

Genome-editing applications of CRISPR–Cas9 to promote in vitro studies of Alzheimer’s disease

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Abstract: Genetic variations play an important role in the clinical presentation and progression of Alzheimer’s disease (AD), especially early-onset Alzheimer’s disease. Hundreds of mutations have been reported with the majority resulting from alterations in β -amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), or presenilin 2 (*PSEN2*) genes. The roles of these mutations in the pathogenesis of AD have been classically confirmed or refuted through functional studies, where the mutations are cloned, inserted into cell lines, and monitored for changes in various properties including cell survival, amyloid production, or A β 42/40 ratio. However, these verification studies tend to be expensive, time consuming, and inconsistent. Recently, the clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9 (CRISPR–Cas9) system was developed, which improves sequence-specific gene editing in cell lines, organs, and animals. CRISPR–Cas9 is a promising tool for the generation of models of human genetic diseases and could facilitate the establishment of new animal AD models and the observation of dynamic bioprocesses in AD. Here, we recapitulated the history of CRISPR technology, recent progress, and, especially, its potential applications in AD-related genetic, animal modeling, and functional studies.

Keywords: Alzheimer’s disease, CRISPR–Cas9, mutation, A β 42/40 ratio

Genetic and current functional studies on Alzheimer’s disease

Alzheimer’s disease (AD) is defined pathologically by extensive neuronal loss and the accumulation of intracellular neurofibrillary tangles and extracellular amyloid plaques in the brain. It comprises 50%–75% of all dementia cases and affects 23–35 million people worldwide (<http://www.alz.co.uk/>). Age is the most prominent biologic risk factor known,¹ and it is used to classify AD patients into early-onset (EOAD, ≤ 65 years) and late-onset (LOAD, > 65 years) groups. Large-scale genome-wide association studies (GWAS) have identified more than 20 genetic loci, including apolipoprotein E (APOE), associated with increased susceptibility to LOAD through pathways involved in A β production and clearance.² On the other hand, the majority of EOAD cases are caused by dominantly inherited mutations in β -amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*). APP is a transmembrane neuronal protein that is sequentially cleaved by β -secretase and γ -secretase to produce amyloid beta (A β), while *PSEN1* and *PSEN2* are critical components of the γ -secretase complex. To date, more than 300 mutations have been discovered in *APP*, *PSEN1*, and *PSEN2* genes worldwide (<http://www.alzforum.org/mutations>). Most of these mutations impair amyloid metabolism, resulting in an elevated A β 42/40 ratio, elevated A β 42 levels, and/or reduced production of A β 40. Except a few cases,

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these mutations followed autosomal-dominant inheritance pattern, and associated with positive family history of disease. In addition, several patients were described with de novo case of mutations, where no affected family members were observed. However, autosomal-dominant EOAD is quite rare, because it appeared in <5% of all AD cases. Pathogenicity of sporadic LOAD could be complex, and the main cause remained unclear. Several genes, nongenetic factors, and gene–environment interactions may contribute to its pathogenesis. In LOAD, no exact genetic factor was identified, but several genes were discovered, which could increase the risk for disease onset. The strongest-risk allele for LOAD is the apolipoprotein E4, in both homo- and heterozygous stages. However, only APOE itself could not be the only genetic risk for AD, because several AD patients were negative for E4 allele. GWAS and next generation sequencing (NGS) approaches identified several possible genes, which could contribute to disease progression, such as clueterin,³ complement component (3b/4b) receptor 1 (CR1), sortilin-related receptor-1 (SORL1), ATP-binding cassette subfamily A member 7 (ABCA7), CD33, or triggering receptor expressed on myeloid cells 2 (TREM2).⁴ Nongenetic factors, such as oxidative stress, inflammation, and lipid metabolism, could also contribute to diseases. Inflammation was suggested to amplify to be involved in neural damage. Over- and underexpression of pro- and anti-inflammatory molecules, respectively, may result in neuroinflammation and, thus, disease initiation and progression. In addition, levels of several inflammatory factors were reported to be altered in the brain or bodily fluids of patients with AD, reflecting their neuropathologic changes.⁵ Enhanced microglial activation and overexpression of inflammatory cytokines could be significantly associated with oxidative stress and cognitive impairment, especially in the elderly. Aging could also be related with impaired brain- and lipid metabolism, which also could contribute to neurodegeneration.⁶

Probably, pathogenic mutations usually result in elevated A β 42 levels and A β 42/40 ratio. The detection of altered A β metabolism is used as evidence to suggest the pathogenicity and support the role of these mutations in disease progression.³ A knock-in study of a familial *PSEN1* mutation showed increase in A β deposition that resulted from a high A β 42/40 ratio caused by a reduction in A β 40 production.⁷ In addition, studies performed to measure APP metabolism by site-directed mutagenesis in different cell lines found that most pathogenic mutations were associated with elevated levels of A β 42 in the plasma, showing that in vitro studies of these mutations usually correlated with in vivo findings.⁸ Different cell models have been used for *APP*, *PSEN1*, and

