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Environmental Stress Drives Transgenerational Epigenetic Inheritance through miR-212/132 Expression Alterations

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Abstract:

Transgenerational Epigenetic Inheritance (TEI) is a phenomenon that is deeply affected by the presence of environmental stress and disease. This phenomenon has been proposed to accelerate the evolution of many animal species. However, most current studies commonly focus on the C. elegantly which is frequently presented as deviating a lot from humans and the studies on mice still fail to propose a plausible explanation for the detailed mechanism of TEI in mammals. In this study, we primarily focused on the effect of environmental stress on mice and the possible TEI characteristics it may perform. The miR212/132 was chosen as the main target for studying and the role it play in the TEI is verified. The hippocampus-sperm communication mechanism of miR212/132 will also be focused and verified which revealed why the differential expression happen in the germ-line cell.

Keywords: *Transgenerational Memory, miR212/132, TEI, Environmental Stress*

1. Introduction

Transgenerational memory or transgenerational epigenetic inheritance (TEI) is a phenomenon the offspring of certain generations of animals have a specific response to certain environments that their parents may have faced before and often this certain response is only present for a few generations then lost if the same environment stimulus is not occurring again. Scientists have proposed several mechanisms and hypotheses to explain why TEI occurs in animals and among all these mechanisms proposed, there are three most popular mechanisms: histone modification, DNA methylation, and small non-coding RNA differential expression in the germline, which all are verified by several experiments and focus on the change in epigenetic trait. Moreover, scientists also identified that environmental stress results in the occurrence of transgenerational epigenetic inheritance such as the heat and food supply are the cause for transgenerational change in the offspring, which these stress induces some extent of genetic content change and eventually leads to offspring phenotype change for several generations [1]. Moreover, the maternal effect is also focused in several research which many of them stated that the maternal effect has the effect of magnify the effect of environmental stress, as the central role of womb provide a pathway for environmental stress to have a more direct contact with the offspring. The environmental stress is found to choose the pathway of changing the maternal mitochondria to enlarge the overall effect [2]the temporal and mechanistic origins of these changes, and whether they are reversible, are unknown. We now show that, in obese female mice, cumulus-oocyte complexes exhibit endoplasmic reticulum (ER. However, for most of the current research, the TEI effect is found to occur in only a few generation and several researches even only point out intergenerational effect and cannot promote this to a broader aspect [3]. This problem has also led to another question, why some animals give up their learned trait through TEI eventually but not keep this trait as natural instinct even though this trait is beneficial for their future development and survivorship. This phenomenon is verified by many researches which one of them has identified that the nematode pathogenetic avoidance behavior towards the PA14 would only perform in F1-F4 generation but not extend to the F5 generation[4] the germline and the ASI neuron are all required for avoidance behaviour induced by bacterial small RNAs, and for the transgenerational inheritance of this behaviour. A single P. aeruginosa non-coding RNA, P11, is both necessary and sufficient to convey learned avoidance of PA14, and its C. elegans target, maco-1, is required for avoidance. Our results suggest that this non-coding-RNA-dependent mechanism evolved to survey the microbial environment of the worm, use this information to make appropriate behavioural decisions and pass this information on to its progeny.”,”container-title”:”Nature”,”-

DOI":"10.1038/s41586-020-2699-5","ISSN":"1476-4687","issue":"7829","journalAbbreviation":"Nature","language":"eng","note":"PMID: 32908307\nPMCID: PMC8547118","page":"445-451","source":"PubMed","title":"C. elegans interprets bacterial non-coding RNAs to learn pathogenic avoidance","volume":"586","author":[{"family":"Kaletsky","given":"Rachel"},{"family":"Moore","given":"Rebecca S."},{"family":"Vrla","given":"Geoffrey D."},{"family":"Parsons","given":"Lance R."},{"family":"Gitai","given":"Zemer"},{"family":"Murphy","given":"Coleen T."}],"issued":{"date-parts":[["2020",10]]}}],"schema":"https://github.com/citation-style-language/schema/raw/master/csl-citation.json"} .

