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Transgenerational Epigenetic Inheritance Through miR-212/132 Expression Alterations Induced by Environmental Stress

Hanlin Wang

Qingdao No.58 High School of Shandong Province, Qingdao, 266071, China
whl200773@163.com

Abstract:

Transgenerational epigenetic inheritance studies suggest that environmental stress can have lasting effects across generations. It proposes the hypothesis that the expression changes of miRNA-212/132 induced by environmental stress will lead to transgenerational epigenetic inheritance. The validity of this hypothesis is demonstrated through experimental behavior changes, RNA expression differences, nucleotide change experiments, and qRT-PCR analysis, and the influence of environmental stress between generations is confirmed again. This will facilitate the application of relevant practical aspects in the future.

Keywords: Transgenerational Epigenetic Inheritance; miR-212/132 Expression Alterations; Environmental Stress.

1. Introduction

1.1 Background Information

The study of transgenerational epigenetic inheritance shows that environmental stress can produce lasting effects between generations. This phenomenon has been observed in various species, including mice, and has aroused great interest in its potential impact on humans. The study of the offspring of people who have experienced famine is one of the most convincing evidence of human intergenerational inheritance. For example, individuals born to pregnant mothers in the hungry winter of 1944-1945 in the Netherlands showed higher obesity rates and metabolic disorders in later life. These findings suggest that severe environmental stress, such as starvation, can lead to epigenetic changes and pass them on to offspring [1].

The recent studies have focused on understanding the mechanism behind this type of inheritance. An important research area is the role of small RNAs, especially microRNAs (miRNAs) in mediating these effects. MiRNAs are non-coding RNAs that play an important role in regulating gene expression. Studies have shown that specific miRNAs can be differentially expressed under environmental stress, and these changes can be inherited to the next generation. For example, miR-212 and miR-132 are involved in the regulation of synaptic plasticity and cognitive function in mice, and increased expression is observed in environmental enrichment (A2 transgene presentation) [2]. These miRNAs are also associated with changes in feeding behavior, suggesting that there may be a mechanism for the observed intergenerational effects [3].

1.2 Literature Review

The role of small RNAs in transgenerational epigenetic inheritance has been widely studied in various model organisms. In Caenorhabditis elegans, piRNAs (piwi-interacting RNAs) and Argonaute protein PRG-1 mediate cross generational learning pathogenic avoidance, indicating that small RNAs can carry information and influence behavior across generations [4]. Similarly, studies on mice have shown that miRNAs (such as miR-212/132) can be transferred from the brain to the reproductive system, which may affect the behavior and physiology of offspring [5]. These findings suggest that miRNAs may play a central role in mediating the effects of environmental stress on offspring.

A significant previous study has shown that environmental enrichment can lead to increased synaptic plasticity, which is passed down from generation to generation through RNA-dependent mechanisms. This study emphasizes the role of miR-212/132 in this process, indicating that these miRNAs are upregulated in both the hippocampus and sperm of enriched mice [6]. In addition, relevant studies have found that miRNAs-mediated TGF - β signaling pathways are crucial for the transgenerational epigenetic inheritance of learning behavior in C. elegans [7]. Then further research has explored the molecular mechanisms

behind the transgenerational epigenetic inheritance of stress memory [8].Based on a series of research findings, they found that environmental stress can induce specific changes in miRNAs expression, which are then transmitted to offspring through the reproductive system. There are also relevant studies providing evidence of intergenerational transmission of reproductive and metabolic dysfunction in male offspring of polycystic ovary syndrome patients, emphasizing the broader significance of miRNA-mediated inheritance [9].

Therefore, this paper propose the hypothesis that changes in miRNA-212/132 expression induced by environmental stress will lead to transgenerational epigenetic inheritance. MiRNA is transferred from the hippocampus to sperm and then passed on to the next generation. Relevant research provides important theoretical support for the argumentation of hypotheses.

