The Study of the Relationship between Or8a1 (M71) Demethylation and Trans-generational Odor Fear Reaction

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ABSTRACT

The transmission of changes in phenotype from the parental generation to their offspring after experiencing environmental stress is termed transgenerational memory. Trans-generational epigenetic inheritance (TEI), which involves DNA modification, histone modification, and miRNAs, is the cause of trans-generational memory. This is because they can regulate the expression of genes and relative phenotypes downstream. This article explores the mechanisms under trans-generational memory of an increase in acetophenone sensitivity in mice after the training of odor fear conditioning in the P0 generation. We designed a series of experiments to detect the demethylation status in olfactory sensory neurons (OSNs) and their precursor cells and an artificial edition of the demethylation status by CRISPR d-Cas9 system in either olfactory sensory neuron and sperms. We aim to gain some insights into whether the artificial demethylation of olfactory sensory neurons is sufficient to cause demethylation in sperms (from somatic to germ cells) and whether the artificial demethylation in sperms could lead to demethylation in OSNs in the following generations (from germ cells to somatic cells).

Keywords: Olfactory Fear Conditioning, Trans-generational Memory, Trans-generational Epigenetics Inheritance (TEI), DNA Methylation, Or8a1 (M71), CRISPR d-Cas9 System

1. INTRODUCTION

The environmental factors experienced by the parental generation before reproduction could affect not only the phenotype themselves but also those in their offspring [1]. This phenomenon appears because it positively affects their fitness to the environment. This is termed trans-generational memory, and it could help organisms deal with food shortage, heat stress, change in the amount of light, develop olfactory fear conditioning, bacteria, and pathogen avoidance, and so on [2]. Trans-generational Epigenetics Inheritance (TEI) is the most prominent reason for this transmission of information across generations [3] because the change in phenotype would revert to as it was after a few generations; the mechanism leading to trans-generational inheritance is not a permanent change like genetic alteration [1,3-5].

Instead, epigenetic mechanisms are the cause of TEI. There are currently three major types of epigenetic mechanisms: DNA modification, histone modification, and miRNAs, which can be exported by extracellular vesicles [2]. These modifications could regulate the expression levels of certain genes, leading to the change of physiological and behavioral characteristics downstream. Since parental germ cells could transmit the epigenetic information (change in the expression level of genes) experienced by their somatic cells to the next generation, the Weismann barrier between germ cells and somatic cells is challenged [1].

Gene Or8a1 (M71) codes for the odor receptors of acetophenone, and it is expressed in the olfactory sensory neurons (OSN) specifically for acetophenone. Mice showed trans-generational memory of acetophenone specifically after odor fear to the condition of P0 generation, since the number of OSNs specific for acetophenone increases, and the avoidance behavior and the increase in OSNs could be transmitted to four generations afterward [4]. To explore the mechanisms under the trans-generational memory, the methylation level of M71 in sperms was examined, and there was demethylation of the gene [4]. However, the epigenetic status of M71 in olfactory sensory neurons and its precursor cells were undetected.

The study aims to confirm our expectation of the epigenetics status of M71(demethylation) in olfactory sensory neurons and their precursor cells. Then, we use the CRISPR d-Cas9 system to genetically edit embryos, which leads to demethylation in the sites of olfactory sensory neurons, their precursor cells, and the sperms of our experimental mice. Then, find out whether merely artificial demethylation of M71 in neurons is sufficient to cause odor fear conditioning of acetophenone and demethylation in sperms of experimental mice. We also aim to determine whether artificial demethylation of M71 in sperms leads to demethylation in neurons of following generations.

2. RESEARCH METHODS

Thirty 2-month-old sexually inexperienced and odor-inexperienced male and female mice in a mixed background of 129/Sv × C57Bl/6J will be used [4]. In our experiment, they will live in group cages with 12-hour light-dark cycles and reproduce to provide germ cells used for IVF [4].

The mice will be equally divided into three groups: control group (naive mice), mice naturally trained by odor fear conditioning with acetophenone (FC group), and artificially demethylated mice with CRISPR-dCas9 system (CRISPR group).

1) Confirm the epigenetics status of OSN and its precursor cells (use control group and naturally trained group):

Experiment 1:

It aims to detect the methylation level of M71 in OSN and its precursor cells in the FC and control groups.

Firstly, use olfactory fear conditioning to train the FC group using 2-month-old sexually inexperienced and odor-naive C57Bl/6J male mice [4]. Collect the neuronal stem cells and OSN in both groups and use HMW DNA extraction to gain HMW DNA. Then, repair the HMW DNA and ligate them on adapters. After that, they are loaded on Nanopore for sequencing to determine out's methylation level of M71 [6]. If artificial demethylation of M71 in neurons is sufficient to cause odor fear conditioning and demethylation in sperms (from somatic to germ cells) (control group, FC group, and CRISPR group).

