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**Review Article**

# Multiplex Genome Modifications of Astrocytes Through CRISPR Dead Cas9: A Novel Candidate Therapy for Chronic Ischemic Stroke

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## Abstract

A stroke is an enervating injury to the brain that occurs from a stoppage in blood supply (ischemic stroke) or bleeding (hemorrhagic) in a hemisphere of the brain. Globally, about 10 million deaths per year are recorded because of stroke. There has been no definitive FDA-approved treatment for chronic ischemic stroke without any side effects so far. Therefore, the search for new therapies is necessary. In this paper, after investigating several studies online, on Google Scholar, PubMed, and Scopus, we hypothesized improving the complications of chronic ischemic stroke in induced Sprague-Dawley rat model by intraluminal suture middle cerebral artery occlusion (MCAo), utilizing the combination of cell therapy and gene therapy.

A new version of astrocytes is proposed by making some changes in their genome. To gain this goal, a gene profile including IL-38 (the most modern anti-inflammatory agent, which barricades inflammatory response factors), BRAG-1 (an anti-apoptotic gene from BCL-2 family), IL-38 and BRAG-1's complementary scaffold RNAs for their expression by deadCas9 (dCas9), complementary scaffold RNAs of LZK and MST-1 for their deletion, and deadCas9 gene is used.

Based on studies and documents, we hypothesized using modified astrocytes by dCas9, which is the most accurate genome-editing technology

**Keywords:** Astrocytes; CRISPR; Cell Therapy; Chronic Ischemic Stroke Gene Therapy; Scaffold Rnas

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## Introduction

Stroke is the second most common cause of death after cardiovascular disease (CVD). It produces severe pressure on the health care system in the US. Approximately 795,000 people have a stroke in this country each year. In 2013, stroke was the reason for 1 in every 20 deaths in the US. Besides, it accounts for 889,000 hospitalizations each year (1). Stroke falls into three categories: 88% are ischemic, 9% are intracerebral hemorrhage, and about 3% is a subarachnoid hemorrhage (2). Therapeutic strategies in stroke have been extended based on two fundamental purposes; the amendment of cerebral blood flow and the reduction of the injurious effects of ischemia on neurons (3, 4). A vast amount of research has been done over the past two decades, which has led to remarkable therapeutic advances, including neuroprotective factors, carotid endarterectomy, anti-platelet factors, thrombolytics, anticoagulant treatment, and treating associated risk factors such as hypertension and hyperlipidemia (5). However, there is not any successful treatment without any side effects for improving the chronic and acute ischemic stroke.

The current therapeutic method for acute ischemic stroke is the tissue plasminogen activator (tPA). However, it has stayed undesirable because of its confined useful therapeutic window of 4.5h after the first stroke and its side effects, including blood-brain barrier (BBB) breakdown, inflammatory reactions, and hemorrhagic transformation (3, 4).

Gene therapy and cell therapy are not FDA-approved therapeutic methods for chronic ischemic stroke, but both of them raise some degree of improvement in animal models of stroke (6-8). Several investigations have been done to determine the therapeutic potential of different kinds of stem cells like, neural stem cells (NSCs), mesenchymal stem cells (MSC), embryonic stem cells (ESC), and human-induced pluripotent stem cell (IPSC) in ischemic stroke, which their results were fascinating, despite contradictory results (9). Moreover, among the many potential side effects, the most grisly effect is the generation of tumors (8, 9).

Despite these therapies, stroke has remained as a prominent cause of long-term incapacitation. The development of new therapeutic methods to replace neurons following stroke bypassing the restrictions and risks negotiated above is indispensable in moving cell-based therapies into the clinic. Based on the Stroke Treatment Academic Industry Roundtable, stroke is an intricate illness; so, its therapy must be multiplexed (10).

We hypothesize the application of modified astrocytes through CRISPR dCas9 for alleviating the deficits of chronic stroke could be practical with the least side effect.

