Research Proposed: Further study into the Pathway Downstream Sperm miR212/132 in EE-induced Phenotype Inheritance

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Abstract:

It has been shown that some environmental factors can lead to changes in an organism's genetic materials and, therefore, be inherited by the following generations. This inheritance form shows evolutionary and clinical implications, suggesting value for research. In a previous study (Benito, 2018), it has been proved that miR212/132 plays an important role in mediating Environmental Enrichment (EE)-induced phenotype inheritance. Proposing for further research, this article aims at studying the pathway downstream of miR212/132. Two targets of miR212/132, Dnmt3a and Rsf1, are considered possible downstream factors, and some changes in DNA methylation or histone modification are regarded as possible downstream effects that lead to EE phenotype in offspring. Experiments are designed accordingly.

Keywords: Inter-generational memory, Environmental Enrichment-induced phenotype, microRNA, DNA methylation, histone modification.

1. Introduction

Studies show that environmental changes can not only affect the organism's phenotype but also be inherited by the next generation through Epigenetic change, leading to a mild change in the understanding of Darwinian evolution theory (Skinner and Nilsson, 2021). However, the mechanisms that mediate the inheritance of environment-induced phenotype vary greatly, and a huge part of them is still unknown.

Studies in understanding the molecular mechanisms of environment-induced phenotype inheritance will provide insight into understanding genetics and evolution biology, which plays a crucial role in understanding the basic logic underlying Biology. In past studies, the understanding of these two fields was brought to a new stage by research into molecular mechanisms as well, when the identification of DNA and chromosomes has promoted the development of Mendelian genetics and adaptation of classic Darwinian evolution theory with molecular genetics, leading to the concept of the "Modern Evolutionary Synthesis" (Huxley, 1942). From an evolutionary point of view, such direct inheritance of environmentally induced phenotype has its advantages, saving reproduction energy that was to be wasted in the offspring that did not inherit the advantageous phenotype from their parents in the classic Darwinian evolution model and leading to a general improvement of fitness in offspring under the same environment. The evolutionary significance of this type of inheritance makes it a field worth paying attention to.

Furthermore, studying inter- or trans-generational inheritance of environment-induced phenotype has important clinical implications. Since a lot of the cases in this field involve changes in synaptic plasticity in CNS and brain function (Fusco et al., 2019; Salamon et al., 2022), understanding the underlying molecular mechanisms can provide insight into the prevention and treatment of many nervous system related diseases, such as Depression, Alzheimer's Disease, Huntington's Disease, Parkinson's Disease and so on.

Specifically, the proposed research aims to study the downstream pathway of sperm miR212/132 in mediating the inheritance of Environmental Enrichment (EE)-induced hippocampal LTP enhancement (Benito et al., 2018).

2. Previous Research

Various studies have been done relating to the inter- or trans-generational inheritance of environment-induced phenotype, including the inheritance of parental olfactory experiences in mice (Dias and Ressler, 2013), trans-generational learned pathogenic avoidance in C. elegans (Kaletsky et al., 2020; Moore et al., 2019), the trans-generational transmission of stress-induced abnormalities in human (Cecere, 2021; Hime and Pang, 2021; Vialou et al.2013). The inheritance of phenotype induced by an aversive environment is often observed to be trans-generational, which can be inherited for 4 generations or up. On the contrary, the inheritance of the EE-induced phenotype is observed to be inter-generational, which means it can be inherited only by the next generation (Benito et al., 2018).

According to Benito et al. (2018), miR212/132 in sperm plays a crucial role in the inter-generational inheritance of EE-induced hippocampal LTP enhancement. In their study, it is shown that although hippocampal LTP level is enhanced in both EE father and their offspring, the increased presence of miR212/132 in the hippocampus can only be detected in the father mice, suggesting that in the F1 offspring born to EE fathers, there is unknown pathway downstream of miR212/132 in regulating hippocampal LTP enhancement.

3. Research Design and Methods

Due to miR212/132's instability and absence in offspring hippocampus and its long-lasting effect on hippocampal LTP enhancement, some changes in DNA-methylation or histone modification during embryogenesis could be a possible downstream pathway. However, few studies show that miR212/132 can cause Epigenetics to change directly. On the contrary, a great number of studies confirm that miR212/132 regulates mRNA translation by binding to its 3'-UTR, leading to mRNA degradation or translation inhibition (Galagali and Kim, 2020; Lei et al., 2020; Maya-Vetencourt and Pizzorusso, 2013; Remeny et al., 2013; Vo et al.,2005). We, therefore, hypothesize that miR212/132 mediates the inheritance of EE-induced phenotype by inhibiting the expression level of a protein that plays a role in DNA methylation or histone modification.