Experiment 2:

The experiment aims to use the dCas9-demethylase system to demethylate M71 in OSN and its precursor cells by manipulating zygotes produced by naive parents through IVF.

The enzyme we will use for demethylation is called TET1, and it is collected in mice. We connect the enzyme to the dCas-9 system. Our dCas9 system has a guide RNA, which is complementary to the targeted sequence of M71, and it has a Cas9 protein linked to our enzyme [7].

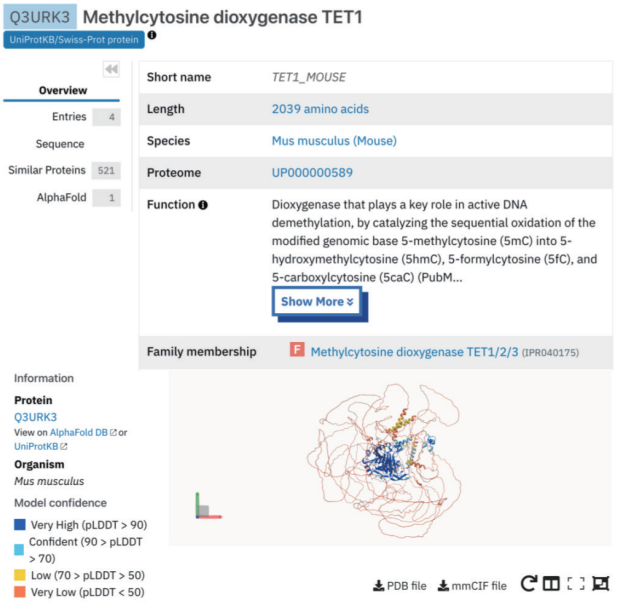

Figure 1. The Information of Methylcytosine Dioxygenase TET1 We Use in d-Cas9 System for Demethylation (Screenshot from InterPro) [8]

Below is the design of the gene we will insert into the zygotes: it contains M71 promoter, sequence coding for dCas9 protein, TETI protein to conduct demethylation of M71, a linker sequence between them, and the guide RNAs. Because it has an M71 promoter, only where M71 is expressed can the system be expressed since genes containing M71 promoter are regulated to be expressed in these cells.

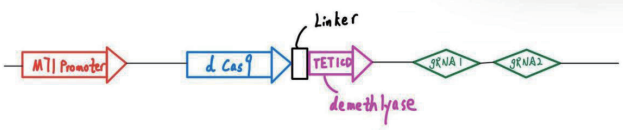

Figure 2. The Gene Sequence We Insert into the Zygotes of Mice

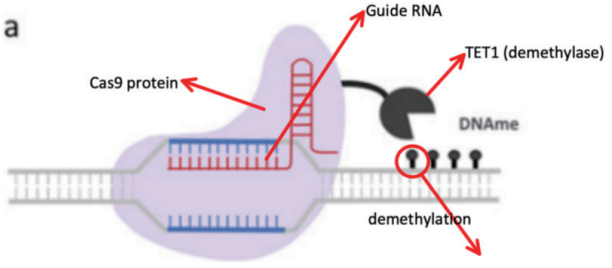

Figure 3. The CRISPR d-Cas9 System We Use for Demethylation

Experiment 3:

It aims to confirm the effectiveness of our dCas9-

demethylase system in the CRISPR group by measuring the methylation level, transcription level, and protein concentration of M71 in olfactory stem cells/ OSN.
Use Nanopore Sequencing for methylation level, RT-qPCR for gene transcription level, and Western Blot for M71 protein.

Experiment 4:

Observe the behavior of the three groups by fear-potentiated startle test and compare their aversive index. It is defined as an increase in the magnitude of the startle reflex in the presence of a stimulus that was previously paired with an aversive event [9].