### Astrocytes

Astrocytes can be the best option for gaining the goal of this hypothesis because of their multiple functions in the healthy CNS, such as providing structural and metabolic support, regulating neurotransmitter uptake, synaptic transmission, and the composition of neurotransmitters (11, 12). It has recently been identified that astrocytes contribute to adult neurogenesis (13-16), which makes them highly complicated cells. Moreover, astrocytes have a critical role in transient physiological activation, ligand-evoked elevations in intracellular calcium ([Ca2+] I), interactions with synapses, regulation of blood flow, buffering the ion (17-19), and supervising cerebral blood flow (20-22). Besides, they have a crucial role in redeploying the water (12, 23, 24), secreting antioxidant materials (25), immunomodulating the brain environment (26), and maintaining the blood-brain barrier (BBB) (27-30).

Astrocytes activation or astrogliosis has a useful role at early time-points following the stroke. Astrocytes have shown a gradient of responses centered on the lesion site for glial scar formation, which limits ischemic site and prevents immense brain damage. There is persuasive evidence that astrocytes in the glial scar are deleterious for the regeneration of the adult brain (31, 32). It is thought that the glial scar is a physical hurdle and releases chondroitin sulfate proteoglycans (CSPGs), which inhibits axon growth and regeneration. However, recent evidence showed that not only glial scar formation has a critical role in confining the ischemic site, repairing the blood-brain barrier, and limiting the spread of inflammation, but also it may even assist in axon regeneration.

Leucine zipper-bearing kinase (LZK or MAP3K13) is responsible for glial scar formation,

and it has been revealed that the deletion of the LZK gene in astrocytes of adult mice decreases astrogliosis and glial scar formation in spinal cord injury (33). After a stroke and glial scar formation, in a standard and natural environment of the brain, astrocytes degrades glial scars because they have a vital role in the regulation of plasminogen activator by preparing a surface for tissue plasminogen activator, which stimulates pro-BDNF (brain-derived neurotrophic factor) activation, which has a critical role in fibrinogen degradation (34). So, they can make the ischemic area ready for the natural migration of NSCs (neural stem cells), which can amplify motor functions amelioration. Due to all these characteristics, astrocytes are the best option for cell therapy of chronic ischemic stroke.

It should be mentioned that what enables us to modify and augment astrocytes functions for improving the deficits of chronic ischemic stroke through multiplexed therapy is the most accurate, most comfortable, cost-effective gene-editing tool CRISPRi (inhibitor) or deadCas9 with complementary scaffold RNAs for each gene (35).

**Clustered regularly interspaced short palindromic repeats (CRISPR) deadCas9**

CRISPR is the novel, the most accurate, and the most cost-effective genome engineering method, which is composed of two components, including a guide RNA (gRNA), which is specific to the target DNA or RNA sequence, and a non-specific CRISPR associated endonuclease protein or Cas protein. There are several problems with Cas9 protein as it cleaves the specific site of the DNA; so, if some mismatches or off-targets (cleaving the host DNA at a random location, which has not been programmed for that) happened it can cause mutations and lead to tumorigenesis (36). Moreover, although Cas9 protein often cuts the right location, which has been programmed for, the cellular repairing system does not work based on what we want. It should be mentioned that there are two repairing ways after cleavage containing non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ is active in replicating and post-mitotic cells, which may make impressive gene disruption through composing a mixture of insertion or deletion (indel) mutations, which could result in frame-shifts in protein-coding genes. However, HDR is highly restricted to replicating cells and is mediated through host pieces of machinery (37).

However, there are not any of the problems mentioned above with the application of dCas9, as it cannot cut the specific site of the DNA, it can just bind to that particular site and inhibit or activate it. Moreover, if any off-targets happen, it is possible to switch off the whole system by anti-CRISPR proteins (38).

**Animal model**

In this hypothesis, the Sprague-Dawley rat model of chronic ischemic stroke induced by Intraluminal suture MCAo (middle cerebral artery occlusion) is employed as it is small, with a reasonable cost and less ethical problems than other animal models (39).

**A new version of astrocytes**

The global occurrence level of stroke seemed to be steady between1990-2010, whereas there are some gain of 68, 84, 12, and 26%, in orderly in the occurrence of primary stroke, an outbreak of the stroke disability-adjusted lifespan lost, and the mortality rate of stroke (9). As mentioned before, stroke is the second cause of death universally, and there is no FDA-approved therapy and medication without any side effects for chronic ischemic stroke; for acute ischemic stroke, there is only one FDA-approved therapy, tissue plasminogen activator (tPA) with many side effects. So, research for a novel and safe therapy for chronic ischemic stroke is significant.