PSEN2 gene mutation studies, including human embryonic kidney cells 293 (HEK293), green monkey kidney cells (COS-1), Chinese hamster ovary cells (CHO), and neuroblastoma (N2a) cell lines. The mutations were typically transfected to cell lines after polymerase chain reaction (PCR) generated mutagenesis and analyzed for alterations in amyloid metabolism by enzyme-linked immunosorbent assay (ELISA). Many techniques have been employed to determine whether these mutations result in disease phenotype through mechanisms such as altering γ -secretase activity. Several cell-based studies have been performed to determine the roles of *APP*, *PSEN1*, and *PSEN2* mutations in AD. The most well-modeled mutations of the *APP* gene are KM670/671NL (“Swedish APP”) and V717I (“London APP”); however, the more stable APP695 isoform is usually used in functional studies of *APP* mutations. The “Swedish APP” mutation was first analyzed by Citron et al in HEK293 cells. Normal and mutant APP-expressing cells were labeled with S35-methionine and monitored for amyloid metabolism, A β expression, and total A β levels. They found significantly higher levels of amyloid expression (six- to sevenfold greater) in the mutant cells compared to control cells.⁹ Cai et al transfected the “Swedish APP” mutation into human neuroblastoma cells (M17) and obtained similar results with 6 times more amyloid being found in the media of mutant cultures.¹⁰ Johnston et al screened the changes in amyloid production in skin fibroblast cells from patients with the “Swedish APP” mutation as well as from unaffected family members and revealed a threefold elevation in total amyloid levels in the affected group.¹¹ Because of the significant increase in amyloid production due to Swedish APP, it could also be used for studying presenilin mutations. APP V717I is one of the most well-known mutations associated with EOAD and has been transfected into several different cell lines. Studies on M17 cells revealed an increased A β 42/total A β ratio as well as elevated A β 42 expression.¹² De Jonghe et al transfected the wild-type and mutant APP695 into different cell lines, including CHO-K1, human neuroglioma cells, and HEK293 cells.¹³ These approaches detected a significant increase in A β 42 levels and decrease in A β 40. Murayama et al used COS-1 cells as a model and studied 28 *PSEN1* mutations. They performed co-transfection with “Swedish APP” and measured the A β 42/total A β ratio.¹⁴ Several *PSEN1* and *PSEN2* mutations (*PSEN1* A79V, I143T, A231V, L262F, C263F, L282V, G384A, and d9, and *PSEN2* N141I) were analyzed by Kumar-Singh et al. They generated stable double mutant cell lines (HEK293), which contained the “Swedish APP” mutation and the selected *PSEN* mutation. All these mutations resulted in an elevated A β 42/40 ratio.

However, several mutations did not result in elevated A β 42 levels, suggesting that these mutations may be loss-of-function mutations. Reduced levels of A β 40 suggest that these mutations could be protective and may enhance A β 42 clearance.¹⁵ Functional analyses of such mutations as demonstrated allow to determine the putative pathogenic nature of some of these mutations in the patients.

Such functional study may also be crucial to fill part of the existing gap in AD genetic research. Hence, in vitro models of AD may provide more extensive information about disease-associated pathways, which could be important for genetic, diagnostic, or drug discovery studies. However, these studies are expensive and require special equipment and settings to be conducted. In addition, technical errors may occur during mutagenesis and transfection.^{5,14,16–18} Fortunately, recent developments in genome editing with new technologies now make it possible to establish animal models to investigate neurodegenerative diseases as well as AD. Newer gene editing techniques that are widely employed to enhance the mutagenesis in molecular biology may facilitate in vitro AD studies. Since the first reports of the successful use of clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR–Cas9) in mammalian cells in early 2013, genome editing has captured the attention of the lay public to an unusual degree for a scientific advancement.¹⁹ How much of the CRISPR–Cas9 story is due to the hype that is commonly witnessed with the introduction of any new and promising technology, and what are the realistic prospects of genome editing, a promising tool for the generation of models of human genetic disease-causing gene mutation in human in the near future? In this review, we outline the basic principles underlying the use of CRISPR–Cas9 for genome editing, potential applications for a new avenue for biomedical and AD research through the generation of new in vitro models, and the substantial obstacles that will need to be overcome if CRISPR–Cas9 is to be used in understanding the pathogenic mutations associated with AD.

History and principles of CRISPR–Cas9

CRISPR was discovered in 1987 by Ishino et al in K-12 strains of *Escherichia coli*. While sequencing an isozyme of the alkaline phosphatase gene, they uncovered a unique sequence nearby with a set of repeat sequences and spacer regions.^{20,21} Later, homologous DNA sequences were identified in several bacterial species, including different *Mycobacteria*, *E. coli*, *Streptococcus*, and *Campylobacter* species. The CRISPR structure in bacteria contained viral (bacteriophage) sequences and could acquire additional repeats inside the

sequence. The *Yersinia* species were found to have 29–132 spacers, with most of them being inactive prophages or conjugative plasmids. The homology between the sequences of the phages/plasmids and the spacers led researchers to believe that CRISPR might be involved in “bacterial immunity” against foreign DNA.^{22–24} The CRISPR–Cas9 system is an adaptive bacterial and archaeal defense mechanism that recognizes and disables invading bacteriophages or other foreign nucleic acids (Figure 1).²⁵ This system presumably functions via the high nuclease activity of CRISPR–Cas9 that induces highly efficient, targeted double-stranded breaks (DSBs).^{19,26,27} This can be repaired by nonhomologous end-joining (NHEJ) resulting in nonspecific insertions, deletions, or other mutations (indels).²⁸ DSBs may also be repaired through homology-directed repair (HDR) using a DNA repair template, through which single-stranded oligo DNA nucleotides can be introduced into the target gene, thus allowing knock-ins of specific mutations.¹⁹