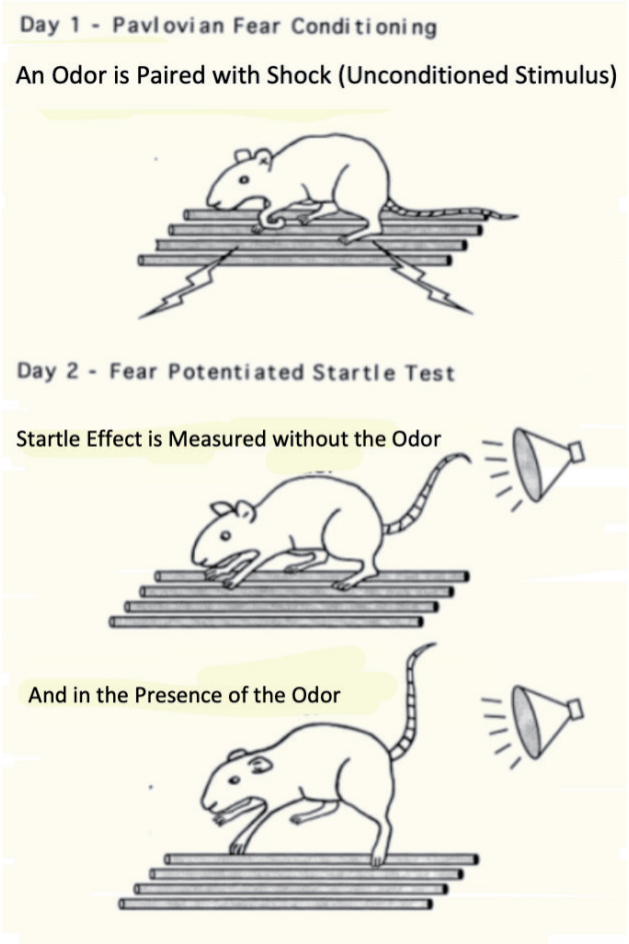

Figure 4. The Process for Training and Testing Odor Fear Conditioning

Experiment 5:

Detect the methylation level of M71 in sperms of the three groups. Collect the sperms and test their methylation levels by Nanopore.

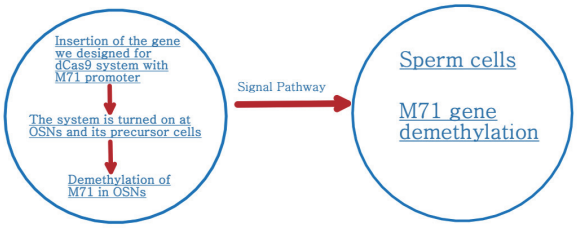

Figure 5. Whether the Artificial Demethylation in Olfactory Stem Cells could Lead to the Demethylation in Sperms (from Somatic Cells to Germ Cells)

1) The next part of the experiment aims to find whether demethylation in sperms is sufficient to cause demethylation in neuronal cells (from germ cells to somatic cells) (control group, FC group, and CRISPR group).

Experiment 6:

Use dCas9-demethylase system to generate M71 demethylated sperms (F0-dCas9-edited). We insert plasmids with the d-Cas9 system. Change the inserted promoter from M71 into sperm specific promoter. Gene AKAP4 is sperm-specific and related to sperm quality and fertility, so its promoter is used [10].

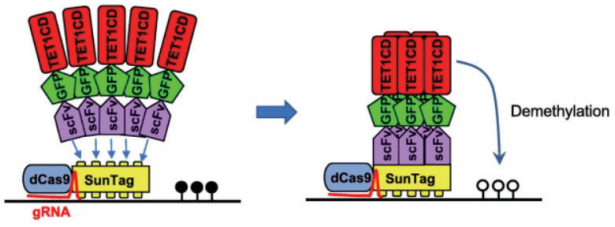

Figure 6. The d-Cas9 System We Use for the demethylation

Experiment 7:
Detect methylation level of M71 in the control group, FC group, and CRISPR group in OSN/sperm. Observe the behavior by fear-potentiated startle test.
Repeat the detection and fear-potentiated startle test in F1-F2.

3. EXPECTED RESULTS

Experiment 1:

Table 1. Possible Epigenetic Status of OSN and its Precursor Cells Collected from FC Group and Control Group and Their Interpretation

Expected Results from Control Group	Possible Results from FC Group	Interpretations
methylation level: + (no change)	methylation level: - (decrease)	Training of odor fear conditioning could decrease the methylation level in neural stem cells and OSN.
	methylation level: + (no change)	Training of odor fear conditioning could not decrease the methylation level in neural stem cells and OSN.

As shown in the table, if the demethylation level decreases in the fear conditioning group, it confirms that the training of odor fear conditioning could decrease the methylation level. If there is no change in the demethylation level, odor fear conditioning training cannot lead to this consequence.

Experiment 3: It aims to test if our dCas9-demethylase system for the M71 gene in neuronal stem cells and olfactory sensory neurons is successful.

Table 2. The Expected Results if our Application of dCas9-demethylase System is Effective

Types of Data Collected	Expected Results	Conclusion if the Expected Results are Met
Methylation level of M71 gene	- (decrease)	The dCas9-demethylase system is effective
Transcription level of M71	↑ (increase)	
Protein M71 level	↑ (increase)	

Table 3. Possible Results from Fear Conditioning Startle Test of CRISPR Group and Corresponding Interpretations

Results from the Fear Conditioning Startle Test	Interpretations
+ (Positive)	Demethylation of M71 gene is enough to cause fear-conditioning-startle behavior.
- (Negative)	Mere demethylation isn't sufficient to trigger the behavior. There might be other pathways involved.