To test the hypothesis, the whole experiment process is divided into two big parts (shown in Figure 4), with the first part studying whether and at what stage miR212/132 affects certain protein levels in the fertilized mouse oocytes and the second part focusing on the effect of the specific protein on Epigenetic changes in the adult mice hippocampus.

In the first part of the experiment, to confirm that certain proteins are downstream of miR212/132 in the inheritance of EE-induced phenotype, we first conducted a miRDB search to find out possible targeting mRNAs for miR212/132. Among the targets listed, we found two strong candidates, DNA methyltransferase 3A (Dnmt3a) and Remodeling and Spacing Factor 1 (Rsf1), with a target score of 90 and 86 respectively and a high expression in CNS (RPKM for Dnmt3a: 11.1, RPKM for Rsf1: 4.7). Dnmt3a is responsible for the establishment of DNA methylation patterns in mouse embryos (Khazaei et al., 2023; Kibe et al., 2021; Li et al., 2022; Loiko et al., 2022), and Rsf1 is predicted to enable histone binding activity and transcription coregulator activity (Min et al., 2018; Zhang et al., 2017).

Second, we want to determine at what developmental stage the impact of miR212/132 on EE-induced phenotype inheritance is completed and already irreversible. We inject sperm RNA from EE mice into fertilized oocytes. Scrambled RNA is included in the first group, which allows us to inject miR212/132 inhibitor at different developmental stages of the embryo, including E1.0, E4.5 (Blastocyst), E7.5 (when neural plate starts to form), E8.0, E9.0, E10.0 (advanced development of brain tube), E11.0 (development of CNS completed), and E18.0 according to the Theiler Stage Definition of mouse developmental timeline (Hill, 2023), shown as in Figure 1. We measure the hippocampal LTP when the mice are adults (3-4 months old). The stage at which miR212/132 inhibitor does not affect the enhanced hippocampal LTP is recorded.

Third, to test whether the expression level of the two targets is regulated by miR212/132 during mouse embryo development, we inject RNA from the sperm of HC or EE mice into fertilized oocytes. For both groups, RNA is co-injected with scrambled RNA, which allows us to include a third group in which we inject into fertilized oocytes RNA from sperm of EE mice and miR212/132 inhibitor (Figure 2). For all three groups, the gene of Dnmt3a is fused to the GFP gene, allowing us to detect the Dnmt3a protein level throughout the embryo's developmental stages. We record the time when there is a significant difference in fluorescent level between the experiment group (group 2) and the control group; we also record the protein expression level (deduced from the fluorescent signal) of the groups. Whether Dnmt3a's function is affected by the fusion with GFP must also be tested before this experiment. Hippocampal LTP in adult mice (3-4 months old) will be measured and compared if the previous testing shows that the tagging of GFP does not affect Dnmt3a's function. The same set of experiments will be repeated for Rsf1.

In the second part of the experiment, we want to find out whether some epigenetic change is related to EE phenotype. If the level of Dnmt3a is found to be related to the miR212/132 level in the previous experiment, we will first conduct WGBS-seq for the hippocampus and sperm of EE mice, HC mice, and their offspring. The DNA methylation pattern is compared, and there is expected to be a significant difference in certain gene loci between HC mice and EE mice offspring hippocampus. Suppose there is a difference in DNA methylation as expected. In that case, we inject Dnmt3a mRNA inhibitor, exosomes carrying Dnmt3a mRNA, and scrambled RNA respectively into fertilized oocytes (or the mice embryo) according to the time stage and amount recorded in the earlier step (Figure 3). Hippocampal LTP and DNA methylation patterns are measured when the mice are

adults (3-4 months old).

Suppose the level of Rsf1 is related to the miR212/132 level in the previous experiment. In that case, the same set of experiments in this step will be done for Rsf1, except that we measure histone modification by conducting naive chromatin immunoprecipitation (N-ChIP) or super-resolution microscopy instead of measuring DNA methylation.