In 2007, this theory was confirmed after studies found correlations between the presence of spacers and phage sensitivity and resistance. The presence of several additional spacers in bacterial strains was found to impart more resistance to foreign DNA, and thus adding and removing different phage sequences is thought to alter bacterial defense.²⁹ CRISPR also prevents conjugation and foreign DNA transformation, as well as limits horizontal gene transfer and antibiotic resistance in bacteria. In 2010, it was discovered that the CRISPR–Cas (in *Streptococcus*) system could cleave bacteriophage and plasmid DNA inside the spacer region, at spacer-specific and orientation-dependent sites.³¹ Small guide RNAs or trans-activating crRNA (tracrRNAs), discovered the following year, were suggested to be involved in the maturation of CRISPR RNAs (crRNAs), which are important in RNase-III and CRISPR activity against foreign RNAs. In the same year, it was suggested that the Cas9 gene may play a significant role in crRNA maturation and DNA cleavage.³² In 2012, Gasiunas et al showed that the CRISPR/Cas9 RNA-guided DNA endonuclease could cleave double-stranded DNA, thus playing an important role in foreign DNA interference.³³ The authors suggested that the CRISPR/Cas9 system could be manipulated for use in DNA engineering. Cong et al further revealed that Cas9 nucleases can induce precise cleavage of human DNA and could induce HDR. In addition, they also could simultaneously edit different sites in human or mouse genomes.¹⁹ This system was revealed to be useful for in vitro studies of different mutations involved in human diseases. CRISPR–Cas9 is currently being widely used in a variety of biologic applications for genome engineering, which makes the

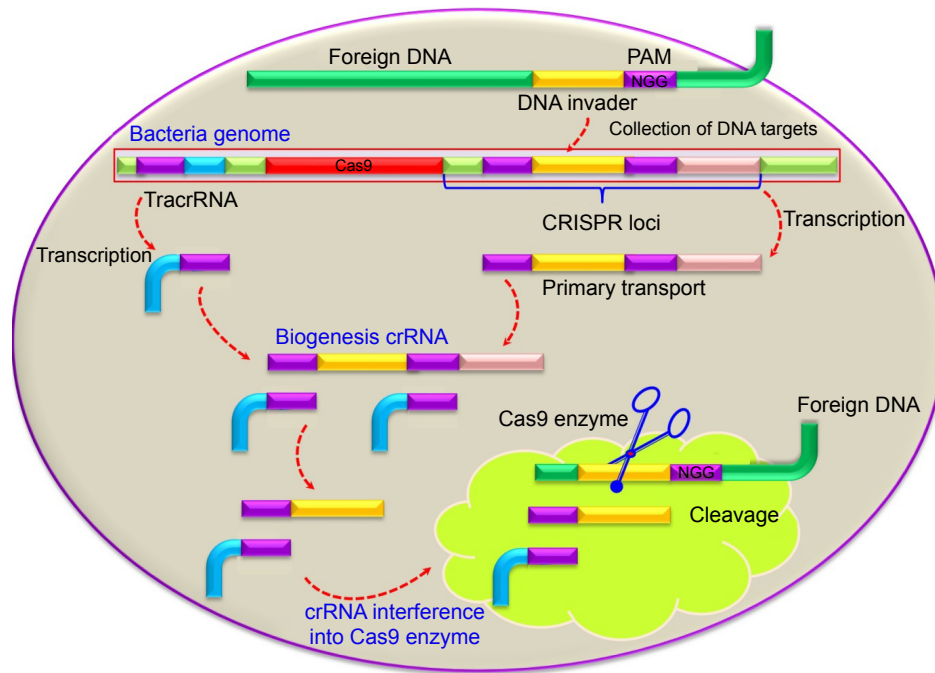


Figure 1 Cas9 in vivo: bacterial adaptive immunity.

Notes: When foreign DNA enters the bacteria, Cas enzymes acquire new spacers from the exogenous sequence and conjugate this spacer unit into the leader end of the CRISPR locus within bacterial genome. The transcription of the CRISPR array is onward processed, and when another corresponding inundation occurs, this mature crRNA acts as a guide for the Cas complex to degrade matching DNA.

Abbreviations: Cas9, CRISPR-associated protein 9; crRNA, CRISPR RNA; CRISPR, clustered regularly interspaced short palindromic repeats; PAM, protospacer adjacent motif; NGG, any base-guanosine-guanosine.

creation of mutant mouse models or mutant cell lines easier and more reliable. Recent advances in genome engineering technologies based on the CRISPR–Cas9 are enabling the systematic interrogation of mammalian genome function. Since these initial studies, Cas9 has been used by many laboratories for genome editing applications in a variety of experimental model systems.³⁴ Genome editing tools play an important role in genetic engineering, and that is the reason why the CRISPR–Cas9 system has been operated and applied in various formats, such as medicine and biology, pharmacology, and biotechnology engineering based on the knock-out, knock-down, and activation screens approaching. The in vivo and in vitro delivery system of CRISPR–Cas9 has been well developed for several important purposes, such as studying the gene functions and designing new therapeutic strategies.³⁵ As illustrated in Figure 2, a large number of publications based on the CRISPR–Cas9 technology have appeared in such a short time (2010–2017).³⁵

CRISPR–Cas9 sequence-specific gene editing strategies

Genome editing, the process of precisely modifying the nucleotide sequence of the genome, has been performed with custom nucleases designed for specific loci, such as zinc finger nucleases or transcription activator-like effector

nucleases. These systems are limited because the molecules are cumbersome, expensive, and time consuming to produce because each target site needs a specific design. Subsequently, several studies adapted the CRISPR–Cas mechanism to create sequence-specific genomic mutations or knock-outs in a variety of eukaryotic cell types and tissues by optimizing the codons in a particular Cas protein, Cas9.^{19,35–37} Off-target mutagenic effects have also been characterized.^{28,38,39} To edit the eukaryotic genome, cells are transfected with a plasmid carrying the Cas9 gene and the engineered sequences, which produce specifically targeted RNA guides. Upon cleavage by Cas9, cellular DNA repair mechanisms are relied upon to complete the editing process. There are two general repair pathways: NHEJ and HDR. HDR allows an exogenous “donor” sequence to be provided to the cell and swapped into the genome, causing specific changes in the sequence. NHEJ is a repair mechanism that is useful when disruption of any sort at the DSB site will give the desired effect. As expected, the cell is now a stable genomic knock-out.