Currently, many researchers also pointed out that mammals could perform TEI. For example, research has pointed out that the parent that experienced the Dutch winter famine have demonstrated an increased risk of disease including coronary artery disease, elevated cholesterol, altered clotting, and increased obesity in the offspring for two generation [5]by factors in germ cells that remain largely uncharacterized. As evidence for germline non-genetic inheritance of phenotypes and diseases continues to grow in model organisms, there are fewer reports of this phenomenon in humans, due to a variety of complications in evaluating this mechanism of inheritance in humans. This review summarizes the evidence for germline-based non-genetic inheritance in humans, as well as the significant challenges and important caveats that must be considered when evaluating this process in human populations. Most reports of this process evaluate the association of a lifetime exposure in ancestors with changes in DNA methylation or small RNA expression in germ cells, as well as the association between ancestral experiences and the inheritance of a phenotype in descendants, down to great-grandchildren in some cases. Collectively, these studies provide evidence that phenotypes can be inherited in a DNA-independent manner; the extent to which this process contributes to disease development, as well as the cellular and molecular regulation of this process, remain largely undefined.","container-title":"Clinical Epigenetics","DOI":"10.1186/s13148-020-00929-y","ISSN":"1868-7083","issue":"1","journalAbbreviation":"Clin Epigenetics","language":"eng","note":"PMID: 32917273\nPMCID: PMC7488552","page":"136","source":"PubMed","title":"Evidence for germline non-genetic inheritance of human phenotypes and diseases","volume":"12","author":[{"family":"Senaldi","given":"Liana"},{"family":"Smith-Raska","given":"Matthew"}],"issued":{"date-parts":[["2020",9,11]]}}],"schema":"https://github.com/citation-style-language/schema/raw/master/csl-citation.json"} . Even more, some kinds of rice were also proposed by the research team to have TEI response to the heavy metal environmental stress which the stress leads to locus specific site expression change of rice HPMA gene and other low-copy cellular gene change [6]considerable interest has been placed on deciphering the mechanisms that allow plants to combat heavy metal stress. Strategies to deal with heavy metals are largely focused on detoxification, transport and/or sequestration. The P1B subfamily of the Heavy Metal-transporting P-type ATPases (HMAs. These phenomena make people wonder more about whether humans and other mammals will perform more TEI in different aspects, especially in the face of different kinds of environmental stress.

For the small non coding RNA, it has also been selected as one potential target for TEI effect. Due to its characteristics in regulating the specific gene site transcription, sncRNA is especially suited as a candidate to be changed during the transgenerational inheritance process as only a few paternal RNA sequence could be passed to offspring and the extent of effect on offspring should also be large enough to generate the overall effect which is demonstrated by its ability to affect the developing of cancer [7]. Moreover, due to its regulatory effect, the small non-coding RNA could serve to eliminate some effect of maternal womb pressure on the offspring during pregnancy as it is not involved in the maternal mitochondria and hence could make sure them effect is only from the paternal side but not blend in the maternal womb influence.

Recently several research has proposed the function of small non-coding RNA in which the modifications of their corresponding genes would result in the occurrence of TEI in several animals. In one research, the team has proposed that the piRNA and corresponding regulating gene PRG-1 would result in a change in C. elegans aversive behavior in responding to the lethal PA14 bacteria and this behavior could pass to future generations without the need to learn again [8]enabling progeny to better survive dangerous conditions. We discovered that, after C. elegans have learned to avoid the pathogenic bacteria Pseudomonas aeruginosa (PA14 Another research focus on the mice has demonstrated that the mice's cognitive behavior would increase if their parents had been raised in an enriched environment and the change in expression of miR212/132 in the germline cells causes this change even though the main change was demonstrated in mice hippocampus [9]. The small non coding RNA differential expression in germline cell is being especially focused for research, some researches point out that the small RNA such as tRNA has the effect in regulating the maturation process of mice's sperm and eventually effect the healthiness of the offspring and the endogenous retroelements

active in the preimplantation embryo[10]we investigate the mechanism by which paternal diet affects offspring metabolism. Protein restriction in mice affects small RNA (sRNA. For both small non-coding RNA in the experiments, the researchers found the change in expression of the gene (both upregulated and downregulated) that would transcript into these RNA and this may be the internal mechanism for the TEI in the mice in the face of environmental stress. The miR212/132 has been reported to have an effect in regulating the cardiac rhythm, cardiac hypertrophy, and cardiomyocyte autophagy and demonstrate to play key role in initiating the rhythm system by regulating the gene transcription which are all vital to the animal and hence highly conserved in most mammal species [11][12] However, this miRNA has also been reported to function in the hippocampus to improve the cognitive behavior of future generations and hence may result in other functions in the offspring and may also modify their behavior to face environmental stress like starvation.

However, there are still some deficiencies in the small non-coding RNA experiments and these experiments leave some mechanisms unexplained. For the miR212/132 experiment which is about environmental enrichment, the experiment found the increased expression of miR212/132 in germ-line cells which deviates from the position it has been previously stated to be expressed which is the brain but didn't explain this phenomenon [9]. The mechanism of the communication between the brain and the sperm hence was not proposed and left unclear. Moreover, the research team has also found that the increased cognitive behavior of mice didn't eliminate the removal of the miR212/132 which has also pointed out that other potential miRNA would be responsible for the TEI in mice [9] Also, in current state, most research primarily focused on the TEI in nematode species and different kinds of plant and relatively few are focused on the mammal like mice, therefore, the vacancy here should be filled up. Moreover, as many researches focus on the nematode on different possible mechanism of TEI, many of them couldn't be applied to other kinds of animal and more general scenario for TEI to happen but solely applied to PA14 condition like a research states that the nematode could identify the bacteria's sRNA then perform the aversive behavior and lots of researches on nematode still confine to PA14 area but not focusing on wider possible cause of TEI [4]the germline and the ASI neuron are all required for avoidance behaviour induced by bacterial small RNAs, and for the transgenerational inheritance of this behaviour. A single P. aeruginosa non-coding RNA, P11, is both necessary and sufficient to convey learned avoidance of PA14, and its C. elegans target, maco-1, is required for avoidance. Our results suggest that this non-coding-RNA-dependent mechanism evolved to survey the microbial environment of the worm, use this information to make appropriate behavioural decisions and pass this information on to its progeny.","container-title":"Nature","DOI":"10.1038/s41586-020-2699-5","ISSN":"1476-4687","issue":"7829","journalAbbreviation":"Nature","language":"eng","note":"PMID: 32908307\nPMCID: PMC8547118","page":"445-451","source":"PubMed","title":"C. elegans interprets bacterial non-coding RNAs to learn pathogenic avoidance","volume":"586","author":[{"family":"Kaletsky","given":"Rachel"},{"family":"Moore","given":"Rebecca S."},{"family":"Vrla","given":"Geoffrey D."},{"family":"Parsons","given":"Lance R."},{"family":"Gitai","given":"Zemer"},{"family":"Murphy","given":"Coleen T."}],"issued":{"date-parts":[["2020",10]]}}],"schema":"https://github.com/citation-style-language/schema/raw/master/csl-citation.json"} .