In this hypothesis, we appraise the effects of transducing the gene profile into the ex vivo astrocytes from the brain of Sprague-Dawley rat models before induction of chronic ischemic stroke in them by Intraluminal suture middle cerebral artery occlusion (MCAo). The gene profile consists IL-38 as an anti-inflammatory agent, BRAG-1 as an anti-apoptotic factor, IL-38 and BRAG-1's complementary scaffold RNAs for their expression by dCas9, complementary scaffold RNAs of LZK and MST-1 for their deletion, and deadCas9 gene. Leucine zipper-bearing kinase (LZK) has a crucial role in glial scar formation, while macrophage-stimulating 1 (MST-1) has a crucial role in the pathophysiological process of various neurological disorders and oxidative stress-induced

neuronal cell decease.

One problem in amending the deficits of chronic ischemic stroke is inflammation. Although astrocytes have an immunomodulatory function, IL-38 is transfected into ex vivo astrocytes to augment their anti-inflammatory action, which is the newest anti-inflammatory agent from the IL-1 family. It acts like IL-1 receptor antagonist (IL-1Ra) and IL-36Ra, which prevents the production of T-cell cytokines like IL-17, IL-22, and IL-8; so, it barricades inflammatory responses (40, 41).

LZK is a conserved mitogen-activated protein kinase kinase kinase (MAPKKK) upstream of c-Jun N-terminal kinase (JNK) in the mitogen-activated protein kinase (MAPK) pathway. It has been reported that the omission of LZK in adult mice astrocytes decreases astrogliosis and prevents scar formation in spinal cord injuries; so, by LZK deletion in astrocytes, their glial scar formation function could be inhibited. Also, based on the brain environment, astrocytes have different functions. Immediately after the stroke, they accumulate around the ischemic site to confine its propagation, repairing the blood-brain barrier, and confining the spread of inflammation and brain damage by producing a glial scar. Even though glial scar acts as a physical barrier and releases chondroitin sulfate proteoglycans (CSPGs), which prevents axon growth and regeneration, the recent pieces of evidence have demonstrated that glial scar helps in axon growth, but prevents the natural migration of NSCs. However, after stroke, astrocytes act differently. They have a significant role in fibrin devastation in CNS by preparing a surface for tissue plasminogen activator, which stimulates pro-brain-derived neurotrophic factor (pro-BDNF) and fibrinogen devastation, which prepares the ischemic area for the natural migration of NSCs. Another problem in the ischemic area is hypoxic death due to lack of circulation below 50%, which results in the primary decrease of migrant NSCs, injected cells, and their derived cells (8). For preventing this hurdle, BRAG-1 (an anti-apoptotic agent from the BCL2 family) is transduced to astrocytes and MST-1, necessary for oxidative stress-induced neuronal cell death, is inhibited.

Moreover, pieces of evidence showed that specific deletion of MST-1 in microglia relieves stroke-induced brain injury. Besides astrocytes have a crucial role in controlling circulation and angiogenesis. This simultaneous expression and inhibition of these genes are just available by the newest, the most precise, cost-effective, and the easiest genome editing tool deadCas9 and complementary scaffold RNAs of each gene (35) **(Figure 1)**. Another advantage of the CRISPR system is possibility of switching the system off by anti-CRISPR proteins, whenever there are scarce side effects (42).

As a result, a new version of astrocytes is produced, which their glia scar formation function is omitted, which makes the area ready for NSCs migration and following motor function improvement; they are resistant to hypoxic death and apoptosis; their anti-inflammatory role is amplified, and they have their angiogenesis function by releasing vascular endothelial growth factor (VEGF) (43). Therefore, it is expected that they will be a novel multiplexed therapy of chronic ischemic stroke through a multiplex CRISPR system in SD rat models.