Our experiments so far are enough to tell whether miR212/132 regulate certain protein (Dnmt3a or Rsf1) levels and whether changes in that certain protein level lead to Epigenetic change and EE-induced phenotype. However, we have not yet proved that the two things, miRNA212/132 and Dnmt3a/Rsf1, function in the same pathway in mediating the inheritance of EE phenotype. If all the results obtained from the steps above support our hypothesis, we should take a step forward to confirm this. We prepare three groups of fertilized oocytes. The first group will be injected with sperm RNA from HC mice, and the second and third groups will be injected with sperm RNA from EE mice. At the developmental stage recorded in the earlier experiment, when the target protein level is down-regulated by miR212/132, we inject into the fertilized oocytes (or developing embryo) in group 3 with exosomes carrying a suitable amount of mRNA of Dnmt3a or Rsf1. The other two groups will be injected with scrambled RNA at the same stage, as shown in Figure 5. We measured the hippocampal LTP level and epigenetic pattern in adult mice in all three groups. Suppose the effect of miR212/132 on LTP enhancement and Epigenetic pattern in group 3 is reversed to the control group level by the up-regulation of Dnmt3a or Rsf1. In that case, we can confirm that miR212/132 and Dnmt3a (or Rsf1) are in the same pathway in regulating EE-induced phenotype inheritance.

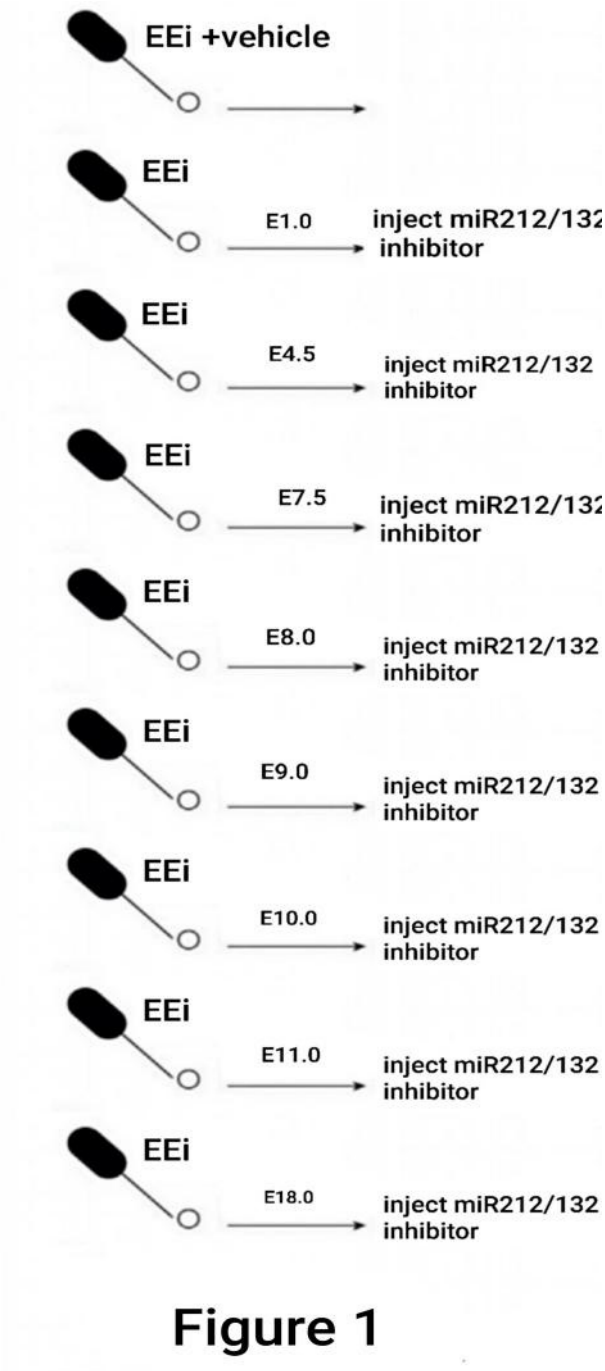

Figure 1: the experiment to study when the effect of miR212/132 is fixed and irreversible.

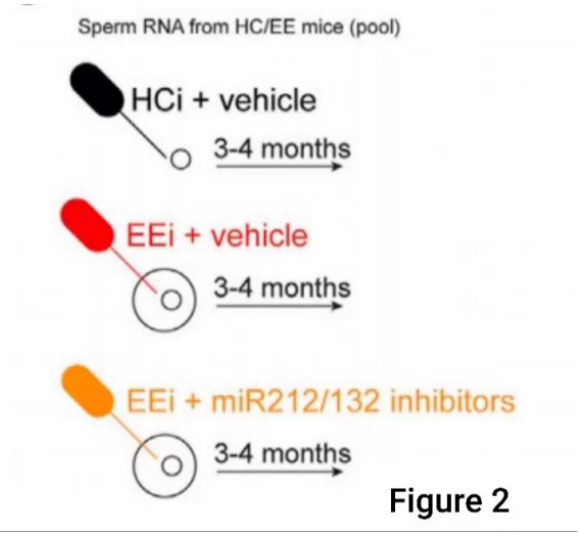

Figure 2: the experiment to study miR212/132's effect on specific protein levels.