2. Experiments

2.1 Behavioral Assays

2.1.1 Design and Methods

The first design experiment is behavioral tests on different groups of mice (control and starved) to observe changes in food consumption and aggression. The method is mice will be divided into control and starved groups. The starved group will receive half of the normal food (maybe 10 Calories) amount every two days. Food consumption and aggression will be recorded for each mouse. Average food consumption per mouse and the number of aggressive incidents will be documented. The second design experiment is cross-fostering to verify social influence (P0 control + F1 starved group, P0 starved + F1 control group). The method is that cross-foster the control and starved groups and record the food consumption and behavior of their offspring (F1 generation). Then it aims to analyze whether cross-fostering affects behavioral changes. The third design experiment is that verification of sex influence (P0 male starved + P0 female control, P0 male control + P0 female starved). The method is to observe the F1 generation mice from P0 male starved + P0 female control and P0 male control + P0 female starved groups. Then it aims to record their food consumption and behavior to analyze the impact of sex on the results.

2.2 miRNA Differential Expression Measurement

In order to verify whether behavioral changes are due to germline miRNA, RNA will be extracted from the sperm of different groups, and miRNA expression levels will be measured using quantitative real-time PCR (qRT-PCR).

Firstly, total RNA will be extracted from collected sperm samples by homogenizing them to release RNA. TRIzol reagent will be added to each sample, mixed well, and incubated for 5 minutes at room temperature. Chloroform will then be added, shaken vigorously for 15 seconds, and incubated for 2-3 minutes. The mixture will be centrifuged at 12,000 x g for 15 minutes at 4℃, and the aqueous phase will be carefully transferred to a fresh tube. Isopropanol will be added, mixed, and incubated for 10 minutes, followed by centrifugation at 12,000 x g for 10 minutes at 4℃. The supernatant will be removed, and the RNA pellet will be washed with 75% ethanol and centrifuged briefly. The RNA pellet will be air-dried and dissolved in RNase-free water.

Secondly, the reverse transcription reaction mixture is prepared and incubated at 37℃ for 60min, and then heat inactivated at 95℃ for 5min to convert RNA into cDNA. The storage temperature of cDNA is controlled at -20℃ until it be used. In order to better detect the expression level of miRNA, the study could prepare qRT-PCR reaction mixture with cDNA, miRNA-specific primers and qRT-PCR reagents. The qRT-PCR reaction will be set up in the qRT-PCR machine.By running the program, this paper collect the CT values of miR-212, miR-132 and U6.

Thirdly,this paper uses ΔCt method (Ct_miRNA - Ct_U6) to standardize the miRNA expression level to U6. By using the 2^-ΔΔCt method, ΔΔCt (ΔCt_sample - ΔCt_control) will be calculated, and relative expression levels will be determined.

2.3 Sperm Communication Mechanism

In order to explore the transgenerational epigenetic inheritance mechanism of miR-212/132 and verify whether it is directly upregulated in sperm or hippocampus, a single nucleotide in it is modified. Then it could be injected it into F1 and F2 embryos, and sequence the expression of miRNA in sperm by qRT-PCR. As shown in Fig.1.

Firstly,by using CRISPR/cas9 technology, single nucleotide mutations are introduced into miR-212 and miR-132 gene sequences.

Secondly, the modified gene sequence is injected into F1 and F2 mouse embryos.

Thirdly, qRT-PCR is used to detect the expression changes of miRNA in the sperm of F1 and F2 mice to verify whether these changes are consistent with the behavioral changes.

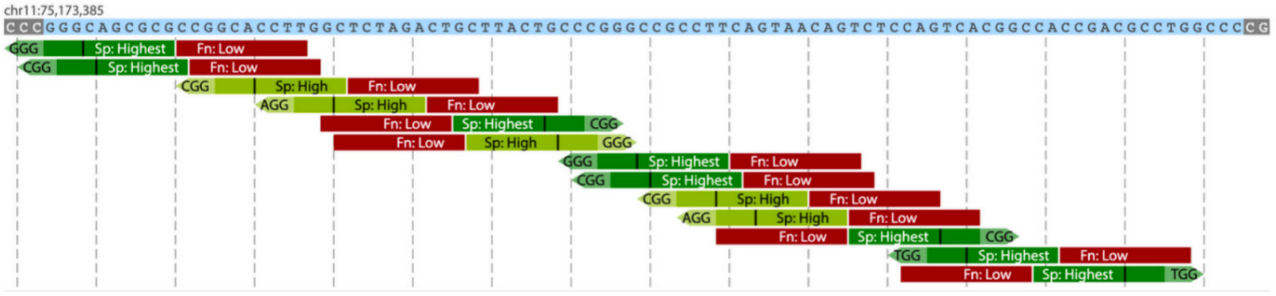

Figure.1 miRNA expression sequencing (Created by Biorender)