In this work, the main focus is the vacancies about the miR212/132 and other miRNA leads TEI effect on mammal like mice and the corresponding communication mechanism between the hippocampus and germline cell. Based on the result of the former experiments that completed by other research teams, in this study, we present the hypothesis that the differential expression of miR212/132 would result the behavioral change in the offspring if the parent faces starvation as environmental stress which the behavioral change also success through two steps which is the parental learning step and offspring inherited step. To prove this hypothesis, behavioral assay and related gene deletion experiment will conduct to reveal the detail mechanism that miR212/132 and other potential miRNA regulate the TEI in mice. Also, we proposed the hypothesis that the miR212/132 differential expression would be presented inside the hippocampus and would transmit to the germline cell but not directly produced in sperm. This hypothesis also prove in the study by observing the whether the changing sequence miR212/132 is presented in mice germ-line cell after inserting into the hippocampus.

2. Experiment Methods & Materials

2.1 Subject Training and Pair Model

In this study, the normal laboratory C57BL/6 mice are used. The P0 generation of mice are brought and selected based on the weight of individual mice. The weight of male mice should be in the range of 30g (±5g) and the weight of female mice are in the range of 25g (±5g). This selection aims to minimize the effect of weight on the amount of food the mice ingest and the weight of food required to fill their stomachs. The selected mice are

separated into two groups which are the control group with a normal feeding scheme and the starvation group with an inefficient food supply. The number of male and female mice in each group should be equal. For the P0 control group of mice, 6g of food is provided per day, and sufficient nutrition is ensured. But the P0 starvation group of mice experience starvation which is 1g of food provision for 2 days and 1 day interval with normal 6g of food provision. After generating the F1 and F2 generation, all the F1 & F2 mice should be treated equally for 6g of food per day and to feed the mice with 6g of food before the experiment to ensure they have full stomachs before the experiment. This equal treatment was to ensure that the starvation as environmental stress would only affect one generation but not spread to the second generation to put potential change in mice behavior. For the starvation group mice female, when the female gets pregnant, stop conducting the starvation feeding scheme and change to the normal feeding scheme to ensure that the environmental stress is not transferring through the female uterus.

For the process of generating the F1 generation, there are three ways for three research groups. The first group of F1 generating is the normal comparison treatment group, in which the P0 mice from the control and starvation group mate together within the group to generate two kinds of F1 mice which are control group F1 mice and starvation group F1 mice. The second group of comparison is aimed to investigate the effect of different gender on the transgenerational. Epigenetic inheritance. This comparison generates three groups of F1 mice, which one group being the control group P0 male mice mated with the starvation group P0 female mice, and another group corresponding be the starvation group P0 male mice mated with the control group P0 female mice. These two mating groups generate two kinds of F1 mice and label them with male starvation and female starvation. For the last group of F1 mice, they are mating within the P0 control group to generate a control experiment. The last sector of large comparison is aim to investigate whether there is a social effect on the TEI observed in the mice. In this comparison experiment, P0 generation are first mate within each group to generate two groups of F1 mice. After that, the starvation group F1 mice are fostered by the control group P0 mice, and the control group F1 mice are fostered by the starvation group P0 mice. Also, a control group of P0 control group mice are fostering the F1 control group mice to ensure enough comparison.

For both 3 large comparison experiment groups, the behavior assay is done on the F1 generation. However, the first comparison group treatment will only be used to generate the F2 generation mice. But only the first simple comparison starvation group and the second paternal effect investigating group is carried out to do the differential expression gene analysis. The simple starvation comparison group is selected to conduct the further experiment. We ensure that all operations on the mice are approved by the corresponding ethical committee.

2.2 Mice Behavioral Assay

To verify our hypothesis, we mainly designed two experiments on mice behavior which aim to reveal the effect of starvation on the P0 generation and whether this effect is passing down to the F1 & F2 generation. The first behavioral assay (n=3) would mainly focus on the amount of extra food ingestion by different groups of mice. According to the experiment that demonstrates the parental chronic high fat diet affect the function of ß-cell of the pancreas of the next generation, we want to investigate whether the starvation in parental generation causes the offspring to be more likely to obese and this may result due to the more food ingestion compare to the normal mice's offspring [13] the extent of any contribution of obese fathers is unclear, particularly the role of non-genetic factors in the causal pathway. Here we show that paternal high-fat-diet (HFD. The extra food ingestion behavioral assay is exerted on P0, F1 & F2 mice that both ensure to fed full before the experiment. Then 20g of food is presented in front of two different groups of mice and the weight of extra food that intake by both groups of mice.