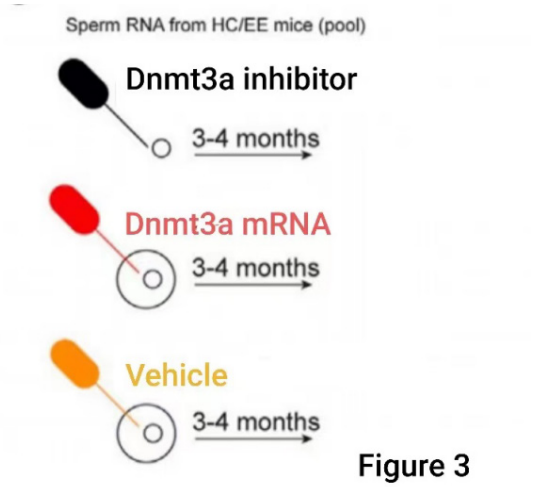

Figure 3: the experiment to study Dnmt3a's role in DNA methylation.

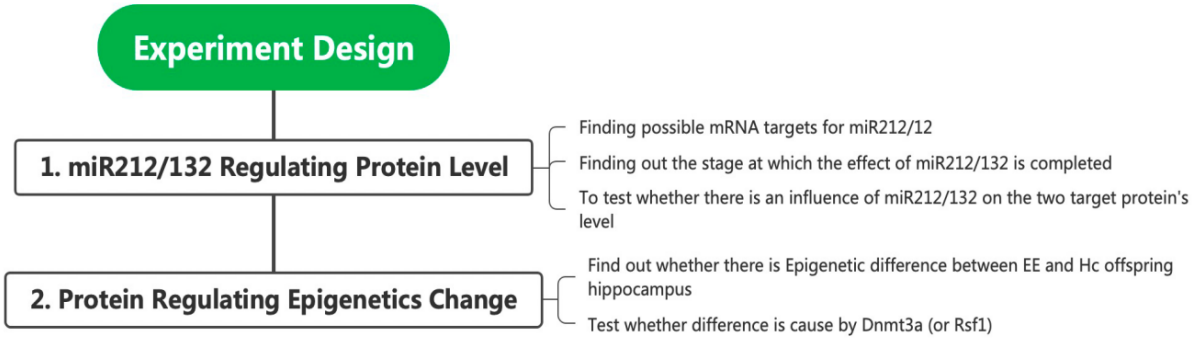

Figure 4

Figure 4: experiment design outline

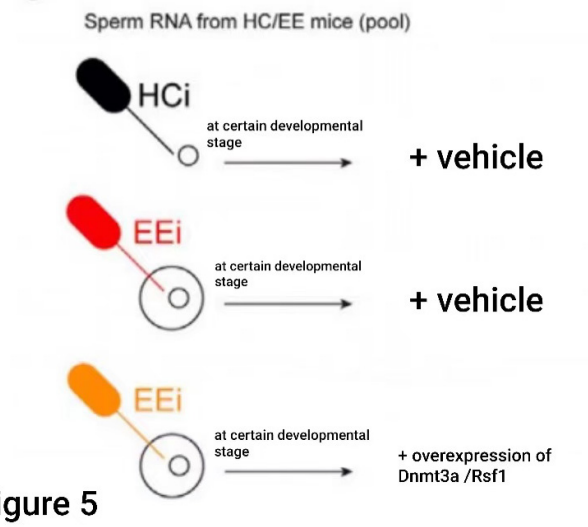

Figure 5: Experiment to confirm that miR212/132 and the protein function are in the same pathway.

4. Expected Results

As mentioned in the theoretical framework, the experiment design for testing the hypothesis can be divided into two parts: the first part focuses on the mRNA target for miR212/132 in mediating EE-induced phenotype inheritance, and the second part focuses on the protein translated from the target mRNA leading to Epigenetic change.