2.4 Nucleotide Change and Injection

In order to verify the effects of specific nucleotide changes in miR-212/132 on behavior and miRNA expression, the single nucleotide in miR-212/132 is modified and injected the modified sequence into mouse embryos. Meanwhile, the study will design and synthesize modified miR-212/132 sequences with targeted single nucleotide variations, insert them into Escherichia coli for amplification, and use restriction endonucleases to cleave the amplified DNA fragments. Then they will be injected into mouse embryos. By measuring the miRNA expression levels of F1 and F2 mice using quantitative real-time PCR (qRT-PCR) and conducting behavioral analysis, the study aim to verify whether behavioral changes are caused by germline miRNAs. As shown in Fig.2.

Firstly, the study identifies the specific miR-212/132 sequences to be modified and rigorously designs single nucleotide variations. By continuously synthesizing modified miRNA sequences, the synthesized DNA fragments are combined with plasmid vectors to form recombinant plasmids. Then, the recombinant plasmid is introduced into Escherichia coli through transformation. By cultivating the transformed bacteria, colonies containing recombinant plasmids are screened, and then the recombinant plasmids are amplified to obtain a sufficient number of DNA fragments.

Secondly, research process selects appropriate restriction endonucleases to cleave and amplify the DNA fragments. By preparing mouse embryos and performing microinjection under a microscope, DNA fragments are delivered into the embryos. Then, the injected embryos are implanted into female mice with false pregnancy, waiting for the offspring to be born.

Finally, RNA is extracted from F1 and F2 mice, cDNA is synthesized using a reverse transcription kit, and the expression levels of modified miRNAs are analyzed using qRT PCR specific primers. By designing and conducting behavioral experiments, the behavioral changes of F1 and F2 mice are recorded and analyzed, and the differences between the experimental group and the control group are compared.

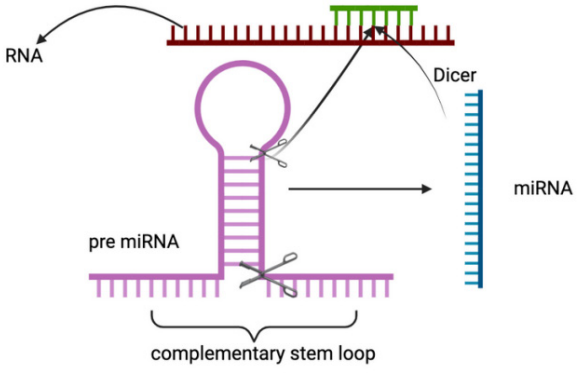

Figure.2 Schematic of miRNA primer design structure(Created by Biorender)

2.5 qRT-PCR Experiment

By using real-time fluorescence quantitative PCR (qRT-PCR) technology to measure the expression levels of specific miRNAs, RNA is extracted from different groups of mice and it will be converted into cDNA, and then qRT PCR is used to detect the expression of miRNAs.

Firstly, the research process uses a reverse transcription kit to convert total RNA into cDNA.

Secondly, qRT PCR analysis is performed using specific primers and miScript primers. Because each miRNA specifically binds to miRScript universal primers.

Thirdly, RNU6B is used as a reference gene to calculate the relative expression level. 2-5ng of cDNA is used for each experiment. During the design process, the GC content range is 30-80% and the Tm of the probe is 67℃. Then the method of 2^-ΔΔCt is used to calculate the relative expression.

2.6 RNA sequencing of miRNA modification

The study combines existing literature analysis to extract RNA and perform high-throughput sequencing to analyze the expression of modified miRNAs in mouse, in order to verify the expression changes of modified miRNAs in different groups of mice and determine their effects on gene expression and behavior [10]. The specific flowchart is

shown in Fig.3.
Firstly, total RNA is extracted from mice.
Secondly, high-throughput technologies such as Illumina are used for RNA sequencing.
Thirdly, analyzing sequencing data is to determine the expression levels of modified miRNAs.
The fourth is to use bioinformatics tools to analyze RNA sequencing data and identify differentially expressed genes. By comparing the miRNA expression differences between the control and the starved group, the study aims to determine the effect of modifications on gene expression.