They have been widely studied, both in vitro and in vivo. Several variations in the CRISPR–Cas system have been created, such as the single-guide RNA (sgRNA) design,^{40–42} Cas9 nickases and dCas9-FokI fusion proteins,^{41–43} CRISPR/Cas9 knock-in mice,^{26,44} and mutation of genes in a living animal^{45,46} to determine the most efficient means of introducing

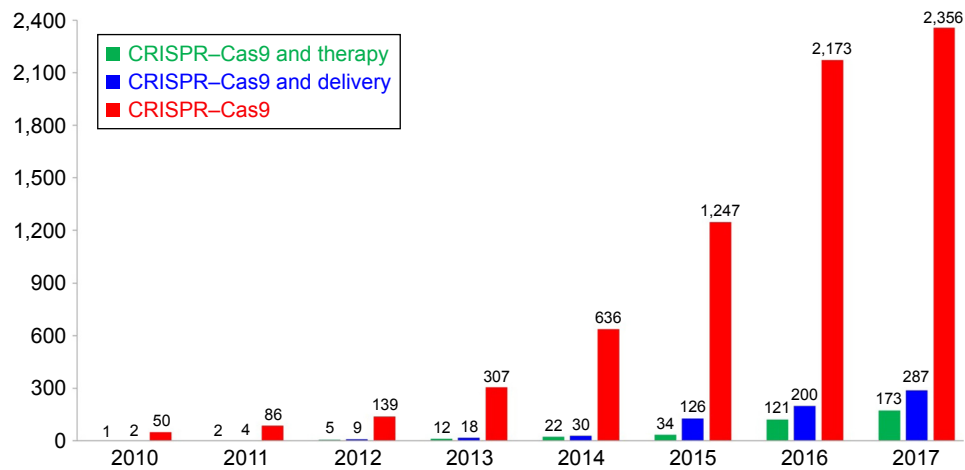


Figure 2 Articles were searched with NCBI Global Cross-database, including PubMed, PMC, Gene, PubChem, UpToDate, among others, as well as Google Scholar by using “CRISPR–Cas9”, “CRISPR–Cas9 and therapy”, and “CRISPR–Cas9 and delivery” in title/abstract for each year.

Abbreviations: Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats.

CRISPR–Cas9 components into cells. Currently, the sgRNA system, in which chimeric molecules of a specifically designed crRNA template are ligated to the scaffold-like tracrRNA and form a complex with Cas9, has been widely adopted.⁴² CRISPR/Cas9 allows the genome to be efficiently edited without residual sequences (loxP sites) or the need to breed transgenic animal strains. It also offers the ability to multiplex mutations in order to observe combined effects, and to work directly with cell lines without designing specific enzymes for each cleavage site. Another advantage lies in the way the cleavage sites are determined.

Many researchers have explored ways of managing the off-target effects created when using CRISPR/Cas9. Disrupting gene expression is a common approach to study gene function and understand loss-of-function neurodegenerative disease-associated mutations. CRISPR can enhance sequence-specific gene editing models by correcting or introducing genetic aberrations linked to a particular disorder. Many of these models have been made for multiple neurologic disorders, including Alzheimer’s, Parkinson’s, schizophrenia, and autism as a mapping way affected by disease-causing mutations (Table 1). Cho et al reported that off-target cleavage was nearly undetectable when the cleavage sites were unique with no homologous sequences elsewhere in the genome.⁵⁶ A more recent study revealed that reducing the target sequence length from 20 nt to 17 or 18 nt decreased the off-target effects without drastic reduction of on-target efficiency.³⁸ When inserting a Cas9/sgRNA plasmid into eukaryotic cells, transcription is often under the control of a U6 promoter, which requires a leading G nucleotide on the transcript. This mechanism was also demonstrated to increase target fidelity via the leading G nucleotide.⁴²

Interestingly, synthetic GG(N)20 sgRNAs produced by T7 enzymes had greater discriminatory abilities compared to plasmid-encoded sgRNAs, though some were then less active at their on-target sites.⁵⁷ However, it seems difficult to thoroughly eliminate the off-target effects of CRISPR genome editing. One strategy could involve the use of a reporter gene construct that is affected by the on-target mutation, or one could alternatively attempt to distinguish wild types and mutants by phenotypes. The use of various sgRNAs to edit the same target area in separate cell populations has also been suggested by Sander and Joung.³⁴

Recently, some studies have attempted to improve HDR efficiency by biochemically altering the HDR or NHEJ pathways. The treatment of various mammalian cells with Scr7, a DNA ligase IV inhibitor, resulted in up to 19-fold increase in efficiency of the HDR pathway.⁵⁸ Likewise, Song et al demonstrated that the HDR enhancer RS-1 increased the knock-in efficiency both in vitro and in vivo by two- to fivefold in rabbit embryos.¹⁸ Most recently, a study in human cells showed that the use of asymmetric ssDNA donors of optimal length increased the rate of HDR by up to 60% for a single nucleotide substitution.⁵⁹ Interestingly, Liang et al achieved up to 56% efficiency in precise genome editing in HEK293 cells and 45% in induced pluripotent stem cell (iPSC) lines after normalizing for the cutting efficiency of gRNAs, though the HDR pathway remains a rate-limiting step for seamless genome editing.⁴³ To explore in detail how specific genetic errors can lead to disease, researchers need to perform experiments in cells that carry these exact mutations. Figure 3 shows the general workflow of CRISPR–Cas9-mediated gene knock-ins in cultured cells including CRISPR design (<http://crispr.mit.edu/>), sgRNA design

Table 1 Neurodegenerative diseases, genes, mutations, and CRISPR/Cas9 editing ability

