様　式　Ｃ－１９、Ｆ－１９、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

平成 ２８ 年　 ６ 月　 ２ 日現在

機関番号：８２４０１
研究種目：若手研究(B)
研究期間：2014～2015
課題番号：２６８６０９５５

研究課題名（和文）The exploration of potential treatment for Angelman syndrome by CRISPR-Cas9 system

研究課題名（英文）The exploration of potential treatment for Angelman syndrome by CRISPR-Cas9 system

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交付決定額（研究期間全体）：（直接経費）　　3,000,000円

研究成果の概要（和文）：本研究では、CRISPR/ Cas9を使用してアンジェルマン症候群を治療する可能性を模索しました。また、 sgRNAのシーケンスの特徴とCRISPR/ Cas9のオンターゲットの活性との相関性を検討しました。本研究により、sgRNAのPAM-distalおよびPAM-proximalでのヌクレオチドが大幅にオンターゲット効率と相関していることがわかりました。さらに、標的DNA、GCの割合、およびsgRNAの二次構造のゲノムコンテキストが効率的に関わる重要な因子であることを実証しました。この研究では高いオンターゲット効率でsgRNAsを設計するための重要なパラメータを明らかにした。

研究成果の概要（英文）：In this study we evaluated the potential of using CRISPR/Cas9 as a novel method to rescue the paternal imprinted UBE3A gene to rescue Angelman syndrome. We also examined how the sequence features of sgRNA correlate with the on-target activity. We found that nucleotides at both PAM-distal and PAM-proximal regions of the sgRNA are significantly correlated with on-target efficiency. Furthermore, we also demonstrated that the genomic context of the targeted DNA, the GC percentage, and the secondary structure of sgRNA are critical factors contributing to cleavage efficiency. In summary, our study reveals important parameters for the design of sgRNAs with high on-target efficiencies, especially in the context of high throughput applications.

研究分野：精神神経科学

キーワード：CRISPR/Cas9　Angelman syndrome

１．研究開始当初の背景

Angelman syndrome (AS) is a severe neurodevelopmental disorder affecting every 1 in 12000 children. AS patients exhibit intellectual disability, seizures, ataxia, speech deficiency and usually with developmental delay. Due to the lack of cure or effective treatment, AS causes huge emotional and financial burden to the patients, their families and the society.

The causal reasons for most AS are due to the deletion or mutation of the maternal allele of *UBE3A* gene. *UBE3A* is located in the 15q11.2 region - one of the chromosomal rearrangement hot spots. Unlike most genes, UBE3A is an imprinting gene, with paternally transmitted copy being silenced and only the maternal allele expressed. Furthermore, the imprinting of *UBE3A* is only restricted to neurons - raising the possibility that the paternal allele of *UBE3A* could be re-activated like other tissues and leads to lessening or rescue of the AS symptom.

It is now clear that neuron-specific silencing of *UBE3A* is achieved by the paternal expression of a large antisense RNA transcript named UBE3A-ATS, which interacts with the sense transcript and lead to the repression of paternal *UBE3A*. In the maternal chromosome, due to the hyper-methylation of the imprinting center, the transcription of UBE3A-ATS cannot be initialized and thus enables the full expression of UBE3A.

Recently, topoisomerase inhibitors have been found to reactivate the paternal UBE3A and became promising therapeutic for AS (HS Huang., et al. *Nature* 2012). Follow-up study demonstrated that topoisomerase inhibitor un-silence the UBE3A by blocking the elongation of UBE3A-ATS (King, I. F., et al. *Nature* 2013). However, topoisomerase inhibitor has a broad repression effect on large genes with length greater than 60 kb - many of which are autism candidate genes. The off-target and strong side effects rule out the possibility of topoisomerase inhibitor as candidate drug for AS treatment.

Based on studies above, it is clear that the key to re-activate the paternal *UBE3A* is to repress the UBE3A-ATS transcription. We have performed preliminary study to knockdown Ube3a-ATS by using antisense oligonucleotide, however the recovery effect is negligible (1%-2%), illustrating the limitation of the oligonucleotide or RNA mediated knockdown strategy.