For performing and evaluating this hypothesis several steps must be done **(Figure 2)**. The first step is the elicitation of the astrocytes from the brain of SD rats. The second one contains the induction of chronic ischemic stroke in the SD rats by intraluminal suture middle cerebral artery occlusion (MCAo) and the composition of the gene profile, including IL-38, BRAG-1, dCas9, and four complementary scaffold RNA sequences for IL-38, BRAG-1, LZK, and MST-1 by using the dCas9-VP64-GFP plasmid (it is about 14,547bp) at the same time. The next step is the plasmid transfection into the ex vivo astrocytes by Electroporation as it is fast, easy, and high efficient, and can be used for numerous cell types (44). After that the transfection must be confirmed by identifying the gene profile in the astrocytes by polymerase chain reaction (PCR) or using the green fluorescent protein (GFP), which exhibits bright green fluorescence when exposed to light in the blue to the ultraviolet range (45). Then the modified astrocytes must be cultured, in which the most common manner is Flow Cytometry sorting, Percoll Gradient, and Mixed Primary glial culture (46). There are two categories of SD rat models, each one contains three ones. The first category is the control group and the second one is the intervention group. At this step cultured ge-

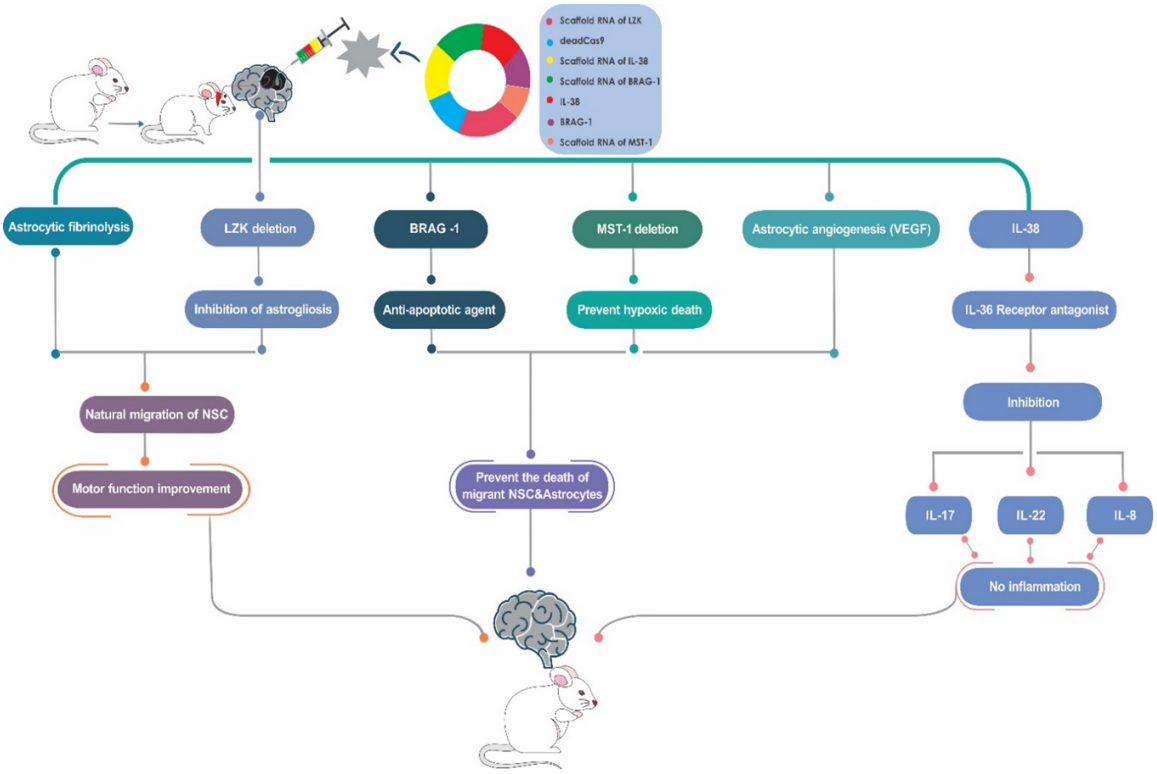


**Figure 1.** Mechanism of the hypothesis. The hypothesized performance of the modified astrocytes has been shown here.

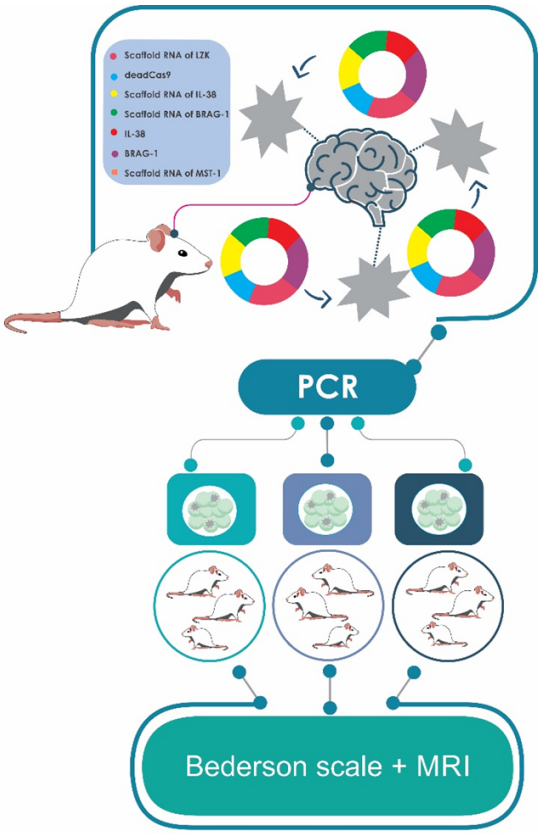


**Figure 2.** Evaluation of the hypothesis. All the steps for evaluating the hypothesis have been summarized in this figure.

netically modified astrocytes should be injected in to the brain of SD rat models by intracranial injection manner. The last step is the investigation of the result of the injection in ameliorating the chronic ischemic stroke deficits in the rat models by magnetic resonance imaging (MRI) assessing, as well as evaluating motor function changes according to the Bederson scale (47).

Many preclinical investigations applying adult stem cell-based strategies report positive results, but there is a considerable need to develop transplantation approaches on the experimental level before clinical application (48). Cell therapy in ischemic stroke in terms of clinical and experimental aspects revealed variable outcomes. However, there are several problems in using stem cells in the clinic like the stem cell type, including the route of cell administration, time interval following the ischemic insult, and harmful interactions between therapeutic cells. Some other adverse effects are immunological and neoplastic complications over seizures, cell clotting, cell-induced embolism, and microembolism after systemic stem cell injection; for example, systemic MSCs injection might lead to embolus formation (49).