Genetic diseases	Mutations that can be corrected with CRISPR/Cas9	CRISPR/Cas9 gene targets	Model system	Significance	References
Alzheimer's	MetI46Val	<i>PSEN1</i>	Human neuroblastoma SH-SY5Y cell line	More efficient introduction of specific homozygous and heterozygous mutations	8
Alzheimer's ³⁰	N141I	<i>PSEN2</i>	Basal forebrain cholinergic neurons	Increased A β 42/40 was also normalized following CRISPR/Cas-mediated correction of the <i>PSEN2</i> N141I mutation	
Alzheimer's	Several mutations (T48P, L52P and K53N)	<i>APP</i>	γ -secretase-APP recognition model	Successfully modeled the impact of <i>APP</i> mutations in γ -secretase cleavage and notch processing	47
Alzheimer's	ϵ 4 allele to ϵ 3 allele	<i>APOE</i>	HEK293T cells	Conversion of Arg158 to Cys158 in 58%–75%	48
Parkinson's disease	Loss-of-function mutations	<i>Parkin</i> , <i>DJ-1</i> , and <i>PINK1</i>	Bama miniature pigs	The gene-modified piglets remain healthy and display normal behavior at the age of 10 months	49
Huntington's disease	Insertion of 97 CAG repeats into exon 1	<i>HTT</i>	293F cells line	May be a successful model for polyglutamine diseases	50
Major mental illness	Frameshift mutation in exon 2 (homozygous), frameshift mutation in exon 8 (homozygous and heterozygous)	<i>DISC1</i>	Induced pluripotent stem cells in modeling	<i>DISC-1</i> mutations may disturb the Wnt signaling	51
Epilepsy	Insertion of tdTomato into <i>GAD67</i> to fluorescently label GABAergic neurons	<i>SCN1A</i>	iPSC-based disease model	tdTomato was expressed in GABAergic neurons and this insertion did not interfere with the function of <i>GAD67</i> in GABA synthesis	52
Autism	Knock out heterozygous	<i>CHD8</i>	iPSCs	<i>CHD8</i> may regulate several genes in autism spectrum disorder and could be possible risk factor for these diseases	53
Fragile X syndrome	Deletion of CGG repeats at the 5'-untranslated region of <i>FMR1</i> gene	<i>FMR1</i>	iPSC-derived neurons	Removal of CGG repeats may induce the epigenetic changes and gene expression	54
Recurrent microdeletion and microduplication syndromes	575 kb deletion, 740 kb deletion, 740 kb insertion	16p11.2 and 15p13.3 copy number variants	iPSC-derived neurons	Copy number variations may result in genomic dose imbalance	55

Abbreviations: Cas, CRISPR-associated protein; CRISPR, clustered regularly interspaced short palindromic repeats; iPSC, induced pluripotent stem cell.

(<http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design>), cloning, delivery of the genomic alteration, validation of regeneration, screening of transgenic cells for gene editing events, and in vitro measurement of protein levels in CRISPR/Cas9-edited HEK293T cells using techniques such as ELISA and Southern blot.

Over the past few years, gene sequencing has uncovered numerous genes that are important in brain development and neurologic diseases like AD and Parkinson's disease. Most diseases are known to be associated with dominant mutations in single or multiple genes. However, new cellular functional models need to be developed to address whether these mutations can affect the phenotype. The CRISPR/Cas9 system has the potential to create better cellular replicas of these mutations with greater ease and precision in inserting the disease-causing genes into the genome, thereby developing

models that mimic the conditions of the disease more closely. This creates more opportunities for AD research to explore a wide range of potential mutations that lead to debilitating or fatal neuronal damage. Specific mutations from an EOAD patient that are suspected to be causative of dementia could be introduced into the cell via the CRISPR–Cas9 system and measured for amyloid production and A β 42/40 ratio, because several of these mutations are associated with an elevated A β 42/40 ratio. Cell models and many other animal models using CRISPR/Cas9 can also be developed to study AD at a cheaper, faster, and more precise rate.

CRISPR–Cas9 in AD

An area where the CRISPR–Cas9 system has provided its advantages was that of genetic recessive disorders. Because of its highly successful genome editing abilities, several