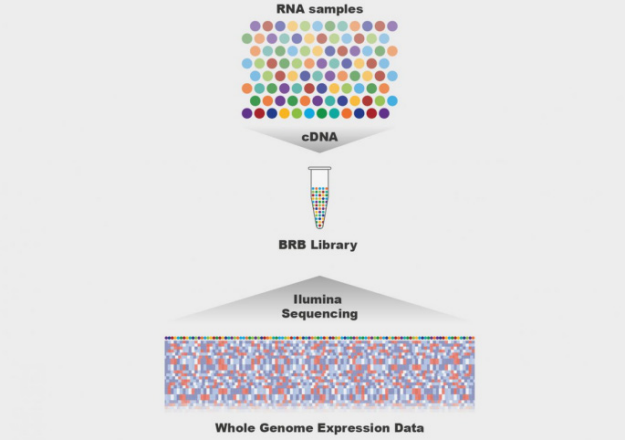

Figure.3 RNA sequencing for modifying miRNAs (Created by Biorender)

3. Expected Results

3.1 Behavioral Differences

3.1.1 Food Consumption and Aggression

By comparing the control and the starved group, it is expected that the food consumption of mice in the starvation group will significantly increase. In addition, due to the pressure of food scarcity, starved group may exhibit stronger aggression.
Through hybrid foster care experiments, it is expected that the offspring of the hybrid foster population (F1 generation) will exhibit behaviors influenced by foster parents. Specifically, the P0 starved+F1 control group may exhibit similar increases in food consumption and aggression as the starved group, while the P0 control+F1 starved group may show reduced signs of these behaviors.
Through gender impact validation, this experiment is expected to reveal that the gender of parents who experience hunger affects the behavioral outcomes of their offspring. For example, the offspring of the P0 male starved+P0 female control group may exhibit different behavioral patterns compared to the offspring of the P0 male control+P0 female starved group.

3.1.2 Behavioral Change Verification

Inreased food consumption: Even under normal feeding conditions, the offspring of the starved group may exhibit a trend of increased food consumption, indicating the existence of transgenerational behavioral adaptation.
Aggression level: A significant difference in aggression level can be observed between the offspring of the starved and the control group, indicating that the stress of starvation can be inherited through behavior.

3.2 miRNA Expression Levels

3.2.1 Expression in Brain and Sperm

Starved Group: It is expected that miR-212 and miR-132 will be significantly upregulated in both the brain and sperm of the starved mice compared to the control group. This would indicate that starvation induces specific miRNA expressions that can be transmitted to the next generation.
Control Group: Relatively stable or baseline levels of miR-212 and miR-132 are expected in the brain and sperm.

3.2.2 qRT-PCR Results

Verification: The qRT-PCR analysis should validate the RNA-seq findings, showing higher expression levels of miR-212 and miR-132 in the starved group compared to the control group.
Cross-Fostering Impact: Offspring from cross-fostering experiments may exhibit miRNA expression patterns reflective of their foster parents' conditions, indicating the social and environmental influence on genetic expression.

3.3 Mechanistic Insights

3.3.1 Hippocampus-Sperm Communication Mechanism

Upregulation Verification: The anticipated result is the identification of a direct mechanism by which miR-212 and miR-132 are upregulated in the hippocampus and subsequently transported to the sperm.
Nucleotide Change and Injection: Altering single nucleotides in miR-212/132 and injecting them into F1 and F2 embryos should demonstrate changes in miRNAs expression, confirming the role of these miRNAs in transgenerational inheritance.

3.3.2 Behavioral and Genetic Correlation

Correlation Analysis: A strong correlation between altered miRNAs expression levels and observed behavioral changes in the offspring is expected. This would support the hypothesis that environmental stress-induced miRNAs

alterations contribute to transgenerational epigenetic inheritance.

4. Discussion

(1) Behavioral Changes

The experimental results show that the offspring (F1-F2 generations) of the starvation group mice consumed more food under normal feeding conditions. This finding supports our hypothesis that environmental stress (such as starvation) can influence the expression of miR-212/132, leading to adaptive behaviors in subsequent generations.

(2) RNA Expression Differences

RNA expression differences are analyzed using RNA-seq and qRT-PCR, revealing significantly upregulated miR-212/132 expression in the brains and sperm of mice in the starvation group. Subsequent RNA experiments further validate the crucial role of miR-212/132 in transgenerational behavioral inheritance, elucidating the mechanism behind miR-212/132's involvement in stress responses and adaptive behaviors.