The second behavioral assay (n=3) is related to the aggressive behavior demonstrated by mice when facing competition on the food source from the presence of other different mice. This behavioral assay wants to investigate whether the effect of parental starvation is extended to the F1, and F2 generation social behavior and interaction with another group of mice, and the P0 generation mice are able to learn to become aggressive after starvation when facing potential competitors. Before the experiment, both groups of mice are fed to full stomach status. One mouse from each group is presented in the same area and facing the same source of food which all of them have the same distance and same view towards the food. We measure the amount of aggressive behavior towards other mice demonstrated by each mouse when facing the food source and the amount of loot behavior towards the food source. All these two factors are included and measured by the aggressive index to demonstrate the relative difference between the groups of mice. Our measurement is based on the mice aggression detector model derived from the study on the territorial aggression behavior demonstrated by resident mice which hence help us to quantitative standardize our measurement of the aggression behavior on the food resource. [14]such as chronic social defeat stress. However, quantifying mouse aggression in a systematic,

representative, and easily adoptable way that allows direct comparison between cohorts within or between studies remains a challenge. Here, we propose a structural equation modeling approach to quantify aggression observed during the resident-intruder procedure. Using data for 658 sexually experienced CD-1 male mice generated by three research groups across three institutions over a 10-year period, we developed a higher-order confirmatory factor model wherein the combined contributions of latency to the first attack, number of attack bouts, and average attack duration on each trial day (easily observable metrics that require no specialized equipment.

2.3 MiR212/132 and Other miRNA Differential Expression Gene Analysis

In this miR212/132 corresponding gene differential expression analysis experiment (n=3), we want to make sure that the transgenerational event occurs due to the change in expression of miRNA corresponding gene and also help the research to find more potential miRNA that may be involved in the whole mechanism. The experiment is mainly separated into two steps which are the preparation of miRNA library and the gene of miRNA differential expression analysis.

In the first step, the miRNAs are isolated from the male mice's testis and sperm to prepare the miRNA library that is used for differential expression gene (DEG) analysis. Both mice from the control group and starvation group prepared in the library separately. The isolation process uses the Trizol process which is relatively advantageous when the separation of cytoplasmic RNA from nuclear RNA is impractical or when cells or tissues are enriched for endogenous RNases. The TRI reagent is used during the extraction and 1ml of TRIzol is used per 100mg of tissue [15]. The Nanodrop-1000 is used to determine the quality and quantity of miRNA gathering and miRNA library construction uses a total of 500ng of RNAs and then apply them to multiplex small RNA library set to achieve the target. These steps ensure enough miRNAs are used for the DEG analysis and the miR212/132 is specifically focused during the DEG analysis and other genes that has been upregulated will also be focused.

The next step would be the DEG analysis for the miRNA library that we prepared. The main focus is the miR212/132 and also measure other potential miRNA in the sperm line and find whether these miRNAs are upregulated or downregulated. For the gene differential reading obtained the UPARSE method is used, the miRNA will organize in OTU (groups of sequences that are intended to correspond to taxonomic clades or monophyletic groups) de novo by using the next-generation reads and then the UPARSE will filter according to the quality, cutting the gene into a fixed sequence, optionally discarding the singleton read and grouping the remaining reads to achieve enough accuracy [16]but they suffer from a high level of sequencing and amplification artifacts. The UPARSE pipeline reports operational taxonomic unit (OTU. For the analysis process, the standard edgeR flow is used and the log2 fold change >1 followed by the p-value <0.05 is considered as a candidate for analysis and for the further experiment selected potential miRNA [17], [18]we ask whether sons born to mothers with PCOS (PCOS-sons. The heatmap hence be plotted according to the calculation from log2.