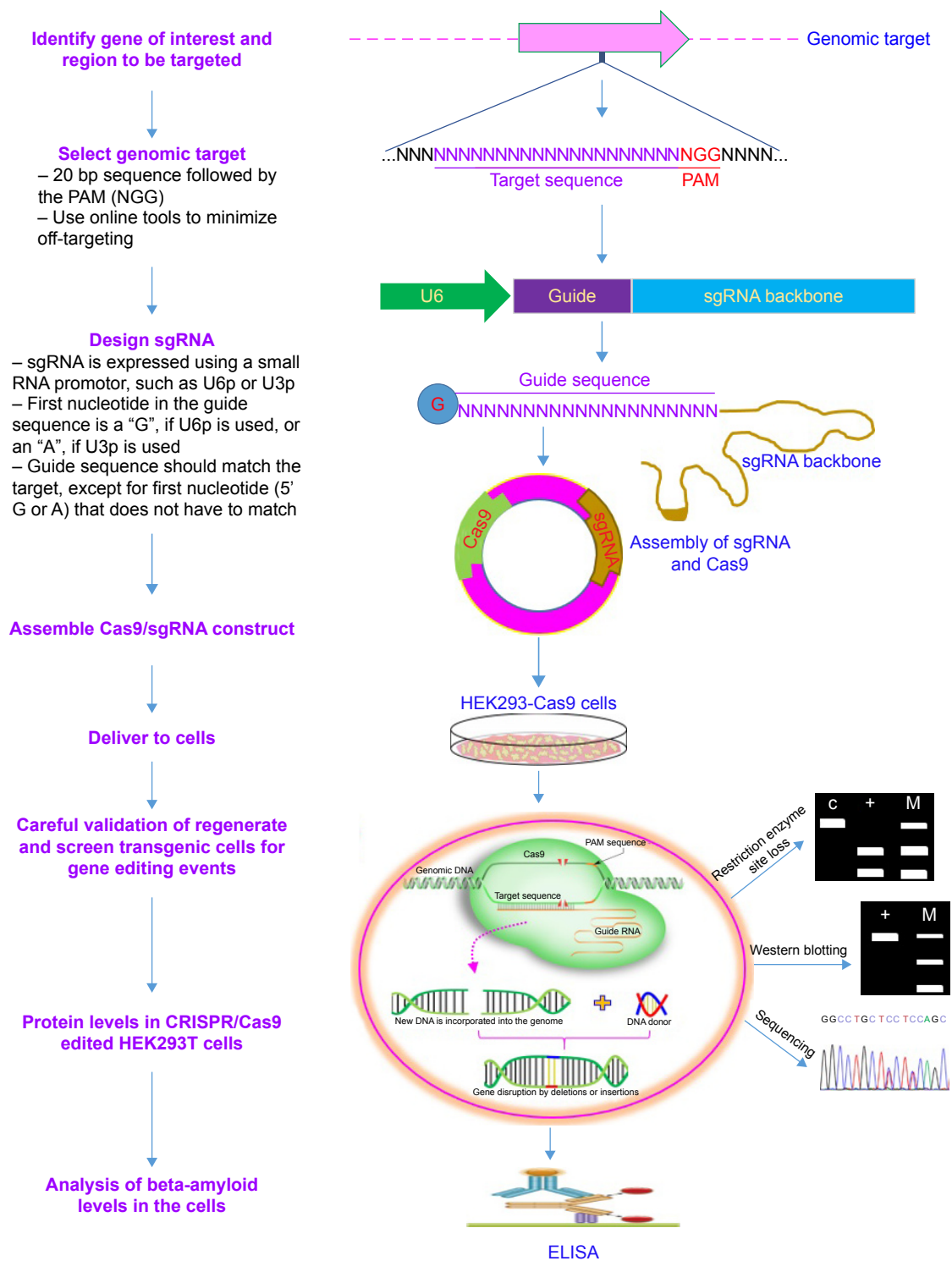


Figure 3 A CRISPR–Cas9 gene engineering workflow is described in three major steps that include vector construction, cell culture, and analysis.

Notes: Note that this workflow includes the minimal procedures to establish a knocked-in cell line (eg, HEK293T), and additional analyses such as ELISA and copy number analysis are often required to confirm the detailed genotype using restriction enzyme site loss, Western blotting, or next generation sequencing.

Abbreviations: Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; ELISA, enzyme-linked immunosorbent assay; sgRNA, single-guide RNA.

studies have used CRISPR/Cas9 to generate genome-edited animals carrying the genetic mutations responsible for a number of human diseases including mouse models of tyrosinemia⁴⁶ and lung cancer⁶⁰ and nonhuman primate⁶¹

models of muscular dystrophy. The CRISPR–Cas9 system was successfully used in the modification of the genomes of different animals and cell types, including mice, zebrafish, pigs, human pluripotent cells, and human somatic cells.

The ability of CRISPR/Cas9 to directly target any gene in the embryo genome opens up a new avenue for us to generate animal models of neurodegenerative diseases. As we mentioned above, CRISPR/Cas9 can cause mutations in one or two alleles, which can mimic heterozygous or homozygous knock-out of a specific gene. This can generate mutations anywhere in the genome resulting in homozygous and heterozygous mutations, which could be useful in exploring neurodegenerative diseases. For example, CRISPR–Cas9 could facilitate the generation of animal models that mimic Parkinson’s disease-associated mutation phenotypes such as loss-of-function mutations observed in PINK1 or Parkin gene mutations. The CRISPR–Cas9 system can also be used for modeling gain-of-function mutations, for example, in Huntington’s disease and PD. CRISPR–Cas9 could also target the nerve cells in adult animals. However, the knock-in rate of genome editing still remains low with this approach, and it may be difficult to model mosaic mutations.⁶² Swine disease models may reflect the phenotypes of human diseases more closely than mouse models. Holm et al attempted to generate disease-expressing mutations (AD, PD, and amyotrophic lateral sclerosis) in transgenic pigs in 2016 using the CRISPR–Cas9 technology. Although this method may also facilitate the development of larger nonhuman vertebrates, further studies on swine models are needed, especially for complex diseases such as AD or PD.⁶³

iPSCs were established as a useful approach for studying different diseases including AD. They were suggested to reflect certain aspects of the AD phenotype more clearly than rodent models and could be used more effectively to study AD genetics, biomarkers, and the therapeutic candidates against the disease.⁶⁴ In 2016, Paquet et al used the CRISPR/Cas9 system to model the *APP* and *PSEN1* mutations, demonstrating a more precise genome editing method using gRNA synthesis.⁶⁵ In addition, the CRISPR-based gene knock-outs in mammalian cells could be created with high efficiency and precise insertion of genetic elements through the cellular HDR pathway.⁴³ The bacterial CRISPR–Cas9 system is capable of sequence-specific gene editing in many organisms and holds promise as a tool to generate models of human diseases, for instance, in human pluripotent stem cells,^{28,35} as well as AD-causing mutations such as *APP* (*APP*^{Swe}) and *PSEN1* (*PSEN1*M146V).⁶⁵

Modeling AD-like phenotypes via CRISPR–Cas9 may also be useful for analyzing disease progression, phenotypes, and therapies, because the new genome editing technique improves reproducibility among researchers.⁶⁶ Komor et al managed to transform APOE E4 to APOE E3 in mouse

astrocytes by generating a C->T exchange at codon 158 (R->C exchange). The success rate of Arg158 conversion to Cys158 was 58%–78%. This study revealed that gene editing by CRISPR–Cas9 could correct several point mutations involved in human diseases.⁴⁸ So far, many of the causal genetic mutations in AD have been discovered through high-throughput sequencing technologies (<http://www.alzforum.org/mutations>). Gene editing with CRISPR/Cas9 can be used to demonstrate whether these identified mutations are indeed directly responsible for the disease phenotypes (Figure 4).