In the first part, we focus on finding the exact target of miR212/132 in mediating the EE-induced phenotype. If the hypothesis that miR212/132 mediates EE-induced phenotype by inhibiting Dnmt3a mRNA translation is correct, in the experiment that studies whether the level of the protein translated from target mRNA is affected by the

presence of miR212/132 (shown in Figure 2), the level of Dnmt3a protein (fluorescent signal) in group 2 is expected to be significantly lower than the other two groups at a certain period during the embryo's development. However, there should be no significant difference in Dnmt3a protein level (fluorescent signal) between groups 1 and 3. If it is confirmed that the tagging of GFP does not affect the Dnmt3a protein's function, hippocampal LTP enhancement should also be observed in group 2 adult mice (3-4 months old). In contrast, the hippocampal LTP level in groups 1 and 3 should be similar to each other and lower than group 2. If miR212/132 inhibits Rsf1 mRNA translation, similar results should be obtained for Rsf1 protein.

One limitation in this experiment design is that since the mRNAs we found are already confirmed as targets for miR212/132, their expression level should be impacted, more or less, by the level of miR212/132, no matter whether they are related to EE or not. However, finding our possible targets from miRDB also indicates that the candidate mRNA we obtained should be directly bound and regulated by miR212/132, which means that the change observed in protein level translated by the mRNA should happen before the effect of miR212/132 on EE-induced phenotype is already fixed. This gives us a means to test whether our finding of target mRNA is correct from another perspective, which is to record the developmental stage during which the effect of miR212/132 on EE-induced phenotype is already fixed and irreversible (shown in Figure 1) and comparing it with the stage at which the protein level shows significant difference between experiment and control groups. If the former is chronologically and significantly earlier than the latter, the experiment should be repeated, or troubleshooting should be done. If the possibility of experimental procedure producing error is excluded, we should conclude that the mRNA is not downstream of miR212/132 in this pathway. If the time stage of protein level changing is within that of miR212/132 inhibitor expiring, this result, along with that obtained in the experiment shown in Figure 5 (if we are lucky enough to obtain another positive result) together, will be a very strong proof for miR212/132 and the protein acting in the same pathway that we are studying.

Another question might arise from this experiment design, so we measure protein level instead of the target mRNA level in the experiment shown in Figure 2. We have two reasons for doing so. Firstly, some previous studies show that some miRNAs inhibit translation by binding to target mRNA but do not promote the degradation of that mRNA (Vo et al., 2005; Galagali and Kim, 2020). In this case, directly measuring the level of the final product, protein, would be a better choice to obtain a correct result. Secondly, the biotechniques used for detecting mRNA levels in cells, such as RNA-seq or microarray, will require us to obtain cells from the mice's body throughout its growth stage, influencing their development. On the contrary, detecting protein levels using fluorescent tagging does less damage for the mice, enabling us to compare their hippocampal LTP between groups and EE offspring with irrelevant variables controlled.

For the second part, we are trying to see whether the correct change in the level of protein translated from target mRNA is enough to induce EE phenotype. In the experiment shown in Figure 3, a significant enhancement in hippocampal LTP level is expected to be observed in the adult mice in group 1 (the group injected with Dnmt3a mRNA inhibitor); the hippocampal LTP level of group 3 adult mice should be similar with that of HC mice. We can not predict the hippocampal LTP level in group 2 mice (with Dnmt3a over-expression). However, it will be interesting to see the results since it will allow a better understanding of Dnmt3a's role in DNA methylation and EE-induced phenotype inheritance. The hippocampal DNA methylation pattern is compared between the three groups and with that of EE mice offspring. A significant difference in certain gene loci between group 2 and group 1 and a resemblance between group 2 and EE mice offspring is expected. These data, along with that obtained in the experiment shown in Figure 2, would be strong evidence that supports the hypothesis that miR212/132 mediates EE-induced phenotype inheritance by inhibiting Dnmt3a gene expression, which leads to further change in DNA methylation.

If no significant difference is observed for hippocampal LTP level and DNA methylation pattern, we need to confirm that some Epigenetic change is induced by miR212/132. We inject sperm RNA of HC and EE mice into fertilized oocytes. We also inject scrambled RNA into the two groups, which allows us to include a third group in which we inject sperm RNA of EE mice and miR212/132 inhibitor. We measure hippocampal LTP and DNA methylation patterns when the mice are adults. If a significant difference is observed between the EE mice sperm RNA injected mice (second group) and the other two groups and the DNA methylation pattern of the second group resembles that of EE mice offspring, we should conduct troubleshooting or move on to study other protein that might be downstream of miR212/132.

The logically same result and result analysis should be expected for Rsf1 if the protein showed a significant difference, as expected in the experiment in Figure 2.

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