CRISPR (clustered regularly interspaced palindromic repeats) - Cas9 system is a newly available genome-editing tool, in which the small-guide RNA (sgRNA) binds to the complementary DNA region and subsequently recruits Cas9 to cleave the DNA strand. The ability to target at any genome region mediated by single-guided RNA (sgRNA) makes it a versatile tool beyond genome editing. It has been reported that by inactivating the endonuclease function of Cas9, the CRISPR system could be repurposed to interfere with transcriptional elongation as a block for RNA polymerase (Qi, Lei S, et al. *Cell* 2013). Further, by adding the

chromatin-modifying domain to the inactivated Cas9, the system could be tuned to change the epigenetic state to inhibit the transcriptional initiation (Gilbert, L. A., et al. *Cell* 2013). We think the above system will be a potential tool to reactivate the silenced paternal *UBE3*a by either blocking the elongation or epigenetically modifying the chromatin status of Ube3a locus

２．研究の目的

In our preliminary study, we found that knockdown of Ube3a-ATS has little effect in recovering the paternal expression of Ube3a, which may caused by the inability to fully knockdown the long non-coding RNA. We consider that blocking the transcription elongation of Ube3a-ATS is the most practical way to un-silence the Ube3a. We propose by using a modified CRISPR-Cas9 system, in which the Cas9 is mutated to lose the DNA cleavage function, the CRISPR-Cas9 complex could specifically bind to Ube3a region and block the transcriptional elongation and consequentially abandon the Ube3a-ATS and re-activate the silenced paternal Ube3a.

３．研究の方法

We aimed to create an artificial epigenetic silencing effect using a different modified CRISPR-Cas9, to repress the transcription of Ube3a-ATS. We fused the CAS9 with transcriptional repression domain from EZG2 and HDAC4, which have been recently recognized as key chromatin remodelers for transcription repression. Theoretically, the CAS9-EZG2 chimeric protein could recruit other components to form the chromatin-modifying complex, and mediate the trimethylation of histone-3 on lysine-27 (H3K27me3) - the mark of histone mark associated with gene silencing. By utilizing the same oligonucleotide library, we could quickly assay the reactivation effect by changing the vector type.

４．研究成果

We first generated a library of CRISPR/Cas9 sgRNAs to specifically target at the long non-coding UBE3A-ATS. We used the Surveyor assay to identify the highest active ones, which presumably to have the high affinity with the Cas9 protein. We then sub-clone the guide sequences into the dCas9 vector to target at the SNORD116 repeat region and intron of Ube3a. We evaluated the sgRNA knockdown efficiency for Ube3a using the NEURO2A cell culture. Combination of sgRNAs specific SNODR116 and intron region of Ube3a have positive effects in up-regulating the Ube3A. We then tested on the primary neutron collected from the Angelman syndrome mouse that bearing a missing exon. We measured the expression of paternal Ube3A upon the transfection of candidate sgRNA vectors. However, the rescue of the paternal allele could not be observed. We reason that this may be related with the chromatin status of the imprinted paternal genomic region which is not accessible for the sgRNA. We suggest the sgRNA should be positioned at the open chromatin region in order to reactivate the paternal Ube3A. Following this hint, we utilized our dataset to examine how the sequence features of sgRNA correlate with the cleavage activity. By evaluating the cleavage activities of 218 sgRNAs using *in vitro* Surveyor assays. We found that nucleotides at both

PAM-distal and PAM-proximal regions of the sgRNA are significantly correlated with on-target efficiency. Furthermore, we also demonstrated that the genomic context of the targeted DNA, the GC percentage, and the secondary structure of sgRNA are critical factors contributing to cleavage efficiency. In summary, our study reveals important parameters for the design of sgRNAs with high on-target efficiencies, especially in the context of high throughput applications.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　1　件）

(1) Xiaoxi Liu, Ayaka Homma, Jamasb Sayadi, Shu Yang, Jun Ohashi, Toru Takumi. Sequence features associated with the cleavage efficiency of CRISPR/Cas9 system. Scientific reports, 2016 Jan 27; 6:19675. doi: 10.1038/srep19675. PubMed PMID: 26813419; **査読(有)**

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