In 2016, the CRISPR–Cas9 system was used for the first time to model AD mutations (co-transfection of *APP* Swedish mutation and *PSEN1* M146V mutation).⁶⁵ The authors used iPSCs, neural precursor cells, and neurons for their analysis and analyzed the A β 42 levels and A β 42/40 ratio in human in *APP*^{Swe} and M146V knock-in cell lines. M146V was examined in both homozygous and heterozygous stages, and the data were compared with controls.⁶⁵ Recently, Sun et al modeled 138 mutations in *PSEN1* from neuroblastoma (N2a) cell lines and used the CRISPR–Cas9 system to generate mutant cells. This study analyzed the gain- or loss-of-function effects of mutations depending on amyloid production and the A β 42/40 ratio. Several of these mutations were associated with an elevated A β 42/40 ratio; however, there was no significant correlation between amyloid levels or ratio and age at the onset.⁶⁷

AD was known as a complex disease, and several mutations affect through alternative mechanisms, which may be

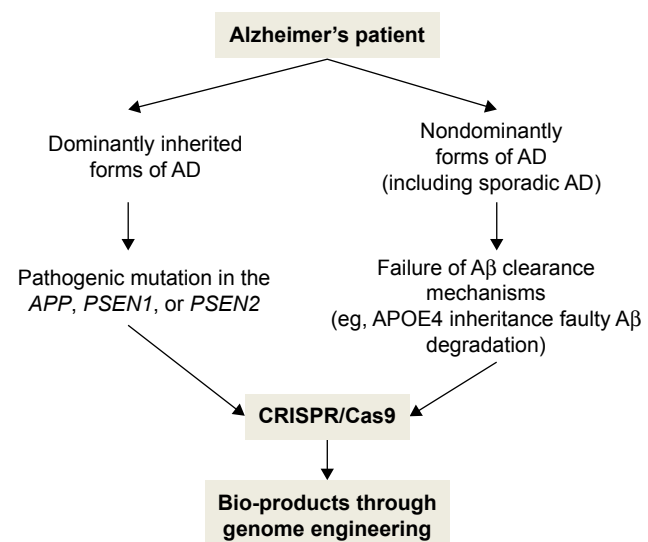


Figure 4 Gene editing with CRISPR/Cas9 can be used to demonstrate that the identified mutation is indeed directly responsible for AD phenotypes.

Abbreviations: AD, Alzheimer’s disease; APOE4, apolipoprotein E4; Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats.

independent from amyloid pathway. Impairment in immune and metabolic pathways was established to contribute in AD progression. Several genes, associated with these mechanisms (ABCA7, SORL1, and CR1), may be genetic risk factors for neurodegeneration. As the mutations in the classic familial AD-associated genes are rare, mutations in genes should be screened to verify their possible involvement in AD. Recently, they were also suggested that they could contribute to EOAD.⁶⁸ Several novel mutations were discovered in these genes, whose possible effect remained unclear. Genome-specific engineering could be helpful for mapping the impairments in inflammatory mechanisms, in terms of mutations, and the expression of immune molecules in terms of mutations.^{69,70} CRISPR–Cas9 could facilitate the screening of the possible impairments in inflammatory and metabolic pathways, associated with mutations, in cell models or mouse models. In addition, CRISPR CAS9 could be involved in epigenetic modifications too. These approaches may be beneficial in treatment by regulating the expression of inflammatory molecules.⁷¹ For example, microRNA 155, edited by CRISPR–Cas9, was verified to downregulate the proinflammatory cytokines.⁷²

New model systems in vitro approach and CRISPR/Cas9 can be used to generate genetic mutations that can faithfully mimic pathology in AD patients. CRISPR/Cas9 is a new genome modification tool that can efficiently and readily target any gene in the genome in germline cells and somatic cells of different species. It should also be pointed out that analysis of the phenotypes of animal models requires further development of functional assays that can be applied to the AD genetic research. All these obstacles remain to be overcome so that this model can be more widely utilized. The newly developed CRISPR/Ca9 technology will support to develop models of AD, a genetic disease, and enhance our understanding of the pathogenesis of these important diseases.

Challenges in CRISPR/Cas9 for generating new model systems in vitro

Our understanding of brain function at the cellular and circuit level has been greatly advanced by functional genomics and the availability of a variety of genetic tools to uncover variants function based on model human brain disorders. For example, many neurologic disorders, such as AD, are associated with genetic risk factors that can be introduced and studied in animal models.⁷³ CRISPR–Cas9 seems to be a promising approach in human genomics as it simplifies genome editing and could facilitate the study of various

genes and the discovery of novel gene functions. However, there are several issues associated with the system for which further studies are required. This approach appears to have a high risk of off-target mutations because of the recognition of similar or homologous sequences. The CRISPR–Cas9 system can cleave identical and homologous DNA sites resulting in mutations at undesired sites, which could result in enhanced toxicity or dysfunction in target cells. Target sequences with high GC content could increase the risk of off-target effects. The use of recently developed Cas9 variants (spCas9), more powerful guide RNAs, and the use of Cas9 proteins could improve the precision of genome editing and reduce off-target effects.⁷⁴ Optimizing the delivery methods for CRISPR–Cas9 is also important for designing more successful genome editing systems. Combinatorial approaches based on iPSC technology and genome editing offer another approach to model human neurologic disorders in vitro. A key advantage of this approach is that genetic modifications can be studied in different human genetic backgrounds because iPSCs retain all of the individual donor's genetic information. Several neurologic disorders including Parkinson's,^{75,76} Alzheimer's,⁷⁷ and Huntington's⁷⁸ disease, which have been generated through iPSC-based disease models, have been proven to closely mimic cellular and molecular features of human diseases.