2.4 Preparation of CRISPR Vector and CRISPR Experiment

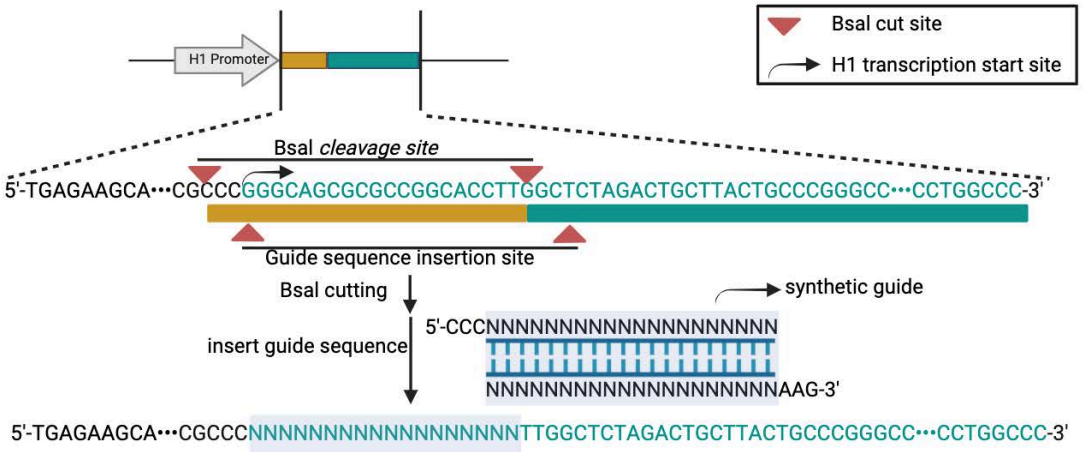

Figure a

Figure b

Figure 1: The excision points of enzyme on the miR212/132 gene and the corresponding process of gRNA insertion, Fig. a, the miR212 excision site and both gRNA insertion process, Fig b. miR132 insertion site and excision site

For the CRISPR experiment, the embryonic stem cells that produce from the P0 generation starvation internal mating group is selected as the experiment subject which operate by the CRIPSR Cas9 for miR212 and miR132 deletion. For both miR212/132 the plasmid construction process will use same H1 promoter and is amplified to suit the mice genome sequence. Target oligonucleotide that contain the H1 promoter and having two specific Bsal site that use for the enzyme restriction is used for PCR amplification to ensure for enough amount for Cas9 to eventually generate. For the miR212 target gene guide RNA vector building process, the embryonic stem cell will first be seeded in a 24 well plate which has the density of 100,000 cells per well and each well is supplied with Cas9 plasmid after 48h culturing to ensure enough cell has growth. Also, the targeting gRNA T1 site is inserted into the well and the operation is based on the manufacturer's protocol to ensure enough accuracy. The Fig.1a demonstrate the 2 gRNA target selecting site and demonstrate the position where Bsal restriction enzyme were selected to remove the DNA and insert the guide sequence which manifest the detail step.

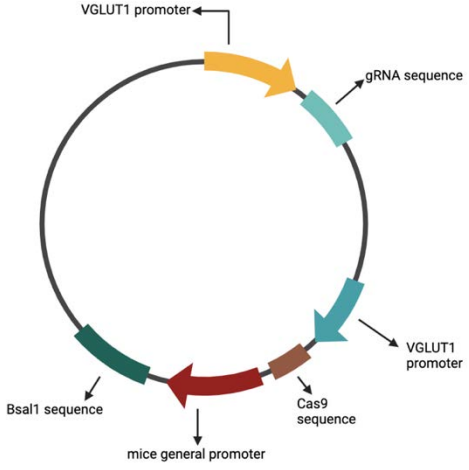

Figure 2: Plasmid for CRISPR Experiment

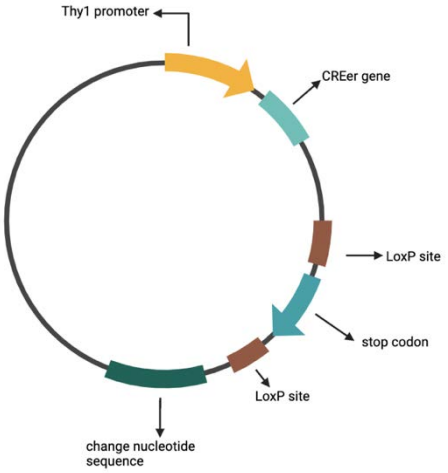

Figure 3: Plasmid Construction for Insertion

After the two guide sequence has been inserted, the Cas9 enzyme will perform gene deletion. For the miR132 guide sequence inserting process, same procedure is repeated but the site is reselected and the. Fig. 1b. demonstrate the process of site selection and gRNA inserting. The plasmid construction process is built based on the pBR322 vector which then injected in the E. coli proliferate into large amount that enough to inject into mice embryonic stem cell to generate enough number of Cas9 for excision process. The whole plasmid is constructed using the mice gene promoter VGLUT1 follow by the Cas9 gene sequence for mice, then on the same plasmid, contain the gRNA corresponding DNA sequence which prior have the mice gene promoter VGLUT1 to transcribe for the excision process locating on the mice sequence. Also, on the same plasmid, Bsal gene is located after the general promoter of mice transcripts to form Bsal enzyme for gRNA original DNA removing. The whole construction of plasmid is indicated by the Fig.2. For both miR212/132, the gene deletion process is similar as same mechanism has been used. The genomic DNA is extracted after 48h of transfection using the QuickExtract solution and Cas9 performs the extraction process according the gRNA site after the extraction. The effect of truncating is revealed by the gel electrophoresis and compare to the result of wild-type DNA sequence. After deletion, both will inject into the embryonic stem cell of mice embryo and move it to a female mice from P0 starvation group.

2.5 Hippocampus-sperm Communication Verification Experiment & Preparation of CREer-LoxP Vectors

For the hippocampus-sperm communication verification experiment, we intended to use this experiment to decide whether the upregulation of miR212/132 is from the hippocampus or in the sperm. For this verification process, we decide to change one nucleotide in gene that made the miR212/132 which the change nucleotide aim to not effecting the normal function of the miR212/132 which are required for the further experiment. For the nucleotide that decide to change, we determine to change the nucleotide of position 9 that from U to A for both miR212 and miR132.