One major issue in preclinical cell therapy for stroke is safety. Despite the lack of ethical issues, a critical problem of using IPSCs in stroke treatment is their high teratoma-forming property. They may have a greater risk for tumorigenesis than ESCs due to genetic and epigenetic differences. Moreover, there is a need for a long-term study to evaluate the risk of tumorigenesis due to using IPSC-NSCs (50). Besides, another side effect is extensive growth from the graft at the expense of the local host tissue; however without the generation of germ cell tumors. Although the risk of tumor genesis is reduced, when employing adult stem cells and particularly allogeneic populations and systemic delivery since the majority of them will be ultimately rejected by the host's immune system, long-term culture can lead to chromosomal alteration and clonal growth in neonatal and adult stem cells (51).

Therefore, there is a strong demand for solving these problems and finding an alternative therapeutic approach. It is a fact that there are various complications with each cell-based therapeutic methods for chronic ischemic stroke recovery; however, we hypothesize that using modified astrocytes can be more useful with the least side effects than other therapeutic options of chronic ischemic stroke. It worth evaluating the effectiveness of this hypothesis in improving complications of chronic ischemic stroke, because this hypothesis is based on autograft transplantation of modified astrocytes by the most precise genome editing tools CRISPR dCas9.

## Conclusion

The new version of astrocytes is proposed, which their glia scar formation function is omitted, which makes the area ready for NSCs migration and following motor function improvement. Besides, they are resistant to hypoxic death and apoptosis, their anti-inflammatory function is amplified, and they have their angiogenesis function by releasing VEGF. So, this could be the novel multiplexed therapy of chronic ischemic stroke in SD rat models, which has the potential to be used in the clinic, if further evaluation is performed.

## Conflict of interests

There is no conflict of interests.

## Authors' contributions

ML contributed to the data gathering, writing the initial draft of the manuscript, and designing figures. NR contributed to study design, scientific and structural editing, and verifying the manuscript before submission.

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