(3) Nucleotide Change Experiment

By using CRISPR/Cas9 technology, specific single nucleotide changes are introduced into cells during the experimental process, it verifies their effects on miRNAs expression and function. This experiment further supports the mechanism of miRNAs transmission from body fluids (such as blood) to sperm in related studies.

(4) qRT-PCR Analysis

The qRT-PCR analysis process validated the RNA-seq data, and the results shows a significant upregulation of miR-212/132 expression in the mice's starved group. It supports the key role of miR-212/132 in transgenerational epigenetic inheritance.

5. Conclusion and Prospect

Based on the results and discussions presented above, the conclusions is that changes in miR-212/132 expression induced by environmental stress will lead to transgenerational epigenetic inheritance. The research process proves that the hypothesis is valid.

With the continuous deepening of the research on transgenerational epigenetic inheritance mechanism, it may achieve more practical promotion. For example, its development in personalized medication may allow doctors to design personalized prevention and treatment programs according to the patient's family history and environmental background. It can improve the therapeutic effect by reducing the impact of environmental pressure on patients and their offspring. For another example, in the field of public health policy-making, research on it can enable public health decision-makers to better design intervention measures to reduce the long-term impact of adverse environmental factors such as natural disasters, wars or economic crises on human health. This is a long-term and more challenging promotion process.

References

[1] Lumey, L. H., Stein, A. D., Kahn, H. S., van der Pal-de Bruin, K. M., Blauw, G. J., Zybert, P. A., & Susser, E. S. (2007). Cohort Profile: The Dutch Hunger Winter Families Study. International Journal of Epidemiology, 36(6), 1196-1204. https://doi.org/10.1093/ije/dym126

[2] Benito, E., Kerimoglu, C., Ramachandran, B., et al. (2018). RNA-Dependent Intergenerational Inheritance of Enhanced Synaptic Plasticity after Environmental Enrichment. Cell Reports, 23(2), 546–554. https://doi.org/10.1016/j.celrep.2018.03.047

[3] Risal, S., Li, C., Luo, Q., et al. (2019). Transgenerational transmission of reproductive and metabolic dysfunction in the male progeny of polycystic ovary syndrome. Cell Reports Medicine, 1(2), 100024. https://doi.org/10.1016/j.xcrm.2019.100024

[4] Moore, R. S., Kaletsky, R., & Murphy, C. T. (2019). PIWI/PRG-1 Argonaute and TGF-β mediate transgenerational learned pathogenic avoidance. Cell, 177(7), 1827-1841.e12. https://doi.org/10.1016/j.cell.2019.04.015

[5] Guo, L., Zhang, Q., & Ma, X. (2017). miRNA-212/132 Cluster: A Potential Diagnostic Marker and Therapeutic Target for Cancer. Frontiers in Oncology, 7, 255. https://doi.org/10.3389/fonc.2017.00255

[6] Kim, V. N. (2005). Small RNAs: Classification, Biogenesis, and Function. Molecular Cells, 19(1), 1-15. https://doi.org/10.1007/s10059-005-0095-2

[7] Gapp, K., Jawaid, A., Sarkies, P., Bohacek, J., Pelczar, P., Prados, J., Farinelli, L., Miska, E., & Mansuy, I. M. (2014). Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. Nature Neuroscience, 17(5), 667-669. https://doi.org/10.1038/nn.3695

[8] Zhang, Q., & Tian, Y. (2020). Molecular Insights into the Transgenerational Inheritance of Stress Memory. Journal of Genetics and Genomics, 47(2), 89–98. https://doi.org/10.1016/j.jgg.2020.02.001

[9] Painter, R. C., Roseboom, T. J., & Bleker, O. P. (2005). Prenatal Exposure to the Dutch Famine and Disease in Later Life: An Overview. Reproductive Toxicology, 20(3), 345-352. https://doi.org/10.1016/j.reprotox.2005.04.005

[10] Rechavi, O., Houri-Ze'evi, L., Anava, S., et al. (2014). Starvation-induced transgenerational inheritance of small RNAs in C. elegans. Cell, 158(2), 277-287. https://doi.org/10.1016/j.cell.2014.06.020