To insert the changing sequence into mouse, we need to make sure that the CER method insertion is inserted in the position that after a promoter that specifically represent in mice hippocampus. We will first to use dicer enzyme to cut a strand of miR212/132 and change the nucleotide of the sequence. Them, we send the miR212/132 to the company to make complementary gene sequence that has suitable promoter and LoxP position that are available in plasmid form. The plasmid design is indicated by the Fig. 3 and the promoter that specifically express in mice hippocampus is Thy1 which was chosen based on the experiment that indicate the Thy1 was specifically express in the mice hippocampus [19]higher-order interactions between components govern emergent dynamics. Here we tested whether contextual threat memory retrieval in mice relies on higher-order interactions between dorsal CA1 hippocampal neurons requiring learning-induced dendritic spine plasticity. We compared population-level Ca2+ transients as wild-type mice (with intact learning-induced spine plasticity and memory. The mice hippocampus transcribe the Thy1 promoter and activate the CREer and it binds to tamoxifen to enable the cutting of LoxP site which leads to the transcription of the change nucleotide miR212/132. To transfer the plasmid to the mice embryo, the bacteria is used and it will bring the plasmid to mice's cells and eventually express in the hippocampus.

2.6 qRT-PCR Experiment

The qRT-PCR experiment aims to determine whether the changed nucleotide miR212/132 was presented in the sperm if we first insert it into the hippocampus to verify whether the communication between the hippocampus and sperm is present. The first step of qRT-PCR is synthesizing the cDNA process. For the cDNA synthesis process, the reverse transcription kit is used to first treat 300 ng of miRNA extracted from the mice sperm after growing up for the DNase-I-treated. After cDNA synthesis, the probe first be designed to contain CG content from 30-80% which have temperature of 67 ºC approximate. For the sequencing process, use the miScript Primer Assays method in which each miRNA binds with miScript universal primer and the corresponding miRNA level is normalized to RNU6B. For the miR212/132, both active arm and inactive arm is assessed and for other potential miRNA that identified upregulated in the DEG analysis experiment, only the active arm is assessed.

3. Expected Result

3.1 Behavioral Change

For the several operations on the different groups of mice, different behavior changes and the degree of differences may be varied. For the normal starvation-treated group and control group P0, F1, and F2 generation, the mice from the starvation group are expected to demonstrate more extra food ingestion, P0 generation starvation group of mice are able to demonstrate learning to be aggressive towards other mice to obtain more food available and the F1, F2 generation starvation group mice also demonstrate to become more aggressive compared to the control group F1, F2 generation mice. For the social effect investigation cross-fostering group, the F1 generation starvation group mice which have parent being starvation group but fostered by the control group P0 generation also expect to demonstrate an increase of aggressiveness and extra food ingestion weight compared to the F1 generation control group mice which foster by the P0 generation starvation group mice. However, the difference between these two groups is expected to become smaller compared to the average value of the previous experiment which hence manifests that fostering will cause some extent of social effect on the offspring behavior but most of the behavior was determined by the genetic information. For the paternal and maternal effect experiment group, the F1 generation mice that have a P0 father in the starvation group and a mother in the control group expect to demonstrate more aggressive behavior and more food ingestion compared to the F1 mice that have a P0 father in the control group and mother in the starvation group. As environmental stress is restricted from passing through the uterus during pregnancy, the gene differential expression in the sperm likely lead to more behavior change. But this experiment also demonstrate less difference than the simple comparison group as the genetic information in the maternal oocyte would also cause a change in a small extent of behavior.

For the behavior change after CRISPR deletion of miR212/132 and other potential miRNA that demonstrated upregulated in the DEG analysis, we expected several different results to occur. For the F1 generation that has deleted the miR212/132 we expected to see no change of behavior on both the aggressive behavioral assay and the

extra food ingestion assy. But for some other potential upregulated miRNA deletion, we expected to see some mice demonstrate three different results some caused only one assay to have no change and others caused both assays to demonstrate no change. Therefore, based on the result, we could group all the miRNAs that we have tested into three different categories to reach the final mechanism of behavioral change.

For the mice that have been inserted of miR212/132 that has one nucleotide change, the mice are expected to demonstrate a normal degree of changing behavior in both assays which demonstrates increased aggressiveness and also more food ingestion to prove that the mice haven't been affected by the nucleotide and hence are available for the future experiment.

3.2 Differential Expression Gene Analysis

For the DEG analysis, the mice that demonstrated more aggressive behavioral change would demonstrate upregulated of the miR212/132 which is represented by the higher heat value in the heatmap. For the other miRNAs, some were expected to demonstrate upregulated while others were expected to demonstrate downregulated which depends. The extent of differential expression would determine by the difference of behavior change that demonstrated by experiment group and the control group. For the log2 fold change volcano diagram, the potential miRNA and miR212/132 should be expected to appear beyond the cutoff edge and hence demonstrate the highest upregulated change.