Plasmids containing guide RNAs and *Streptococcus pyogenes* Cas9 (SpCas9) sequences have been also developed, which can target several cell lines. However, these plasmids could also be inserted randomly into the host genome, which could result in detection of problems. Epigenetics-associated immune responses could also be induced in the case of bacterial sequences, which may disturb the genome editing process. Plasmids may also cause toxic effects in host cells. As methods using plasmids pose several challenges, other methods have been developed including the delivery of Cas9 proteins with guide RNAs through electroporation or microinjection. CRISPR/Cas9 has been used in several cell lines such as zebrafish, mice, or pigs. However, this method could induce stress inside the host's cells. Chemical conjugation of the Cas9 protein and sgRNA may be an alternative approach that could result in less off-target effects. However, it could be limited by an increased risk of chimera formation and insufficient efficiency. Viral vectors for the system are also under development and were suggested to be less toxic and more successful at genome editing. However, viral and eukaryotic genomes are very different and their chromosomal integration may be less effective.⁷⁹ Among the repair pathways for the DSBs generated by CRISPR–Cas9, HDR seems

to be a precise and high-fidelity mechanism for error-fixing, but NHEJ could result in unwanted insertions or deletions. One of the major challenges of genome editing is to induce HDR mechanisms and reduce the degree of NHEJ. However, there is no specific method to measure HDR and NHEJ, but digital PCR-based methods are currently under development for the simultaneous detection of both. Conditions of genome editing also need to be optimized for the cells to favor HDR genome editing.⁸⁰ An additional issue with CRISPR–Cas9 is that it is uncertain how genetic editing could affect future generations. Germline editing may be problematic, because it could result in a loss of “eugenics” and human diversity.⁸¹

Taken together, in addition to generation of new model systems as *in vitro* models, genome editing in combination with single-cell transcriptomics provides an approach to understanding a specific mutation function within animal cells, which could be also allowing for precise dissection of genetic networks in the brain, and understanding the molecular pathologic changes that occur throughout the AD patients. To realize these advances, however, several open challenges have to be addressed. Currently, methods for delivering Cas proteins and RNA guides to the cells need to be optimized and new methods developed to achieve sufficient levels of specificity and efficiency. In addition, insertion and correction of the gene in target cells need to be more controlled. Nevertheless, precise and efficient gene editing using CRISPR–Cas systems has the potential to advance both basic and translational research in the AD as well as neuroscience.

Conclusions and future perspectives

Similar to other genetic disorders, AD has been studied by deriving iPSCs from patients with EOAD mutations,^{77,82–85} but this approach requires several months to culture and is limited by the availability of the patients' cells and variability of genetic backgrounds. Moreover, this disease has mostly been studied in animal models relying on nonphysiologic mutant gene overexpression,⁸⁶ and only TALEN-mediated gene editing has been used to knock-in an EOAD mutation.⁸⁷ The rapid progress in the development of Cas9 into a tool for cell and molecular biology research has been remarkable, likely due to the simplicity, high efficiency, and versatility of the system. Since Paquet et al successfully introduced knock-ins of specific homozygous and heterozygous mutations of EOAD mutations (APP^{Swe} and PSEN1^{M146V}) into iPSC cells using the CRISPR/Cas9 system,⁶⁵ this approach now opens up the possibility of developing models for potentially pathogenic mutations from other genes of interest in AD research including both EOAD- and LOAD-associated genes.⁸⁸

As GWAS and NGS studies introduced several possible risk genes for AD progression, CRISPR–Cas9 could be helpful to screen their AD progression. Several genes could act through different mechanisms than amyloid pathways, like Tau mechanism or inflammation or metabolism. These approaches could facilitate the improvement of therapies against AD. Transcriptomics could be a promising target for gene editing, because RNA editing, or editing the RNA-binding molecules, could be important in regulating the gene expression and in epigenetics.⁸⁹ In addition, CRISPR–Cas9 could also modify the epigenome, by recruiting enzymes, involved in histone modifications, DNA methylation/demethylation, or RNA interference. It could be important to study the structure–function relationships, associated with epigenetic changes. However, further research may be needed on refining the mechanism of acetylation or chromatin accessibility. Epigenetics could play a role in several diseases, including AD, and studying the epigenetic modifications could be helpful in understanding the disease mechanism.^{90,91}

In addition, the generation of new model systems based on CRISPR/Cas9 including iPSC-derived *in vitro* models will provide a route to understanding cell type-specific gene function and its networks in the brain with more precision. Furthermore, along with whole/exome genome sequencing and GWAS, *in vitro* genome editing holds potential for personalized therapeutic applications for patients with AD. Recently, several commercially available products for CRISPR–Cas9 are beginning to provide convenient and effective methods to generate gene knock-outs/ins using programmable nucleases. The newly developed CRISPR–Cas9 technology will not only support the development of genetic models of AD but also enhance our understanding of the pathogenesis of the related disorders including Parkinson's and Huntington's diseases, all of which have become more prevalent as the life expectancy of humans has increased.⁹² Novel genome editing technologies based on the CRISPR–Cas9 system together with powerful readout methods might help us better understand the logic of neuronal circuits and unravel some of the mysteries of AD and dementias in general in the near future.

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Disclosure

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