If there is a reduction in the methylation level of the M71 gene measured by Nanopore Sequencing, an increase in the transcription level of M71 measured by RT-qPCR, and an increase in the M71 protein level measured by Western Blot, our dCas90 demethylation is considered effective. We should repeat the process or adjust our methods if our expected results are unmet.

Experiment 4: If our demethylation process is successful, we aim to determine if the artificial demethylation of M71 could lead to positive results from the odor fear conditioning startle test.

Table 4. Possible Results of Methylation Levels of M71 in Sperms Produced by CRISPR Group and Interpretations

Methylation Level of M71 in Sperms	Interpretations
- (decrease)	Demethylation in neurons could lead to demethylation in sperms.
+ (no change)	Mere demethylation in neurons could not lead to demethylation in sperms. There might be other unknown pathways to pass on this information in naturally trained mice.

Experiment 5: We want to determine if mere demethylation in neurons could lead to demethylation in sperms. If not, the demethylation in sperms found by Dias et al. is due to other unknown mechanisms [4].

Experiment 7: The next goal is to find out if artificial demethylation of M71 in sperms could decrease its methylation level in targeting OSN-related cells. And if the demethylation could further cause positive results odor fear conditioning startle test. We will also collect sperms produced by our epigenetically edited mice and test their methylation level.

Table 5. The Expected Results of the Artificial Demethylation of M71 in Embryo is Sufficient to Cause Demethylation of M71 in Neuronal Stem Cells, Olfactory Sensory Neurons, and Sperms, and Positive Results in Odor Fear Conditioning Startle Test

M71 Methylation Level of	Naive (Control)	Fear Conditioning (FC)	dCas9-edited (CRISPR)
Neuronal Stem Cells	+	-	-
Olfactory Sensory Neurons	+	-	-
Sperms	+	-	-
Odor Fear Conditioning Startle Test	-	+	+

For the dCas9-edited (CRISPR) group, the decrease in the methylation level of neuronal stem cells, and olfactory sensory neurons could testify that the artificial demethylation of M71 in sperm cells is sufficient to lead demethylation of M71 in somatic cells in the next generation; the decrease in the methylation level of M71 in sperm cells could proof that the artificial demethylation in sperms could be passed on to sperms for the next generation. The Naive (Control) group and Fear Conditioning (FC) are used for control.

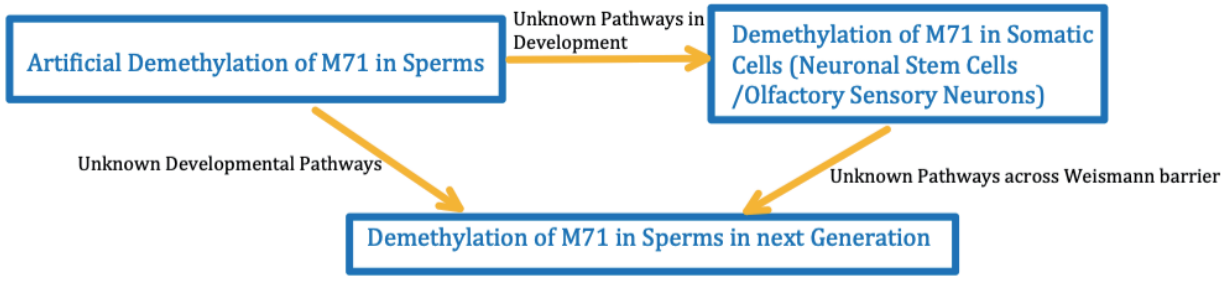

Figure 7. Logical Interpretations for Expected Results of dCas9-edited (CRISPR) Group in Table 5

4. POSSIBLE PROBLEMS AND LIMITATIONS OF OUR PROPOSAL

Using the M71 promoter to express the dCas9 system could result in the expression of this demethylation system in other locations where the M71 gene is expressed, which could lead to unpredictable results. A better solution is to check the expression level of M71 in different tissues in mice. If it is not OSN specific, we could find olfactory stem cell or OSN-specific promoters to avoid these unpredictable results.

Epigenetic effects leading to trans-generational epigenetic inheritance can be caused by various molecular mechanisms, including DNA methylation, histone modification, and snRNA. Altering DNA methylation alone does not necessarily result in complete phenotypic changes and genetic effects. In conclusion, we should also explore other mechanisms involved in TEI of odor fear conditioning.

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Chengyu Liu contributed equally to this work and should be considered co-first authors.

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