3.3 Presence of the Change Nucleotide Sequence in Sperm

In this experiment, after inserting the change nucleotide gene sequence in the mice, the qRT-PCR sequence should detect the change sequence miR212/132 in the hippocampus first to verify the successful insertion process. As the animal that demonstrated the behavior change, the change sequence miR212/132 should both be expected to be found in the mice's sperms and should be present in large abundance which is at least 70% to verify the enough communication was built between the hippocampus and sperm producing. But for the mice that don't demonstrate the behavioral change, the change sequence nucleotide should only demonstrate to present in small amounts which is less than 10% to verify that this communication was only established due to environmental stress like starvation.

4.Discussion

The hypothesis about whether the miR212/132 differential expression cause TEI on mice is verified by the result from behavior assay that the changing behavior offspring mice demonstrate different expression scale compare to the offspring that is have no change, while this would be further proved by the CRISPR experiment that demonstrate deletion of miR212/132 gene would result disappear of behavior change of offspring. The hippocampus-sperm communication is also verified by finding the change sequence miR212/132 in the sperm-line cell after injection and only express in mice hippocampus.

For the hinder mechanism about why miR212/132 could affect the mice behavior, in the study some possible explanation is proposed. In some previous testing on the function of miR212/132, it demonstrates to play an important role in regulating the growth and development of neuronal plasticity in the hippocampus and another brain area which hence could present an effect on the memory or motor neurons and cause action change in the mice [20]. Therefore, it could probably be considered that the neuronal change and the plasticity change would cause the change in the behavior of the F1 to F2 generation. Due to the starvation of the parent generation the F1 and F2 generations have a higher sensitivity to food resources and eating food builds the reward system faster compared to other normal individuals and hence acquire the food more urgently. Furthermore, a study has demonstrated that the miR132/212 performed a function in regulating seasonal adaptation of the animal and the spine density on SCN neurons which is responsible for the animal's physiology and therefore may be the reason for the change in mice behavior [21]. Moreover, other studies have demonstrated the function of miR212/132 function in binocular orientation identification which may also explain the behavior change of our miRNA which affects the visual preference of the food and forces the mice to do action to pursue the food stimulation [22]but their role in postnatal brain development is still poorly explored. We show that the expression of many miRNAs is dramatically regulated during functional maturation of the mouse visual cortex with miR-132/212 family being one of the top upregulated miRNAs. Age-downregulated transcripts are significantly enriched in miR-132/miR-212 putative targets and in genes upregulated in miR-132/212 null mice. At a functional level, miR-132/212 deletion affects development of receptive fields of cortical neurons determining a specific impairment of binocular matching of orientation preference, but leaving orientation and direction selectivity unaltered. This deficit is associated with reduced depth perception in the visual cliff test. Deletion of miR-132/212 from forebrain excitatory neurons replicates the binocular matching deficits. Thus, miR-132/212 family shapes the age-dependent transcriptome of the visual cortex during a specific developmental window resulting in maturation of binocular cortical cells and depth perception.","con-

tainer-title":"Nature Communications","DOI":"10.1038/ncomms15488","ISSN":"2041-1723","issue":"1","journalAbbreviation":"Nat Commun","language":"en","license":"2017 The Author(s. The whole mechanism may also be accompanied by other potential miRNAs and we propose several possible subjects that should be specifically focused on for the experiment. The miR29 has been investigated for its function in regulating the spatial expression of the mammals and the miR378i which is reported to be responsible for FUS may also cause the TEI changing in animal motor neuron areas to lead to behavior change [23], [24]highly complementary miRNA-binding sites within viral and artificially engineered transcripts induce miRNA degradation in vitro and in cell lines. Here, we show that a genome-encoded transcript harboring a near-perfect and deeply conserved miRNA-binding site for miR-29 controls zebrafish and mouse behavior. This transcript originated in basal vertebrates as a long noncoding RNA (lncRNA. Overall, if our CRISPR experiment does verify the multistep learning process, we could according to the miRNA group propose a possible mechanism for the whole learning processing behavior and the mechanism for its transmission to the next generation. If our experiment does verify the communication process between the hippocampus and the sperm, the corresponding explanation that we supposed for this phenomenon is that the first expression of the hippocampus would be a verification step that ensures that the environmental stress is sufficient to threaten the life of the mice and make sure that the TEI which change behavior would not occur on the offspring in the unnecessary environment and may lead to the survival disadvantage.

