptosis. In addition to this, induced DSBs have also resulted in unnecessary massive deletions and rearrangements of sequences, suggesting a significant safety concern for clinical uses of DSB-inducing CRISPR therapy. Given the challenges involved in using these gene editing techniques, gene therapy is still a distant therapeutic approach in at least *in vivo.* Considering all limitations and the need for improvising CRISPR

technology, studies using genomic editing tool is limited to cultured cells or animal models, and extrapolation of such experiments in patients has not been yet achieved. Therefore, the application of results from pre-clinical studies to the clinical treatment of ASD will require extreme care.

Figure legends:

Figure 1 Schematic diagram describing the structure and functioning of CRISPR-Cas9 technique in autism spectrum disorder. In this schematic, we have highlighted the mechanism of CRISPR-Cas9 in recognizing a target using PAM sequencing and causing cut at specific point. Following cleavage and forming double stand breaks, repair systems like NHEJ and HDR come into the play for avoiding any unspecific mutations. Diverse application of CRISPR-Cas9 has been explained in this diagram for investigating the mechanism involved in ASD pathophysiology. Various mentioned potential therapeutic targets for ASD could be worked upon using CRISPR-Cas9 technology.

Note: sgRNA-single guide RNA; ASD- Autism Spectrum Disorder; CRISPR- Clustered regularly interspaced short palindromic repeats; Cas9- CRISPR associated protein 9; PAM- Protospacer adjacent motifs; DSB- double strand breaks; NHEJ- Non-homologous end joining; HDR-Homology directed repair.

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