The advantage of our experiment is that it focuses on the possible detailed mechanism for TEI in mice species and also tries to propose the mechanism of the communication between the hippocampus and the germline cell which probably directly results the TEI occurring in the mice. The additional miRNA upregulated identified in the DEG analysis would provide clues for the next step of investigation and also provide a potential target for the human medical treatment the diseases that have family inheritance characteristics and also reveal the inner possible mechanism for some TEI occurrence in the human body such as the review summaries the non-genetic inheritance of human disease [5]by factors in germ cells that remain largely uncharacterized. As evidence for germline non-genetic inheritance of phenotypes and diseases continues to grow in model organisms, there are fewer reports of this phenomenon in humans, due to a variety of complications in evaluating this mechanism of inheritance in humans. This review summarizes the evidence for germline-based non-genetic inheritance in humans, as well as the significant challenges and important caveats that must be considered when evaluating this process in human populations. Most reports of this process evaluate the association of a lifetime exposure in ancestors with changes in DNA methylation or small RNA expression in germ cells, as well as the association between ancestral experiences and the inheritance of a phenotype in descendants, down to great-grandchildren in some cases. Collectively, these studies provide evidence that phenotypes can be inherited in a DNA-independent manner; the extent to which this process contributes to disease development, as well as the cellular and molecular regulation of this process, remain largely undefined.","container-title":"Clinical Epigenetics","DOI":"10.1186/s13148-020-00929-y","ISSN":"1868-7083","issue":"1","journalAbbreviation":"Clin Epigenetics","language":"eng","note":"PMID: 32917273\nPMCID: PMC7488552","page":"136","source":"PubMed","title":"Evidence for germline non-genetic inheritance of human phenotypes and diseases","volume":"12","author":[{"family":"Senaldi","given":"Liana"},{"family":"Smith-Raska","given":"Matthew"}],"issued":{"date-parts":[["2020",9,11]]}}],"schema":"https://github.com/citation-style-language/schema/raw/master/csl-citation.json"} . However, the limitation of this study is that in this study, we only focused on the miRNA that demonstrated differential expression in the germ-line cell but did not focus on other small non-coding RNA that would also be a part of the whole mechanism. Also, in this study, we ignore other genetic modification methods that would result in the TEI change in mice such as gene methylation on the CpG island indicated by one study and the virus-like particle CER-1 that causes the TEI in the nematode[25], [26]and unstable genomic elements threaten from within. C. elegans protects itself from pathogens by \"reading\" bacterial small RNAs, using this information to both induce avoidance and transmit memories for four generations. Here, we found that memories can be transferred from either lysed animals or from conditioned media to naive animals via Cer1 retrotransposon-encoded virus-like particles. Moreover, Cer1 functions internally at the step of transmission of information from the germline to neurons and is required for learned avoidance. The presence of the Cer1 retrotransposon in wild C. elegans strains correlates with the ability to learn and inherit small-RNA-induced pathogen avoidance. Together, these results suggest that C. elegans has co-opted a potentially dangerous retrotransposon to instead protect itself and its progeny from a common pathogen through its inter-tissue signaling ability, hijacking this genomic element for its own adaptive immunity benefit.","container-title":"Cell","DOI":"10.1016/j.cell.2021.07.022","ISSN":"109

7-4172","issue":"18","journalAbbreviation":"Cell","language":"eng","note":"PMID: 34363756\nPMCID: PMC8812995","page":"4697-4712.e18","source":"PubMed","title":"The role of the Cer1 transposon in horizontal transfer of transgenerational memory","volume":"184","author":[{"family":"Moore","given":"Rebecca S."},{"family":"Kaletsky","given":"Rachel"},{"family":"Lesnik","given":"Chen"},{"family":"Cota","given":"Vanessa"},{"family":"Blackman","given":"Edith"},{"family":"Parsons","given":"Lance R."},{"family":"Gitai","given":"Zemer"},{"family":"Murphy","given":"Coleen T."}],"issued":{"date-parts":[["2021",9,2]]}},{"id":647,"uris":["http://zotero.org/users/14612718/items/78IG9B39"],"itemData":{"id":647,"type":"article-journal","abstract":"Transgenerational epigenetic inheritance in mammals remains a debated subject. Here, we demonstrate that DNA methylation of promoter-associated CpG islands (CGIs. The more different RNAs that are involved in the TEI mechanism and another kind of transmission method was potentially present in the mice and affect the final result but not revealed by the current study could both be the possible direction for the future experiment and study on mice and other animals.

5. Conclusion

To conclude, this study mainly wants to prove the function and mechanism of miR212/132 in mice TEI effect when facing environmental stress such as starvation and also verify the possible hippocampus-germline communication mechanism within the mice body. The predicted result will verify the differential expression of miR212/132 in the sperm cell of mice which hence proves the important role of miR212/132 in mice TEI effect. Also, if the change nucleotide sequence is presented in mice sperm which is demonstrated by the qRT-PCR experiment, this will verify that it is true that there is the presence of a communication pathway between the hippocampus and the sperm. The overall experiments are still not perfect, we only tested on one specific mechanism of mice TEI but ignored the other possible mechanisms that may lead to a similar result, also oversimplified the learning and transmission process which may have other miRNAs play a role in the whole mechanism. Even though there are drawbacks presented, this study could provide a pathway for future research about the mice and provide a possible site for mice behavior investigation and may also promote it to human medical treatment as a site for psychiatric disease. For future research, I recommend that the future research team put more focus on the other potential mechanisms for TEI and stay closer to mammals. The research in the future should explain in more detail the mechanisms of TEI and how it changes the brain of mice whether this brain structure change would happen in humans and how it will affect our lives and health. The basic mechanisms could also be used to treat diseases that have family inheritance potential.

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